

A comprehensive study and process development to treat oil refinery waste

A thesis

Submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

by

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Under the supervision of

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DEDICATED

TO

MY FAMILY

SHRIMATI GEETA BHATTACHARJEE (GRANDMOTHER)

LATE SHRI MANINDRA CHANDRA BHATTACHARJEE (GRANDFATHER)

LATE SHRIMATI KATYAYANI CHATTERJEE (GRANDMOTHER)

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SHRIMATI POULOMI CHATTERJEE (MOTHER)

SHRI PARBATI CHARAN CHATTERJEE (FATHER)

SHRI PREETAM CHAYAN CHATTERJEE (BROTHER)

MY TEACHERS

MY FRIENDS

THE ENVIRONMENT



पर्यावरण केंद्र, भारतीय प्रौद्योगिकी संस्थान गुवाहाटी

गुवाहाटी - ७८१०३९, भारत

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Declaration

It is to hereby declare that the content of this thesis entitled “**A comprehensive study and process development to treat oil refinery waste**” was carried out by me at **Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India** under the supervision of **Prof. Sanjukta Patra** and **Prof. Mohammad Jawed**. The contents are thoroughly investigated and validated and are solely used to present for the award of the degree of **Doctor of Philosophy**. The same has not been submitted to any other institute for obtaining any other degree, diploma or fellowship from any other University or Institute. The information provided is accurate to the best of my knowledge and understanding, and every effort has been made to avoid duplication of any document or article. In accordance with accepted practices, proper acknowledgment has been mentioned while assessing, reporting and citing scientific documents and research findings of other investigators.

Date: April 24, 2024
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Certificate

This is to certify that the thesis entitled “**A comprehensive study and process development to treat oil refinery waste**” submitted by **Mr. Prithwi Chayan Chatterjee** (Roll No. 166152002), is an authentic record of work carried out under our supervision at **Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India**. The thesis is recommended as per the institute regulations for the degree of **Doctor of Philosophy** and the same in part or whole has not been submitted to any other institute for obtaining any other degree or diploma.

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List of abbreviations and symbols

%	Percentage
~	Approximately
</≤	Less than/ less than or equal to
>/≥	Greater than/ greater than or equal to
µg/mL	Micrograms per milliliter (equivalent to mg/L)
3D	Three-dimensional
AD	Anaerobic digestion
AFEX	Ammonia fiber extension
ANOVA	Analysis of Variance
APHA	American Public Health Association
API	American Petroleum Institute
ASTM	Formerly American Society for Testing and Materials
BIS	Bureau of Indian Standards
BLAST	Basic Local Alignment Search Tool
BMC	Bacterial Mixed cultures
BOD	Biochemical oxygen demand
BPC	British Petroleum Corporation
BSA	Bovine Serum Albumin
BTEX	Benzene, toluene, ethylbenzene, xylene
BTX	Benzene, Toluene, Xylene
C	Carbon
C1,2DO	Catechol 1,2 dioxygenase
C2,3DO	Catechol 2,3 dioxygenase
CCD	Central Composite Design
CDOs	Catechol dioxygenases
CFU/mL or CFU/g	Colony Forming Unit per unit milliliter or grams
CHAPS	3-(3-cholamidopropyl dimethylammonio)-1-propanesulfonate
cm	Centimeter
C_n	Hydrocarbon with “n” carbon chain
CO₂	Carbon dioxide
CoA	Coenzyme A
COD	Chemical Oxygen Demand
COTBS	Crude oil tank bottom sludge

CPCB	Central Pollution Control Board
CPCL	Chennai Petroleum Corporation Limited
C.V	Coefficient of variance
CYP	Cytochrome P450
d.c.w	Dry cell weight
d⁻¹ or h⁻¹	Unit of specific growth rates (μ/μ_m) or hydrocarbon utilization rates (q/q_m) or reaction rate constant (k) as time ⁻¹ in “days” or “hours”
DAF	Dissolved air flotation
DCM	Dichloromethane
DDW	Deionized distilled water
DMSO	Dimethyl Sulfoxide
DO	Dissolved Oxygen
DoE	Design of Experiments
EC	Enzyme Commission
EK	Electrokinetic
FAD	Flavin adenine dinucleotide
FAs	Fatty acids
FCC	Fluid catalytic cracking
F-test	Fisher test
FY	Fiscal year
g	Relative centrifugal force (RCF)
g/Kg	Grams per kilogram
g/L	Grams per liter (equivalent to mg/mL)
GoI	Government of India
GW	Groundwater
H	Hydrogen
H₂O	Water
H₂S	Hydrogen sulfide
H₂SO₄	Sulfuric acid
HCs	Hydrocarbons
HPAM	Hydrolyzed polyacrylamide
HRT	Hydraulic retention time
IOCL	Indian Oil Corporation Limited
KEGG	Kyoto Encyclopedia of Genes and Genomes
L/ mL/ μL	Litre/ millilitre/ microlitre
LPG	Liquified petroleum gas

M/ mM/ μM	Molar/ millimolar/ micromolar
max	Maximum
MEK	Methyl ethyl ketone
mg/L	milligram per liter (equivalent to ppm)
mg/mL	Milligram per milliliter (equivalent to g/L)
min	Minimum
mMSM	Modified Minimal Salt Media
MMTPA	Million metric tons per annum
MoEF	Ministry of Environment, Forest and Climate Change
MoPNG	Ministry of Petroleum and Natural Gas
MoSPI	Ministry of Statistics and Programme Implementation
MSM	Minimal Salt Media
MT	Metric tons
MTC	Maximum tolerance concentration
mV	Millivolts
N	Nitrogen
NA	Nutrient Agar
NADH	Reduced Nicotinamide dinucleotide
NAPs	Naphthenic acids
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NDO	Naphthalene dioxygenase
nm	Nanometer
nmol/ μmol	Nanomoles/ micromoles
NO	Nitric oxide
O	Oxygen
O₂	Molecular Oxygen
°C	Degree Celsius
OD/OD_x	Optical density/ optical density measured at “x” nm wavelength
OFAT	One-factor-at-a-time
OH•	Hydroxyl free radical
ORP	Oxidation-reduction potential
P	Phosphorus
<i>p</i>(<i>F</i>)	Probability of the occurrence of <i>F</i>
PAC	Power activated carbon
PAHs	Polyaromatic hydrocarbons

PBD	Plackett-Burman Design
PCR	Polymerase chain reaction
ppm	Parts per million (equivalent to mg/L)
PSB	Photosynthetic bacteria
ROS	Reactive oxygen species
rpm	Rotation per minute
RSM	Response Surface Methodology
SD	Standard deviation
SDI	Sludge density index
SLP	Slurry phase
SO₂	Sulfur dioxide
SOP	Solid phase
sp.	Species
SVI	Sludge volume Index
SW 204	Scheduled Waste
TCA	Tricarboxylic acid
TDS	Total dissolved solids
TOC	Total Organic Carbon
TPH	Total Petroleum Hydrocarbons
TSS	Total suspended solids
U mg⁻¹	Enzyme activity in Unit per milligram protein complex
USA	United States of America
USEIA	United States Energy Information Administration
USEPA	United States Environmental Protection Agency
UV-Vis	Ultraviolet Visible
v/v	1 milliliter volume per 100 milliliters
vs	Versus
w.r.t	With respect to
w/v	1 gram-weight per 100 milliliters
w/w	1 gram weight per 100 grams
WHO	World Health Organization
WWTP	Wastewater treatment plant

Abstract

Petroleum refineries have proved to be an important sector for energy consumption. Being one of the largest sectors of energy generation, India, along with the whole world, depends heavily on petroleum refinery products to meet daily requirements. Due to the severe complexity of raw crude oil mixture, refining through various operating units is carried out in a petroleum refinery to convert it into usable components. Alongside the essential products and byproducts, refineries also generate an enormous quantity of waste in the form of oily sludge either during the process of refining or while treating toxic effluents. The oily sludge containing primarily hydrocarbons (HCs), contributes to high chemical oxygen demand (COD), alongwith total suspended solids (TSS), heavy metals, trace elements, and anions. Some of these components are reported as persistent, thereby combating such contamination has become a challenging environmental agenda. The range of HC pollution may spread to soil and aquatic systems, and hence, for the biotic to sustain, removal of these highly toxic chemical compounds is a necessity. Whereas several physicochemical techniques are available, bioremediation has been identified as an eco-friendly and cost-effective avenue as viable microorganism adept at surviving such harsh environments are employed to reduce overall toxicity. To attain the best efficiency of solutions for such a complex problem, a simplified strategic development of an optimal nutrient medium and microbial consortia could be an effective approach that enhances the removal of harmful contaminants and minimizes the detrimental effect of sludge spillage or improper disposal into the environment. The primary objective is to harness the indigenous microbes and obtain an optimum proportion. Amendment of nutrients in the form of nitrogen (N) and phosphorus (P) enhance the bioavailability and further result in higher degradation and removal efficiency of the pollutants. Studying the effect of micronutrients and optimum conditions also aid in identifying the optimized parameters and consortia that would render best microbial performance for minimizing oily sludge contamination and attaining maximum reduction in toxicity.

In this study, we presented a comprehensive overview of the process of utilizing the capabilities of indigenous microorganisms and engineering their efficacy of survival in different classes of HCs under controlled laboratory conditions. Experimental works were initiated by studying the physicochemical characteristics of the sludge sample from the Indian Oil Corporation Limited (IOCL) Guwahati refinery. The pollution level evaluated through

COD >46 g/L and TSS ~9.8 g/L of the sludge was high, and the habitat was abundant in nutrients such as Total nitrogen (TN) ~0.45 g/L and $\text{PO}_4^{3-}\text{-P}$ ~8.5 mg/L and free ions, making it conducive to microbial growth. 7 indigenous strains GR1, GR2, GR3, GR4, GE1, GE2 and GS1 were isolated and identified using enrichment methods. The strains collectively belonged to predominantly three genera: *Aquamicrobium* sp. *Bacillus* sp. and *Staphylococcus* sp. Their survivability was checked in different classes of HCs such as *n*-hexadecane (straight chain aliphatic), benzene/toluene/xylene (aromatic), and anthracene/phenanthrene (polyaromatic) that are expected to be present as derivatives in a typical refinery waste. The isolated strains exhibited sluggish growth, however, contributing significant bacterial count even after over a month-long (39 days) incubation. The strains could collectively utilize *n*-hexadecane, benzene, toluene, and xylene more efficiently than anthracene and phenanthrene contributing to the reduction in toxicity by >80% in certain cases. All the isolates except *Aquamicrobium* sp. GR3 were further selected after evaluating the biomass growth and bacterial counts, alongside COD removal collectively in different HC supplemented media, and were subjected to two rounds of statistical optimization with a target of achieving maximum biomass concentration after incubation. The effectiveness of increased microbial was also investigated by evaluating the COD removal and correlated to maximizing the reduction in toxicity of the sample. Micronutrient factors $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl were added to the original minimal salt media (MSM) to obtain a modified media at their optimized levels (65.34 mg/L and 334.88 mg/L respectively) by virtue of their positive influence on the biomass production of the strains. The individual strains were statistically optimized in the amended media to obtain three possible optimized microbial combinations, C1, C2, and C2, that were used for further experiments. Biomass composition and enzyme activities were also studied as indicative of HC degradation to proficiently gain insights into the role and efficiency of the microbial strains in metabolizing the pollutant components during the bioremediation process. After harnessing the biomass obtained at the end of each incubation period in different HC media, the lipid, carbohydrate, and protein concentrations (collectively high as 30-40%) indicated the quality of biomass and the adaptability of the strains in a harsh HC-contaminated environment. *alkB*, *ndo*, *catA* and *catE* activities collectively higher to ~105 U mg^{-1} in crude protein complex pertaining to alkane, aromatic and polyaromatic HCs (PAHs) metabolism also indicated the ability of the strains to metabolize such substrates in their cell. The addition of nutrients in the form of nitrogen (N) and phosphorus (P) depicted higher bioremediation efficiency, citing the importance of such amendment to improve bioavailability. Optimum C:N:P of 100:5:1 and 100:10:1 were collectively employed to check for the efficiency of the optimum settings

(modified media, 48 days incubation) that were obtained through vigorous laboratory experimentation and statistical validation. The final application rendered significant biomass of 1.536 g/L along with presence of 7.73×10^9 CFU/mL bacterial cells, and a ~70% reduction in COD after 48 days of incubation. Considerable N and P utilization of ~70% was also observed in addition to obtaining significant biomass yield ~218 mg/g COD consumed and decrease in half-life (<2 days) of the sludge sample. The biomass obtained at last also possessed a higher deposition of lipid and protein content (19-34% dry biomass), justifying the possibility of usage in further applications and value addition. The microbial strains isolated showed the presence of necessary machinery in the form of enzymes to metabolize the toxic components of the waste. After 48 days of incubation, enzymes pertaining to alkane (*alkB*), aromatic, and PAHs (*ndo*, *catA*, *catE*) metabolism were found to depict tremendous activity (3-33 U mg⁻¹) in the crude protein complex, citing their potential to convert the harmful components into simpler non-toxic forms. Overall, the indigenous isolates depicted the better adaptability and acclimatization making them promising candidate for application in bioremediation of refinery waste sludge contaminated site.

Keywords: Biomass, Bioremediation, Enzyme, Nutrients, Optimization, Petroleum, Sludge

1

Introduction and literature review

1.1. Background

Whereas the world is moving towards implementation of renewable sources, fossils fuels are still the biggest contributor towards energy consumption in the year 2024. With increasing challenges of enhanced energy requirements due to population growth, overconsumption, unequal distribution, and energy production by fossil fuels, which include coal, petroleum and natural gases, are monitored closely as per the International Energy Agency, of which India is an Associate member since 2017, to derive suitable patterns for sustainable consumption and resource usage. While India, alongwith the whole world, saw a significant decline in energy usage due to the COVID-19 pandemic in 2020, there has been steady growth in energy production and consumption during the year 2021 (+10.39%), and considerable recovery of energy production was seen in the fiscal year (FY) 2022-23 [1–5]. Over 10 years ago, Kumar and Mohan, 2013 reviewed that petroleum product consumption in India had grown by 4-5% over the past 20 years and the oil demand in India was expected to rise upto 368 MMTPA by 2025. In another report, the demand is predicted to increase by 28% within 2015-2040 [6,7]. Although concerns related to greenhouse emissions from fossil fuels have been discussed over the years by various organizations and strategies are being made to limit climate change, upgradation to renewable sources face severe challenges such as financial and infrastructural reforms. Hence, over the next few decades, dependence on energy production and consumption must be relied upon the existing fossil fuels. However, extensive reviews by different researchers highlight the several challenges to meet the global energy consumption of petroleum-based products of 34%. Current governing factors such as climate change, increase in anthropogenic activities, and the limited reserves of these natural resources pose deeper concerns of a significant increase in energy demand, and the sustainability of these resources requires thorough investigation! [8–10].

Crude oil, one of the fossil fuels associated with the production of petroleum products, contributes significantly to the total energy production of an economy. As per the bp Statistical Review of World Energy, 71st edition, 2022, and bp Energy Outlook 2023, the total primary energy production and consumption of crude oil was the highest in 2022, of which mostly ~70% are used up for refining and the rest (~25%) is being used up for transportation. Only ~6% is used for non-energy petrochemicals. In India, crude oil has been reported as the second most important energy source (24%) after coal (44%), producing energy above 250 metric tonnes (MTs) by the end of FY 2022-23. It is found in the alluvial deposits of tertiary rocks in the extra peninsular India. These areas include the northern plains of Ganga-Brahmaputra

valley, the Thar desert, coasts of Bombay, Godavari, Krishna, Kaveri and Mahanadi and offshore areas of Gujarat, Rajasthan and Andaman and Nicobar Islands. In North-Eastern India, Assam contains the oldest Digboi refinery alongwith trace reserved found in Tripura, Nagaland and Arunachal Pradesh. Important petroleum products such as diesel, petrol, liquified petroleum gas (LPG), kerosene etc. are generated that are used daily in transportation, agriculture, industry and domestic sectors. Whereas there is an increase in consumption of oil products by 10% by the end of 2022-23, amendment in energy policies have contributed to the decreased usage of kerosene as fuel to as low as 16.42% by the end of FY 2022-23 [4,11–16].

Petroleum, a fossil fuel, was formed in ancient times by the gradual deposition of dead organic biomass of prehistoric plants and animals beneath the earth crust. The formation of petroleum and other fossil fuels was dependent on the depth to which the dead matter was buried, the temperature and pressure prevailing to that depth of burial, the order of burial, etc. [17–21]. Crude oil is a naturally occurring, thick, dark greenish fluid that occurs under the uppermost strata of the earth. It indicates all types of HCs obtained in a natural state or by natural condensation on gas. Crude oil consists of simple to complex structures such as resins, asphaltenes, etc. Crude oil is formed by bacterial decomposition of organic matter (carbohydrates/ proteins/ fats from plants/ animal origin) under the presence/ absence of air into HC-rich sediments by undergoing physical, chemical as well as biological alterations. At typical temperature and pressure conditions, components with a higher molecular weight (pentane and higher) can exist as liquids and solids. Crude oil also contains some chemical compounds that may contain N, sulfur (S), P, and some heavy metals such as nickel (Ni), lead (Pb), and chromium (Cr), etc. Crude oil is insoluble in water (H_2O). It is less dense than water, and thus, it floats over the surface as slicks [20,22,23]. It is a product originated from heating and/or compression of naturally decaying biomass occurring at considerable subsurface depth beneath layers of sedimentary soils deposited over geological time, and hence, it is believed to be found at shallow depth from ground level, that is easily recovered by drilling and then processed to consumer-friendly daily essentials like kerosene, pharmaceuticals, plastics etc. [24–27].

Petroleum recovered from different reservoirs varies widely in compositional and physical properties. Various products are available in the markets that are the result of the processing and downstream of petroleum, natural gas, and coal. Crude oil primarily occurs similarly in most places and the composition mainly consists of HCs, most of that comprise

13% weight of hydrogen (H) and 85% of that carbon (C). In addition to these, essential elements such as 0.5% N, 0.5% S, 1% oxygen (O), and <0.1% trace metals comprising iron, nickel, and copper are also found.

The useful components of crude oil are summarised from various sources such as Demirbas and Bamufleh, 2017; Eneh, 2011:

1. Carbon (C) (84%) and Hydrogen (H) (14%): These are the primary components of various fuels like gasoline, jet fuel, diesel, and heating oils. They are also used for manufacturing synthetic rubber, fertilizers, plastic, latex paints, drugs, synthetic fibres, and explosives.
2. Sulfur (S) (1 to 3%): Sulfur compounds in crude oil include hydrogen sulfide (H_2S), sulfides, disulfides, and elemental sulfur. Sulfur is a key component in the production of sulfuric acid (H_2SO_4), one of the most important chemicals used in industrial processes.
3. Nitrogen (N) (<1%): Nitrogen compounds with amine groups are present in crude oil. Nitrogen is a crucial element in the creation of fertilizers.
4. Trace metals (Fe, Ni, and Cu (<0.1%)): As stated earlier, these metals are found in small and are often removed during refining processes [20,28].

The exact composition can vary depending on the source of the crude oil. Depending on the extraction, crude oil is also characterized based on the sulfur content. Crude oil having very high sulfur content are classified under young/ shallow. This type of oil is viscous and sour. This type of oil contains a high amount of naphthenes and paraffins. Young/ deep crude oil has moderate sulfur content and is less viscous as compared to young/ shallow oil whereas old/ shallow oil has lesser sulfur content and is lesser viscous than young/ deep. This type of oil has moderate amount of paraffins, naphthenes and aromatics, all contributing equally to composition. The old/ deep crude has the least sulfur content and is least viscous and is lighter in appearance primarily existing as solid. The paraffins content is the least in this type of oil, whereas the aromatics fraction is the highest [29–31].

Due to the complex composition and unpredictability of occurrence, readymade usage of crude oil is not recommended. Instead, energy that is produced from crude oil is processed to make it suitable for consumption by end users. Petroleum refining is defined as processing of raw crude oil into different useful products. The series of complex industrial reactions for complete processing of crude oil together constitutes a petroleum refinery. On a simpler note,

several types of fuels are the result of crude oil refining and liquefied gases such as propane and butane are the resultant of refining >85% of raw crude oil. According to the latest Petroleum Supply Monthly released till date by the US Energy Information Administration (USEIA) on March 29, 2024, crude oil produces around 48% gasoline which is used to fuel cars. Other proportions include heating oil (14%) useful for heating buildings and jet fuel (5.2%), light fuel (11%), asphalt (0.8%), residual oil (~1%) used for powering factories, fueling large ships and making electricity. Other products such as petrochemical feedstocks, lubricants, naphtha, coke, wax (20%) are essential cosmetics medical supplies, plastics etc. Oil-based products are used for packaging applications, three-dimensional (3D) printing, drug delivery, etc., to manufacture clothing products, non-flammable and colorful furniture, and kitchen items. Hangers are also strengthened by petroleum resins. Oil also finds use in making car bumpers and insulation at home [12,32–36]. An overview of the whole process is depicted in Figure 1.1.

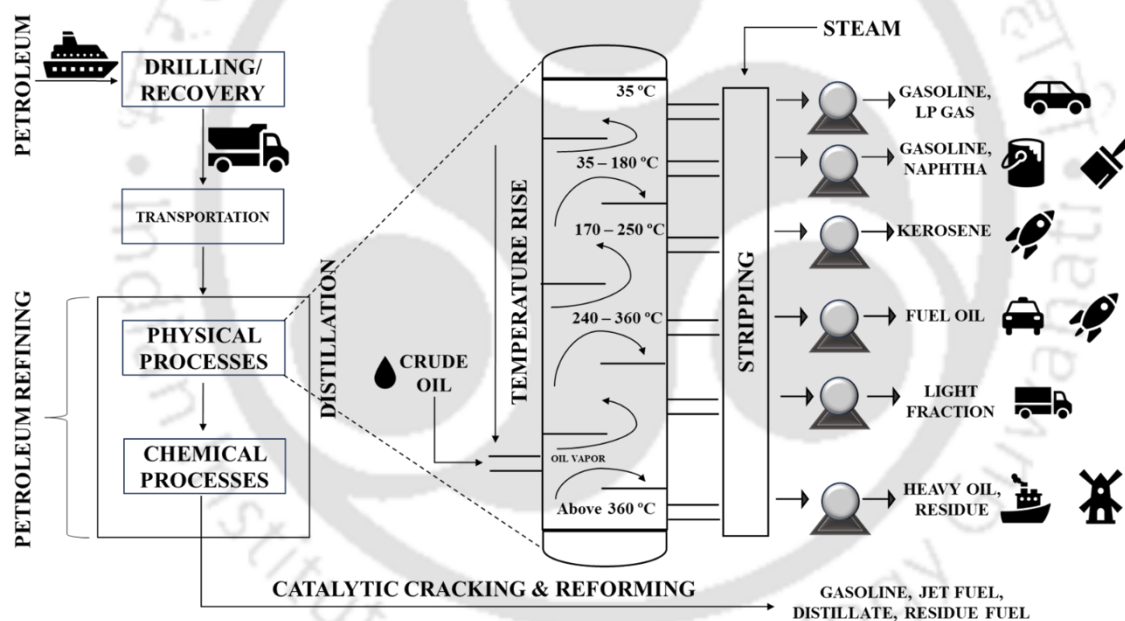


Figure 1.1: Petroleum refining process. The petroleum industries process the crude petroleum after drilling and transporting to the refinery unit. Essential products such as LPG, kerosene etc. are generated along with other by-products.

In general, the petroleum industries have three main units namely upstream unit that mainly deals with exploration and production, the downstream unit that deals with refining and marketing and the midstream unit mainly deals with transportation of refined petroleum oil products [37,38]. Although higher production of refinery products is desirable, the process of energy supply initial production and final consumption includes various operations and processes, causing significant loss of heat energy due to conversion in thermal electricity plants and in own in-house industry use. Moreover, decrease in profit due to insufficient refining

capacity, increasing oil prices, environmental regulations and market competitions have made the refineries to modify their technologies for operation and production. However, these advanced process engineering varies greatly in their use, and hence, their existence and usage for business benefits remains an unanswered question [6,39–41].

New oil fields, a proportion of which are likely to be located offshore, permit a greater capacity for petroleum pollution to impact upon the oceans as well. In addition, oil and gas production processes also account for huge quantity of solid and liquid wastes. Around 0.002–0.1% processed crude are wasted by means of oil spills during drilling, refining, isolation and transportation. Moreover, underground water and soil are also greatly polluted due to discharge of these toxic waste that contain harmful organic and inorganic components. [42–44]. Since crude oil is toxic, carcinogenic and a potent immunotoxin compound, improper disposal of crude oil results in huge contamination in groundwater (GW), and hence, it has become very important to search for the effective means to reclaim such contaminated environment [45]. Due to the complex structure and difficulty in understanding crude oil movement into systems, assessment of toxic effects to the external environment are sometimes limited. Crude oil components such as polar HCs, *n*-alkanes cyclo-alkanes and their derivatives, aromatics, resin and asphaltene residues are considered persistent organic pollutants and thus, their presence affects soil quality. Some constituents of crude have the potential to bioaccumulate within susceptible aquatic organisms and can be passed to other levels of the food chain, eventually causing death or mutation in lifeforms. Apart from these, non-living things are also affected by action of crude oil. The toxic effects may range from acute toxicity to chronic toxicity depending upon the extent of subjectivity to crude oil and dosage and understanding the nature of organisms and the time period to which they are exposed to crude oil is important [46–48]. In the adverse effects, recent reviews have also revealed that prolonged exposure and high oil concentration may cause the development of liver and kidney disease, possible damage to the bone marrow and increased chances of cancer. Oil stains can decrease the ability of birds to fly, dive and swim leading to starvation. The feathers may be less insulated and hence the birds can die. According to an article from United States Environmental Protection Agency (USEPA), birds swallow oil while cleaning the feathers, that could cause serious intoxication. In the long-term effects, intoxication can lead to cell damage, reproduction problems and developmental disorders and investigating the dynamics to prevent not only the ecosystem, but humans, animals and vegetation from the adverse effects of crude oil and components is crucial

[49–52]. Due to the hazardous nature and impact of crude oil, several countries including India have resolved legal regulations of safe handling, storage and disposal of crude oil [38,53].

Apart from the accidental contamination, that is reported to be the primary mode of oil spills (Figure 1.2), accumulation of oily materials from the vast amount of oily sludge generated in refineries pose greater challenges due to the expensive disposal techniques. The most notable oil spills in sea occurs due to accident and imbalance of large tankers, such as Deepwater Horizon oil spill of British Petroleum Corporation (BPC) Gulf oil. These spills can cause problems to sea and shoreline organisms in addition of contamination of land and soil, thereby posing multiple challenges related to hazardous waste and environment management [54,55].

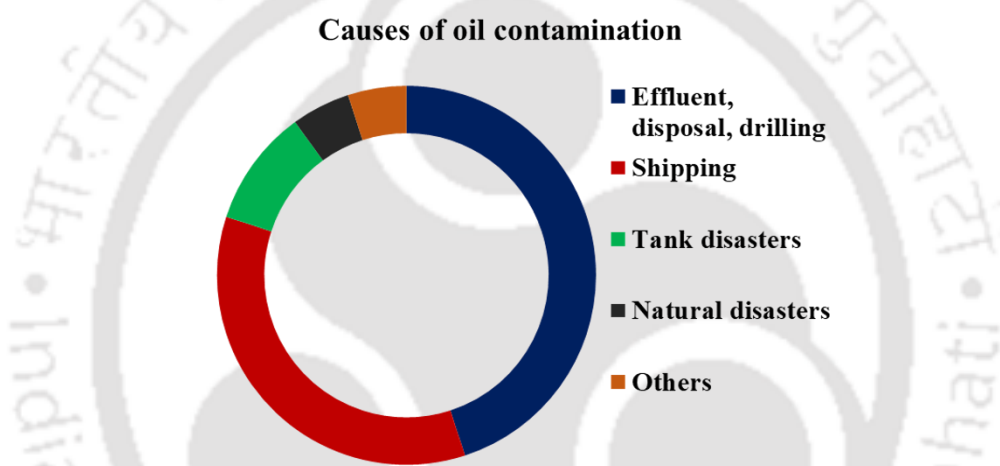


Figure 1.2: Causes of oil contamination. A comparative study of various causes of crude oil contamination is shown. Most oil spills occur due to improper disposal techniques or error in drilling operations in addition to accidents related to transportation.

The BPC oil spill at Gulf Mexico in April 2010 is considered to be one of the largest oil spills in United States of America (USA) history an estimate of 210 million gallons of oil was reported to have spilled. Around 800000 birds were believed to have died and a compensation loss of around 65 million US Dollars (USD) had to be lost. Some other examples of spillage accidents include Ixtoc Oil Well incident in 1979, Castillo de Bellver accident in Capetown, South Africa in 1983 etc. The biggest crude oil spillage ever in history was thought to be in 1910, when the Lakeview well in California, USA erupted causing a spillage of over 370 gallons of crude oil. Whereas it was believed that the oil paved back in causing minimal damage, the effect of land soaked in oil is still felt even after over 110 years of the accident. Another notable reported spillage is from the Gulf war of 1991 in Persian Gulf where around 240 million gallons of oil was spilled [56–61]. The extent of the damage was unknown;

however, experts believed that extensive damage to marine habitats was caused [62,63]. In Indian Scenario, the recent Ennore oil spill of Kamarajar port near coast of Chennai has been extensively studied. The incident happened due to the collision of two cargo ships named BW Maple and MT Kanchipuram carrying 32000 tons of heavy oil. Around 34000 square feet area was reported to be affected by the spillage of 75 MT of oil. Similarly, an earlier incident of *Chitra* oil spill of Mumbai in 2010 was also reported that was caused due to collision of two cargo ships. Around 200 containers were believed to have dropped into Arabian sea [64,65]. Recently, Ennore oil spill on December 4, 2023, during cyclone Michuang at the Chennai Petroleum Corporation Limited (CPCL), the Tobago oil spill on February 7, 2024, was controlled to minimize the potential impact within the Atlantic coast of Tobago and nearby Caribbean islands. In Northeast India, a heavy oil leak in Manipur was reported during January 2024 [66–69].

Oil spills are different in their severity and extent of damage. This difference occurs due to variation in habitat, temperature, oil types, location, climate etc. The spread and behavior are generally governed by physical, chemical and biological processes. These processes also depend on the oil properties, hydrodynamics, meteorological and environmental conditions.

Some of the physical processes (Figure 1.3), include diffusion, advection, spreading, and chemical processes include extraction, excavation, and *in situ* chemical degradation, while major biological process include phytoremediation and bioremediation [64,70]. There are several ways with several advantages and disadvantages of their own. Hence, to comply with the environmental regulations and to abide by the recommendations, formulation the current technologies in combination to remove such contaminations are necessary. Since, all these factors are interrelated, the effects of crude oil spill in any form are detrimental to the economy and environment, and thus, proper clean up methods are needed to be established. It has also been reviewed how remedial techniques could be developed by identifying the current treatment methodologies available for such treatments, a source characteristic and parameter relating to the best way of their disposal and to develop the future plans in order to cope up with such problems [71–73].

In biodegradation, complex oil components are metabolized by bacteria and other microorganisms thereby reducing the toxicity by forming non-toxic inert components such as fatty acids (FAs) and carbon dioxide (CO₂).

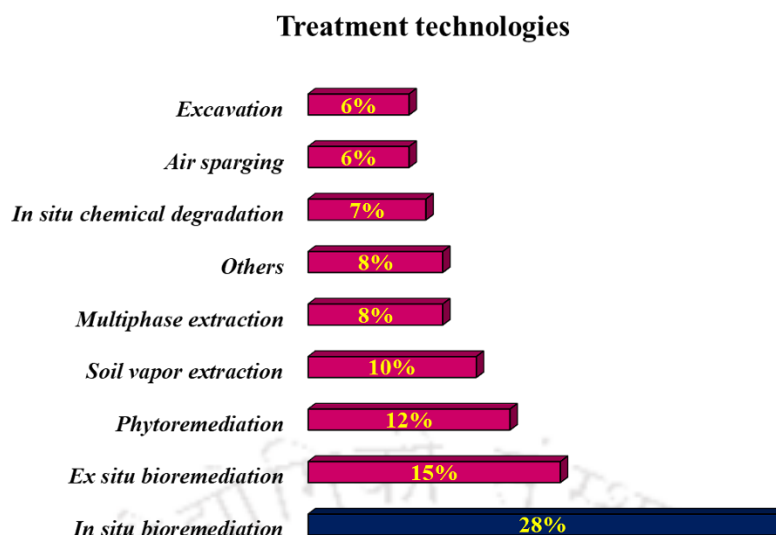


Figure 1.3: Overview of treatment technologies of oily sludge. Various clean-up strategies have been reviewed and compared. *In situ* bioremediation has been reported the most effective due to the application of viable microorganisms and its cost-effectiveness.

Microbes, specifically bacteria, are considered the most potent towards hydrocarbon degradation due to their versatility and adaptability into stress environments. Bacteria are well equipped with the necessary inbuilt mechanisms in the form of enzymes which generate a cascade of reactions to sequentially degrade hydrocarbon contaminants. Whereas, some metabolic intermediates might be toxic, these are further transformed by microbial enzymes to a form which becomes non-toxic to environment [74–76]. The extent of spillage and composition of the spilled oil are some of the governing factors affecting the rate of biodegradation and are important criteria to implement a treatment technique. Whereas the natural process of biodegradation can be accelerated by adding nutrients like N and P, these nutrients, henceforth, stimulate growth of the microorganisms concerned [71,77,78]. Reports suggest that virtually all types of HCs are good habitat for microbes. Microorganisms are seen to easily use HC, particularly alkane as their sole nutrient source and hence the bioremediation solution of such hazardous waste is well justified. Bioremediation is an eco-friendly and cost-effective treatment that uses microbes to degrade HCs. Microorganisms capable of breaking down different components of crude oil aliphatic or aromatic HCs are easily enriched and isolated from various pollutant sites such as a typical petroleum refinery contaminated site.

In this chapter we highlight a brief overview on the refining process and subsequent waste generation, their possible avenues of clean-up, the implications of application of microbial consortia as a mode of biological treatment, presence of enzymes for hydrocarbon breakdown and the fate of biomass for further value addition and application.

1.2. Literature review

1.2.1. Overview of oil refining process and waste streams

The refining process is a complex operation that converts crude oil into various useful products. It consists of series of unit operations and processes, with different units operated simultaneously to generate different products. For example, cracking, coking, and viscosity reduction unit help in minimizing residual oil; gasoline is obtained through isomerization, alkylation and reforming unit; and fluid catalytic cracking (FCC) contributing to maximizing fuel production [27,38]. Refineries can generate huge volume of waste containing significant proportion of HCs. These contents are predominantly generated from rejected streams from boiler processes and cooling tower. Wastes are also contributed from process wastewaters streams originating from desalting, sour water stripping, tank bottom, and spent caustic operations. Important products obtained from the refining processes of crude oil include jet fuel, diesel oil, petrol, gasoline or LPG [35,37]. Typical processes and their means of generation of several types of waste streams are discussed below:

1. Waste stream from cooling tower: This is one of the four types of refinery units contributing towards waste streams. It is used in removing impurities of larger concentration of organic matter, total dissolved solids (TDS), TSS, hardness etc. Water availed for blowdown operation for cooling systems also result in fouling, scaling and corrosion, and regulatory steps result in discharging excess water into the waste streams.
2. Waste streams generating from process water streams: This kind of waste stream is generated from the desalting, stripping, tank bottom discard or caustic soda operations. These streams contain high fractions of petroleum HCs require extensive treatment. Such streams are directly in contact with the generated HC mixture and is considered as one of the most heavily contaminated waste streams in the whole refining process that requires serious treatment. Since direct contact with petroleum fractions occur, the volume of wastes generated from this unit are considerably higher than the other units.
3. Wastes in the form of storm water: This is another type of refinery waste. Surface water runoff is also generated.
4. Sanitary sewage waste: This is another type of waste generated due to sewage activities. Daily sewage loads originating from various sources are also treated separately disposing into the discharge unit.

5. Other waste sources: These are originated from laboratory works, wastes generated from experimental consumables and discards. Any other kinds of operational defects and faults may also contribute to generation of additional streams that might be due to other external factors out of control of human intervention. In addition to the above, a refining operation may also account for different streams of HCs. Mixing of several additives such as detergents, antioxidants, metal deactivators, octane enhancers, rust-inhibitors, anti-knock agents etc. into petroleum streams are also generated as waste and require vigorous involvement in toxicity reduction.

The treatment of these waste streams involves several stages, including primary oil-water separation, secondary oil-water separation, and biological waste treatment [79–81].

Refinery waste treatment plants generate a by-product known as oily sludge during the treatment process. In India, more than 28000 tons of oily sludge is generated every year. Typically, oily sludge exists as oil-water emulsion, mostly composed of water. Other fractions include asphaltenes, light fraction of HCs, wax etc. Since, the viscosity is high, this kind of sludge is difficult to be dewatered [82,83]. Oily sludge wastes from various sources, primarily American Petroleum Institute (API) separator and effluent treatment plant settles into the heavier and lighter fractions during shipping and storage. The heavier fraction, mainly the tank bottom sludge found at the bottom is a result of such separation. Other sources of oily sludge include boiler sludge, desalter sludge etc. Oily sludges be of two types: Paraffin-based oily sludge that is formed as a result of flocculation of heavier straight chain of HCs. This type of sludge accumulates at the bottom of a vessel and gets stratified once the volatile fractions are emitted thus decreasing the fluidity and increasing the viscosity and density. On the other hand, asphaltic sludge is formed due to precipitation of asphaltenes, resins and polymeric compounds. These are colloidal in nature and based on their physical properties, they can occur as high molecular weight aromatic HCs insoluble in alkalis and acid (neutral resins) or insoluble in gasoline or petroleum ether (asphaltenes) [84–86]. Oily sludge is also formed from precipitation of inorganic salts, sediments, or introduction of a non-compatible batch of crude.

The composition of oily sludge is highly variable and is dependent on the in-plant treatment conditions and external environmental factors. The list of sludge composition reviewed by various investigators at different geographical locations is provided below in Table 1.1.

Table 1.1: Comparison of composition of waste sludge obtained from different refinery sites.

Location	Reported composition	Reference
Haifa Oil Refinery's open storage impoundments	An average of 15% HCs, 30% soil, and 55% water (w/w).	[87]
Part of the Barauni refinery situated in the state of Bihar, India	Water 11%, 15% solvent-extractable total petroleum hydrocarbons (TPH). Aromatics, at 46%, alkane fraction (37%), asphaltene fraction (10%), and NSO (7%).	[88]
Mathura Refinery south of Delhi	Water 6%, Organic matter 41%, TPH 18%, alkane fraction 52%.	[89]
Flocculation-flotation unit of a wastewater treatment plant (WWTP) at the Shell refinery in Gothenburg, Sweden	Dry matter 10.2; TPH 145 g/Kg, total organic carbon (TOC) 24.87%, Total N 4.05%, Total P 1.87 g/Kg.	[90]
Settling tank of the second oil plant in Daqing of China	Moisture content, is approximately 14.81% high oil content, approximately 40.78% high sand content, approximately 44.41%.	[91]
Arabian Gulf sludge from an environmental service company in the United Arab Emirates	Water 3.2%, C residues 10.1%; Organic materials 95%, asphaltenes, 10.1%.	[92]
Sludge storage tank of an oil reservoir facility of the Bharat Petroleum Corporation Limited, located at Rajbandh, Durgapur, India	Total N 598 mg/L; Total P 41 mg/L; TOC 3.6%; TPH 54%.	[93]
Crude oil tank bottom sludge (COTBS) generated from Azzawiya oil refinery in Libya	42.08% ($\pm 1.1\%$) oil composed of light HCs ($30.7 \pm 0.07\%$) and heavy HC ($69.3 \pm 0.4\%$) fractions. Water and solid contents were 2.9% ($\pm 0.2\%$) and 55.02% ($\pm 0.6\%$) respectively.	[94]

Rolling steel process of an iron and steel company in Wuhan city, China	Moisture 32.4%, Oil content 48.6%, Fixed C 9.35%, Solid residue 19.3%.	[95]
Settlement tanks of the Second Oil Plant in Daqing of China	HCs 60.36%. The remaining is 29.26% moisture and just 10.38% solid.	[96]
Crude oil storage tank in Niger delta area of Nigeria	51.8 to 57.1% of oil, 9.3 to 23.4% solid and water content of between 15 and 21%.	[97]
Pure beam oil production plant of Sheng Li Oilfield	The oil and water content 40.71 and 10.70%, respectively. the initial COD and TPH content were 597,164.3 mg/g and 40.71%, respectively.	[98]
Ankleshwar oil fields of Oil and Natural Gas Commission (ONGC), Gujarat, India	5% moisture. The saturates and aromatics were 77.90% and 16.50%, Organic C 3.61%, Total N 0.36%, Total P 362 mg/Kg respectively.	[99]
API separator placed in a petroleum refinery wastewater treatment plant, located in Spain	TPH 26%, COD 196 g/Kg, Total Solids 213 g/Kg, Total N 2.8 g/Kg, TOC 0.2 g/L, Phosphate 1 mg/L.	[100]
Sludge waste lagoon of the Guwahati refinery	Oil content 1.5%, TPH 15.37%.	[101]
Bharat Petroleum Corporation Ltd., furnace oil tank bottom sludge in Chennai, India	Solid content 90%, Total C 60%, Total N 4.5% Moist content 114.4 units.	[102]
Karamay Oilfield, Xinjiang Uyghur Autonomous Region, China	Water 3.5%, solid: 74.36%, Total C, 76.74%.	[103]
Numaligarh Refinery Limited (NRL), Golaghat district of Assam, India	Moisture content (39.59%), dissolved oxygen (DO) (5.5 mg/L), TDS 494 mg/L, Total C 53.5%, Total N 19.89%.	[104]

1.2.2. Waste generation from refineries

Refinery wastes are typically collected in a segregated form based on their type and constituents. These are generally solid wastes and are obtained as sludges containing solids, water, or oil. The storage as well as disposal method depends on the type and composition. Dewatering of sludge is usually carried out in sludge thickeners or ponds through gravity settling. Sludges originating as spent contact clays and percolation clays from lubricating oil treatment, are dewatered and de-oiled by centrifugation and settling. In addition to this, there are also advanced techniques used for effluent treatment include adsorption, coagulation, chemical oxidation, biological techniques as well as contemporary technologies like membranes and microwave-assisted catalytic wet air oxidation [105–107]. Hence, the collection and treatment of refinery wastes are done in a systematic and segregated manner to ensure effective waste management and minimize environmental impact.

The generation of waste from a typical refinery is represented in Figure 1.4.

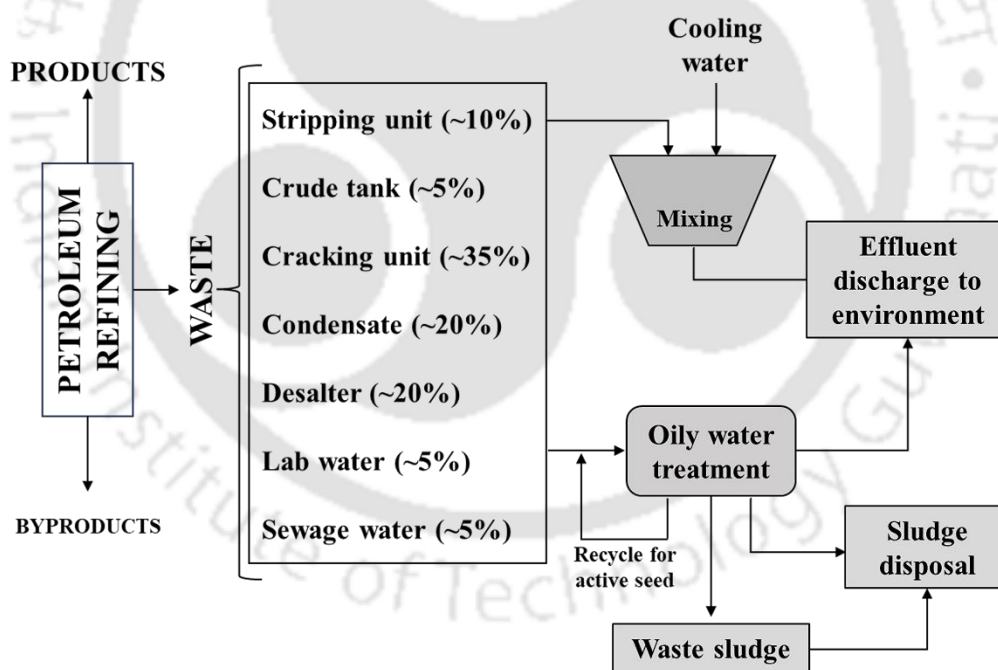


Figure 1.4: Typical generation of oily sludge waste. Alongwith the important products and byproducts, refineries generate waste from various units. Wastes in the form of effluent and sludge are treated and disposed as per regulatory guidelines.

In Indian refinery industries, the management of refinery sludge waste has to be a priority as the mixture is HC-rich and contains various types of pollutants like phenols, heavy metals, etc., and is treated as hazardous waste [53,108,109].

Some of the methods being used are:

1. Liquid waste management: Refineries contain underground network for segregated collection of various waste streams, with each stream subjected to extensive treatment in the designated treatment facilities. The treatment processes involve physical, chemical, and biological operations prior to their regulated disposal.
2. De-oiling the Sludge: Melting pits with skimming pumps, sophisticated hydro-cyclones, centrifuges etc. are employed for de-oiling the sludge.
3. Residual Sludge Treatment: Recycling of residual sludge after de-oiling is considered environmentally safe and are used for reclamation of highly contaminated sites.
4. Solid waste Management: Generation of enormous amount of tank bottom oily sludge pose major challenge in terms of hazardous waste treatment and disposal as well as environment management.

These methods help in minimizing adverse environmental impact from refinery activities and conserving scarce natural resources by continually optimizing their consumption. In Indian scenario, several treatment plants work within the prescribed regulations laid by the pollution control board fulfil discharge and disposal regulations.

The management and disposal of such hazardous waste remain a challenging problem worldwide. Over the years, various physical and chemical methods have been developed for the treatment of oil-impacted sludge. In addition to these methods, bioremediation strategies are also being explored. For example, a study analysed three petroleum refinery sludge samples from Northeastern India using next-generation sequencing technology to explore the diversity and functional potential of inhabitant microorganisms and scope for their on-site bioremediation [101,104,110].

Legally, waste management in India is taken care of as per the governance discussed in National Environment Policy, 2006. Since, refinery sludge is termed as hazardous waste, they are taken care of as per “The Hazardous Wastes (Management, Handling and Transboundary Movement) Rules”, 2008. Whereas Schedule 1 mentions petroleum and petrochemicals within the class of hazardous waste, Schedule 5 laid down regulations related to recycling, transportation, accident prevention and awareness [13,53,109]. Although, the Ministry of Environment, Forest and Climate Change, Government of India in conjunction with State

Pollution Control Boards of different states administers the waste management regulations, there is no strict conditions mentioned for all stakeholders, and this has resulted absence of compliance monitoring. Moreover, there does not exist exact audit statistics to provide data for predictive analysis of hazardous waste management, although guidelines are laid only at the industry level varying from state to state. Lack of professional reports of risk assessments also contribute to generation of confusion among the investors. Lastly, all the data related to different industries, waste generation and disposal norms are yet to be properly documented on digital platforms [111–113]. However, devising exact strategies for managing refinery waste can vary depending on several factors, including the specific characteristics of the waste, the microbial community present, and the environmental conditions and monitoring these factors during waste treatment are necessary to ensure effective bioremediation [110,114].

Treatment technologies studied by the researchers for the biological treatment of petroleum refinery sludge include lagoon, landfilling, incineration, and composting. After pre-treatment of oily sludge, the second most appropriate technique is reported to be land treatment. Oily sludge could be remediated by three-tiered oily sludge waste management strategy where technologies are used to reduce the quantity of oily sludge generation from operation in petroleum industry, recovery, and reuse of valuable fuels extracted from oily sludge [115–117].

1.2.3. Overview of physico-chemical techniques of waste clean-up

Physico-chemical techniques are often used in the treatment of refinery waste sludge. There are many ways that oil spillage can be cleaned.

Some of the methods are described:

1. Solvent extraction: This method uses a solvent to separate or extract different components based on their solubility [82]. In this process, the sludge is mixed with organic solvents in a suitable ratio. Distillation and Centrifugation are used as an aid of separation method, as water and impurities in oily sludge are immiscible. The most commonly used extraction solvents include *n*-hexane, dichloromethane (DCM), acetone, propane trimethylamine, trichloromethane, methyl ethyl ketone (MEK), etc. [46,92].
2. Froth flotation: This is a process for selectively separating hydrophobic materials from hydrophilic ones. This process is achieved by forcing the miscibility of oil-water emulsions.

External foaming agents such as detergents are used to attain a good quality froth, that are skimmed off after the desired separation is achieved [82].

3. Ultrasonic and microwave irradiation: These methods use sound waves and electromagnetic waves, respectively, to break down the sludge. The ultrasound produced strips off the oil particles from the solid surface. Mechanical agitation is the underlying principle of the process that increases the collision among the oil droplets and decreases the interfacial forces within liquid molecules to achieve the separation [117].

Microwave generates radiation with frequencies between infrared and radio frequencies that bring out di-electric heating among the molecules through electromagnetic penetration [82].

4. Pyrolysis: This is a thermal decomposition process that occurs at elevated temperatures without the participation of oxygen. Sludge composition, temperature, reaction time are some important factors that define the efficiency of the process. The advantages of this method lie in the potential of high resource recovery, production of useful byproducts such as bio-oil, syngas, and biochar, all of which can be treated as an alternative source of various applications. Moreover, due to application at anoxic conditions, probability of generation of any toxic compound is very less, that makes this process more on demand [54,95,102].

5. Electrokinetic (EK) method: This method uses an electric field to move the charged particles in the sludge. In this technique, electrodes in soil are applied to generate current of low intensity to aid in pollutant removal. The three main mechanisms involved in EK method are: (1) Electrophoresis, that helps in migration of colloidal aggregates towards the anode; (2) Electro-osmosis, that helps in the movement of oil and water towards the cathode, and (3) Electro-coagulation through which the solid phase is deposited near the anode as aggregates [118,119].

6. Centrifugation: This technique uses centrifugal force to separate different components of the sludge. Rotation at high velocity is used to separate out impurities of variable densities. Optimum speed and feed dosage are some of the important parameters governing effective pretreatment of oily sludge using centrifugation [117,119].

7. Surfactant: In this process the separation is based on the principle of change in surface tension through vigorous agitation. Water-oil emulsions are generally taken care of using this method. Viscosity and surface tension measurement are the major controllables that describes the efficiency of this method [119–121].

8. Freeze/thaw: This method involves freezing the sludge and then thawing it, that helps to break down the structure of the sludge. The recovery and separation are based on the temperature, rate of freezing, impurities concentration etc. At different temperatures, the oil water blend can be destabilized and hence, the separation process is affected [122].
9. Incineration is a process of complete combustion of oil wastes in the presence of excess air and auxiliary fuels. Generally, Rotary Kiln and Fluidized bed incinerators are used. The residence time is in the order of days in fluidized bed incinerators and hours in case of rotary kiln. The temperature is in the range of 900-1200 °C in rotary whereas the temperature is lower at 700-750 °C in fluidized bed incinerators [116].
10. In situ burning is also applied at some places. *In situ* burning is the controlled burning of oil in a place. The method of burning can be a very effective method of cleaning oil pollution that occurs in open water bodies, such as an oil spill in the ocean. It is also very effective on land areas with limited access. However, this method is often limited by wave and wind conditions and by the proximity of the spill to populated areas [123].
11. Solidification and stabilization are process of immobilizing the contaminants by converting into a less toxic insoluble component. It is cheap and are widely accepted. The insoluble and less toxic component is captured within a durable matrix with high structural integrity. This method has been reported mostly for the disposal of inorganic wastes [118].
12. Oxidative treatment has also been employed in the removal of a number of organic wastes through chemical treatment or enhanced oxidative treatment. Chemical oxidation involves usage or application chemicals over oil wastes, thereby converting into CO₂ and H₂O or to a component or compound that is less toxic and hazardous to the environment. The oxidation can be brought by Fenton's reagent, hypochlorite, permanganate, persulfate, and by generating enough free radicals such hydroxyl radicals (OH•) which can react very fast with oil and organic wastes [116].
13. Mechanical cleaning is the process in which the oil is captured and stored until it can be disposed of properly. There are various types of oil booms that can be used either to surround or isolate a slick, or to block the passage of a slick to vulnerable areas such as fish-farm or other environmentally sensitive areas. Reports suggest application of variable but buoyant material boom types during the process [84,116].

14. Clays contain large quantities of Ca^{2+} , Mg^{2+} , K^{+} , Na^{+} , NH_4^{+} : Clay can attract these cations to retain their lamellae structure and avoid leaching of water through them. Chemical dispersants help in reducing the surface tension preventing oil-water mixing. Formed small droplets aids in further rapid dilution of the oil by increasing the surface area resulting in increased exposure to natural attenuation and soil microbial activity thereafter [124].

15. Sludge gasification technique is also similar to pyrolysis except gasification occurs at higher temperature ($>700\text{ }^{\circ}\text{C}$) and presence of oxygen. The advantages are like that of pyrolysis. In this technique the combustible primary product is syngas is obtained and is further used as heating fuel and electricity generation. Operating cost and inability for application in moist sludge are the disadvantages of this method [125].

These methods significantly contribute to devising proper treatment techniques of the waste and also aid to the recovery of polluting materials. However, challenges of lower treatment efficiency, and enhanced logistical constraints are required to be addressed. Moreover, to combat the complexity of refinery waste, development of more environmentally friendly process to reduce generation of secondary pollutants and sludge formation have become a necessity. The current state-of-art improves the scope for application of coupled systems for long-term stability and sustainability [126].

1.2.4. Biological treatment and challenges

Biological waste treatment is a process that uses bacteria, protozoa, and other microorganisms to clean up waste (Figure 1.5). This process is centuries old and is still being investigated and experimented with all over the globe. It is a complex process at the intersection of biology and biochemistry [100,116].



Figure 1.5: Illustration of microbial degradation of oily sludge. Microbes capable of utilizing the components of oily sludge metabolize HCs as their source of nutrient and survival.

The biological treatment processes include:

1. Aerobic treatment: This refers to a process in which oxygen is present. Aerobic waste treatment involves septic tanks, oxidation ditches, activated sludge, trickling filters, ponds and lagoons, and aerobic digestion. Oxygen supplied through aeration helps in decomposition of organic matter in wastes by bacteria and other organisms.
2. Anaerobic treatment: In this biological process, treatment is carried out in absence of oxygen. Anaerobic processes are typically used for the treatment of waste with high concentrations of organic matter.
3. Bioremediation: This includes both aerobic and anaerobic treatments.
4. Phytoremediation: This involves using plants to remove contaminants from waste.
5. Mycoremediation: This involves using fungi to remove contaminants from waste.

Biological treatments are based on application of viable bacteria, or other microorganisms to catabolize organic wastes using regular metabolic activities. Refinery waste typically contains a high content of organic matter in the form partially degraded food, or garbage wastes, in addition to heavy metals toxins and other heavy metals. Biological treatment techniques are implemented to attain proper disposal and set up a uniform and streamlined strategy for collection of decomposed products after treatment [100,116,127].

As mentioned earlier, during biodegradation process, components of the oil are broken down by bacteria sequentially through a series of enzymatic reactions into simpler non-toxic substances including FAs and CO₂. The natural process of biodegradation can be intensified by adding nutrients like N and P. These nutrients stimulate growth of the microorganisms concerned [75,76,78,99]. It could be argued that all classes of HCs are not quite potential C-sources and are governed by the composition of oily sludge. Although, various reviews and reports suggest most of the petroleum components are degraded by indigenous microorganisms under natural circumstances, nearly all classes of HCs are considered C-source which could also be accepted by the existing population thereby breaking to simpler molecules. Alkanes are most easily degraded, whereas asphaltenes are found to be lesser prone to microbial utilization and hence the research of developing application strategies by manoeuvring biotechnological processes of utilizing the versatility of microbes for bioremediation of the oil contamination is justified [99,116]. Some of the most efficient technologies studied for bioremediation of oily sludge are landfarming, bio-slurry treatment and biopile/composting [100,106,116,119,128]. However, the major challenge lies in getting a good or better result in the laboratory. Strategies

of cost-effective bioremediation process include types of natural attenuation, biostimulation, bioventing, bioaugmentation, landfarming, composting and phytoremediation. Some of these are techniques of biological treatment are listed:

1. Land treatment involves the incorporation of wastes into soil and then the use of various processes to degrade contaminants in that soil. Biological activity usually accounts for most of the degradation of organic pollutants, while physical and chemical removal mechanisms such as evaporation and photo-degradation may also be important for some compounds. Landfarming is a widely employed land treatment approach, and it spreads the well-mixed oily sludge and fresh soil on the ground surface of a treatment site. The treatment efficiency can be improved by maintaining appropriate sludge application rate, aeration, fertilization, moisture content, and pH to maintain microbial density and enhance their activity in the sludge/soil mixture [87,128].
2. Biopile/composting of petroleum wastes has received increased attention as a substitute technology for landfarming that often requires a large land area. Biopile refers to the turning of waste materials into piles or windrows usually to a height of 2-4 m for degradation by indigenous or extraneous micro-organisms. The piles are static in design with equipped with aeration piping or regulated by special devices. The bio-treatment efficiency can be improved with moisture adjustment, air blowing, and the addition of bulking agent and nutrients. Bulking agents usually include straw, saw dust, bark and wood chips, or some other organic materials. Addition of bulking agents results in increased porosity in soil-sludge piles, that leads to better air and moisture distribution in the matrix. This technology is termed as composting if organic material is added [90,114].
3. Slurry-phase (SLP) bio-treatment has been successfully used to clean up oil-contaminated soils and has been shown to have a faster pollutant removal rate than solid-phase treatment methods (such as landfarming and composting). This technology mixes sludge-associated solids with water (i.e. 5-50% w/v) and dissolves contaminants into aqueous phase to obtain a larger number of solubilized pollutants. The pollutants are thereby transformed to lesser toxic intermediates (e.g. organic acids and aldehydes) or end products of CO₂ and water by the action of microbial degradation. The objective of this operation is maximizing the contact between microorganisms, HCs, nutrients, and oxygen [104,128].

Several cases of efficacy of preferred biological treatment have been highlighted with respect to TPH and COD removal. For example, degradation potential of immobilized

hydrolysed polyacrylamide (HPAM) degrading bacteria S1, S2 isolated from a petroleum contaminated soil was assessed. The TPH was reported to have decreased 98.7% and at the same time HPAM was also utilized to 79.6% [129]. In another instance, a study of mixed culture with used engine oil and diesel oil were checked. The study showed the bacterial consortium A2457 comprising strains *Stenotrophomonas maltophilia*, *Bacillus cereus* and *Bacillus pumilus* could yield maximum degradation to 99.77% of used engine oil and 94.13% of diesel oil alongwith significant lipase activity during 28 days of incubation [130]. For another mixed system of *Bacillus cereus*, *Bacillus thuringiensis*, *Geomyces pannorum*, and *Geomyces sp.* isolated from soil collected from oil fields of the Ecuadorian Amazon rainforest, TPHs after 30 days incubation was significantly found higher in slurry phase (SLP) than in solid phase (SOP) [131]. Tao et al., 2017 checked the combination of indigenous bacterial consortia with *Bacillus subtilis* in the ratio 2:1 to obtain a TPH degradation of 85% [132]. Liu et al., 2016 also isolated a HC-degrading bacterium Y-1 from petroleum contaminated soil in the Dagang Oilfield and investigated for the potential effect in biodegradation of crude oil. *Bacillus licheniformis*, as was identified in the study accounted for degradation of 60.2% of TPH within 5 days of incubation [133]. In another study, a separate crude oil degrading marine *Acinetobacter sp.* strain HC8-3S had affected 94% of degradation of saturated HCs within 5 days of incubation [134]. Various other investigators have identified indigenous microbes to track the biodegradation of TPH from contaminated soil or oily sludge [135–138]. As highlighted, oily sludge is a mixture of toxic recalcitrant components and is considered hazardous due to predominance of petroleum hydrocarbons. Several investigators have emphasized that attempts to establish optimized biological treatment technique is crucial to enhance bioavailability of nutrients for microbial decomposition in order to attain maximum biodegradability reclamation of the contaminated site [139–141].

In terms of combination of treatments, there are several methods that can be used. For instance, a study showed that a combination of heat application (80 °C), biosurfactant (thermophilic emulsifier), and bioaugmentation (using a consortium containing *Bacillus lentus* and *Pleurotus tuberregium* as members) enhanced the biodegradation of asphaltene. Another study showed that a combination of bioaugmentation, biostimulation, bioreactors, and biosurfactant treatment should be considered for biological treatment of oil sludge before disposal into the environment. Several nutrient stimulated studies have been carried out and significant degradation patterns were noticed [142–145]. In another case, a biosurfactant producing *Bacillus subtilis* was employed to obtain TPH degradation of 60% within 10 days

[146]. A study to check *n*-alkane degradation by application of both biosurfactant and nutrient was also reported earlier by Rahman et al., 2003. Recently, another nitrate stimulating microbial consortium to treat oily sludge was reported by Sarkar et al., 2020 [142,147].

Whereas, combination of treatment techniques is helpful, information on scaling up is also important. Whereas various reviews have been published related to biological treatment of petroleum sludge and their recent advances, limitations related to handling high volume wastes remains a critical point of research [148–150]. However, studies at reactor level have been conducted and the results seem promising [151,152]. For example, an indigenous bacterium, *Stenotrophomonas multophila* isolated from the contaminated sites of Jordan Petroleum Refinery was used to check for bioremediation in a lab scale aeration reactor. The overall kinetic constant k' based on food-to-microorganisms ratio was found to be 0.02 d^{-1} [153].

In some past studies to degrade HCs, a laboratory scale rotating biological contactor with biodrum system that was constructed to treat a mixture of *n*-alkanes using *P. zopfii* in a batch operation [154]. Jou and Huang, 2003 designed a fixed-film bioreactor system with highly porous polyurethane foam to incubate microorganisms at concentrations up to 8000 mg/L. COD removal of 85-90% was observed within 8 hours of hydraulic retention time (HRT) [155].

Whereas, these studies mainly constituted liquid mixture, however, the same may be found suitable in case oily sludge suspension in water are allowed to be treated. A submerged membrane bioreactor (MBR) technique was used and COD removal of >80% was observed at different cases [156,157]. Xu et al., 2017 investigated Fenton pre-oxidation and a subsequent bioremediation phase of 80 days to obtained upto 58% TPH degradation [158]. Similarly, several other studies where significant COD, TPH removal and nutrient removal were observed are also reported for reference [159–163].

Apart from studying oily waste, several reports about biological treatment of refinery components, specifically, *n*-alkanes and PAHs also available. This helps devising suitable removal techniques in addition to understanding their uptake and metabolism inside a microbial cell [164–166].

The oily sludge biodegradation can be affected by a variety of factors, such as the type of microorganisms, treatment duration, temperature, nutrients, concentration and

characteristics of oily sludge and efficiency of bioremediation process highly rely on these parameters. Moreover, soil characteristics such as water content, presence of oxygen, nutrients, co-substrates, the redox potential, sunlight, organic matter, pH, Na^+ , Cl^- , CO_3^{2-} , and SO_4^{2-} content, porosity, bioavailability, bioaccessibility and toxicity are all known to affect the removal of HCs from soil [78,100,167] and several feasibility studies are required to be carried out before proceeding for field applications. Short term studies are also helpful in reducing the cost of operations and identifying the factor limiting bioremediation performance for a site under study.

As illustrated in Figure 1.6, microorganisms, mainly bacteria and fungi, are capable of degrading HCs, but there is no single microbial strain having the metabolic capacity to degrade all of the components found in oily sludge.

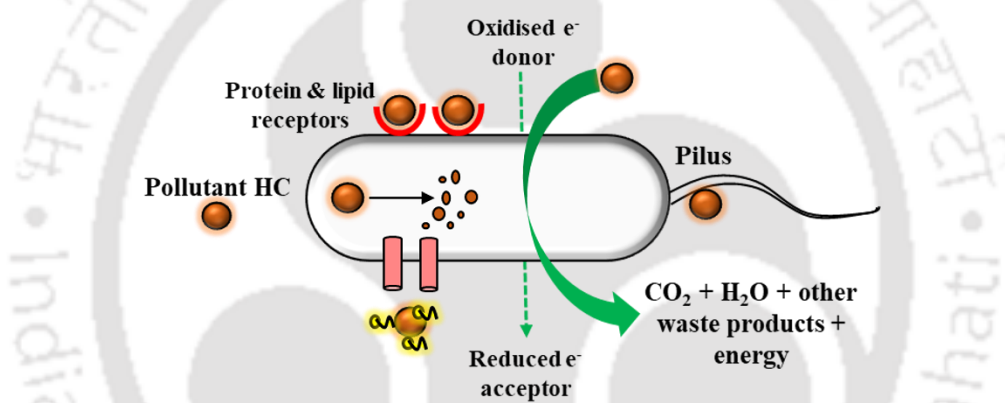


Figure 1.6: Microbial uptake of HCs. Microbes degrade HC components through series of catalytic reactions and convert them into other non-toxic forms, CO_2 , H_2O and energy.

The degradation of HCs in sludge may involve progressive or sequential reactions where a group of micro-organisms may initially degrade the petroleum constituents into intermediate compounds, and these intermediates are then utilized by a different group of microorganisms for further degradation. As a result, the biodegradation of oily sludge typically needs a microbial consortium with a succession of species. Whereas evidence shows efficient bacterial strains helping in remediation of oil contaminated sites and employment of a group bacterial culture was more advantageous due to enhanced synergy among different microbial populations [99,104,130,144,168], some past reports have suggested that the bioremediation performance of the combined bacteria sometimes were not so satisfactory. In some studies, other than micro-organisms, earthworms and plant assemblages were reported under microcosm study to determine their efficiency towards petroleum oil degradation [46,169,170].

Bioremediation is generally promoted by introducing an engineered enriched inoculum (bioaugmentation) or through addition of formulated external nutrients in the forms of N and P (bioaugmentation) [78,110,147]. In addition, the degradation processes usually restricted by their high hydrophobicity or low solubility and hence some investigators have also highlighted the importance surfactants are used to enhance their desorption and solubilization, thereby enhancing their bioavailability and facilitating their assimilation by microorganisms [119–121,142,145]. The C:N:P ratio is a critical factor in bioremediation of oily waste. It represents the ratio of C, N, and P in the waste. The optimal C:N:P ratio can vary depending on the specific microorganisms involved in the treatment process. When the removal efficiency is not 100% and the yield is not 0.41, it indicates that the conditions are not optimal for the microorganisms to fully metabolize the nutrients [171]. This could be due to an imbalance in the C:N:P ratio or other environmental factors. For instance, when the C:N ratio is too high, the lack of N can inhibit microorganism growth; when the C:N ratio is too low, the removal of N and the final N concentration of the effluent cannot meet the required amount. In one of the studies, it was found that photosynthetic bacteria (PSB) bioconversion was efficient when the wastewater C:N ratio was within the range of 400-0.1 [172].

Some instances where addition of N and P has been found beneficial are described below in Table 1.2.

Table 1.2: Different C:N:P for biodegradation of refinery waste.

N and P composition	Remarks	Reference
Optimized as 100:50:10	At 20% initial sludge with the optimum C:N:P and rhamnolipid addition of 400 mg/Kg, TPH reduction of 50.77% was observed after 40 day incubation.	[173]
C:N:P = 100:5:1 using NH_4Cl and KH_2PO_4	Sludge amended with compost in the ratio 1:2, 1:4, 1:6 and 1:8 showed TPH degradation of 67%, 73%, 75% and 80% respectively.	[174]
C:N:P = 100:10:1 using $(\text{NH}_4)_2\text{SO}_4$ and KH_2PO_4	Addition of microbial consortium and nutrients yielded 84.1% TPH reduction for 6% oily sludge contaminated soil after 90 days of monitoring	[175]
C:N:P = 100:5:1 using NH_4Cl , Amendment with compost in the ration 1:5 to 100	Degradation of 1:5 oily acidic sludge was observed as 71.6% whereas the same in 1:100 was 48.6% after 70 days of incubation.	[176]
C:N:P = 120:10:1	TPH and PAH degradation obtained as 47% and 59% after 80 days of incubation.	[177]
C:N:P = 100:10:1, Addition of 1.5g NH_4Cl , 0.1g KH_2PO_4	Degradation of petroleum HCs were stimulated and TPH reduction was observed upto 48.5% after 10 days of incubation.	[178]

Apart from NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$, Sodium nitrate (NaNO_3) is often used as a N source. During bioremediation, the N in NaNO_3 serves as a nutrient for the bacteria that are breaking down the HCs in the waste. During this process, the N in NaNO_3 is converted into various forms by the bacteria. For example, some bacteria can convert nitrate (NO_3^-) into nitrite (NO_2^-), and then into nitrogen gas (N_2), that is released into the atmosphere. This process is known as denitrification. Potassium dihydrogen phosphate (KH_2PO_4) is often used as a P source, and similarly, P in KH_2PO_4 also aids in developing metabolic machinery in bacterial cells. During this process, the P in KH_2PO_4 is converted into various forms by the bacteria. For example, some bacteria can convert phosphate (PO_4^{3-}) into polyphosphate or other organic forms of P [110,147,168]. However, the exact fate of NaNO_3 and KH_2PO_4 can depend on several factors, including the specific characteristics of the waste, the microbial community present, and the environmental conditions. Therefore, monitoring these factors during waste treatment are necessary to ensure effective bioremediation. Moreover, achieving optimal removal efficiency

and yield often requires careful optimization and adjustment of these ratios and other environmental conditions such as temperature and pH [116,128].

Before biological treatment is carried out, pre-treatment processes are often applied to the waste. These can include stages such as screening, grit removal, and oil and grease trap. Pre-treatment aims to reduce certain constituents in the waste that might interfere with the subsequent biological treatment process. Biological treatment is carried out in either aerobic or anaerobic systems and to decide upon the precise option between these systems depends on various factors, including the specific characteristics of the waste and the treatment objectives. Aerobic systems require oxygen and are often used for the degradation of easily biodegradable organic matter. Anaerobic systems do not require oxygen and are typically used for the treating waste with low to moderate concentrations of organic matter [127,179–181].

Experiments can be conducted in either batch or continuous processes. In a batch process, a fixed volume of waste is treated for a definite interval of time. In a continuous process, waste stream is continuously fed into the system and treated effluent is continuously discharged. Operational factors such as target sludge quality; the amount and composition of waste are some of the driving parameters to decide application of batch or continuous mode of treatment [144,180,182].

Biological treatment of refinery waste can face several challenges, particularly when dealing with specific chemical components of the waste. For example, improper biotreatment quality could occur due to insufficient presence of nutrients. Microbial growth is greatly affected due to minute presence of N and P which otherwise help in building necessary machineries to food intake and metabolism. Additionally, poor distribution of DO in sludge flocs or films can hinder the biological treatment process.

Some of the other challenges of biological treatment of oil refinery sludge based on different reports [168,181,183] alongwith other references are mentioned here:

1. Inability to remove sludge: Biological treatment methods often struggle with the removal of sludge [82,105].
2. Long and elaborate treatment: These methods can require a long time to fully treat the waste.

3. High land requirement: Biological treatment processes often require a large area for the treatment processes [118].
4. Foaming sludge tanks: This can lead to spillage due to deposition of excessive oil.
5. Improper solid-liquid separation: This can be due to over bulking of sludge [84,118].
6. Presence of excessive amines in waste: This can lead to excessive oxygen demand, process failure, and permit violation [184].
7. Regulatory requirements related to hazardous waste treatment and disposal: Bioremediation of oily sludge poses significant challenges to meet regulatory requirements. Additional physicochemical treatments are employed to achieve a desirable treatment quality [53,112,114].
8. Sludge blinding due to grease: Blinding effect occurs due excessive grease in the waste stream and causes difficulties in sludge pressing. It can also affect the aerobic biological waste treatment processes by reducing the oxygen transfer rates.
9. Persistence of certain compounds such as PAHs and aliphatic compounds: PAHs and long chain aliphatic HCs are highly persistent in the environment and can pose a big challenge to microbial degradation of petroleum oil sludge [145,166].
10. Poor treatment quality: This might arise due to inadequate presence of nutrients in terms of N and P. Whereas considerable nutrients may be found at some instances, a typical refinery wastes mainly consists of water, solids and HCs [80,185].

These challenges highlight the complexity of treating oil refinery sludge and underscore the need for ongoing research and development in this area. It is also important to address how recovered oil, or sludge could be used directly for applications, such as energy, water treatment and soil restoration, respectively, without having to disturb the natural ecosystem [126].

1.2.5. Consortia design - challenges and methodologies

Microbial consortia are defined as a group of two or more microbial species that exists in conjunction in a diversity of environmental conditions. Interests related to designing consortia has seen an upward trend by virtue of their appalling advantages and usage [186,187]. The status of consortia design is promising, with significant advancements in both laboratory and field applications. At the laboratory scale, consortia design has been successful. For

example, microbial consortia have been engineered to distribute complex tasks among multiple populations. This has led to substantial progress in programming microbial consortia for basic understanding and potential applications. Consortia are designed using a number of strategies, that involve studying mutual interactions among a population, or understanding introducing population control by inhibiting the growth of any one class of microorganisms in a group, by programming the segregation of different groups in a 3D space to reduce competition or by statistical approaches such as one-factor-at-a-time (OFAT) or randomized full factorial design of experiments (DoE) [147,186,188,189]. An illustration of a hypothetical consortia action in a typical refinery waste is illustrated in Figure 1.7.

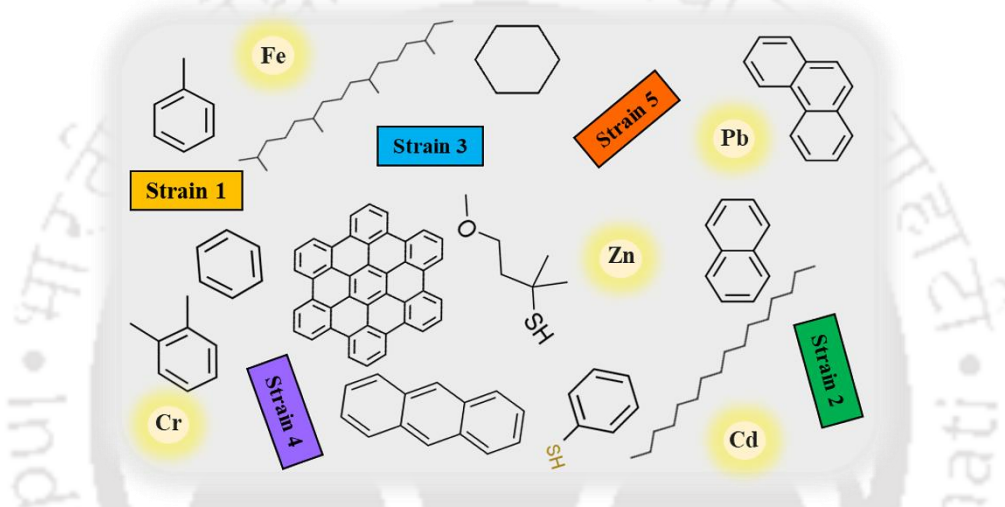


Figure 1.7: Microbial consortia in HC degradation. Different groups and classes of microbes specialized in metabolizing different classes of components in refinery waste are employed to achieve maximum bioremediation efficiency.

Even if the microbial consortia are applied for different application, challenges that coexist with these promising developments. Lack of insufficient insights related to the inherent molecular changes of microbial interactions greatly affect the performance analysis of a defined consortia further posing contradictions pertaining to the rational design and application. Currently, research of such fundamental knowledge bases would further help removing any technical obstacles, and noble and simpler avenues for synthetic microbial consortia designing might pave important breakthrough in redesigning biological and environmental engineering processes [186,190,191].

Bacterial consortia design techniques have evolved significantly over the years, with a focus on improving the efficiency and effectiveness of microbial communities for various applications. Here are some of the existing techniques and their advantages and disadvantages:

1. Programming bidirectional interactions: In this type, a bidirectional design is followed such that microbial consortia that benefit from the presence of each other. The consortia design is based on mutualism, competition and predation. Several synthetic experiments and mathematic modelling are required to be carried out and modelling such interaction is a key to the versatility of such interaction-based consortia design. Whereas the community obtained may be robust and stable, the community is susceptible for disintegration under minimal change in the surrounding ecosystem [190,191].

2. Programmed population control: This technique is also used to designing synthetic consortia based on synthetic circuit and mathematical modelling. In such type of design, individual population are inhibited of overgrowth within the consortium and involves three or four bacterial strains being subjected to modelling in complex culture conditions. The community may exhibit complex hierarchy of higher order relationships among themselves and pairwise interaction data can predict the interactions within a community of more than two strains with enhanced precision and accuracy. Apart from the listed complexity, the design helps in keeping microbial population in balance, which is crucial in understanding the interaction-based consortia design of such type [190].

3. Spatial segregation: In this type of consortia design, the genetic circuit system is not used and competition among species is reduced by applying spatial separation and is based on understanding symmetric and systematic interactions such as competition, mutualism and neutralism. The underlying principle of such set of design is that every complex habitat offers space to microbial strains where interactions are based on diffusion of molecules. Hence, the rate of interaction is modulated by visualizing strains as droplets and estimating the distances between them. This can readily enhance the stability of the community and help understanding the measures to reduce domination by one single species. The only disadvantage the technique poses are the complexity in design and analysing huge set of experimental results upto their minute details [186].

4. Bacterial consortia design by response surface methodology (RSM), is a statistical method used for obtaining an optimum inoculum/dosage of different species of microbes present in a culture by optimizing processes with several variables to obtain an optimized design. The feasibility and ease of use would depend on the specific application and the complexity of the microbial community being designed [74,192]. The use of statistical approaches like RSM and

central composite design (CCD) in microbial consortia design are generally accurate and statistically validated. Ease of use and efficient prediction are some of the advantages. This saves time of conducting a three-level factorial experiment in order to understand a second order quadratic polynomial. Moreover, the optimization involves analyzing multiple variables in a single experiment and a general prediction of an untested condition can be made [189,192–194]. Comprehensiveness and versatility add to the advantages. To conclude, an experiment with specific design can be prepared. In the context of microbial consortia, these statistical approaches can be used to optimize the conditions for microbial growth, interactions, and functionality. This can lead to more efficient and effective microbial consortia for various applications, such as biodegradation.

In terms of field applications, synthetic microbial consortia have shown promise in various engineering applications. There are several other examples of microbial consortia designed for HC degradation that are listed below in Table 1.3.

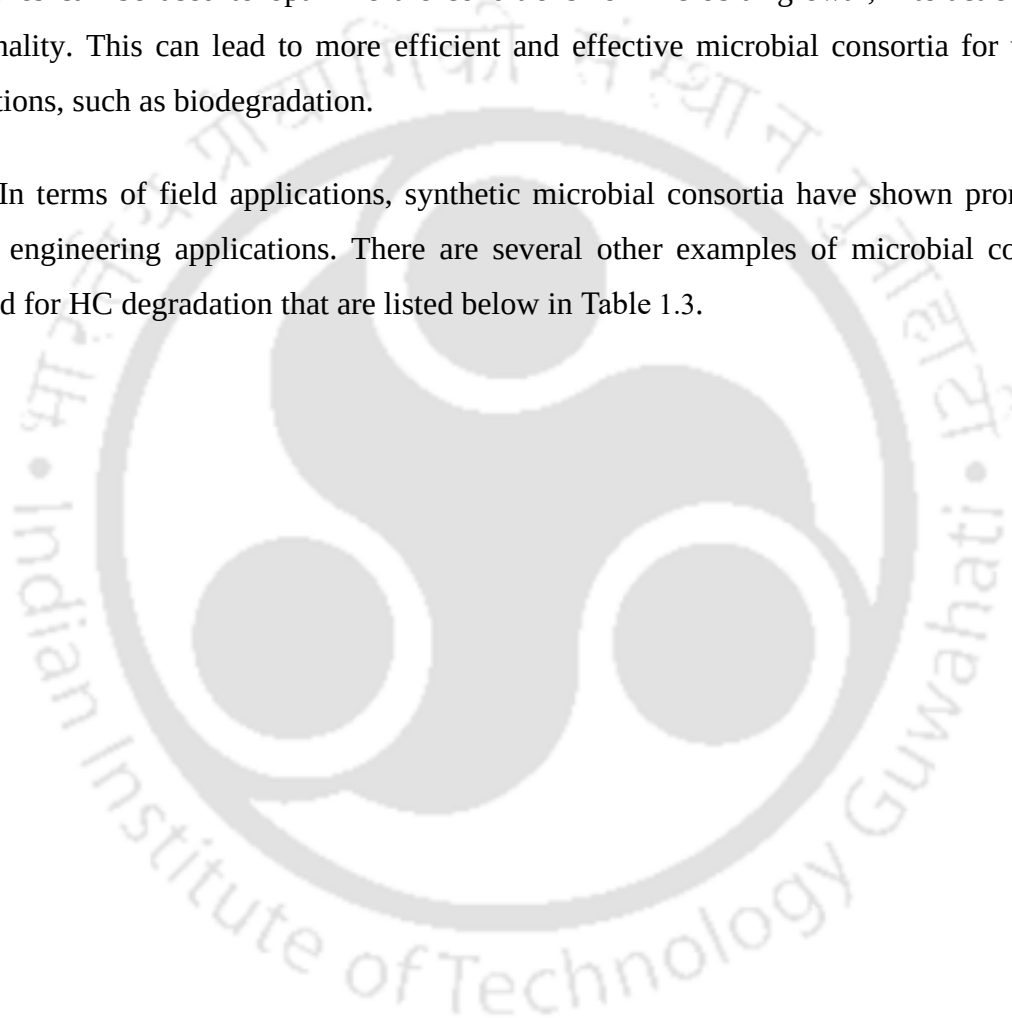


Table 1.3: Application of microbial consortia for HC degradation.

Consortia	Remarks	Reference
Set D consortia of isolated strains of <i>Pseudomonas</i> sp. and <i>Bacillus</i> sp.	100% soil was reclaimed within 30 days of incubation.	[195]
Consortium 10 comprising two <i>Bacillus</i> strains, namely, <i>Bacillus pumilus</i> KS2 and <i>B. cereus</i> R2	TPH degradation of 84.15% were observed after 5 days of incubation.	[196]
A nitrate stimulated consortia	combination of nitrate addition and bioaugmentation resulted in ~86% TPH degradation for a 30-day microcosm.	[147]
Immobilized bacterial consortia consisting of <i>Bacillus</i> sp. DL-13, <i>Brevibacillus</i> sp. DL-1 and <i>Acinetobacter</i> sp. DL-34 on zeolite	Almost 53% degradation of heavy oil was observed after 100 days of incubation.	[197]
Consortia AK6 containing <i>Klebsiella</i> , <i>Enterobacter</i> and <i>Salmonella</i>	99% of degradation of 5% (w/v) oily sludge within 9 days of incubation.	[183]
Combination of strain NYGH, strain T1, and strain T2	depicted higher values of biomass and degradation efficiency of TPH to about 91 % within 5 days in a system supplemented with TPH extracted from the tank cleaning oily sludge as the sole carbon source.	[198]

When two microbial strains, each capable of degrading a specific compound, are mixed for refinery waste treatment containing both these compounds, the outcome can be quite effective. Since each strain can specialize in degrading the specific compounds, such kind of cooperative degradation can lead to more efficient and complete breakdown of complex organic matter. However, the success of the approach can depend on several factors, including

the compatibility of the two strains, the specific conditions in the waste (such as pH and temperature), and the concentrations of the compounds to be degraded. The efficiency of bacterial consortia has also been reported by various other investigators [131,199–201]. These examples demonstrate the potential of microbial consortia in degrading HCs, that is crucial for bioremediation efforts.

While a single microbial strain could carry out sequential degradation of complex organics present in refinery wastes, an efficient approach to using a consortium of microbes yields better results. As different microbes are capable of degrading different compounds, a consortium can distribute the metabolic burden among multiple populations. In addition, a consortium can potentially be more robust and resilient to environmental changes. The major advantage that has been extensively discussed is the increased efficiency of a co-culture system where the growth in terms of biomass is higher and there are chances that genes that are silent otherwise, may get expressed because of stress induced by the external environment or competition among the diversity of strains introduced into the culture together [191]. Although, each strategy has its own advantages and challenges, and the choice of strategy should be based on the specific goals and requirements of the study.

Apart from the extensive advantages of the existing techniques, challenges related to optimizing complex responses within a single population could be laborious and inefficient. Moreover, the dynamics of a particular design under study may also be impacted due to implementation of multiple components. This would impose an unnecessary burden on the incubated culture conditions and drastically reduce overall productive response of the engineered design. However, understanding microbial interactions is still considered as an important factor to decide upon the application of consortia for a particular process with maximum insights into the benefits and drawbacks of the methods. Some of the desirable microbial interactions include mutualism or commensalism where at least one of the interacting species is benefitted and the other species is at least not harmed. Other interactions that are mostly seen among co-culture systems are parasitism, amensalism and neutralism and their prospects are greatly discussed [191,202,203]. With the advent of synthetic biology and tools might enable more efficient and robust design of microbial combinations with higher potency in terms of applications in environmental processes. Further exploration of omics tools would provide us with more knowledge base helpful for consortia development to carry out degradation of complex compounds in a refinery sludge waste and value-addition [190,191].

1.2.6. Overview of waste characteristics of typical oily sludge

Refinery waste characteristics differ significantly depending on the specific processes used at the refinery. In terms of how these characteristics relate to refinery wastes produced in Indian conditions, existing reports that the specific characteristics vary depending on the crude oil being processed and the specific processes used at the refinery (Figure 1.8). However, the general characteristics listed above would likely be applicable to Indian refineries as well.

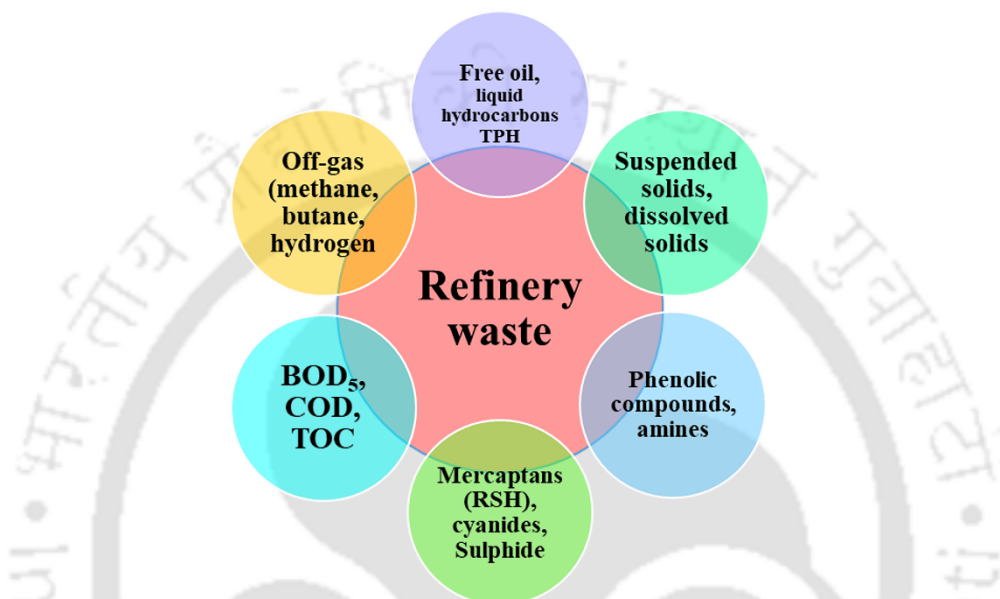


Figure 1.8: Typical characteristics of oily sludge. The composition of oily sludge varies greatly and is dependent on the location of its formation. Primarily, oily sludge is characterized by moisture content, high TSS, BOD, COD in addition to TPH, phenolic compounds etc.

Some general characteristics identified are listed below:

1. Oil impurities: Waste from petroleum refinery contains high amount of oil impurities such as non-oxidized paraffins, undissolved sediments etc. [107,204].
2. High levels of biochemical oxygen demand (BOD) and chemical oxygen demand (COD): Biochemical Oxygen Demand (BOD) and COD are typically high in refinery waste [84,204].
3. Total solids (TS): Refinery waste often contains high total solids [100,106].
4. Hydrocarbons (HCs): HCs are a common component of refinery waste [100,107,204].
5. Inorganic and organic constituents: Refinery waste contain a variety of inorganic and organic constituents. These can include derivatives of aromatic HCs (benzene, toluene, xylene, phenol), or PAHs and other toxic organic pollutants [204,205].

As for the distribution of complex organics present in the refinery wastes, this can also vary widely depending on the specific processes used at the refinery and the type of crude oil being processed. Complex organics in refinery waste can include different forms of HCs (alkanes, aromatic derivatives of benzene/toluene/xylene (BTX), and PAHs), phenols, and other organic compounds. However, detailed information about the specific distribution of these compounds would likely require laboratory analysis of the waste.

1.2.7. Are microbial consortia more efficient?

Rigorous studies confirm that microbes can naturally form consortia based on available food sources, there are several reasons why we might want to design microbial consortia:

1. Complex tasks: Engineering microbial consortia can help organizing complex tasks among different populations. This can be beneficial for tasks that are too complex or burdensome for a single population.
2. Reduced metabolic burden: By distributing tasks among multiple populations, the metabolic burden on individual cells can be reduced.
3. Reduced crosstalk: Engineering microbial consortia can help reduce crosstalk between cellular elements, that might pose difficulties while engineering complex circuits in cells.
4. Robustness: Microbial consortia can strengthen robustness to environmental fluctuations. They also exhibit stronger resistance and resilience for individual members to environmental changes.
5. Sophisticated metabolic capabilities: Microbial consortia exhibit flexible and highly characterized metabolic abilities and can perform complicated functions more efficiently than individual populations.
6. Engineering applications: Synthetic microbial consortia are optimized as per the necessity of different engineering applications, including metabolic engineering, environmental engineering, bioprocess applications, and biosensing.

Figure 1.9 represents the working of microbial consortia for various applications. While natural microbial consortia formation does occur, the design of microbial consortia allows us to harness these communities in a more controlled and effective way for specific applications [186,191,206].

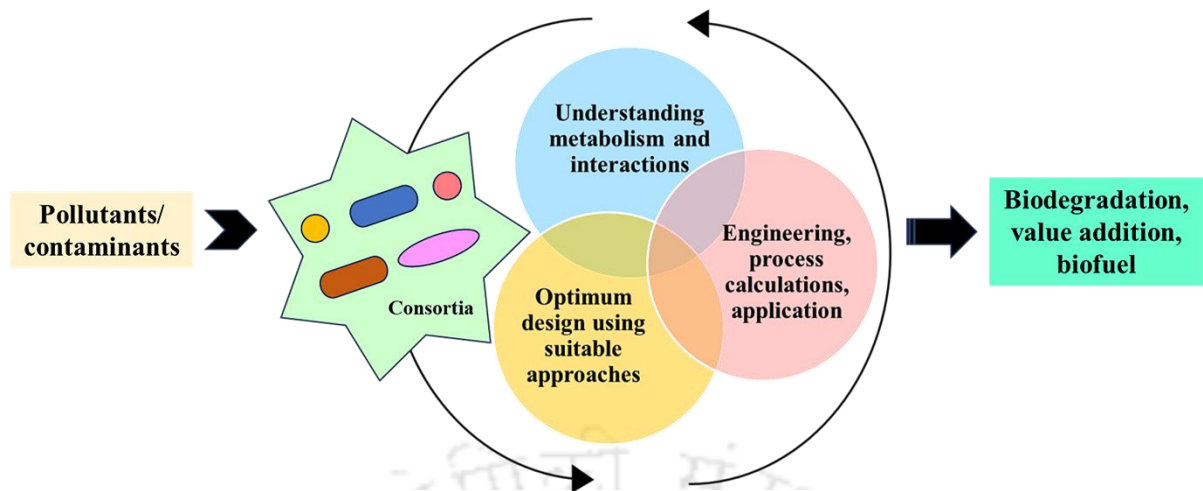


Figure 1.9: Overview of design and working of microbial consortia. Different microorganisms forming consortia depict synergy and participate in breakdown of waste components. Due to the advantages, microbial consortia are employed to attain maximum treatment efficiency.

1.2.8. Oily sludges itself as a source of microbes?

Microorganisms are isolated from sludge for several reasons:

1. Understanding microbial communities: Isolating microorganisms helps us understand the types of microbial communities present in the sludge. This can provide insights into the overall health and efficiency of the waste treatment process [137,150,207].
2. Identifying key microorganisms: Some microorganisms play a crucial role in the breakdown of organic matter in the waste. By isolating these microorganisms, we can better understand their role and potentially enhance their activity to improve the efficiency of the treatment process [130,200].
3. Studying microbial interactions: Isolating microorganisms allows us to study their interactions with each other and with the environment. This can help us optimize conditions for beneficial microbial activity [110,202].
4. Bioaugmentation: In some cases, specific microorganisms might be isolated, cultured, and then added back into the waste treatment system to enhance the performance. This process is known as bioaugmentation [78,110,147].

Sludge collected from the aeration tank of an activated sludge process would contain diverse group of microorganisms. These microorganisms come in direct contact and consume the biodegradable materials in the waste mixture food for their survival and growth. They are reported to form a hydrophobic layer around their cell wall which act similar to glues and

enables them to exist as clumps form a conglomerate union often termed as bio-solids or sludge. This bio-solid is then extracted and separated out from the liquid phase containing microbial populations alongwith waste components.

1.2.9. Uptake of pollutants: an insight into the functioning of microbial enzymes

Enzymes are involved in conversion of complex compounds from the initiation of a cascade of catalytic reactions. Different classes of enzymes present are equipped with the capability to accept the intermediates of a metabolic pathways to convert into a terminal non-toxic product during the biodegradation of refinery waste sludge. Microorganisms present in waste are excellent sources of enzymes with inherent metabolic catalytic activity and are capable of reducing complex compounds into lesser toxic forms. Presence of hydrolytic enzymes such as cellulases, reductases, ureases, proteases, lipases, phosphatases and dehydrogenases also significantly aid to enhanced biodegradation of complex organic compounds. Several refineries have adopted of enzyme-based biocatalysis as their mode of treatment operation over a range of contaminants due to their inherent advantages related to reduced toxicity, mediocre reaction setup, low energy input and reduced amount of unwanted byproduct generation [75,208]. It has been observed that enzyme recovered from an activated sludge plant has shown better compatibility to substrates and improved settleability and digestibility of sludge. Moreover, enzyme extraction offers significant economic benefits by recovering valuable resources from waste. Understanding the enzymatic pattern of metabolism further helps in optimizing important waste treatment processes and parameters, such as the optimum microbial dosages, anaerobic digestion (AD) of sludge and renewable energy production [209]. Further, as is the interest of this study, the potency and adaptability of the microbial community and the ability to process recalcitrant substrates and produce extracellular biomolecules i.e. biosurfactants and biocatalysts such as hydrolytic enzymes add to the efficiency of enzymes for playing a significant role in the degradation of toxic HCs into non-toxic metabolic intermediates [74,76].

The refinery waste mainly consists of primarily *n*-alkanes, aromatic compounds, PAHs and some portions of cycloalkanes (naphthenes). The same are discussed below:

1.2.9.1. Alkanes

Alkanes constitute most parts of crude oil. They are present in contaminated as well as non-contaminated habitats as organisms adhere to alkanes so as to content and protect the cells from water loss. However, the problems arise in their metabolism due to their hydrophobicity

which disables microorganisms to use alkanes as their source of energy. However, studies have revealed that there exists microorganism capable of utilizing alkane as the primary source of their nutrient. Although further studies demand more, but current research have shown the utility of alkanes being a very good C-source [139].

Alkanes are saturated HCs, formed exclusively by C and H atoms. They can be linear, cyclic or branched. Alkanes can constitute upto 50% of crude oil, depending on oil source, but are also produced by many living organisms such as plants, green algae, bacteria or animals [139,166]. Alkanes may serve as chemo-attractants and help organisms in defence mechanisms, protecting against insects, pathogens etc. Alkanes are chemically active with high energy content and hence can be a very good nutrient source for the strains capable of utilizing HCs. Alongwith the other molecules produced during metabolism; alkanes also are hydrophobic. Whereas it is therefore easier for the microbes to accumulate in the cytoplasmic membrane, changes the fluidity of the membrane could also be detrimental for the cell. Because alkanes are apolar and chemically inactive compounds, their metabolism by microbes presents difficulties because of their limited solubility in water, propensity to bioaccumulate in cell membranes, and energy requirement to activate and transfer the molecule through the cell membrane [210,211]. Extensive studies have been carried out over the last few years and researchers have been successful in identifying and characterizing microorganisms that are highly specialized to degrade HCs and hence could efficiently degrade alkanes.

Petroleum oil degradation by bacteria can occur under both aerobic and anaerobic conditions and hence alkanes can be metabolized both aerobically and anaerobically [208,212]. In general, as reviewed by Kumari and Das, 2023, oxygenase enzyme needed facilitates aerobic metabolism of HCs by adding molecular oxygen (O_2) to the reduced substrate. This O_2 is used as a reactant by aerobic alkane degraders to activate the alkane molecule. Simultaneously, production reactive oxygen species (ROS) by the monooxygenases help overcoming the low reactivity of HC [213]. Specific adhesion and extracellular emulsifier productions are the two broad techniques evolved in bacteria to improve interaction with water-insoluble HCs by inducing their growth and attaining breakdown [214].

Alkanes are largely insoluble in water with reduced gradual solubility with rise in molecular weight, thus hampering the uptake of alkanes; however, it is unclear about how alkanes actually enter the cell. It could be supposed that low molecular weight alkanes are relatively soluble in water and hence they are taken in quite easily by the cell and thus there is

sufficient mass transfer. For medium chain and long chain *n*-alkanes, either the transport is facilitated by extracellular surfactants by emulsifying the HCs and enhancing motility of the cells or microbes adhere themselves to the droplets to promote biosorption [139,166,210].

The process of aerobic degradation of *n*-alkanes with two or more C atoms is often initiated with the oxidation of a terminal methyl group, resulting in the formation of a primary alcohol. This alcohol is then further oxidized to produce the matching aldehyde and ultimately transforms into FAs. Acetyl-CoA is produced through the conjugation of FAs to CoA and subsequent β -oxidation processing. Sometimes the ω -hydroxylation of FAs at the terminal methyl group oxidizes both ends of the alkane molecule, resulting in an ω -hydroxy FAs that is then processed by β -oxidation to become dicarboxylic acid [215]. Subterminal oxidation of *n*-alkanes has also been reported resulting in the generation of secondary alcohol. After conversion to the equivalent ketone, the same is oxidized by a Baeyer-Villiger monooxygenase to produce an ester. An esterase hydrolyses the resultant ester to produce FAs and an alcohol. Whereas, both mechanisms are uniquely coded, in certain bacteria, terminal and subterminal oxidation can coexist [216,217].

1.2.9.2. Naphthenes

Naphthenes form the cyclic aliphatic HCs of petroleum with their general formula C_nH_{2n} . They are characterized by the presence of one or more rings of saturated C atoms and constitute considerable proportion of petroleum refinery products. They are also termed as cycloalkanes and exist as heavy complex residues. Naphthenes occur as Naphthenic acids (NAPs) and sometimes exist as acyclic, monocyclic and polycyclic carboxylic acids substituted with alkyl groups in crude oil, refinery wastes and petroleum products. Although, mostly occurring in refinery products, they might occur as minor component in refinery waste. Certain reports suggested that during treatment of oily waste, NAPs were gradually allowed to be sorbed to mixed liquor activated sludge biomass and the persistent components were further captured by introduction of power activated carbon (PAC). More than 80% of removal was observed with gradual desorption observed within 10 days of incubation. Lower fractions of NAPs were reduced even in the absence of PAC in the experimental microcosm [218,219].

The uptake of naphthenic acids into the cell is governed by several factors:

- ✓ Acidic crudes are prone to metal naphthenate formation. In this process, NAPs occurring naturally in crude oil react with metal ions forming organic salts that might inhibit normal processing of oil and distribution of components.
- ✓ Choice of naphthene inhibitors such as sulfonates, succinates and phosphonates may not be advised as it can greatly reduce compatibility of the process thereby decreasing crude oil quality [218,220].

1.2.9.3. Aromatic Hydrocarbons (HCs)

These are circularly structured organic compounds that contain sigma bonds along with delocalized pi electrons. Organic compounds where multiple such rings exist are known as PAHs. Aromatic HCs, particularly PAHs, are persistent organic pollutants that are widely distributed in urban areas and can be transported through stormwater runoff and by sediment facilitated transport. PAHs are introduced into the soil through plants, where root uptake and aerial deposition on leaves are the predominant ways of PAHs accumulation in plants. Subsequently, the transfer occurs from soil to food chain. Similarly, PAHs relocate into the marine environment also by virtue of accidental spills or through gradual PAHs accumulation due to improper disposal for over long period of time. Microbial degradation is mostly employed to facilitate the sequestration of PAHs in contaminated marine sites however, insufficient physical contact and extensive hydrophobicity limits their tendency towards bioaccumulation [221–223].

The uptake of aromatic HCs into the cell is governed by several factors such as availability of HCs dissolved in the aqueous phase, affinity of the cells towards the oil droplets and direct access of larger oil drops [224,225]. Trans-membrane transport occurs after the bacterial cells come within the vicinity of HCs. Because these compounds are very hydrophobic and have limited aqueous solubilities, serum components, such as proteins and lipids, would have a considerable effect on the bioavailability of these HCs for cell uptake [221,225,226].

For both aromatic substrates as well as PAHs, hydroxylation of ring occurs via mono or dioxygenase enzymes in eukaryotes. Several reviews have been published in context to microbial metabolism of aromatic HCs. It has been established that the first step is the introduction of O₂, similar to the metabolism of alkanes. Aromatic HCs are converted to *trans*-dihydrodiols by first dioxygenase attack, that are further oxidized to catechol, a key

intermediate in the biodegradation of aromatics in prokaryotes [164]. Eukaryotes microorganisms use monooxygenase to produce 1, 2-oxide, followed by the addition of O₂, yielding *cis*-dihydrodiol. This is oxidized in turn to catechol, which is opened by *ortho* and *meta* cleavage, yielding muconic acid or hydroxymuconic semi-aldehyde, respectively [216,220,227].

1.2.10. Enzymes involved in metabolism of hydrocarbons (HCs)

Alkane hydroxylases (*alkB*), catechol dioxygenases (CDOs) and naphthalene dioxygenase (NDO) are some of the key enzymes involved in the biodegradation of aliphatic, aromatic and PAH compounds, that are common in refinery waste sludge.

1.2.10.1. Alkane hydroxylase (*alkB*)

Enzymes that degrade higher chain are identified as alkane hydroxylase, sometimes called as alkane monooxygenase (Figure 1.10). Three types of alkane monooxygenase are known: A rubredoxin dependent protein for bacteria encoded by the gene *alkB*, a CYP153 family of cytochrome P450 monooxygenases in bacteria and a cytochrome P450, the NADH dependent CYP52, encoded by the gene CYP52, is found in fungi [228,229].

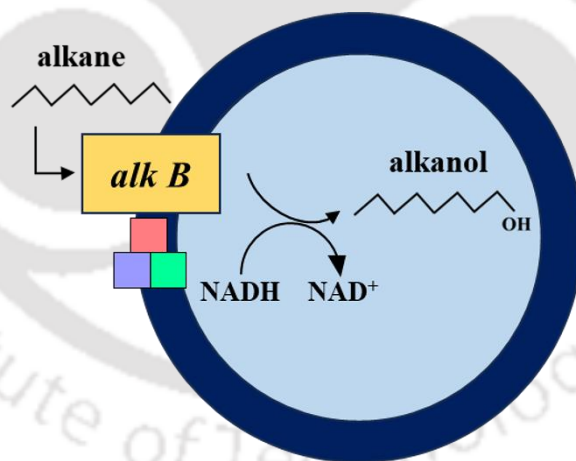


Figure 1.10: Working of alkane hydroxylase (*alkB*). *alkB* is an integral protein and accepts alkane as the primary substrate. At the first step alkane is converted to alkan-1-ol and is released into cytoplasm for further breakdown.

alkB, an integral-membrane non-haem diiron monooxygenase, is the first enzyme in the alkane metabolism pathway. It hydroxylates alkanes at the terminal position. Rubredoxin (*alkG*) and rubredoxin reductase (*alkT*) are two electron-transporting proteins that are necessary for *alkB*. Electrons from NADH are transferred via rubredoxin reductase to rubredoxin, which then transmits the electrons to *alkB* through cofactor FAD. It has been

observed that some strains can digest alkanes bigger than C₂₀. Such alkanes are solid at normal temperature, and the enzymes that oxidize them are still not well understood [210,230–232]. Degradation of branched chain alkanes are poorly degraded than linear *n*-alkanes. There are very few reports that show degradation of simple as well as complex branched compounds.

Analyzing intermediate metabolites have assisted in identifying alternative pathways of degradation involving different mechanisms of *n*-alkane activation in bacteria, however, limited data of molecular mechanisms and biochemical conversion processes might put forward a greater scope of research in future. The organization of genes involved in alkane oxidation varies significantly within alkane degrading microorganisms. There are cases where alkane hydroxylase encodes differently for rubredoxin and rubredoxin reductase. In cases where different alkane hydroxylase coexists in a single strain, they are reported to be present at different locus in the chromosomes. Studies have also revealed the extent of higher variability in degree of clustering of alkane degradation genes among bacterial strains that also influence the presence of necessary enzymes in the cell [233,234].

1.2.10.2. Naphthalene Dioxygenase (NDO)

Naphthalene dioxygenase (NDO) belongs to bacterial Rieske non-heme iron family of oxygenases. This enzyme is involved in the initial catalysis of biodegradation of symmetric PAHs such as naphthalene, anthracene, polychlorinated biphenyls etc., followed by other enzymes encoded by the *nah* gene cluster. Upregulation of degradation enzymes through activation of *nahR* regulator activated by salicylate, thereby enhancing the biodegradation of PAHs and formation of intermediate 1-hydroxy-2-naphthoic acid.

Figure 1.11 depicts the working of NDO. In the first step of catabolism of naphthalene, an O₂ molecule is introduced at the 1,2 positions of the aromatic nucleus by NDO to produce *cis*-naphthalene dihydrodiol. *cis*-naphthalene dihydrodiol is transformed into 1,2-dihydroxynaphthlene and subsequent intermediates, resulting in the production of salicylaldehyde, which is then transformed to salicylate by salicylaldehyde dehydrogenase.

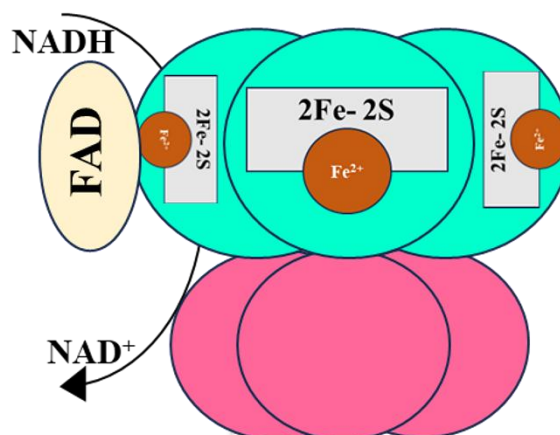


Figure 1.11: Working of naphthalene dioxygenase (NDO). NDO converts certain PAHs such as naphthalene, anthracene etc. into *cis*-dihydrodiol inside the cytoplasm as the first step of metabolism.

The pathway for naphthalene-enhanced phenanthrene degradation in *Pseudomonas* sp. strain SL-6 has been reviewed [220,227,235]. The three components of the dioxygenase system include an iron-sulfur centre-containing flavoprotein reductase, a Rieske [2Fe-2S] ferredoxin that transfer electrons from NADH to the oxygenase complex and the oxygenase component containing a Rieske 2Fe-2S centre and mononuclear iron at the active site. Both redox centres are located in the α -subunit is the location where the redox reaction takes place by facilitating addition of oxygen to the aromatic ring and subsequently forming dihydrodiol enantiomer [236]. Similar mechanism has also been experimentally studied by Jouanneau et al., 2006, wherein the ring hydroxylating dioxygenase (RHD) present in *Sphingomonas* CHY-1 was reported to be responsible for catalysing substrates of upto five aromatic rings [235]. Hence, the existing reports proves the efficacy of application of NDO enzymes for bioremediation of HC contaminated site.

1.2.10.3. Catechol Dioxygenases (CDOs)

These enzymes play a crucial role in the aerobic biodegradation of aromatic compounds. CDOs also belong to RHD class of enzymes. The working of CDOs is similar to NDO except it catalyses catechol to initiate a chain reaction. Catechol, that are central intermediates in the degradation pathway of these aromatic and PAHs, are degraded by CDOs. These enzymes cleave the aromatic ring by introducing two atoms of oxygen into either *cis-cis* muconic acid or 2-hydroxymuconic semialdehyde through the *ortho* or *meta* pathway respectively.

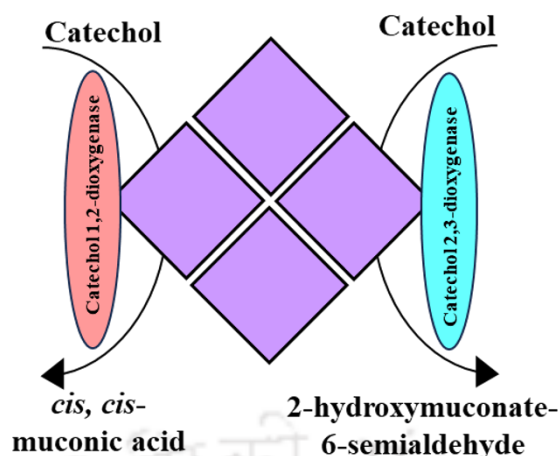


Figure 1.12: Working of catechol dioxygenases (CDOs). CDOs act through *ortho* or *meta* breakdown on substrate catechol. *ortho* breakdown involves oxidation at 1,2 position form *cis, cis*-muconic acid whereas *meta* breakdown results in formation of 2-hydroxymuconate semialdehyde by incorporation of oxygen at 2,3 positions.

For instance, a novel functional trimeric catechol 1,2-dioxygenase from *Pseudomonas stutzeri* has been studied for the ability to cleave the benzene ring of catechol [237]. In another study, the activity of catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* was also checked to understand the metabolic pathway of 2,4-dichlorophenol which subsequently thought to pave better bioremediation strategies [238]. Reports of catechol 2,3 dioxygenase are also widely available. For instance, the mechanism of catechol 2,3 dioxygenase (C2,3DO) with phenol as substrate has been studied from the strain *Pseudomonas putida* KB3 where high activity was reported. However, in another case, no activity was shown by the strain *Pseudoarthrobacter phenanthrenivorans* Sphe3 when phenol was the substrate. Reports related to the bacterium utilizing *m*-xylene as a substrate has also been shown. Figure 1.12 illustrates the working of CDOs. Due to the homotetramer construction of the enzyme complex, it generally accepts small monocyclic compounds as its substrate [239–241]. In an attempt to understand the efficacy towards biomass valorisation alongwith aromatic HC degradation, C2,3DO identified from a novel lignin degrading *Bacillus ligniphilus* L1 was also reported recently [242].

Some reports suggesting the activity of *alkB*, NDO and CDO are listed in Table 1.4.

Table 1.4: Enzyme activities involved in HC metabolism.

Substrate	Remarks	Reference
Oil Contaminated soil from Dagang oil field, China	Abundance of <i>alkB</i> and <i>nah</i> were established. Positive correlation was found with presence of petroleum HCs.	[243]
<i>n</i> -alkanes, PAHs	Catabolic genes of <i>alkB</i> and <i>nahR</i> were metabolically active in all the strains except HRJ6 lacking <i>alkB</i> . 54% TPH degradation was observed as the highest for HRJ6 strain.	[244]
Crude oil, <i>n</i> -hexadecane	Significant enzyme activity of <i>alkB</i> was observed in <i>Pseudomonas synxantha</i> LSH-7'.	[245]
Alkanes (C13-C20), PAHs (anthracene, phenanthrene, pyrene, fluoranthene)	Activity of <i>alkB</i> and C23DO was significantly high. 90% of aliphatic and aromatic fractions were reduced.	[246]
Crude oil, <i>n</i> -hexadecane, <i>n</i> -nonadecane, fluorene, phenanthrene, pyrene	<i>alkB</i> , C12DO and C23DO activity were examined in <i>Pseudomonas aeruginosa</i> san ai. <i>alkB</i> showed significant activity to metabolize alkane, C12DO showed more activity and ortho cleavage was the preferred pathway for PAHs degradation was observed as 45% of aliphatic fraction and 25% of aromatic fraction, 80%, 98%, 96%, 50% and 41% of the individual components respectively.	[166]
<i>n</i> -alkanes, PAHs, BTEX, and naphthalene	Catabolic genes of <i>alkB</i> , C23DO, and <i>nahR</i> were metabolically active. 97% TPH degradation was observed.	[247]

Hence, these enzymes are part of complex biochemical pathways that allow microorganisms to use these compounds as sources of carbon and energy and understanding these mechanisms can facilitate improved strategies to maximize the bioremediation efficiency of PAH-contaminated sites.

1.2.11. Other modes of hydrocarbon (HCs) uptake

In the subsurface, oil degradation is primarily mediated by anaerobes or other sulfur reducing bacteria by employing different electron acceptors. Reports have shown that alkanes and other components, especially benzene/toluene/ethylbenzene/xylene (BTEX) components

are degraded by sulfidogenic or methanogenic bacteria cultures under anoxic conditions also able to deliver significant yield and energy. Anaerobic degradation of crude oil has also been shown in some studies in context to natural environment, but their ecological significance has not been vast [212,248]. Although aromatic derivatives such as benzoates are degraded mainly under anaerobic conditions, Wu et al., 2024 and Zhou et al., 2024 recently reported the biological removal of ethylbenzene and xylene under denitrifying conditions from soil [249,250].

Under strict anaerobic conditions, alkanes are activated through mechanisms that do not involve the presence of oxygen in it and the growth of microbes is relatively slower than in aerobic degradation. On the other hand, it is advantageous as HCs would be easily recycled. The strains show a very narrow range of degradation as there is very little chance of using alkane as their substrate. As reported so far, very few pathways are known supporting metabolism of alkanes by anaerobic means. One pathway mostly believed to exist is the one that involves activation of alkane through formation of a succinate derivative through addition of fumarate to the subterminal position [248]. This reaction is believed to occur through generation of an organic radical intermediate. Through extensive research, it has been believed that the reaction is driven forward through generation of free radical which is an inorganic intermediate. To summarize, the uptake of different classes of HCs greatly var and depends on their chemical structure and environmental factors. Prolonged exposure to these compounds can have significant environmental and health impacts due to their potential toxicity and higher degree of persistence.

1.2.12. Biomass quality as an indicative of treatment efficiency

Biomass obtained post refinery sludge treatment can indeed be a good feedstock for biodiesel production or value-added products. It consists of abundant components with higher renewability for usage in different biochemical processes for the energy production and value addition [209,251,252].

An overview of biomass pre-treatment techniques for biorefinery and value addition are listed in the following Table 1.5.

Table 1.5: Pretreatment of biomass for different applications.

Technique	Description	Reference
Physical methods	This includes mechanical milling, irradiation, extrusion and water hydrolysis.	
Chemical methods	This includes Acid hydrolysis, alkaline hydrolysis, pretreatment using organic solvent and ionic liquid, application of metal salts, oxidative treatment using ozone, hydrogen peroxide.	[253–256]
Physico-chemical methods	This includes Ammonia fibre explosion (AFEX), steam explosion, microwave assisted pyrolysis, CO ₂ explosion, ultrasound.	
Biological methods	This includes usage of certain species of bacteria, white rot fungi and yeast, enzymes such as cellulases, protease, pectinase etc.	

Recovered biomass obtained from a refinery waste sludge has several unique characteristics:

1. Microbial characteristics: The biomass obtained from a sludge treatment facility is composed of microorganisms that produce extracellular polymeric substances, which stabilize granule structure and accumulate P [257].
2. Physico-chemical properties: Biochar derived from waste petroleum refinery sludge (biochar-WPS) has specific pore structure, elemental composition, and surface functional groups [258].
3. Biomass concentration: Biomass obtained from aerobic treatment plant have been reportedly higher in concentration for potential use in further applications. In one of the reports, upgradation of municipal treatment facility to aerobic granular technology yielded an increased two-fold biomass content [257]. The same might also be accounted for a refinery waste sludge treatment facility.

Refinery sludge, being rich in organic content, can be treated and converted into valuable biomass. For instance, during the hydrothermal liquefaction process, the catalytic conversion of lignocellulosic biomass to value-added products takes place. Sludge biomass

also account as potential feedstock due to high availability, excellent lipid profile and lower cost [252,259].

Here are some key characteristics that make it suitable:

1. Variety of feedstock: Biomass is present as biosolids in sludge and Sludge liquidation is an option for production of biofuel in the form of granules or pellets from sludge enriched by waste celluloses. The resultant biofuel quality is comparable to the currently available conventional fuels. This makes it a versatile feedstock for biodiesel production [260].
2. Minimal sulfur (S) and nitrogen (N) residual: Biomass typically contains relatively low S and N content, thereby reducing sulfur dioxide (SO₂) and nitrogen oxide (NO) emissions. This makes it environmentally friendly [258].
3. High combustion performance and efficiency: Biomass has a high flash point and high combustion performance and efficiency, thereby making less prone to explosion and safe to use [261].
4. Lipid composition close to composition of vegetable Oils: Biomass, such as lignocellulosic biomass, can be used for production of biodiesel using oleaginous microorganisms. Lipid content accumulated under various stress conditions were found similar to that of vegetable oil. Biomass containing classes of bacteria, that contain higher lipid share of their biomass might be considered a valuable and sustainable feedstock. These lipids can be converted into biodiesel, a renewable source of energy [262,263].
5. Sustainability: The carbon dioxide released from the combustion process does not impact atmospheric CO₂. This makes it a sustainable source of energy [251,264].
6. Potential for value-added products: Apart from biodiesel, biomass is also used for the production of a variety of chemicals. The biomass can be used for producing high-value fuels and products, serving as a low-C equivalent to fossil-based gasoline, jet, and diesel fuel. Wet wastes, including sewage sludge, have prospects to be converted into biocrude. These sources being identical in composition to petroleum feedstocks might be mimicked to produce high-quality fuels and value-added products such as proteins, carbohydrates, and glycosidases. These products have various applications in industries like food, pharmaceuticals, and biotechnology. This opens up opportunities for creating value-added products [261,262].

7. Metabolic flexibility: Bacteria are versatile and are capable of utilizing innumerable number of substrates for growth, including various types of waste, thus making them ideal for waste-to-energy conversion processes [263].
8. Genetic modifiability: Bacteria present in the biomass are amenable to genetic or metabolic modifications, which can enhance their biofuel production capabilities. Newer and advanced metabolic engineering technologies are highly effective for constructing efficient microbial cell banks for the next-generation bioeconomy [259,265,266].
9. High growth rate: Bacteria generally have high growth rates, that lead to rapid biomass accumulation. This is beneficial for large-scale biofuel production processes [266,267].
10. Waste biorefinery: Sustainable and circular bioeconomy could be established by integrating biomass obtained into bioprocesses to produce metabolites and usable products. By lowering greenhouse gas emissions, this approach would create a low-C economy and offer excellent opportunities for an eco-friendly future [268,269].
11. Waste valorisation: Value-addition by transformation of biomass obtained into products provides environmental and economic benefits. It supports a sustainable and circular bioeconomy by reducing greenhouse gases footprint and optimizing waste treatment processes [261,270].

Moreover, AD is a proven technology for biomass reduction and recycling. Waste and other organic and nutrient-rich sludge is often used as the feedstock for generation of environmentally friendly products such as methane. Although the feasibility and efficiency of using treated biomass from refinery sludge as feedstock can depend on several factors including the type of biomass, the specific treatment process used, the intended end-use application, and environmental considerations [271], the biomass derived from refinery sludge has the potential to be an excellent feedstock for biodiesel production and also suitable for value addition due to the favourable characteristics and sustainability.

1.3. Motivation of thesis work

Petroleum refineries are well equipped with extensive network and facilities of oily sludge collection, treatment and disposal. Whereas, the sequence of these steps is followed in correct hierarchical order, unwanted interruption of operations and sudden disruption of the working units affects the functioning of a plant. Apart from spillage due to inappropriate

handling and transportation, accidents related to fault within either one of the units or the whole system due to overload or congestion greatly pose challenges for maximum recovery of the process. As a result, sludge in the collection tanks is released in between the treatment units causing unusual halt in operation. Several reliable physico-chemical clean-up techniques have been investigated, bioremediation has been reported the most effective environment friendly strategy to avoid generation of any other secondary pollutants, thus achieving the desired result and restore the functioning of the plant. Bioremediation strategy is often employed by application of an engineered group of population that are well versed to cater the toxicity of harsh HC contaminated site. Indigenous microorganisms, that exist within the site and are considered as best acclimatised to the waste environment and hence, the most potent candidate to attain maximum clean-up. Microbes possess the essential machineries for uptake of waste components continuous supply of external nutrients are necessary to enhance the bioavailability of the pollutants to achieve desired reclamation. Hence, to address the challenges sludge accumulation within the treatment units, quicker, simpler and comprehensive strategies of developing and employing microbial inoculum (consortia) are required to be devised in order to reclaim the contaminated site. Moreover, channelizing the biomass for useful applications is essential as harnessing the microbial biomass after exposing to HC stress might also render fruitful characteristics thereby making biomass as a potential feedstock for formulating environment friendly products such as biofertilizer, biofuel and value addition. The biomass composition and quality serve as an indicative of the efficiency of the microbes to readily use the toxic contaminants as their source of nutrient and survival.

1.4. Proposed thesis objectives

In view of addressing the existing reports and the possible research gaps, following objectives were proposed for the thesis work. These objectives were elaborated in the form of chapters as discussed subsequently.

1. Screening, isolation, and identification of indigenous microorganism(s) from the waste sludge.
2. Statistical optimization for designing optimum consortia for remediation of waste sludge.
3. Analysis of biomass characteristics and enzyme activities for an insight into the bioremediation of waste sludge.
4. Application of developed consortia for bioremediation of the waste sludge.

1.5. Organization of thesis

1.5.1. Chapter 1: Introduction and literature review

This chapter was the first step to accumulate the existing reports of the existence of petroleum industries and the pros and cons associated with it. Chapter 1 elaborately introduces to the continuous contribution of petroleum industries towards generation of important products, byproducts and energy, which are the integral parameters governing the daily lifestyle and economy. In addition, it has also been discussed about the quantity of wastes the refineries generate during the processing of raw crude oil into valuable products. Reports related to the distribution of products as well as composition of wastes are also summarised to understand the potential impact and havoc this type of wastes can create. Various existing technologies for refinery waste clean-up have been reviewed and the efficiency of bioremediation has been investigated as the best approach. Following a brief introduction, an elaborate review was put forward to gain deeper insights of the existing scenario and statistics related to waste generation from the refineries, mechanisms approved by regulatory bodies for their mitigation, different avenues available for maximum clean-up and the challenges associated with the promising aspect of application of biological treatment. Further, applying the group of microorganisms to achieve best desirable results have been highlighted as an efficient strategy supported by existing reports. A simpler and comprehensive mode of engineering indigenous microbial population have also been studied. At the end of the chapter, effectiveness of microbial degradation was supported by the presence of necessary inherent mechanisms and machineries such as enzymes to feed upon the toxic components by using HCs as their substrate are highlighted. Enzymes generate a sequence of catalytic reactions to metabolise the HCs into a harmless non-toxic form. Biomass quality and the probable indications of an efficient bioremediation process was put forward citing the efficacy of microbes in reclamation of a refinery waste contaminated site.

1.5.2. Chapter 2: Screening, isolation and identification of indigenous microorganism(s) from waste sludge

This chapter elaborates the sample collection, isolation and identification of indigenous microorganisms, and understanding the dynamics of the isolated strains in different classes of HCs that are expected to be present in the waste as either in pure form or in the form of derivatives by means of substitution by different functional groups. The methodologies and the respective results pertaining to sample characteristics such as temperature, acidity, oxidation-

reduction potential (ORP), conductivity, presence of TSS, COD, TPH, nutrients in the form of N and P etc. was summarized. Isolation of indigenous strains using enrichment method, their identification based on sequencing 16S rRNA conserved regions, optimum growth conditions such as temperature and pH have been discussed in minute details. The experimentation was further explained to understand the growth of these strains separately in nutrient broth (NB) and MSM. Further dynamics of these strains in different classes of HCs such as *n*-hexadecane (aliphatic), anthracene, phenanthrene (PAHs) and BTX (aromatic) supplemented in MSM was studied by analysing their growth in terms of increase in bacterial count, biomass concentration and the extent of their survival in the culture media. Prior to that, a sequential process of obtaining maximum tolerance and optimum inoculum of each strain in each class of pollutants are discussed. The work was further validated using the existing techniques of modelling of microbial growth such as visualizing the growth kinetics using Monod's model or Andrew's growth model of substrate inhibition and understanding reduction in toxicity by analysing the decrease in overall COD of the mixture. The chapter ended with a summary of brief outcomes obtained after the set of experimental procedures carried out pertaining to the ability of the isolated strains to reduce the toxicity of different HCs.

1.5.3. Chapter 3: Statistical optimization for designing optimum consortia for remediation of waste sludge

The primary objective of this chapter was to obtain an engineered media where maximum biomass production of these isolated strains could be expected alongside maximising the COD removal of the different HCs studied in Chapter 2 supplemented in MSM. The concept of maximum biomass production was further utilized to design optimum microbial population (consortia) which alongside providing significantly higher biomass would also contribute to reducing maximum toxicity caused due to the presence of these HCs. The experimentation was started by identifying different micronutrient factors such as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ and NaCl and growth parameters such as concentration of HC, time period of incubation and inoculum concentration that are expected to influence the biomass growth. Based on the kinetic parameters and COD removal, some of the selected strains from Chapter 2 were subjected to series of optimizations. This chapter is elaborately discussed on analysing a set of randomized experiments based of 1st order Plackett-Burman Design (PBD) linear screening followed by 2nd order CCD quadratic model to obtain optimum response of the significant factors. Chapter 3 comprehensively explains the simplicity of availing DoE and their analysis using statistical approaches. Statistical analysis such as

Analysis of Variance (ANOVA) was chosen as an effective means of assessing full factorial models and their reliability and precision were governed by the respective statistical parameters. A sequence of 1st order optimization to obtaining predicted response in terms of optimum media composition and optimum inoculum compositions were explained. The chapter ended with a summary note of overview of the modified media and possible designed consortia that were selected for further applications.

1.5.4. Chapter 4: Analysis of biomass characteristics and enzyme activity for insight into the bioremediation of waste sludge

The working of this chapter was divided into 2 stages: Understanding the biomass quality obtained after incubation in different classes of HCs; and enzyme activities to analyse the catalytic efficiency to metabolize these pollutants inside the cell. The chapter was initiated with a brief background citing the importance of harnessing biomass and the characteristics for an indicative of the quality of bioremediation process followed by brief methodology of the experiments and results. Biomass characteristics such as lipid deposition, carbohydrate content and protein concentrations were chosen, and these macromolecules are gradually expected form in higher concentration in response to the stress created due to presence of toxic HC components. All of these were also compared to the biomass obtained before and after the media optimization where higher biomass concentration was expected to depict higher biomass quality post optimization. The working in this chapter was further proceeded by selecting enzymes pertaining metabolism of aliphatic, aromatic and PAHs. The chapter describes the methodologies in detail to perform enzyme assays and calculate their respective activities of *alkB*, NDO, catechol 1,2-dioxygenase (*catA*) and catechol 2,3-dioxygenase (*catE*). The chapter elaborates the working methodologies towards understanding the optimum condition for the best activity of these enzymes, their substrate specificity and storage stability for maximum and accurate results. The experimental work ended by understanding the maximum activity (V_{max}) and Michelis-Menten's constant (K_m) of the crude complex in terms of the respective enzyme activities for inferring the extent these enzymes are upregulated for metabolising the HCs as their substrate. A brief summary was reported at end of this chapter.

1.5.5. Chapter 5: Application of designed consortia for bioremediation of waste sludge

In this chapter, the applications of all the optimized settings obtained from the previous chapters were applied to check the applicability of the designed consortia in real waste sludge sample. The working of the chapter was initiated by incubation of the microbial consortia in

different classes of HCs supplemented in the modified MSM (mMSM) to compare the change in toxicity reduction in terms of COD removal. The chapter further discussed the fractional increase in enzymes activities and biomass composition that acted as a confirmatory biochemical assay to signify enhanced metabolism. Addition of nutrient components in the form of N as NaNO_3 and P as KH_2PO_4 was carried out to check the effectiveness of nutrient amendment to enhance the availability of the toxic pollutants. The chapter discusses the detailed procedure involved in obtaining optimum C:N:P ratio by incubating strains into synthetically prepared pollutant mixture containing all the HCs upto the maximum tolerance value supplemented in mMSM alongwith necessary proportions of added nutrients. Optimum C:N:Ps obtained for the respective consortia were chosen for final application. Prior to the final application, a trial run of the consortia into a diluted sludge sample was carried out. The bacterial count, biomass concentration, COD removal, N utilization and P utilization were all analysed assess the consortia performance. Enzyme assay was carried out to confirm the active presence and participation of the respective enzymes in the crude protein to metabolize the HC components present in it. Biomass qualities were also checked to obtain a definitive insight of HC uptake. The results obtained from the trial experiment was taken as reference values. In the final part of the chapter, the consortia were applied into the undiluted sludge sample to investigate and compare all of bacterial count, biomass production, COD removal, N utilization, P utilization, biomass quality and enzyme activities for their potential application in bioremediation of refinery waste. The chapter ended with a summarized overview.

1.5.6. Chapter 6: Conclusion and future perspectives

Chapter 6 provides a complete overview of the thesis. The chapter started with a brief summary of the thesis followed by brief chapter wise overviews. Each chapter overview was limited to discussion of outcomes and the probable implications on application of bioremediation of refinery waste. The chapter also allows the reader to comprehensively analyse the working methodologies and address a conclusive synopsis of their outcomes to obtain an overall insight into the working of the thesis. The chapter ended with a brief set of scope and perspectives leaving potential possibility of further research into bioremediation of refinery waste by scrutinizing the inherent characteristics of indigenous microbes.

2

Screening, isolation and identification of indigenous microorganism(s) from the waste sludge

2.1. Background

Studying the presence of indigenous microbes in a waste sample is the first step towards understanding the sample toxicity and the ability of these microbes to survive in such toxic conditions. Thus, identifying and the dynamics of indigenous bacteria play a significant role in the treatment of refinery waste sludge as native bacterial mixed cultures (BMCs) from a mixture of refineries and petrochemical waste can be acclimatised in typically all refinery sludge originating from any refinery [147,272]. The addition of suitable bacterial species may further enhance the treatment potential in the waste medium as the inorganic nutrients are assimilated, while the organic nutrients are utilized by bacteria giving out good quality biomass. Various reports have been discussed of the efficacy of microorganisms for their intrinsic biodegradation ability. These findings highlight that indigenous community of refinery sludge is intrinsically diverse, yet appreciable rate of *in situ* bioremediation could be achieved [132,231,273]. This represents a significant step towards sustainable and effective management of hazardous refinery waste.

As discussed before, refinery waste sludge, also known as petroleum sludge, is a byproduct of the oil refining process and is a complex mixture composed of components such as organic phase which includes HCs, heteroatom compounds, and asphaltene-tar substances, water phase, mechanical impurities which include sand, clay, and other solid particles and a large amount of flocculating agent. However, the composition of the sludge can vary depending on the type of crude oil being processed and the specific processes used in the refinery [6,97,100,104]. Due to high toxicity and potential environmental impact, proper treatment and disposal of refinery waste sludge are crucial.

In this chapter we limit our scope only towards the HC component and will primarily study the effect on growth and survival of the isolated indigenous microbes in separate HCs such *n*-hexadecane (straight chain aliphatic), anthracene, phenanthrene (PAHs), and BTX (aromatic HCs).

2.2. Materials and methods

Chemicals and reagents

The chemicals, solvents, reagents were collectively obtained from Merck Lifesciences Pvt Ltd., Kolkata; HiMedia Laboratories Pvt Ltd., Mumbai; Sigma Aldrich Chemicals Pvt Ltd.,

Bengaluru and Spectrochem Pvt Ltd., Mumbai. Media components K_2HPO_4 , $CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$ was procured collectively from HiMedia and Merck. Nutrient broth and nutrient agar were also procured from HiMedia. $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot H_2O$, $CoCl_2 \cdot 2H_2O$, $Pb(NO_3)_2$, $NiSO_4 \cdot 6H_2O$ and CrO_3 were collectively obtained from Merck and HiMedia. $K_2Cr_2O_7$, $HgSO_4$, conc. H_2SO_4 and Ag_2SO_4 was obtained from Merck. Anthracene, *n*-hexadecane, phenanthrene and xylene were obtained from Sigma Aldrich. Benzene and toluene were obtained from Merck. Ammonium molybdate, brucine sulfate, sulfanilic acid, conc. HCl, $SnCl_2$ was purchased collectively from Merck and Himedia. Phenolphthalein, diphenylcarbazine (DPC), barium sulfate $BaSO_4$ and dextrose was obtained from Merck. $K_2S_2O_8$ and Gram Staining Kit was obtained from HiMedia. Finn timer micropipette of *F2* grades (Thermo Fisher Scientific, India) were used of ranges (μL) 0.2-2, 1-10, 10-1000, 2-20, 10-100, 10-200. Weighing balance of Sartorius, India, Quintix model was used. Autoclave, water bath was from Equitron Medica Pvt. Ltd., India. Biosafety Cabinet Class II laminar was used of Cleanair, India. Plasticware and glassware including filtration assembly for biomass estimation were procured from Tarsons, India and Borosil India. Bacterial incubator was procured from Scigenics Biotech, Chennai, India. Whatman qualitative filter papers (No. 1; pore size 0.7 μm ; 125 mm diameter) was procured from Cytiva Lifesciences, India. DispoVan syringes obtained from local pharmacy were used for better precision.

Physicochemical analysis of refinery waste sludge

In this study, the petroleum sludge sample was collected from Indian Oil Corporation Limited, Guwahati refinery effluent treatment plant located in Assam, India ($26^\circ 10' 54'' N$, $91^\circ 48' 14'' E$). Sludge was obtained as a semi-solid cake and was dark greenish colour in appearance. The sample was collected from the sludge tank was stored at $4^\circ C$ to retain the original characteristics.

The pH of waste (sludge: water suspension) was determined using a pH meter (Hanna Instruments, India, HI2211), and conductivity was estimated using a conductivity meter. Moisture content was measured by evaporating out the liquid and volatile fractions and measuring the final weight [46,136], COD estimation was carried out by closed reflux method and TSS by drying at $103-105^\circ C$ for 2-4 hours in hot air oven followed by cooling and gravimetric analysis, chloride (Cl^-) concentration was estimated using argentometric method, sulfate (SO_4^{2-}) using turbidimetric method and Phosphate (PO_4^{3-}) by adding stannous chloride ($SnCl_2$) as reducing agent using spectrophotometric method [274]. Nitrate (NO_3^-) was estimated using brucine-sulfanilic acid digestion method after completely digesting all of

present forms of N by oxidizing the sample using strong oxidizing agent such as potassium persulfate ($K_2S_2O_8$) [38,275–277]. TPH was estimated according to Das and Mukherjee, 2007. Briefly, 10 grams of sludge sample was extracted sequentially and separately in different solvents such as *n*-hexane, DCM, acetone and chloroform. The extracts were allowed to dry by evaporation under room temperature. Finally, the residual TPH was calculated gravimetrically and expressed as percentage of dried solids [136]. Sludge volume index (SVI) and Sludge density index (SDI) was determined according to APHA, 2005 [136,274]. Bacterial count was obtained as per standard serial dilution method and colony forming unit per unit volume sample (CFU/mL) was obtained as per Equation 2.1.

$$\text{CFU/mL} = \frac{\text{No. of distinct colonies}}{\text{Volume of sample (in mL)}} \times \text{dilution factor} \quad \text{Equation 2.1}$$

2.2.1. Indigenous bacteria from waste

Indigenous bacterial strains were isolated as described by Geetha et al., 2013 and Larik et al., 2016. Briefly, 1 mL sludge: water suspension to be used as inoculum, was transferred to 50 mL NB. The flasks after inoculation were incubated in a rotary shaker at 180 rotation per minute (rpm) [130,278]. Spread plating in nutrient agar (NA) plates was used to obtain viable colonies from the waste by serial dilution technique over a dilution range of 10^{-1} to 10^{-8} [46]. The plates were incubated at 37 °C for one week. Distinct viable colonies were streaked onto NA plates to obtain pure colonies.

2.2.2. Growth profile in nutrient broth (NB) and minimal salt media (MSM)

The isolated strains were subjected to acclimatization separately in NB containing 10 g/L peptone, 10 g/L beef extract and 5g/L NaCl (HiMedia, India) and in MSM containing 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L K_2HPO_4 , and 0.1 g/L $CaCl_2 \cdot 2H_2O$. For both the media, pH was checked and maintained at 6.8 ± 0.2 post sterilization.

To initiate the growth process, a loopful of culture from the preserved pure colony NA plates were inoculated in 50 mL NB in separately in each 100 mL Erlenmeyer flasks. A separate flask with NB medium flask not inoculated with any of the cultures was used as control. The growth was checked at regular intervals viz. 8 hours in case of NB media and 4 days in case of MSM by measuring the optical density (OD) at 600 nm (OD_{600}) [144] using UV-Vis Spectrophotometer (UV-2600, Shimadzu, Tokyo). Subsequently, to clarify whether there were viable cells in the culture medium, CFU/mL was also measured at the same intervals as OD_{600}

was measured [46]. Further, biomass obtained per unit volume sample at the specific intervals were also calculated and a change in biomass concentration profile with time was obtained.

2.2.3. Estimation of specific growth rate

The specific growth rate (μ) was calculated as per the following Equation 2.2:

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad \text{Equation 2.2}$$

Where,

X = Biomass concentration as time, t ,

dX = Change in biomass between times $t = 0$ to $t = t$,

dt = Change in time from time $t = 0$ to $t = t$.

This process was used to calculate specific growth rate for all the isolated strains separately for NB and MSM.

2.2.4. Morphological and 16S rRNA identification of the isolates

Morphological identification was carried out by Gram Staining [272,279] using Gram Stains -Kit (HiMedia). Cultural characteristics were observed from the growth in NA plates [280].

Sequencing services were availed from 1st BASE (Malaysia) and was fulfilled by GeneMatrix LLP, Pune, India. DNA was extracted by the service provider as per their optimized protocol D described in PrimeWay Genomic DNA Extraction Kit (KIT-9020). PCR amplification was conducted for the 16S rRNA gene using universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R to (5'-TACGGCTACCTTGTTACGACTT-3') to confirm the taxonomic identity. Sequencing was carried out using 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') primer pairs. Then, a homological comparison was performed based on basic local alignment search tool (BLAST) available in National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using Neighbour Joining Method with a maximum sequence difference of 0.75 [281–283]. The results were viewed through the BLAST Tree widget and the phylogenetic tree was obtained for identifying unknown strain(s).

2.2.5. Temperature optimization

All the strains were subjected to growth in MSM at different temperatures ranging from 20 °C to 60 °C at an increment of 5 °C. OD₆₀₀ was observed using UV-Vis Spectrophotometer (UV-2600, Shimadzu, Tokyo) after stationary phase is reached. As the real time waste condition would more or less vary between this temperature range, the growth profile was not visualized for very low and very high temperature.

2.2.6. pH optimization

All the strains were subjected to growth in MSM at different pH ranging from 4.0 to 10.0 at an increment of 0.5. OD₆₀₀ was observed using UV-Vis Spectrophotometer (UV-2600, Shimadzu) after the stationary phase was attained by all the strains when subjected to growth in MSM. As the real time waste condition would vary between this pH range, the growth profile was not visualized for very low and very high pH.

2.2.7. Tolerance to heavy metals and salinity

Typical heavy metals reported in oily sludges are Fe, Cu, Zn, Co, Pb Cr, Ni etc. [128]. The tolerance of the isolates was checked by incubating at 10-100 mg/L each of FeSO₄.7H₂O, CuSO₄.5H₂O, ZnSO₄.H₂O, CoCl₂.2H₂O, Pb(NO₃)₂, NiCl₂.6H₂O and CrO₃ supplemented MSM at an increment of 10 mg/L. Similarly, the tolerance was also checked at saline conditions of 50-500 mg/L NaCl with 50 mg/L difference. The experimentation was carried out by measuring OD₆₀₀ after 6 days. Since, the scope of our study was mostly intended towards degradation of HCs, the study was briefly summarised by weighing the growth in terms of + (OD₆₀₀ ≥ 0.1), ++ (OD₆₀₀ ≥ 0.2), +++ (OD₆₀₀ ≥ 0.3) until ++++ (OD₆₀₀ ≥ 0.5). In cases, where OD₆₀₀ < 0.1, the growth was marked negative (-). The tolerance was also deduced by observing visible growth. To limit our studies only to HCs, no significant inferences were drawn out and the experiment was carried out only for reference.

2.2.8. Maximum tolerance concentration (MTC)

The isolated strains were subjected to growth in MSM supplemented with a range of HCs concentrations. *n*-hexadecane, anthracene, phenanthrene, benzene, toluene and xylene were supplemented separately for each strain at concentrations in the range as mentioned in Table 2.1. Maximum tolerant concentration (MTC) was identified as the maximum concentration of HCs where visible growth (OD₆₀₀ ≥ 0.1) was observed.

Table 2.1: Range and classes of different HCs studied.

Pollutant	Hydrocarbon (HCs) type	Concentration range
<i>n</i> -hexadecane	Straight chain aliphatic HC.	10-1000 mg/L
Anthracene	Polyaromatic HC (PAH).	5-100 mg/L
Phenanthrene	Polyaromatic HC (PAH).	5-100 mg/L
Benzene	Single ring aromatic HC with no substitution.	10-1500 mg/L
Toluene	Single ring aromatic with methyl (–CH ₃) at 1' position	10-1500 mg/L
Xylene	Single ring aromatic with two methyl (–CH ₃) substitutions at 1' and either 3' (<i>meta</i>) or 4' (<i>para</i>) positions respectively.	10-1500 mg/L

Since, *n*-hexadecane is a long chain aliphatic compound, it is insoluble in water. Anthracene and phenanthrene, being higher classes of PAHs are also insoluble in water. Benzene, toluene and xylene were sparingly soluble in water. For all the cases, the extended aeration was provided by rotation of incubated flasks. OD₆₀₀ was measured using UV-Vis Spectrophotometer (UV-2600, Shimadzu) the end of 6 days of incubation. As discussed in section 2.3.2 afterwards, the stationary phase was attained by all the strains when subjected to growth in MSM after 6 days on incubation. Hence, OD₆₀₀ was measured after 6 days.

2.2.9. Inoculum preparation and optimization

Bacterial isolates were activated by transferring a loopful of culture from NA plates into NB medium and were induced in HC environment by supplementing NB medium with 1% of the HCs (*n*-hexadecane/anthracene/phenanthrene/benzene/toluene/xylene). All the isolated strains were subjected to growth separately in *n*-hexadecane/anthracene/phenanthrene/benzene/toluene/xylene supplemented MSM at concentrations previously shown in the Table 2.1. Inoculation was carried out from the induced activated culture discussed earlier at different inoculum concentrations on volume by volume (v/v) measurements as 1%, 2%, 3%, 4% and 5% of growth medium (i.e. MSM supplemented with different concentrations of HCs). Cell density was measured for all the set of inoculum and HCs concentrations as OD₆₀₀ using UV-Vis Spectrophotometer (UV-2600, Shimadzu) over an interval of 3 days till two successive evidence of decayed growth was observed. By this time, the cells were assumed to have attained the death phase and the experiment was discontinued.

2.2.10. Shake flask growth study in hydrocarbon(s) (HCs)

Batch study was carried out to check the growth of the strains at different HC concentrations as discussed in the section 2.2.9 above along with the best inoculum concentration obtained from the above study in terms of maximum cell density and the total time the strain could survive in HC supplemented MSM. The strains were inoculated using optimum inoculum (as discussed later in section 2.3.9) in different concentration of the HCs separately. The growth profile was measured as OD₆₀₀ using UV-Vis Spectrophotometer (UV-2600, Shimadzu) over an interval of 3 days till the death phase was observed (similarly as described in the section 2.2.9 above). CFU/mL was also calculated to validate the presence of viable cells in HC supplemented MSM. Biomass concentrations were also measured to obtain the profile of biomass change at each interval of time period of incubation.

To attain maximum bioavailability, and to check maximum degradation efficiency, extended aeration was provided, and hence, volatilization of the hydrocarbons, especially aromatic, could not be separately controlled. To address this limitation, an uninoculated abiotic control was set up and the microbial growth, biomass concentration and COD removal was compared to this control results each time the samples were analysed at regular intervals of time.

2.2.11. Estimation kinetic parameters

The kinetic parameters half-saturation constant (K_s), maximum specific growth rate (μ_m), and substrate inhibition constant (K_i) were calculated by fitting the values of specific growth rate into Monod's/ Andrew's type equation expressed in Equation 2.3 and Equation 2.4 respectively.

$$\mu = \frac{\mu_m [S]}{K_s + [S]} \quad \text{Equation 2.3}$$

$$\mu = \frac{\mu_m [S]}{K_s + [S] + \frac{[S]^2}{K_i}} \quad \text{Equation 2.4}$$

Where,

μ_m = Maximum specific growth rate (time⁻¹),

$[S]$ = Initial substrate concentration (mg/L), and;

K_s = Half-saturation constant (mg/L),

K_i = Substrate Inhibition constant (mg/L).

2.2.12. COD removal of hydrocarbons(s) (HCs)

COD removal for each of the HCs were checked by closed reflux colorimetric method according to EPA 410.4, Revision 2.0 (1993) and ASTM D1252-06 (2020) [284–286]. The calibration curve was prepared according to the method described in SM5220D [274] except glucose was prepared as standard. Stock glucose solution was prepared by dissolving 100 mg glucose in reagent water and diluting upto final volume of 100 mL. 1 L = 1.067 mg COD. Standard potassium dichromate digestion mixture was prepared by dissolving 0.0334 M $K_2Cr_2O_7$, 16.7 mL H_2SO_4 and 3.3 g $HgSO_4$ in reagent water. Final volume was adjusted to 100 mL. Catalyst solution was prepared by dissolving 5 g Ag_2SO_4 in 500 mL H_2SO_4 . Briefly, to 2.5 mL sample, 1.5 mL of digestion mixture and 3.5 mL catalyst solution was added in COD vial and was heated for 55 minutes at 150 °C. After cooling and precipitating down the mixture, absorbance was measured at 600 nm. COD was measured by interpolating the standard curve (Appendix Figure 7).

2.3. Results and discussion

Physico-chemical analysis of refinery waste sludge

The sludge sample was obtained as semi-solid cake. The estimated parameters and characteristics are depicted in Table 2.2.

Table 2.2: Physico-chemical characteristics of refinery waste sludge.

Parameters	Value	Anions and metal ions	
Temperature	29.8 ± 2.23 °C	TPH	27.4 (% w/w)
pH	6.67 ± 0.32	TN	445.4 ± 14.06 mg/L
Density	1.24 g/mL	Sulfate (SO_4^{2-})	198 ± 0.21 mg/L
ORP	-152.3 ± 1.4 mV	Chloride (Cl^-)	306 ± 0.31 mg/L
Conductivity	2.4 ± 0.22 S/m	Phosphate (PO_4^{3-})	8.447 ± 0.05 mg/L
Moisture	56.8 ± 3.71 % (wet sludge)	Chromium (Cr)	3.1 ± 0.07 mg/L
content	5.3 ± 1.1 % (dried sludge)	Total bacterial count	$4.4 \pm 0.23 \times 10^8$ CFU/mL
COD	46017 ± 305.22 mg/L	TSS	9806.57 mg/L

The pH of the sludge (sludge: water suspension) was found to be 6.67 ± 0.32 and temperature was observed as 29.8 ± 2.23 °C. This was in close range as observed by [93,100]. Both pH and temperature were measured on the spot during the collection of the sample. pH

suggested that the sludge sample was acidic in nature. The moisture content of the sludge (both wet and dried) was obtained as $56.8 \pm 3.71\%$ and $5.3 \pm 1.1\%$ respectively. Moisture content was an indicative of heating values of the sludge [205]. Apparent density of the sludge was found to be 1.24 g/mL that validated that the sludge had density more than water and it was taken out as suspension from the bottom of the tank. Both high TSS and COD values were quite above the limit value set by World Health Organisation (WHO) and indicate high pollution levels and require pretreatment. Although toxicity not just depends on TSS and COD, but also on other components in refinery waste such as heavy metals, aromatic HCs. Regulatory organizations set disposal guidelines for all classes of pollutants alongwith TSS and COD to minimize their environmental impact. However, sludge toxicity assessment also involves analysing pollution load and presence of higher concentration of particulate matter is indicative of high TSS which directly affects the concentration of organic and inorganic contaminants as depicted by high COD. Hence, it can be stated that TSS and COD values are associated and can signify toxicity [287,288].

High COD value confirmed higher chances of the waste streams to cause both organic as well as inorganic pollution in the final discharging water bodies and soil. [213,289]. This could result in eutrophication and anoxia in water bodies, reduction of marine organisms etc. Conductivity value suggested free ions available which could mean the incorporation of flocculation and coagulation treatment techniques. Negative ORP confirmed the reducing environment prevailing at the contaminated site. Conductivity value was in close range to the value obtained of the waste from a site in Jorhat, Assam, India [46] which was an indicative of the presence of enough cations and anions, heavy metals etc. [93]. TPH was found to be as $\sim 27.4\%$ of the total sludge content which was less than that obtained by Das and Kazy, 2014 ($\sim 54\%$).

N, P and sulfate concentrations were well above the permissible limits as per the restrictions set by WHO. Chloride content found out in this study (306 ± 0.31 ppm). High N and P content were indicative of high nutrient availability. It could be expected that the concentration of phosphate in the sludge at the bottom most part of the tank could be the minimum and that at the top was maximum. On the other hand, N could be available as nitrates or nitrites that plants absorb [290]. Similar to phosphates, presence of sulfates also in the tank bottom sludge also indicated shallow age of the sludge. Overall, TN was found to be 445.4 ± 14.06 mg/L signifying the abundance of nutrients for microbial growth. In our current sludge

sample, COD:N is 103:1 whereas the ideal growth conditions are generally reported as 100:5 to support microbial growth [176,291]. However, considering our microbial count $>10^8$ cells and comparing with some existing reports, lesser CFU/g was observed. Although nutrient conditions were comparable, it proved abundant for growth of indigenous microbes in this sample [292–294].

Higher bacterial count was obtained along with higher COD value confirmed the presence of higher concentration of pollutants and thus require treatment before disposal. SVI was found to be 64.83 mL/mg suggesting that the sludge could occupy 64.83 mL volume per mg of suspension after 30 minutes settling. SDI was calculated to be 1.54 mg/mL. Among the heavy metals, chromium was found to be higher than as prescribed by USEPA (0.1 mg/L), WHO (0.03 mg/L) and also in Bureau of Indian Standards (BIS) IS - 10500:2012 in its second revision (0.05 mg/L). Presence of chromium could be attributed to the fact that the refinery waste under study could be considered the category of scheduled waste SW204 [38,295]. At a glance, the physico-chemical environment of the sludge has slightly acidic with moderate temperature.

2.3.1. Isolation of bacteria from waste

The enrichment and isolation procedure followed according to resulted in a total of 7 strains (Figure 2.1). The isolates were chosen based on the morphology of the distinct colonies that were obtained post incubation in oily sludge-supplemented NB followed by spread-plate technique in NA plates. Initially the isolates were tentatively named as GR1, GR2, GR3, GR4, GE1, GE2 and GS1 in the order of the isolation. These were used initially until the 16S identification was carried out.

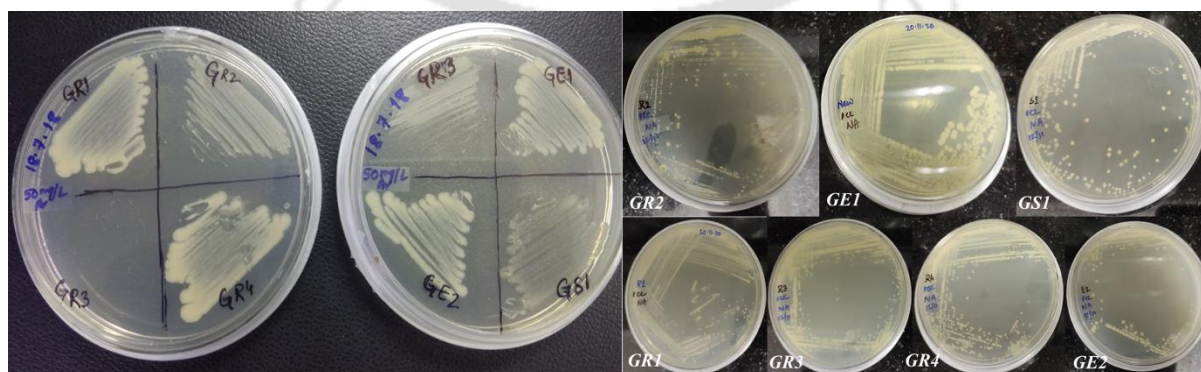


Figure 2.1: Isolated strains from refinery waste sludge. Pure colonies were obtained after observing distinct colonies following the enrichment of nutrient media. The strains were tentatively marked as GR1, GR2, GR3, GR4, GE1, GE2 and GS1.

2.3.2. Growth profile in nutrient broth (NB) and minimal salt media (MSM)

Growth of microorganisms were observed to understand the growth pattern of each of the isolated strains as bacterial growth and the time of their viability is an essential factor for any biodegradation study [280]. The growth of the respective strains is illustrated in Figure 2.2.

In NB, the growth of the strains was in the order $GR2 > GE2 > GS1 > GR1 > GE1 > GR4 > GR3$ whereas in MSM, the growth was $GE1 > GR2 > GE2 > GS1 > GR4 > GR1 > GR3$. The maximum CFU/mL in nutrient media was attained by GR2 (2.1×10^9 CFU/mL) and then the growth was steady. The biomass at this time was found to be 3.69 mg/mL. In case of MSM, maximum CFU/mL was attained by GE1 and GR2 ($\sim 1.9 \times 10^6$ CFU/mL) before stationary phase was observed. The biomass concentration at this time was found to be 0.07 mg/mL.

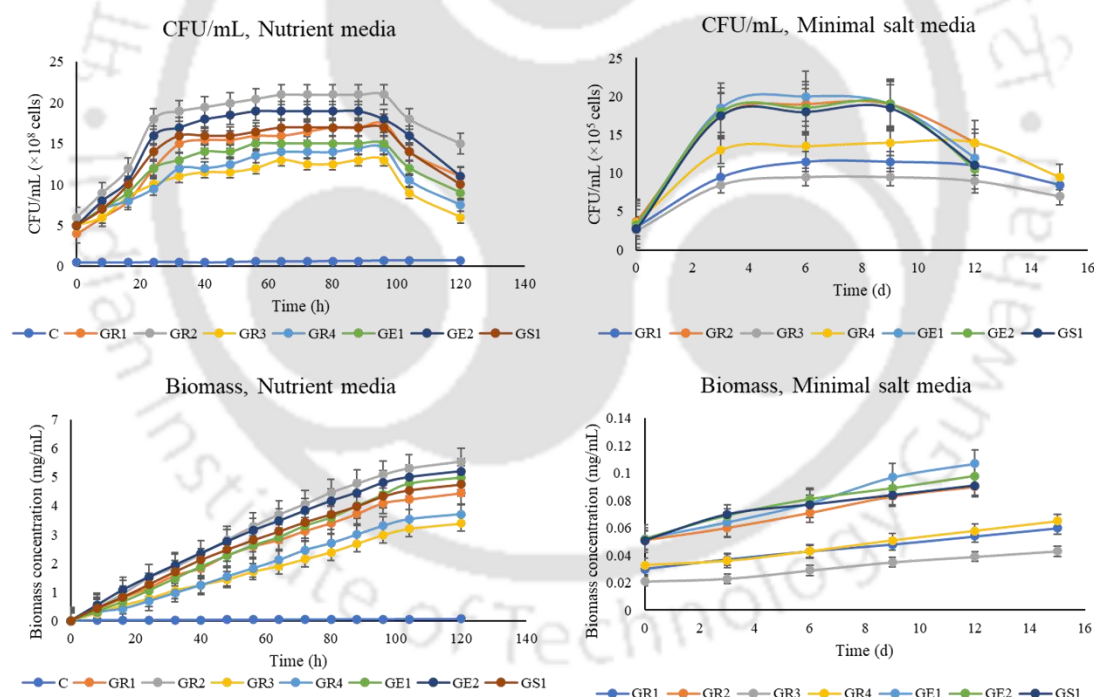


Figure 2.2: Growth profile of isolates in NB and MSM. The CFU/mL and biomass concentration were compared both for NB and MSM. Alongwith CFU/mL, the biomass concentration was significantly lower in MSM depicting sluggish growth as compared to NB. The growth cycle was lower in case NB (96 hours) in contrary to 9 days in MSM. This is attributed to nutrient availability and enhanced metabolisms by the isolates upon incubation NB. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Likewise, the minimum CFU/mL was found to be GR3 in both nutrient media and MSM (8.8×10^7 CFU/mL and 6.5×10^6 CFU/mL respectively). The biomass at this time were

0.837 mg/mL and 0.023 mg/mL for NB and MSM respectively. In nutrient media, the stationary phase was reached at the end of 24 hours that continued till 96 hours of incubation and then decreased whereas in MSM, the exponential growth continued till 6 days and the stationary phase continued till 15 days of incubation and decreased thereafter.

2.3.3. Estimation of specific growth rate

The specific growth rate was in the order $GR2 > GS1 > GE2 > GE1 > GR1 > GR4 > GR3$ in nutrient media whereas that in MSM was $GR2 > GE1 > GS1 > GE2 > GR1 > GR4 > GR3$ which is almost close to the growth order both in NB and MSM. The maximum specific growth rate (of GR2) was found to be 0.071 h^{-1} and 0.029 d^{-1} in nutrient media and MSM respectively and the minimum specific growth rate (of GR3) was found to be 0.022 h^{-1} and 0.01 d^{-1} in nutrient media and MSM respectively. Similar values were also reported where new strains were isolated [296], however, our reported values were lower than that reported elsewhere [297,298].

All the observed characteristics of the strains are tabulated in Table 2.3. It was inferred that the strains could grow very well when there is a C-source available for consumption.

Table 2.3: Typical physiological characteristics of isolated strains.

Test parameter	GR1	GR2	GR3	GR4	GE1	GE2	GS1
Gram staining	–	+	–	–	+	–	+
Cell diameter > 1.0 m	–	–	–	–	–	–	–
Colony shape in NA plate	O	R	I	O	R	O	O
Morphology under microscope	O	CR	NC	O	R	O	R

Symbols: + Characteristics positive; – Characteristics negative; CR = Coccus rod; NC = Not clear; O = Oval, I = Irregular; R = Round

The cells feed on the C-source and increase in numbers till at stationary phase when the nutrient was completely finished. The cells and endogenously respired and competed for their survival afterwards. On the other hand, when there was no C-source available in MSM, the cells had no nutrient to feed on except for the mineral salts. The cells took more time to acclimatise to the mineral salt environment and hence the growth cycle was more than that in case of NB medium.

2.3.4. Morphological identification of isolated strains

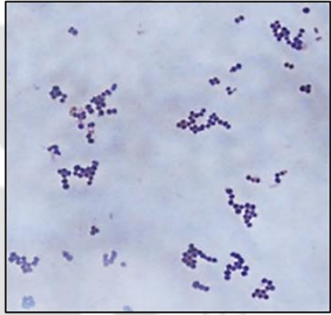
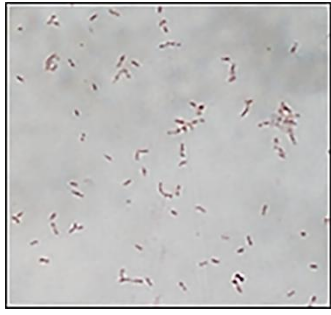
Gram staining images are shown in Table 2.4. Except GR2, GE1 and GS1, all the other strains were gram negative. The morphology of the strains GR1, GR4, GE2 and GS1 were oval shaped, motile and irregular. GR2 was rod-shaped, GE1 was round whereas that of GR3 was not clearly visualized. However, colonies of all the strains were observed as pale white colour irregular flat round shaped colonies. The colonies looked translucent, auriculate and smooth that described the general morphology of any isolated bacterial strain from any source [272]. All the strains showed colony of size 0.5-1.5 mm upto 48 hours of incubation in NA plates. Similar size range was also observed by Liu et al., 2014 which confirmed the precision and accuracy of the method of isolation of the new strains [289].

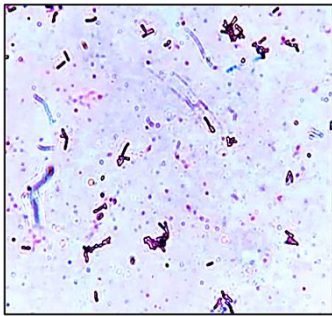
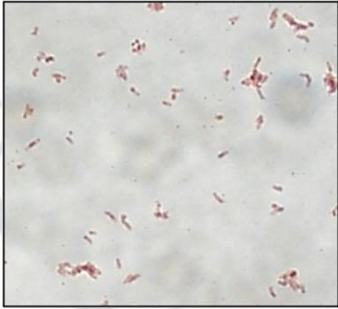
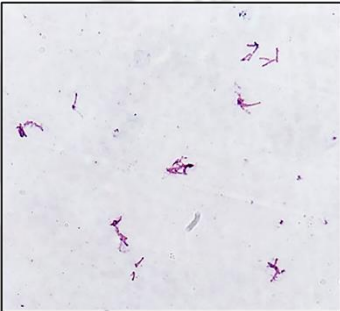
PCR amplification was conducted for the 16S rRNA gene using universal primers 27F and 1492R [281]. Then, a homological comparison was performed based on BLAST and the results indicated that the sequence of the strain showed more than 99% homology with *Aquamicrobium sp.* Subsequently, a phylogenetic tree was constructed using similar 16S rRNA sequences with neighbour-joining methods. It indicated that strain GR1, GR4 and GE2 had the closest relative with *Aquamicrobium sp.* For the other strains GR2, GR3, GE1 and GS1, similar studies were carried out. All these classes of microbes are extensively reviewed and reported by investigators and are considered potent candidates for degradation of xenobiotic compounds [293,299]. All the observed characteristics of the strains are tabulated in Table 2.4 and Appendix Table 1. From here on, unless mentioned or required, we use to address the identified strains with nomenclatures as *Aquamicrobium sp.* GR1 for GR1, *Staphylococcus sp.* GR2 for GR2, *Aquamicrobium sp.* GR3 for GR3, *Bacillus sp.* GE1 for GE1, *Aquamicrobium sp.* GE2 for GE2 and *Bacillus sp.* GS1 for GS1.

Aquamicrobium sp. are rod-shaped gram-negative bacteria and are generally found in aquatic environments. Although considered a potential candidate in utilizing diverse C-sources, the report of their abundance is scanty in a typical refinery waste. They occur as non-pathogenic and can survive both in aerobic and anaerobic conditions. Due to their exhibition of various metabolic pathways and ability to breakdown different organic C-sources, application in PAHs removal, nitrate reduction, sulfide breakdown and esterase enzyme production have been reported [300, 301, 302]. *Bacillus sp.* also occur as rod-shaped structures under microscope and are classified as gram positive bacteria. *Bacillus sp.* is also considered facultative anaerobes and are capable of forming endospore for protection from harsh environmental conditions.

Similar to *Aquamicrobium sp.*, *Bacillus sp.* also produce enzymes to metabolize C-sources. Due to their versatility and abundance, investigators utilize the potential for bioremediation of refinery waste. Some of the advanced studies report *Bacillus sp.* to be an excellent agent for bioaugmentation aiding in enhanced TPH and COD removal in addition to significant reduction of BOD, TDS etc. [303, 304].

Table 2.4: Characteristics of isolates.

Isolate	16S rRNA identification	Specific growth rate	Gram stain
GR1	<i>Aquamicrobium sp.</i>	NB: 0.041 h^{-1} MSM: 0.017 d^{-1}	
GR2	<i>Staphylococcus sp.</i>	NB: 0.071 h^{-1} MSM: 0.029 d^{-1}	
GR3	<i>Aquamicrobium sp.</i>	NB: 0.021 h^{-1} MSM: 0.01 d^{-1}	
GR4	<i>Aquamicrobium sp.</i>	NB: 0.033 h^{-1} MSM: 0.014 d^{-1}	

GE1	<i>Bacillus sp.</i>	NB: 0.053 h ⁻¹ MSM: 0.022 d ⁻¹	
GE2	<i>Aquamicrobium sp.</i>	NB: 0.059 h ⁻¹ MSM: 0.019 d ⁻¹	
GS1	<i>Bacillus sp.</i>	NB: 0.062 h ⁻¹ MSM: 0.025 d ⁻¹	

*NB = Nutrient broth; MSM: Minimal salt medium

Staphylococcus sp. are gram positive, non-motile, spherical structures, often occurring as irregular clusters. Similar to *Bacillus sp.*, *Staphylococcus sp.* is also capable in growing in presence or absence of oxygen. Generally, lesser reported in refinery waste, the change in external environmental condition create a negative impact of the microbiological abundance [305, 306]. Except for strains like *Staphylococcus aureus*, others are generally not harmful. Several investigators report the applications of *Staphylococcus sp.* related to petroleum waste treatment which include a salt tolerating strain involved in removal of organics and N, hydrocarbon degradation etc. [307, 308].

There are various classes of strains that are isolated from sludge. Microbial diversity varies depending on the location it is collected, the time period of exposure to external disturbances and the inherent microbiological construct of the contaminated site. *Pseudomonas sp.* and *Bacillus sp.* are readily reported by investigators [93,136,168,309,310] alongwith

Acinetobacter sp., *Acrobacter sp.*, *Achromobacter sp.*, *Aerococcus sp.*, *Micrococcus sp.*, *Klebsiella*, *Enterobacter*, *Stenotrophomonas sp.* etc. [46,93,139,168,183,289]. However, keeping in view of our interest in studying HC utilization, indigenous microbes were harnessed for understanding their growth and efficiency of COD removal.

2.3.5. Temperature optimization

Figure 2.3 shows the growth of the strains in the temperature ranging from 20 °C to 60 °C. The highest cell density was observed for all the strains at 35 °C except *Aquamicrobium sp.* GR3 which showed highest growth at 30 °C. To maintain uniformity, an average value of 33 ± 2 °C was chosen as the optimum temperature for our experiments. Temperature is always considered as an important parameter for microbial growth, and similar range of optimal conditions for new isolates have been reported too [71,311,312].

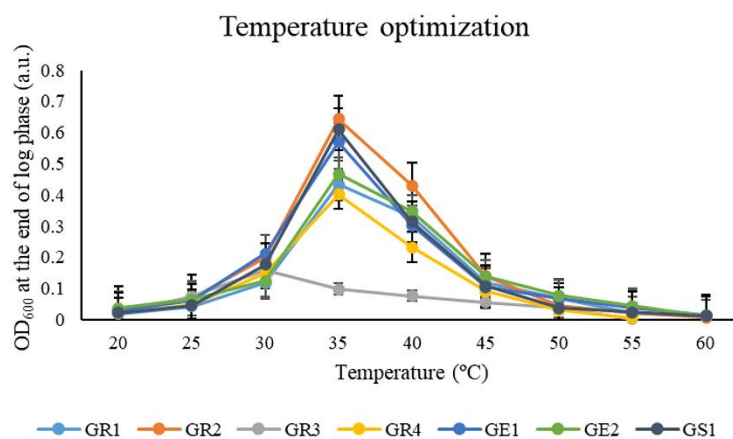


Figure 2.3: Temperature optimization for growth of isolates. Except *Aquamicrobium sp.* GR3, all other isolates showed maximum growth at 35 °C. Average value of 33 ± 2 °C was chosen as the optimum temperature to maintain uniformity in experimentation. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

2.3.6. pH optimization

Figure 2.4 shows the growth profile of all the strains from pH range 4.0-10.0. The highest cell growth was observed at pH 7.0 by *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and *Bacillus sp.* GS1 and the other strains showed highest growth at pH 6.5.

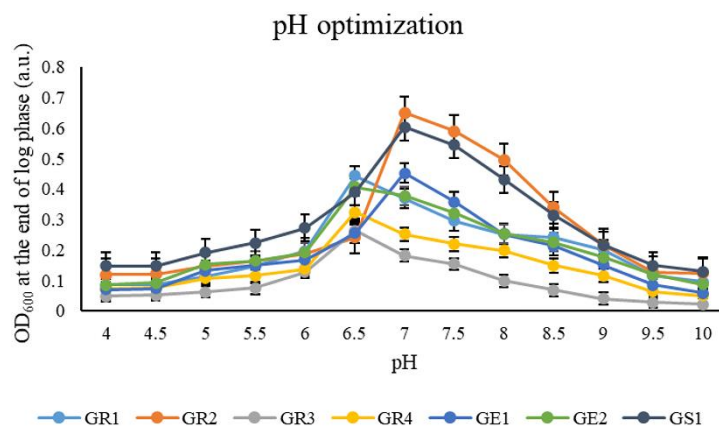


Figure 2.4: pH optimization of the isolates. *Staphylococcus sp.* GR2 and *Bacillus sp.* GE1/GS1 exhibited maximum growth at pH 7.0, whereas all others pH 6.5 as the optimum. Accordingly, average value of 6.75 ± 0.25 was chosen as an optimum pH to minimize experimental error. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

This showed that the isolates could not grow at very highly acidic or basic medium. Typically, similar pH ranges are reported and are considered as best for microbial growth [291,313]. Where all the *Aquamicrobium sp.* GR1/GR3/GR4/GE2 exhibited appreciable growth at moderately low pH, other strains grew at fairly neutral pH. The range of growth of the isolated strains were in pH 6.5 to 7.0. To minimize experimental error and as measure of uniformity, the average value to 6.75 ± 0.25 was chosen as the optimum pH.

2.3.7. Tolerance to heavy metals and salinity

Table 2.5 illustrates the tolerance of the isolates at 50 mg/L of heavy metals and 250 mg/L NaCl. Except *Aquamicrobium sp.* GR3, all the other isolates showed significant tolerance in different heavy metals and saline conditions. *Bacillus sp.* GE1 and GS1; and *Staphylococcus sp.* GR2 showed exceptional survivability in 250 mg/L NaCl owing to their capability to exist in stressful environmental conditions. A summary of OD₆₀₀ obtained against other concentrations of heavy metals and salinity are listed in Appendices.

Table 2.5: Tolerance of isolates to heavy metals and salinity.

Heavy metals/Salinity	GR1	GR2	GR3	GR4	GE1	GE2	GS1
Co	+	++	—	+	++	+	++
Cr	+	+	—	—	++	—	++
Cu	+	+	—	+	+++	+	++
Fe	++	++	+	++	+++	+	+++
Ni	—	—	—	—	—	—	—
Pb	—	+	—	—	+	—	++
Zn	+	+	—	+	++	+	+
NaCl	+	+++	+	++	++++	++	+++

There are reports where higher tolerance to heavy metals (> 500 mg/L) and salinity (> 50 g/L) are also exhibited by strains obtained from waste [314–316], however, growth was diminished ($OD_{600} < 0.05$) for all the isolates above our tested condition (> 50 mg/L heavy metals; > 250 mg/L NaCl). For *Aquamicrobium sp.* GR3, no growth was observed at all.

2.3.8. Maximum tolerance concentration (MTC)

Figure 2.5 shows the growth of all the strains in MSM supplemented with *n*-hexadecane with concentration ranging from 10 mg/L to 1000 mg/L.

The study suggested *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1 significant visible growth at 500 mg/L *n*-hexadecane and could grow steadily in the concentration range 10-250 mg/L. *Bacillus sp.* GS1 alongwith *Staphylococcus sp.* GR2 exhibited highest cell growth at 250 mg/L *n*-hexadecane and appreciable growth was observed at concentration 100-250 mg/L. *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 showed steady growth in the range of 50-200 mg/L *n*-hexadecane, *Bacillus sp.* GE1 exhibiting lesser cell density than *Aquamicrobium sp.* GE2, 100 mg/L *n*-hexadecane being their highest concentration to growth. On the other hand, *Aquamicrobium sp.* GR1/GR3/GR4 also showed maximum growth at 10 mg/L.

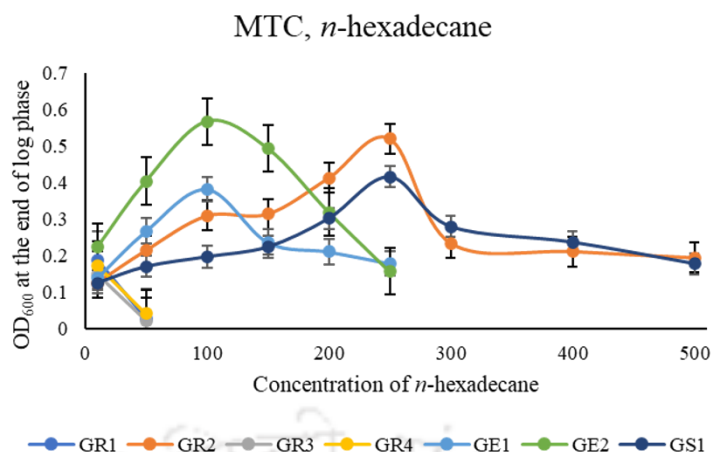


Figure 2.5: MTC of the isolates in *n*-hexadecane. Maximum visible growth was observed at 10 mg/L *n*-hexadecane for *Aquamicrobium* sp. GR1/GR3/GR4. Similarly, for *Bacillus* sp. GE1 and *Aquamicrobium* sp. GE2, MTC was deduced as 250 mg/L *n*-hexadecane, whereas for *Staphylococcus* sp. GR2 and *Bacillus* sp. GS1, the same was 500 mg/L. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Hence, it could be concluded that MTC of *Aquamicrobium* sp. GR1/GR3/GR4 was found to be lower as 10 mg/L *n*-hexadecane. However, the MTC 100 mg/L for *Bacillus* sp. GE1 and *Aquamicrobium* sp. GE2 for *n*-hexadecane, and finally 500 mg/L for *Staphylococcus* sp. GR2 and *Bacillus* sp. GS1 suggesting better capability [310].

For anthracene (Figure 2.6(a)), it was seen that the growth of all the strains in MSM supplemented with anthracene/phenanthrene with concentration ranging from 5 mg/L to 100 mg/L.

Bacillus sp. GE1 showed highest growth at 30 mg/L anthracene, *Aquamicrobium* sp. GR1/GE2, *Staphylococcus* sp. GR2 and *Bacillus* sp. GS1 showed highest growth at 10 mg/L anthracene, whereas *Aquamicrobium* sp. GR3/GR4 could sustain a maximum of 5 mg/L anthracene.

The maximum concentration at which visible growth was observed *Aquamicrobium* sp. GR3 and GR4 was 10 mg/L. *Aquamicrobium* sp. GR1 and GE2, *Staphylococcus* sp. GR2, and *Bacillus* sp. GS1 could exhibit minimum visible growth upto concentration 20 mg/L. *Bacillus* sp. GE1 showed highest resistance at 50 mg/L anthracene. The tolerance was lower than that reported elsewhere, and the isolates could be lesser acclimatized to growth in PAHs [168,317].

On the other hand, for phenanthrene (Figure 2.6(b)), except *Bacillus* sp. GE1 which showed maximum growth at 10 mg/L phenanthrene, all other strains showed minimum visible

growth at only 5 mg/L phenanthrene. *Bacillus sp.* GE1 showed minimum visible growth at 20 mg/L phenanthrene.

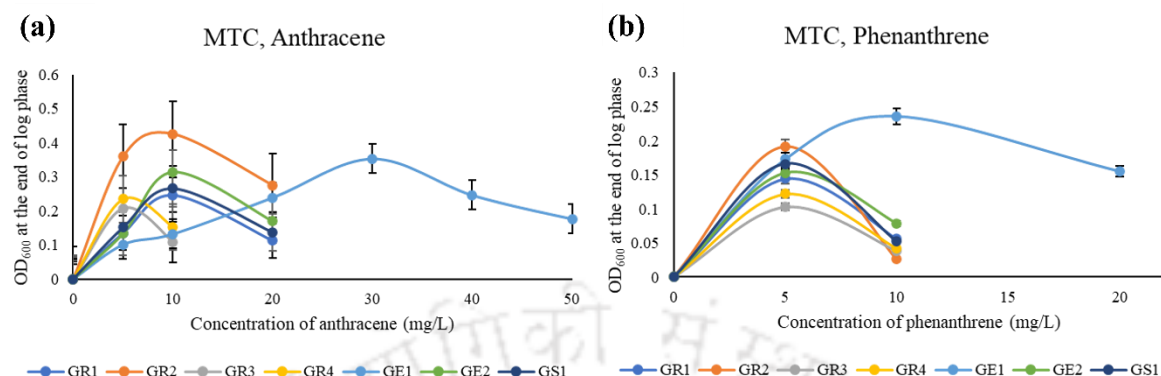


Figure 2.6: MTC of the isolates in (a) anthracene; (b) phenanthrene. On account of maximum visible growth, MTC of 50 mg/L anthracene and 20 mg/L phenanthrene was obtained for *Bacillus sp.* GE1. For others MTCs collectively ranged within 10-20 mg/L anthracene and phenanthrene. Error bars indicate the standard deviation (±SD) of three experimental replicates.

The same study was carried for similar concentration range of benzene/toluene/xylene (Figure 2.7(a), (b) and (c)). All the strains showed highest growth at 500 mg/L benzene/toluene/xylene and could grow steadily in the concentration range 100-600 mg/L. The minimum visible growth for all the strains was observed at 1000 mg/L benzene/toluene/xylene.

Studies have been carried out elsewhere where microbial growth has been reported within such range of BTX concentrations [297,311]. Hence, it could be concluded that MTC of GE1 was found to be 50 mg/L anthracene followed by GR1, GR2, GE2 and GS1 (20 mg/L anthracene). MTC of GR3 and GR4 was found to be 10 mg/L anthracene. For phenanthrene, except for GE1 (MTC = 10 mg/L), all others showed MTC of 5 mg/L. MTC of all the strains in benzene/toluene/xylene was found to be 1000 mg/L.

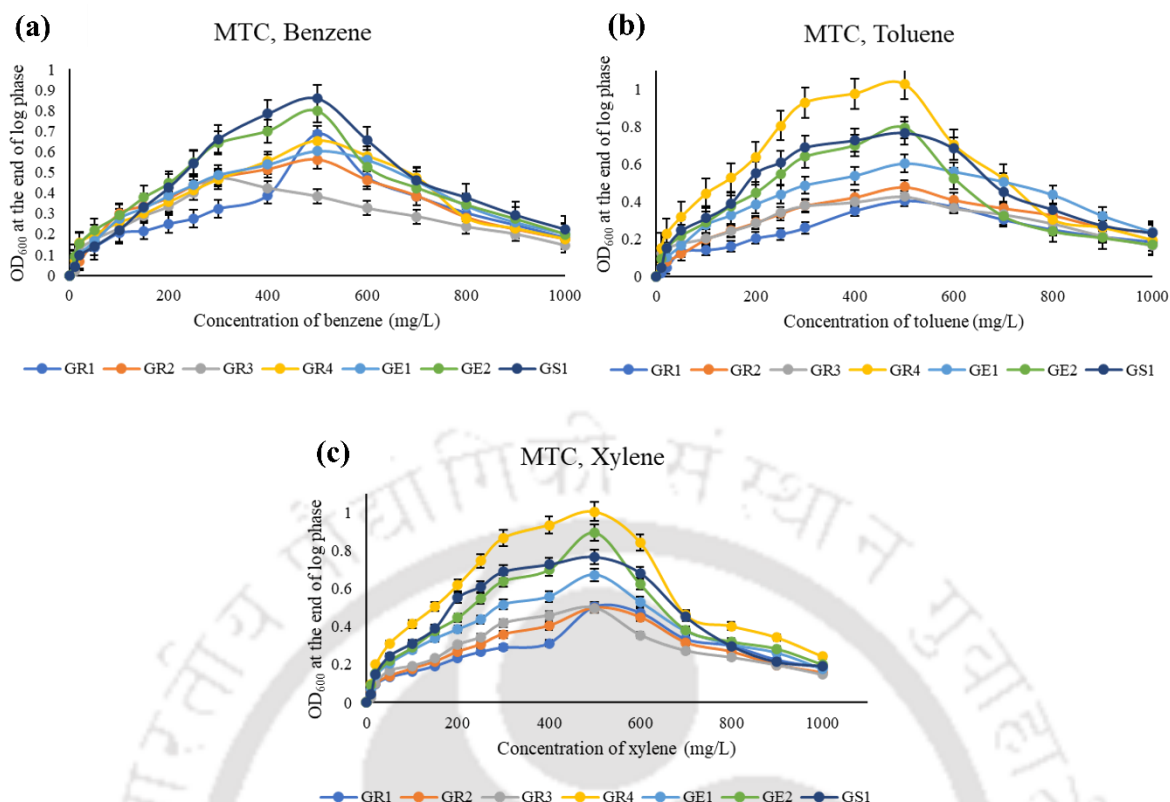


Figure 2.7: MTC of the isolates in (a) benzene; (b) toluene; (c) xylene. For all the isolates in BTX, MTC was obtained as 1000 mg/L benzene/toluene/xylene. The maximum visible growth occurred upon incubation in these pollutants at the said concentration. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For *n*-hexadecane (Figure 2.8), all the strains were subjected to growth in MSM supplemented with MTC values of *n*-hexadecane separately with 1%, 2%, 3%, 4% and 5% inoculum that was prepared in NB supplemented with 1% HC (induction of culture in HC (*n*-hexadecane) environment). The optimization for each strain was carried out at their respective MTC values as discussed in the earlier section 2.3.8.

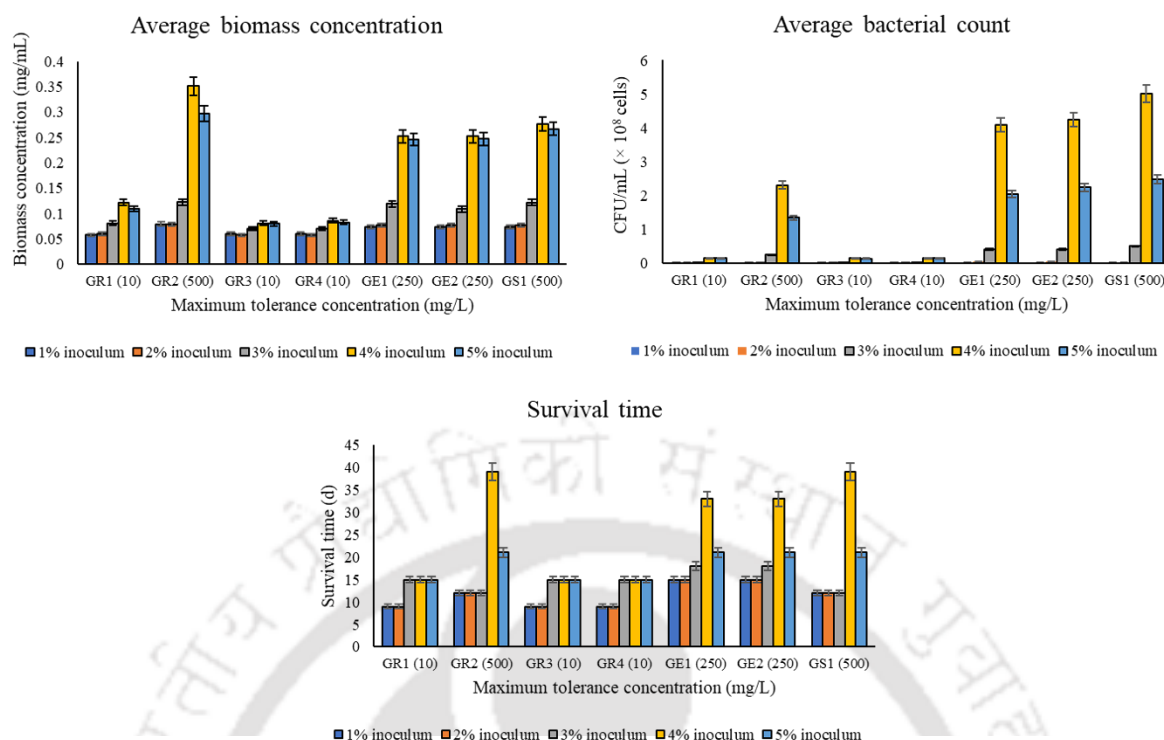


Figure 2.8: Inoculum optimization for growth in *n*-hexadecane supplemented MSM. The maximum survival alongwith biomass concentration and bacterial count was observed at 4% (v/v) inoculum following which the growth was reduced, and survival time was lesser. On account of the observation, 4% initial inoculum (v/v) was chosen as the optimum value. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The average bacterial count and biomass concentration for *Aquamicrobium sp.* GR1, GR3 and GR4 at 1% inoculum was approximately 1.75×10^5 CFU/mL and 0.067 mg/mL respectively and that for *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1, and *Aquamicrobium sp.* GE2 at 1% inoculation was approximately 2.25×10^6 and 0.085 mg/mL respectively. The cells of *Aquamicrobium sp.* GR1, GR3 and GR4 could survive the most for 15 days in 10 mg/L *n*-hexadecane. *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 exhibited best survival time in 250 mg/L *n*-hexadecane (15 days), and then *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1 showed survival time of 12 days in 500 mg/L *n*-hexadecane. Similar results were obtained at 2% inoculum also where average bacterial count and biomass concentration for *Aquamicrobium sp.* GR1, GR3 and GR4 was found to be 1.85×10^5 CFU/mL and 0.072 mg/mL and that of *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1, and *Aquamicrobium sp.* GE2 was found to be 2.45×10^6 CFU/mL and 1.00 mg/mL respectively. Similar to 1% inoculum, the strains *Aquamicrobium sp.* GR1, GR3 and GR4 survived for 12 days, *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 in 250 mg/L for 10 days and *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1 could survive for 18 days when incubated at their respective MTC. For 3% inoculum, the bacterial count and biomass concentration increased 10 times but was still

not in range of bacterial count that was obtained/required for sludge (i.e. 4.4×10^8 CFU/mL). The average bacterial count and biomass concentration was obtained as 1.95×10^6 CFU/mL and 0.083 mg/mL respectively for *Aquamicrobium sp.* GR1, GR3 and GR4 and that for *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1, and *Aquamicrobium sp.* GE2 was found to be approximately 2.5×10^7 CFU/mL and 0.119 mg/mL respectively. The survival time though, apart from *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1, increased to 15 days for *Aquamicrobium sp.* GR1, GR3 and GR4 and 18 days for *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2, as compared to what was found out in case of 1% and 2% inoculum.

In case of 4% inoculum, the bacterial count increased significantly as compared to 3%. The average bacterial count and biomass concentration for *Aquamicrobium sp.* GR1, GR3 and GR4 was found to be approximately 1.7×10^7 CFU/mL and 0.1 mg/mL respectively whereas for *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2, it was found to be 4.6×10^8 CFU/mL and 1.66 mg/mL respectively. *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1 exhibited bacterial count to as high as 0.96×10^9 CFU/mL and biomass concentration of 3.65 mg/mL. The survival time also increased rapidly in case of 4% inoculum for *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1 at their MTC of 500 mg/L *n*-hexadecane to 42 days. However, *Aquamicrobium sp.* GR1, GR3 and GR4 could only survive the most in 10 mg/L *n*-hexadecane (18 days), whereas *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 could survive in 250 mg/L *n*-hexadecane for 33 days. In case of 5% inoculum, average biomass concentration, bacterial count and maximum survival time decreased to 0.8 mg/mL, 1.8×10^8 CFU/mL and 21 days respectively for *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1, and *Aquamicrobium sp.* GE2 at their respective MTCs. Considering the above scenario, using 4% inoculum (v/v) could be used as optimum inoculum.

For anthracene (Figure 2.9), all the strains were also subjected to growth in MSM supplemented with the respective MTCs of anthracene and phenanthrene separately with 1%, 2%, 3%, 4% and 5% inoculum that was prepared in NB supplemented with 1% HC (induction of culture in HC (anthracene/phenanthrene) environment).

Similar to the analysis done for *n*-hexadecane, the average bacterial count and biomass concentration for the cells at 1% inoculum was similar for all the strains. On an average, in anthracene was approximately 7.1×10^6 CFU/mL and 0.013 mg/mL biomass concentration were observed. The cells of *Bacillus sp.* GE1 could survive the most in 50 mg/L anthracene (6 days).

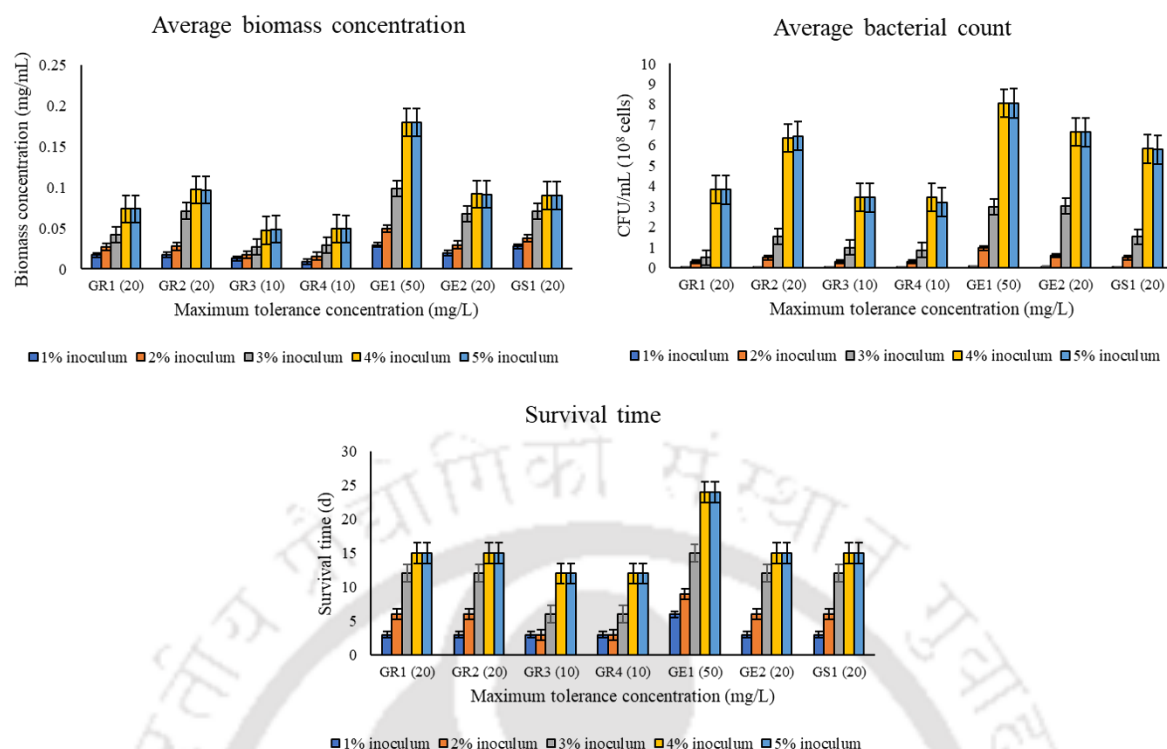


Figure 2.9: Inoculum optimization in anthracene supplemented MSM. The maximum biomass concentration, bacterial count alongwith survival time was almost identical for isolates at 4% and 5% inoculum (v/v) at the respective MTCs. Hence, 4% (v/v) inoculum was chosen as optimum for experimentation. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

At 2% inoculum also the average bacterial count and biomass concentration was found to have slightly increased to 1.85×10^7 CFU/mL and 0.036 mg/mL respectively for all the strains. Except *Aquamicrobium sp.* GR3 and GR4, all other strains could enhance their survival. The maximum survival of 9 days was obtained for *Bacillus sp.* GE1 at 50 mg/L anthracene. For 3% inoculum, the bacterial count and biomass concentration increased by 10 and 2.5 times respectively for anthracene. The average bacterial count and biomass concentration was obtained as 2.55×10^7 CFU/mL and 0.068 mg/mL respectively for anthracene. Similar to 2% inoculum, *Bacillus sp.* GE1 highest survivability of 15 days at 50 mg/L anthracene. The results obtained in case of 4% and 5% were exactly similar. The average biomass concentration and bacterial count increased to 0.135 mg/mL and 6.1×10^8 CFU/mL across all cells, so did the survival time with an average survival of 18-21 days. Hence, considering lower value of inoculum 4% exhibiting same biomass concentrations, bacterial counts and survival times as with 5%, 4% (v/v) was chosen as optimum inoculum for experimentation in anthracene supplemented MSM.

For phenanthrene, the detailed results are indicated in Appendix Figure 1. The results obtained for 4% were exactly similar to 3%, and hence, similar to the case of anthracene, the lower value of inoculum was selected as optimum inoculum, that in case of phenanthrene was 3% (v/v).

For BTX, all the strains were subjected to growth in MSM supplemented with MTC of benzene/toluene/xylene separately with 1%, 2%, 3%, 4% and 5% inoculum that was prepared in NB supplemented with 1% HC (induction of culture in HC (benzene/toluene/xylene) environment). Results for benzene are illustrated below in Figure 2.10. The detailed results for toluene and xylene are depicted in Appendix Figure 2 and Appendix Figure 3 respectively.

The average bacterial count and biomass concentration for the cells at 1% inoculum in benzene/toluene/xylene was approximately 3.5×10^7 CFU/mL and 0.015 mg/mL. Except *Aquamicrobium* sp. GE2, which could survive for 15 days, all the other cells could survive the most for 12 days in 1000 mg/L benzene. Similar results were obtained at 2% inoculum also where average bacterial count and biomass concentration was found to be 3.85×10^7 CFU/mL and 0.0162 mg/mL for benzene/toluene respectively. However, bacterial growth and biomass concentration increased rapidly in case of xylene to upto 40 times and 3 times respectively. The survival time also increased to a maximum of 21 days for *Aquamicrobium* sp. GR1, GR3 and GR4 and *Staphylococcus* sp. GR2 at 1000 mg/L xylene. Other strains *Bacillus* sp. GE1 and GS1 could also enhance their survival to 18 days.

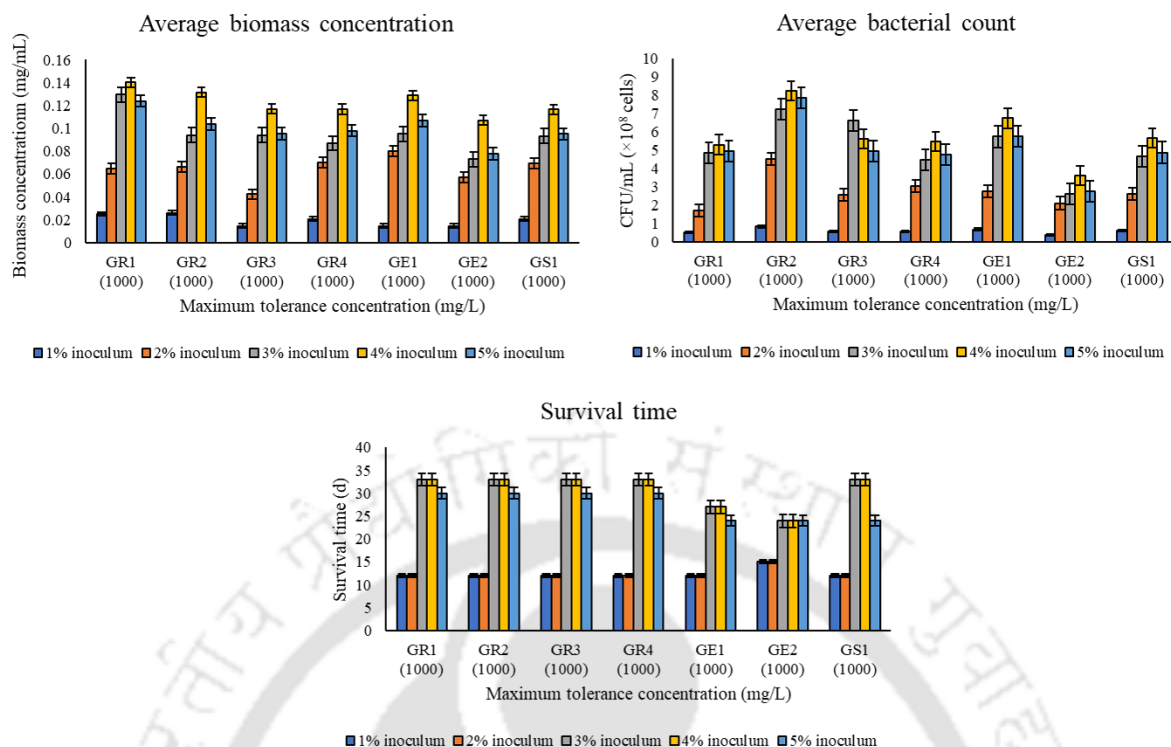


Figure 2.10: Inoculum optimization in benzene supplemented MSM. The biomass concentration, bacterial count and the survival times was maximum at 4% (v/v) and reduced significantly at 5%. Based on the observation, 4% inoculum (v/v) was chosen for further growth studies. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For 3% inoculum, the bacterial count and biomass concentration increased by 40 times and 3 times respectively for benzene and 10 times and 2.5 times for toluene. The average bacterial count and biomass concentration was obtained as 5.95×10^8 CFU/mL and 0.038 mg/mL respectively for benzene/toluene. The survival time also increased for most of the strains. The maximum survival was observed for *Bacillus sp.* GE1 and GS1 and *Aquamicrobium sp.* GE2 (33 days) for 1000 mg/L benzene/toluene. For xylene, both bacterial count and biomass concentration increased only by approximately 15%. However, the survival time increased upto a maximum of 30 days for *Aquamicrobium sp.* GR1, GR3 and GR4; *Staphylococcus sp.* GR2, and *Bacillus sp.* GS1 at 1000 mg/L xylene. *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 could exhibit maximum survival of 27 and 24 days respectively.

Similar results were obtained at 4% inoculum also where average bacterial count and biomass concentration was found to be 6.85×10^8 CFU/mL and 0.047 mg/mL for benzene/toluene respectively. The survival time also remained the same in case of 4% inoculum for benzene/toluene. In case of 5% inoculum, the strains showed detrimental effect in 1000 mg/L benzene/toluene. The average biomass concentration, bacterial count and survival time decreased and hence, was not evaluated for further studies. This similar trend was also observed

when the strains were checked at 4% inoculum for their effect in 1000 mg/L xylene. Hence, considering the scenario, 5% inoculum was not required to be studied for xylene and the optimum inoculum was chosen as 4% for benzene and toluene and 3% for xylene. All inoculum concentrations were measured on v/v basis.

Hence, from this study, it could be concluded that initial inoculum concentration is an important factor to undergo growth in HC contaminated environment. While designing an efficient consortium and in order to maximise growth, biomass concentration and survival time of the strains at different concentrations of a HC, an optimized inoculum could be helpful [291].

2.3.10. Shake flask growth study of isolated strains in different class of hydrocarbon(s) (HCs)

As described earlier, inoculum concentrations 4% (v/v) was found suitable in cases of *n*-hexadecane, anthracene, benzene and toluene whereas the optimum inoculum for phenanthrene and xylene were 3% (v/v). The respective HCs were supplemented in MSM, and the respective strains were subjected to growth in the highest MTC value obtained combined for all the strains. The same has been as discussed in detail in the earlier sections 2.3.8 and 2.3.9. Batch study was carried out for different strains. The study comprised of measurement of cell density (OD₆₀₀), bacterial count (CFU/mL) and biomass concentration (mg/mL) after each 3-day interval. The culture medium was inoculated with the optimum inoculum separately at different concentration flasks of the HCs and were incubated in an orbital shaker at 35 ± 2 °C and 170 ± 5 rpm for all strains except *Aquamicrobium* sp. GR3. The study for this strain was carried at the average optimum temperature of 33 ± 2 °C.

As depicted in Figure 2.11, with 4% inoculum, the survival time of the strains in 500 mg/L *n*-hexadecane was *Staphylococcus* sp. GR2 > *Bacillus* sp. GS1 > *Bacillus* sp. GE1 > *Aquamicrobium* sp. GE2 > *Aquamicrobium* sp. GR4 > *Aquamicrobium* sp. GR1 > *Aquamicrobium* sp. GR3. The highest bacterial count was observed in case of *Staphylococcus* sp. GR2 in 500 mg/L *n*-hexadecane (9.42×10^8 CFU/mL) and the least was observed in case of *Aquamicrobium* sp. GR3 (7.76×10^6 CFU/mL). The highest biomass content was also seen in *Staphylococcus* sp. GR2 (3.05 mg/mL) and the least in *Aquamicrobium* sp. GR3 (0.057 mg/mL). *Bacillus* sp. GE1 and *Aquamicrobium* sp. GE2 could also show satisfactory growth in 500 mg/L *n*-hexadecane despite not their MTC value.

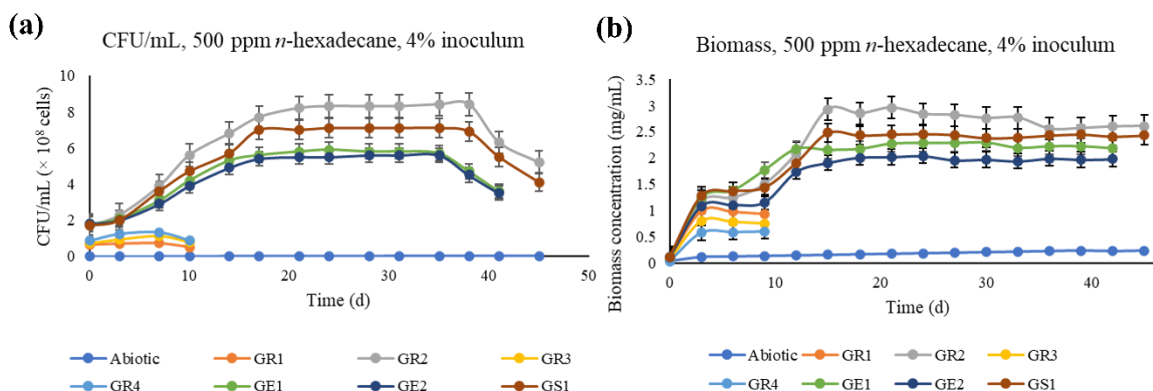


Figure 2.11: Growth of isolates with 4% (v/v) inoculum in 500 mg/L *n*-hexadecane supplemented MSM. (a) Bacterial count in terms of CFU/mL; (b) Biomass concentration in mg/mL. The strains depicted ability to survive in *n*-hexadecane media for a prolonged period of time (39 days). Higher bacterial count ($> 10^8$ cells) and biomass concentration signify the potential of the isolates for application in bioremediation of refinery waste. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

This could be attributed to the fact that the bacterial cells in the inoculum were more in number and hence could utilize the concentration of HC upto their desirable amount. The same can be seen from the survival time depicting the strains took more time to adapt to the excess nutrient condition and hence their rate of uptake were decreased. Moreover, the increased number of cells led to competition among the strains thereby consuming nutrient generating considerable biomass concentration [318,319].

For anthracene (Figure 2.12), with 4% inoculum, the survival time of the strains in 50 mg/L anthracene was *Bacillus sp.* GE1 $>$ *Aquamicrobium sp.* GE2 $>$ *Bacillus sp.* GS1 $>$ *Staphylococcus sp.* GR2 $>$ *Aquamicrobium sp.* GR1 $>$ *Aquamicrobium sp.* GR3 $>$ *Aquamicrobium sp.* GR4. The highest bacterial count was observed in case of GE1 in 50 mg/L anthracene ($\sim 9 \times 10^8$ CFU/mL) and the least was observed in case of *Aquamicrobium sp.* GR3 (3×10^8 CFU/mL). The corresponding biomass content was seen as 0.312 mg/mL and 0.032 mg/mL respectively. The other strains showed very limited growth that could be attributed to the fact that 50 mg/L was not their maximum tolerance value. However, the strains showed potential to survive in higher toxic environment considering extended exposure is provided for the strains to acclimatise [317].

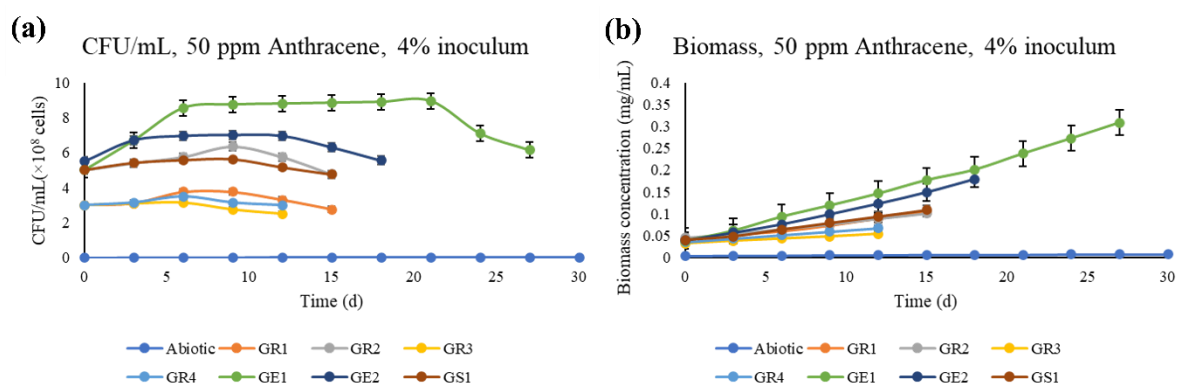


Figure 2.12: Growth of isolates with 4% (v/v) inoculum in 50 mg/L anthracene supplemented MSM. (a) Bacterial count in terms of CFU/mL; (b) Biomass concentration in mg/mL. The strains depicted ability to survive in anthracene media for a prolonged period of time (21 days). Highest bacterial count ($> 10^8$ cells) and biomass concentration signify the potential of the isolates for application in bioremediation of refinery waste. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Similarly, with 3% inoculum, the survival time of the strains in 20 mg/L phenanthrene (Appendix Figure 4) was *Bacillus sp.* GE1 $>$ *Aquamicrobium sp.* GE2 $>$ *Bacillus sp.* GS1 $>$ *Staphylococcus sp.* GR2 $>$ *Aquamicrobium sp.* GR1 $>$ *Aquamicrobium sp.* GR3 $>$ *Aquamicrobium sp.* GR4. As also evident the highest bacterial count was observed in case of *Bacillus sp.* GE1 in 20 mg/L phenanthrene ($\sim 6.75 \times 10^6$ CFU/mL) and simultaneously the highest biomass content was noted as 0.126 mg/mL. The low growth profile suggest that the strains required more time to acclimatise. Moreover, the active induced inoculum needed to have more viable cells during initial inoculation which would enhance competition and may thus increase the intake rate [291,320].

For benzene (Figure 2.13), with 4% inoculum, the survival time of the strains in 1000 mg/L benzene was observed as *Aquamicrobium sp.* GR1 $>$ *Bacillus sp.* GE1 $>$ *Staphylococcus sp.* GR2 $>$ *Bacillus sp.* GS1 $>$ *Aquamicrobium sp.* GR4 $>$ *Aquamicrobium sp.* GR3 $>$ *Aquamicrobium sp.* GE2.

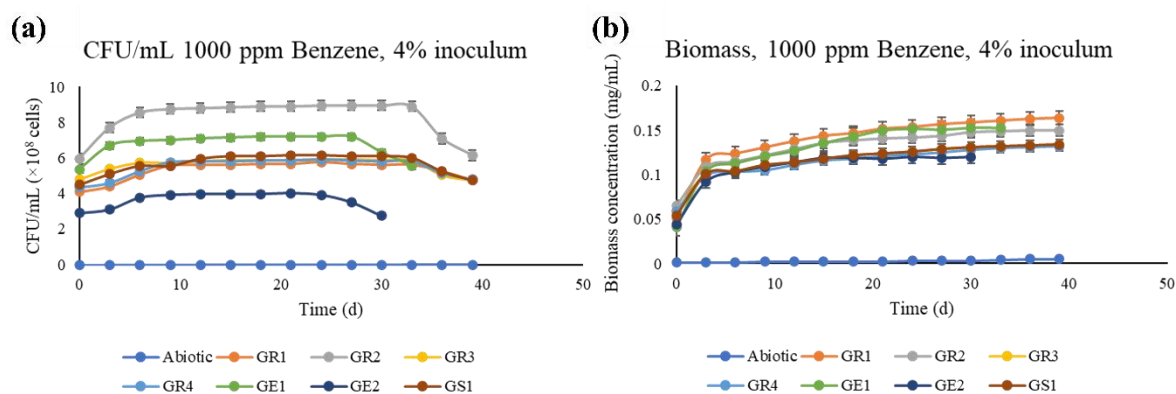


Figure 2.13: Growth of isolates with 4% (v/v) inoculum in 1000 mg/L benzene supplemented in MSM. (a) Bacterial count in terms of CFU/mL; (b) Biomass concentration in mg/mL. The strains depicted ability to survive in anthracene media for a prolonged period of time (33 days). Highest bacterial count ($> 10^8$ cells) and biomass concentration signify the potential of the isolates for application in bioremediation of refinery waste. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The highest bacterial count was observed in case of *Staphylococcus sp.* GR2 in 1000 mg/L benzene ($\sim 9 \times 10^8$ CFU/mL) and the least was observed in case of *Aquamicrobium sp.* GE2 (3×10^8 CFU/mL). The highest biomass content, however, was seen in *Aquamicrobium sp.* GR1. This discrepancy could be due to experimental error obtained during either serial dilution or biomass calculation by gravimetric method.

Similar results were obtained in case of different concentrations of toluene also (Appendix Figure 5). The order of growth and survival time was approximately the same in order as that of benzene. The highest bacterial count was observed in case of *Staphylococcus sp.* GR2 in 1000 mg/L toluene (9.42×10^8 CFU/mL) and the least was observed in case of *Aquamicrobium sp.* GE2 (2.76×10^8 CFU/mL). The highest biomass content was also seen in *Staphylococcus sp.* GR2 in 1000 mg/L toluene (0.155 mg/mL) and the least also in *Aquamicrobium sp.* GE2 (0.087 mg/mL). The discrepancy obtained in case of benzene were not observed. Moreover, bacterial count was only carried out to check the viability of the collected biomass.

For xylene, with 3% inoculum (Appendix Figure 6), the survival time of the strains in 1000 mg/L xylene was observed identical to that observed in case of benzene/toluene. The highest bacterial count was observed in case of *Staphylococcus sp.* GR2 (8.34×10^8 CFU/mL) and the corresponding biomass content was obtained as 0.138 mg/mL. Whereas there lies slight anomaly regarding the viability patterns of bacterial count and biomass content of *Aquamicrobium sp.* GR1 and GE2, the same may also arise as xylene alongwith

benzene/toluene is emitted out quickly during the initial phases which sometimes may leave the culture conditions as nutrient limiting.

The bacterial count, hence, may be obtained as incorrect. Since the higher biomass content was obtained by other strain has similar pattern of bacterial count too, we assumed our experimental results were established with minimal error that could be neglected [320,321].

2.3.11. Estimation of kinetic parameters for biomass growth of isolated strains

The specific growth rate obtained for all the strains were higher than that obtained with strains subjected to growth only in MSM. Using the values of the specific growth rate at different concentrations of HCs, the kinetic parameters (μ_m , K_s and K_i) were calculated by fitting the specific growth rates (μ) obtained for the isolates for each concentration of HCs as initial substrate concentration. The values were obtained using Minitab v19.1 by fitting non-linear curve of specific growth rate, μ vs concentration of hydrocarbons into a suitable model and was represented by the kinetic parameters in Equation 2.3 and Equation 2.4. The complete growth kinetics for 4% inoculum in *n*-hexadecane, 4% and 3% inoculum in anthracene and phenanthrene respectively and 4% and 3% inoculum in benzene/toluene and xylene respectively supplemented MSM are elaborated in the order as follows.

The complete list of kinetic parameters for growth in *n*-hexadecane are shown in the Table 2.6.

Table 2.6: Biomass growth kinetic parameters of isolates in *n*-hexadecane supplemented MSM.

Pollutant: <i>n</i> -hexadecane, Monod's model							
Strains/ parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
μ_m (d ⁻¹)	0.003	0.094	0.003	0.003	0.067	0.057	0.057
K_s (mg/L)	2.11	91.58	0.45	0.59	15.57	4.2	4.84
K_i (mg/L)	-	-	-	-	-	-	-
R^2	0.999	0.999	0.999	0.992	0.999	0.999	0.994

For *n*-hexadecane, as is also evident from Figure 2.14, the highest specific growth was observed for *Staphylococcus* sp. GR2 in 250 mg/L *n*-hexadecane (0.066 d⁻¹) whereas the least was observed for *Aquamicrobium* sp. GR1, GR3 and GR4 in 10 mg/L *n*-hexadecane (0.003 d⁻¹, 0.003 d⁻¹ and 0.003 d⁻¹ respectively). Both these strains did not show any upward trend on increase in *n*-hexadecane suggesting that these strains were intolerant at concentration higher than 10 mg/L *n*-hexadecane. The highest μ_m was observed for *Staphylococcus* sp. GR2 (0.094 d⁻¹) that also agrees to the higher biomass production at 500 mg/L *n*-hexadecane. The

corresponding K_s value obtained was 91.58 mg/L *n*-hexadecane. The results of the other strains *Bacillus* sp. GE1 and GS1; and *Aquamicrobium* sp. GE2 also suggest that these strains are potent in *n*-hexadecane medium and can be used for bioremediation of refinery waste [319,322].

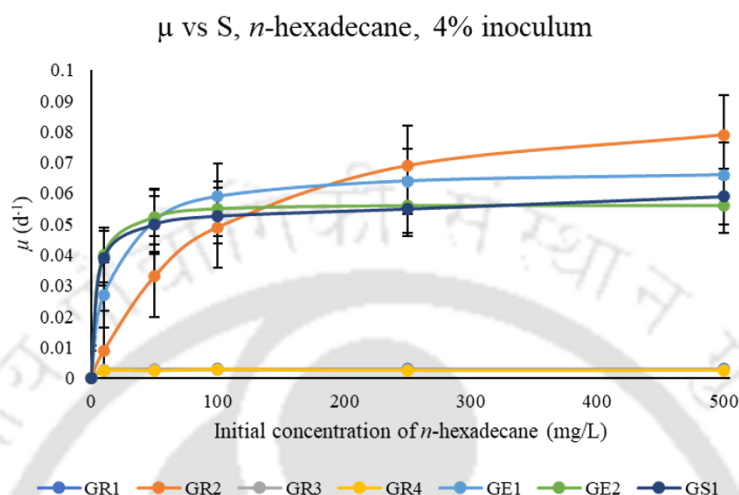


Figure 2.14: Biomass kinetics of isolates in *n*-hexadecane supplemented MSM. All the isolates could depict Monod's kinetic model of growth when incubated in *n*-hexadecane supplemented MSM. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The kinetic parameters obtained for strains in anthracene are listed in Table 2.7:

Table 2.7: Biomass growth kinetic parameters of isolates in anthracene supplemented MSM.

Pollutant: Anthracene, Andrew's model except <i>Bacillus</i> sp. GE1							
Strains/ parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
μ_m (d ⁻¹)	0.037	0.044	0.022	0.02	0.09	0.039	0.053
K_s (mg/L)	11.84	8.58	3.45	1.33	14.33	4.97	3.84
K_i (mg/L)	22.44	51.65	19.77	18.44	-	45.22	69.55
R^2	0.983	0.958	0.99	0.99	0.985	0.987	0.99

As is evident from Figure 2.15, the highest specific growth was observed for *Bacillus* sp. GE1 in 50 mg/L anthracene (0.059 d⁻¹). Except *Bacillus* sp. GE1, all others could fit into Andrew's type kinetics for anthracene. The fitted values obtained shows that the affinity of *Bacillus* sp. towards anthracene utilization could be seen as the highest μ_m was observed as 0.09 d⁻¹ for *Bacillus* sp. GE1 followed by 0.053 d⁻¹ for *Bacillus* sp. GS1. The corresponding K_s values of 14.33 mg/L and 3.84 mg/L indicated that these strains could survive for longer time in anthracene medium. For others, such as *Aquamicrobium* sp. GR1 and GE2, μ_m of 0.037 and 0.039 d⁻¹ respectively suggest that the strains could sustain anthracene upto their tolerance limit, and lower values of K_s 11.84 mg/L and 4.97 mg/L suggest significant affinity towards

anthracene intake. *Aquamicrobium sp.* GR3 and GR4 had the lowest values suggesting these strains are not suitable for anthracene metabolism. On the other hand, *Staphylococcus sp.* GR2 also showed significant potential towards survival in anthracene medium suggesting that this strain alongwith *Bacillus sp.* GE1 and GS1, and *Aquamicrobium sp.* GE2 can be used to bioremediation of refinery waste [317].

For phenanthrene (kinetic parameters depicted in Appendix Table 10), all the strains depicted best fit into Andrew's kinetics only where the highest μ_m and K_s was observed for *Bacillus sp.* GE1. Since the tolerance towards phenanthrene was less for all the other strains, they did not exhibit significant kinetics.

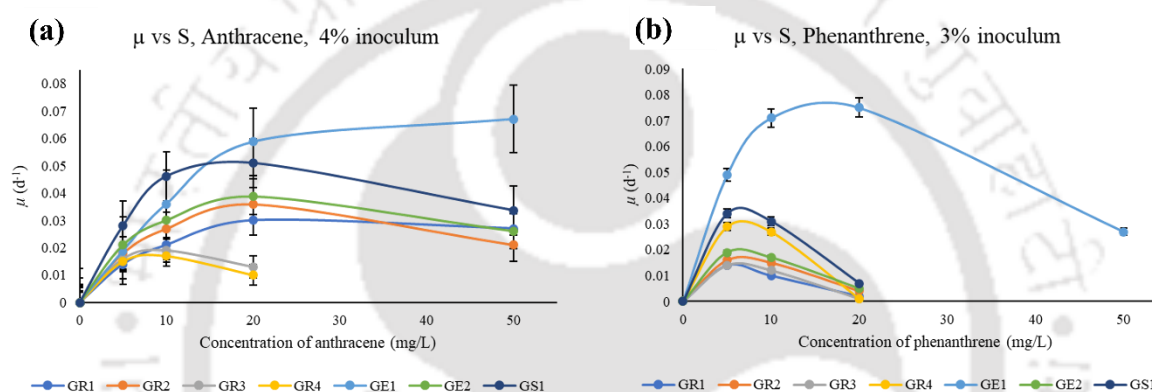


Figure 2.15: Biomass growth kinetics of isolates in (a) anthracene; and (b) phenanthrene supplemented MSM. Except *Bacillus sp.* GE1 in anthracene supplemented MSM depicting Monod's kinetics, all the isolates could exhibit Andrew's inhibition kinetic model of growth when incubated in anthracene/ phenanthrene supplemented MSM. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The kinetic parameters for benzene are listed in Table 2.8, the same for toluene and xylene are illustrated in Appendix Table 11 and Appendix Table 12 respectively.

Table 2.8: Biomass growth kinetic parameters of isolates in benzene supplemented MSM.

Pollutant: Benzene, Monod's model							
Strains/ parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
μ_m (d^{-1})	0.045	0.066	0.048	0.043	0.034	0.038	0.036
K_s (mg/L)	250.62	75.23	87.33	55.99	71.67	114.19	134.66
K_i (mg/L)	-	-	-	-	-	-	-
R^2	0.997	0.993	0.98	0.97	0.988	0.999	0.998

In case of BTX (Figure 2.16), the highest specific growth rate value was obtained for *Staphylococcus sp.* GR2 in 1000 mg/L benzene/toluene ($0.051 d^{-1}/0.025 d^{-1}$), and for *Bacillus sp.* GE1 in 1000 mg/L xylene ($0.037 d^{-1}$). The highest μ_m was observed for *Staphylococcus sp.* GR2 ($0.056 d^{-1}$) in case of benzene, for *Bacillus sp.* GE1 ($0.022 d^{-1}$) in case of toluene and for

Aquamicrobium sp. GE2 (0.031 d^{-1}) in case of xylene supplemented MSM. The least value was obtained for *Aquamicrobium* sp. GR1 in benzene (0.014 d^{-1}), for *Aquamicrobium* sp. GR3 in toluene supplemented MSM (0.006 d^{-1}) and for *Bacillus* sp. GS1 (0.011 d^{-1}). The highest K_s was observed for *Aquamicrobium* sp. GR1 (250.62 mg/L and 120.52 mg/L) in case of both benzene and toluene respectively, and for *Aquamicrobium* sp. GR4 (71.94 mg/L) in xylene, whereas the least was found for *Staphylococcus* sp. GR2 (75.23 mg/L) in benzene, for *Aquamicrobium* sp. GE2 (8.72 mg/L) in toluene and for *Aquamicrobium* sp. GR4 (15.836 mg/L) in xylene suggesting the strains possess potential to collectively survive in BTX contaminated environment.

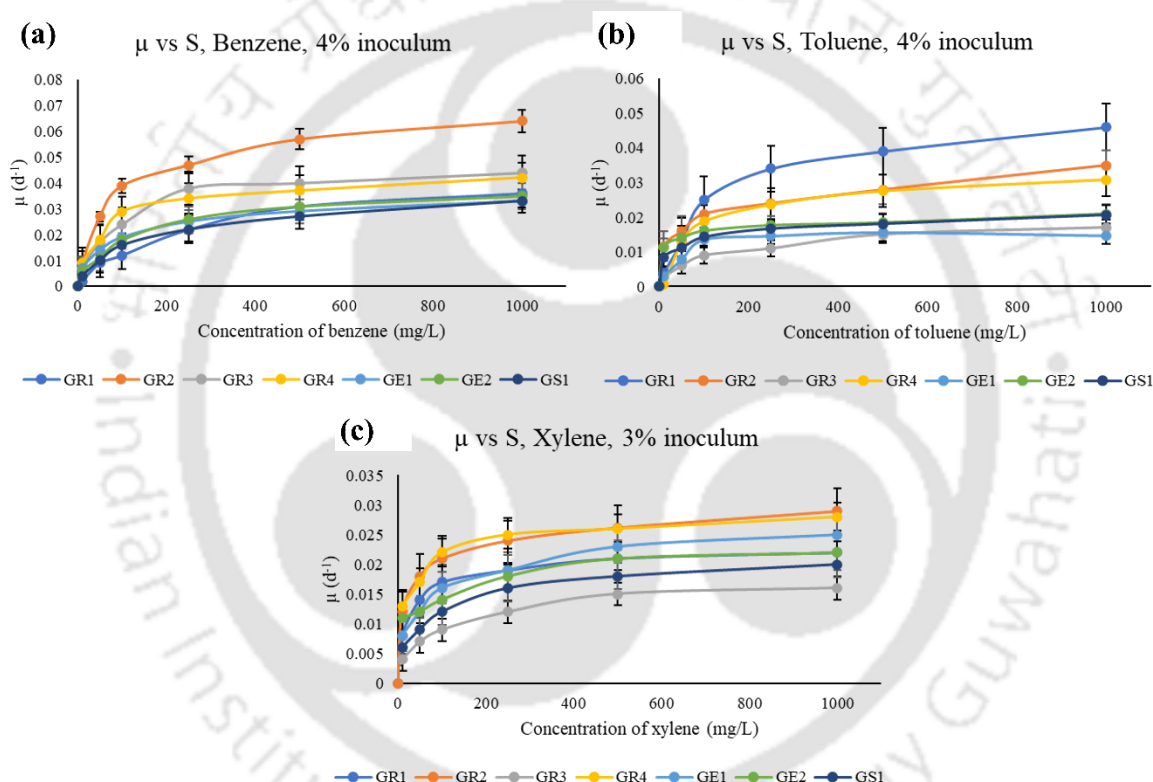


Figure 2.16: Biomass kinetics of isolates in (a) benzene; (b) toluene; and (c) xylene supplemented MSM. All the isolates could exhibit Monod's growth kinetic model of growth when incubated in benzene/ toluene/ xylene supplemented MSM. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

All the anomalies obtained could purely be due to experimental error as BTX are generally emitted out at faster rate during the initial phases, however, biomass accumulation may not have happened in a similar way. However, considering the above scenario, apart from *Aquamicrobium* sp. GR3, all the other strains have shown potency for survival in BTX media and may be helpful in bioremediation of refinery waste [297].

Analysing kinetic parameters is necessary to obtain enhanced and optimum treatment conditions as the values indicate the extent of the ability towards HC remediation. Higher μ_m could suggest faster utilization potential, however, higher saturation constant (K_s) could suggest lesser access to microbial breakdown. Microbes being versatile to adaptation in harsh environmental conditions is an added advantage and understanding their dynamics in such environment would offer deeper insights to attaining greater bioremediation efficiency [297,317,320,321].

2.3.12. COD removal of hydrocarbon(s) (HCs)

COD removal at the end of stationary phase for each strain was studied. The standard curve in terms of glucose is illustrated in Appendix Figure 7.

For *n*-hexadecane (Figure 2.17), the highest removal was obtained for *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1 at 500 mg/L (~67%). Almost 50% of the *n*-hexadecane in terms of COD was reduced by the end of 24 days for all strains. Other strains such as *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 showed approximately 55% removal for initial concentration of 500 mg/L. *Aquamicrobium sp.* GR1, GR3 and GR4 had lower tolerances and evidently did not contribute significantly towards COD removal. Similar degradation was reported with greater extent of survivability suggesting the efficiency of the strains to mineralize *n*-hexadecane [319,322].

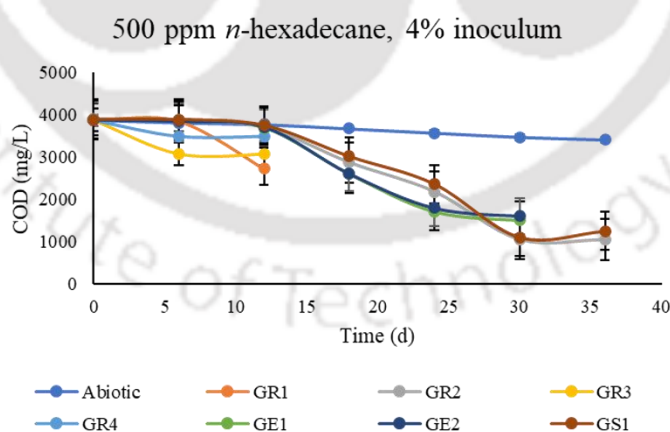


Figure 2.17: COD removal by isolates in *n*-hexadecane supplemented MSM. The isolates depicted significant COD removal, a high of 67% by *Staphylococcus sp.* GR2 alongside capacity to survive in *n*-hexadecane media. The isolates possess the potential for bioremediation of refinery waste. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Figure 2.18 depicts COD removal of both anthracene and phenanthrene. For anthracene, the highest removal was obtained for *Bacillus sp.* GE1 at 50 mg/L (~35%). *Staphylococcus sp.* GR2 showed ~20% removal that were lower than that observed [311,317]. In case of phenanthrene, 17% removal was attained by *Bacillus sp.* GE1 at 20 mg/L within 9 days of their growth cycle. Since the other strains were tolerant to only upto 5 mg/L phenanthrene, they did not contribute significantly towards COD removal of the medium containing MSM supplemented with phenanthrene.

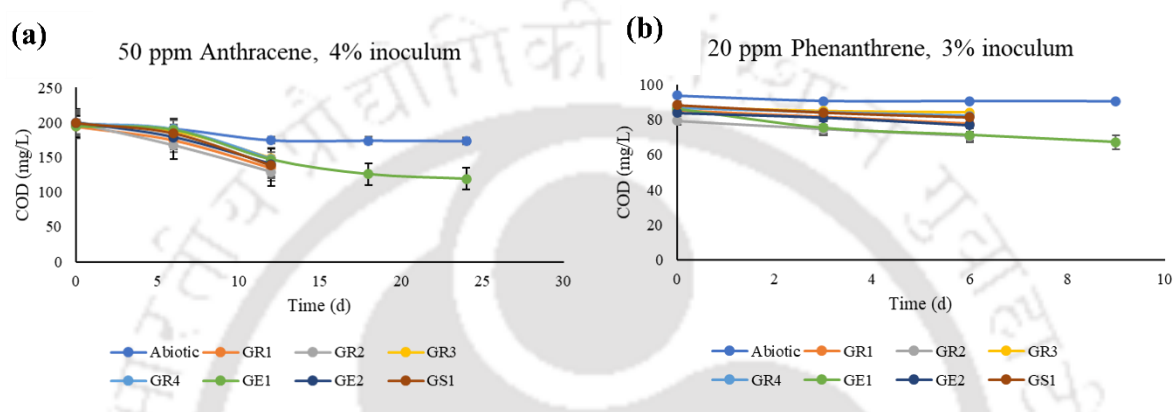


Figure 2.18: COD removal of isolates in (a) anthracene; and (b) phenanthrene supplemented MSM. The isolates depicted COD removal, a high of 35%/17% by *Bacillus sp.* GE1 alongside capacity to survive in anthracene/ phenanthrene media. Error bars indicate the standard deviation ($\pm$ SD) of experimental three replicates.

COD removal at the end of stationary phase for each strain was also studied for benzene/toluene/xylene (Figure 2.19). For benzene, the highest removal was obtained for *Staphylococcus sp.* GR2 at all concentrations. The highest percentage removal recorded was ~93% at 1000 mg/L benzene. *Bacillus sp.* GS1 also exhibited considerable percentage removal as ~92% for 1000 mg/L benzene. Similarly, for toluene, highest percentage removal was obtained for *Staphylococcus sp.* GR2 (~94%) at 1000 mg/L. For xylene, *Staphylococcus sp.* GR2 also exhibited the highest removal at all concentrations. Hence, these strains have greater potency towards BTX removal [297,317].

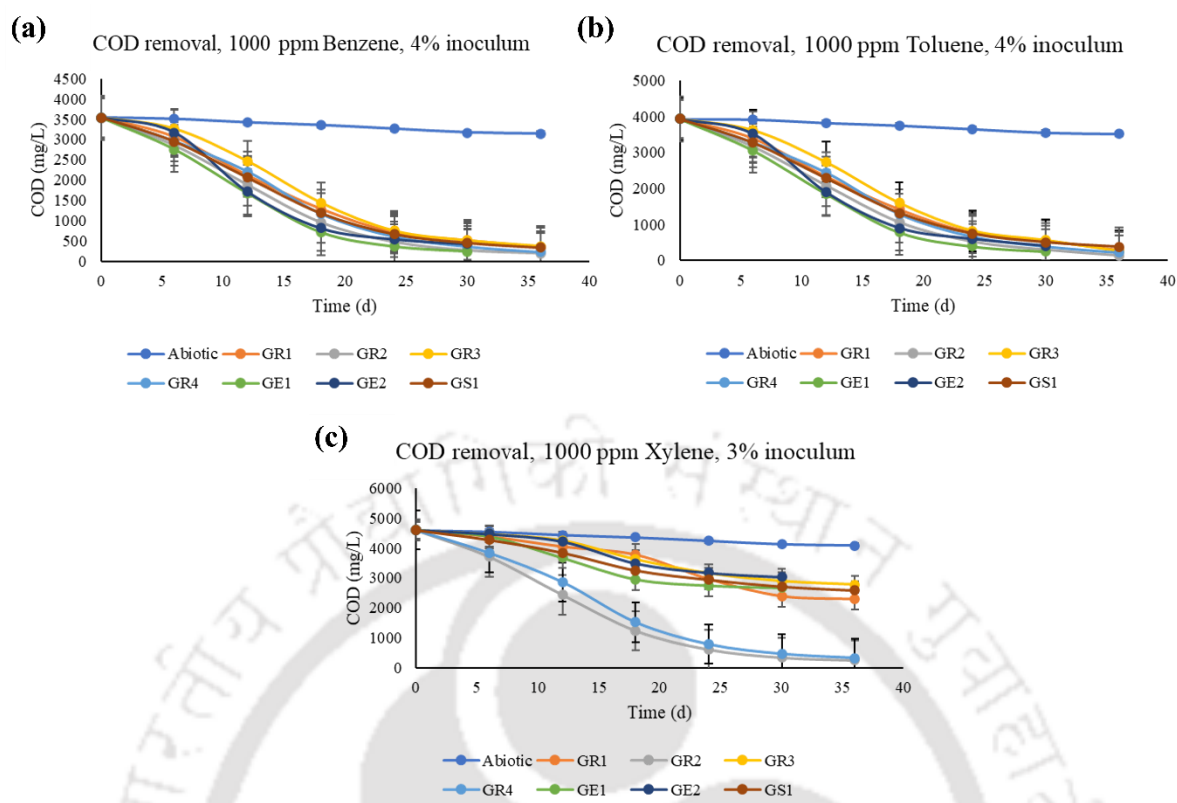


Figure 2.19: COD removal of isolates in (a) benzene; (b) toluene; and (c) xylene supplemented MSM. The isolates depicted high COD removal (>90%) collectively by *Staphylococcus* sp. GR2 and *Bacillus* sp. GS1 alongside capacity to survive in benzene/ toluene/ xylene media. The strains have potential for bioremediation of refinery waste. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

COD removal is a function of the utilization potential of the strains and was assessed to understand the ability to degrade HCs. Typically, microbial strains would breakdown hydrocarbons to obtain nutrition and enhance their chance of survival and gradually the overall concentration of HCs are expected to be lowered on account of microbial biotransformation that could be identified by obtaining the decaying COD profile [285,288,311,317,319].

2.4. Summary

The collected sample was acidic and possessed a typical odour as that would be expected in a petroleum refinery waste contaminated site. The toxicity assessment suggested higher COD and solid content. Availability of abundant nutrient suggested favourable habitat for presence of microbial populations. Indigenous strains were isolated using enrichment method as the pure colonies were separated for use in various growth studies. 16S rRNA sequencing revealed the strains belonging to *Aquamicrobium* sp. for GR1, GR3, GR4 and GE2; *Bacillus* sp. GE1 and GS1; and *Staphylococcus* sp. for GR2. Gram characteristics suggested *Staphylococcus* sp. GR2, *Bacillus* sp. GE1 and GS1 being gram positive. The strains also

showed tolerance towards heavy metals and saline conditions. Collectively, in different classes of HCs namely *n*-hexadecane (aliphatic), benzene/toluene/xylene (aromatic) and anthracene/phenanthrene (PAHs), the isolates exhibited survivability and tremendous reduction in pollutant toxicity in terms of COD removal was observed. MTCs for were high upto 500 mg/L for *n*-hexadecane, and 1000 mg/L for each of BTX, whereas the same for PAHs anthracene/phenanthrene was high upto 50 mg/L. Overall, the strains showed potential in acclimatising in different classes of HCs when facilitated with adequate inoculum and a designated C-source as the nutrient. Kinetic parameters suggested the applicability of the strains for bioremediation of refinery waste sludge. A summary of the studies carried out using various microbial strains in different HCs are summarised in Appendix Table 13 for reference.



3

Statistical optimization for designing optimum consortia for remediation of waste sludge

3.1. Background

Statistical optimization is a powerful tool for culture media optimization. It involves the use of statistical methods to optimize the composition of culture media for improved production of desired metabolites or growth characteristics. Various studies related to using PBD model and CCD which uses RSM optimizing tool has been reported.

3.1.1. Design of experiments (DoE)

PBD is one of the unique and efficient way to obtain various significant factors that could affect a process. It is one of the easiest ways for multivariate optimization [323–325]. PBD was used to screen out the significant nutrients and growth parameters that could influence biomass production by the bacterial strains.

Mathematical modelling for PBD was carried out by using the following first order polynomial dynamics (Equation 3.1) as described by Annadurai et al., 2008 and Priyadharshini and Bakthavatsalam, 2016:

$$Y = \beta_0 + \sum_{i=0}^n \beta_i x_i \quad \text{Equation 3.1}$$

Where, Y = Predicted response (Biomass concentration in g/L), β_0 is the model intercept, β_i is the linear coefficient and x_i is the level of independent variable [323,326].

CCD is one the simplest known experimental designs in order to obtain the optimum significant factors that are predicted to have the maximum effect on a process. It has a unique factorial design and are quite effective to analyse how precisely the process can be explained. CCD was used to obtain the maximum response of the optimized nutrients and growth parameters which contributes to maximum biomass production by the bacterial strains under study.

Mathematical modelling was carried out by using the following second order polynomial dynamics as described in Equation 3.2 as:

$$Y = \beta_0 + \sum_{i=0}^n \beta_i x_i + \sum_{i,j=0}^n \beta_{i,j} x_i x_j + \sum_{i=0}^n \beta_i x_i^2 \quad \text{Equation 3.2}$$

Where, Y = Predicted response (Biomass concentration in g/L), β_0 is the model intercept, β_i is the linear coefficient, $\beta_{i,j}$ is the quadratic coefficient and x_i and x_j are the independent variables.

CCD generally is formed of 2^x factorial runs, $2x$ axial runs and x_c center runs, where x is the number of factors. The number of runs N_r is given as per Equation 3.3. The center points are used to determine the experimental error and the reproducibility of the data.

$$N_r = 2^x + 2x + x_c \quad \text{Equation 3.3}$$

Extensive reports to enhance microalga cultivation, studies based on the PBD and RSM to optimize the composition of media for the cultivation of microalgae have been published. Since the traditional OFAT optimization approach was found to be laborious and time-consuming, using a designed PBD model instead was reported to deliver faster results by Udayan et al., 2022. The designed media components assisted in enhanced FAs production. In another study, Ju et al., 2018 carried out the optimization of nutritional requirements for antimicrobial activity of *Streptomyces rimosus* using statistically based experimental designs. Although significant C and N sources were identified using an OFAT approach, RSM was applied further to optimize the growth medium components. Furthermore, Annadurai et al., 2008 depicted implementation of modern approaches of statistical analysis into the DoE based design of media components for enhancing phenol degradation, and similarly, increasing the production of *Antrodia sp.* was reported by Chang et al., 2006. Software based study to produce microbial-biocarrier matrix has also been reported, enabling optimization of biodegradation of petroleum further proving the simplicity and efficiency of statistical approach that could be helpful to study and optimize complex problems [323,327–329]. Hence, statistical optimization can be significantly relied on to improve the efficiency and effectiveness of culture media optimization.

3.1.2. Laboratory experimental conditions

In this chapter, we tried to understand the effect of certain mineral nutrient factors such as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl and $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ into the biomass production of these bacterial strains. Moreover, growth parameters including concentration of HCs, time period of incubation and inoculum concentrations were also varied alongside the micronutrient to obtain a cumulative effect on biomass production. Optimization studies were carried out on two phases: 1st order screening using PBD based linear optimization followed by 2nd order CCD based quadratic model. Five nutrient factors assigned as concentration(s) of (i) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A), (ii) $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (B), (iii) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (C), (iv) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (D), and (v) NaCl (E) and three growth parameters identified as (i) concentration of HCs (F), (ii) time (G), (iii) inoculum (H) were investigated for enhanced biomass production (response) at 95%

confidence level. In this study, biomass concentration (response) was the average of two trials. The factors with confidence level of more than 95% ($p \leq 0.05$) were considered to have significant effect on biomass production and will be considered for further studies. All the experiments were design based on the randomized set of DoE obtained from Minitab v19.1. The results were validated statistically and after obtaining the optimum media components, individual strains were varied as factors to obtain the optimum inoculums of each consortium with an objective to maximize the biomass production.

3.2. Materials and methods

Chemicals and reagents

The media chemicals K_2HPO_4 , $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, the optimization micronutrient factors $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl and $ZnSO_4 \cdot H_2O$, solvents, reagents were collectively obtained from Merck Lifesciences Pvt Ltd., Kolkata; HiMedia Laboratories Pvt Ltd., Mumbai; Sigma Aldrich Chemicals Pvt Ltd., Bengaluru and Spectrochem Pvt Ltd., Mumbai. $K_2Cr_2O_7$, conc. H_2SO_4 , $HgSO_4$ and Ag_2SO_4 were collectively purchased from Merck and HiMedia. Anthracene, phenanthrene *n*-hexadecane and xylene were obtained from Sigma Aldrich. Benzene and toluene were obtained from Merck. All plasticware and glassware including filtration assembly for biomass estimation were obtained from Tarsons, India and Borosil India. Micropipettes of ranges (μL) 0.2-10, 0.2-2, 0.2-20, 20-100, 20-100, 10-1000 were obtained as F2 grade Finn timer from Thermo Fisher Scientific, India and Research Plus Edition from Eppendorf India, Chennai. Autoclave and laminar air flow was used from Equitron Medica Pvt. Ltd. India and Cleanair, India respectively. Weighing balance Quintix model from Sartorius India was used. Bacterial incubator was used from Scigenics Biotech, Chennai, India. pH was adjusted using pH/MV meter HI2211 from Hanna Instruments, India. Whatman qualitative filter papers (No. 1; pore size 0.7 μm ; 125 mm diameter) was procured from Cytiva Lifesciences, India. DispoVan syringes obtained from local pharmacy were used for better precision.

3.2.1. Screening of microorganisms

Bacterial strains were screened on the basis on their ability to survive in HC contaminated environment and mineralize these complex compounds to simpler and lesser toxic form. The results were corroborated from Chapter 2 and subsequently some of the best strains were selected for their participation in media optimization and consortia design.

3.2.2. Estimation of biomass and COD

The selected strains were subjected to growth in MSM supplemented with MTC of HCs. The biomass growth was analysed by measuring the biomass concentration at the end of each combination of experiment by carrying out dry weight analysis. Briefly, the culture medium was filtered through syringe filter equipped with pre-weighed filter paper. The filter paper was dried at 105 °C overnight and dry weight was measured. Difference in weight per unit volume sample was calculated to obtain biomass concentration in mg/mL (equivalent to g/L). Difference in weight per unit volume sample was calculated to obtain biomass concentration in mg/mL (equivalent to g/L). COD removal of the HCs by the respective strains was checked at their optimized settings using the method as described Chapter 2 in section 2.2.12. The COD at the beginning and end of each incubation was determined by interpolating the OD₆₀₀ from the standard curve of absorbance vs COD (Appendix Figure 7).

3.2.3. Experimentation conditions and variables

The set of experiments were designed using Minitab v19.1. All the set of experiments were incubated at 33 °C at 170 rpm. Unless mentioned, the experiments were carried for the time period as per the DoE laid down by the software.

3.2.4. Optimization of mineral nutrients for enhanced biomass production

In order to enhance biomass production, the growth medium was preferred to be engineered. Briefly, 100 mL Erlenmeyer flasks were used for the study. For nutrient media optimization studies, concentration of FeSO₄·7H₂O, CoCl₂·6H₂O CuSO₄·5H₂O, ZnSO₄·7H₂O and NaCl were varied according to PBD of experiments (Table 3.1). The high concentrations were chosen by virtue of their maximum growth observed in terms of OD₆₀₀ by the isolated strains (data shown from Appendix Table 2 to Appendix Table 9 in Appendices). NiSO₄·6H₂O, Pb(NO₃)₂ and CrO₃ were not chosen for optimization due to lesser growth by the isolates as well as disparity in understanding uniform conditions of maximum growth in terms of OD₆₀₀.

Table 3.1: Level of nutrient factors tested in PBD.

Factors	Symbol	Experimental value	
		Low (–1)	High (+1)
FeSO ₄ .7H ₂ O (mg/L)	A	5	50
CoCl ₂ .6H ₂ O (mg/L)	B	2	10
CuSO ₄ .5H ₂ O (mg/L)	C	5	30
ZnSO ₄ .7H ₂ O (mg/L)	D	1	10
NaCl (mg/L)	E	1	250

3.2.5. Optimization of growth parameters for enhanced biomass production

The media used to optimize growth parameters viz. inoculum concentration, concentration of HCs and survival time to investigate the most significant parameter affecting biodegradation. For optimization of growth parameters, concentration of HCs, time and inoculum were varied in addition to the nutrient factors according to PBD of experiments (Table 3.2). The high values were chosen as per the maximum survival time and optimum inoculum obtained in Chapter 2. The high HC concentrations were limited to the maximum values MTC of each HC also obtained during screening studies (Chapter 2).

As shown, all the variables were varied at two widely spaced levels and investigated as one-way variable technique. The complete set consisted of a set of 20 experiments as design by PBD. Biomass concentration was taken as response. Estimation of biomass concentration was discussed in the section 3.2.2 above. All the experiments were performed under ambient conditions. During the study, 2 mL of cultures were taken at the end of stationary phase of the respective strains.

Table 3.2: Level of growth parameters tested in PBD.

Factors	Symbol	Experimental value	
		Low (–1)	High (+1)
[Hydrocarbon (HCs)] (mg/L)	F	Min.	MTC
Time (days)	G	Min.	Max.
Inoculum (%)	H	Min.	Max.

3.2.6. Optimum nutrient factors and growth parameters for maximum biomass production

In order to obtain the optimum response of the significant to predict the maximum biomass production for the experiment under study, the significant factors obtained from the

1st order optimization i.e. PBD were further subjected to 2nd order optimization. The significant nutrient factors and growth parameters obtained were further varied as per the CCD of experiments. Since the strains under consideration had already been screened earlier based on the maximum concentration of the HCs they can tolerate, the concentration of HC, wherever significant, was not varied and was replaced with the next immediate significant factor obtained from the 1st order optimization studies.

CCD was constructed by assuming a cube of unit sides having 8 factorial points (f), 4 center points (c_c) in cube, 2 center points in axial (c_a) and selecting 6 axial points (a). 3 blocks of 3 variables with replicates of each were formed with a set of 20 runs and form factor α . The significant factors were varied over ranges from the range of $-(1 + \alpha)$ to $1 + \alpha$ (Table 3.3). For each experimental run, α was assigned to allow the cubic blocks together to rotate in 3D space. Considering the 3 blocks structure rotation can be visualized independently, α was estimated to be 1.633 by using the following Equation 3.4.

$$\alpha = \sqrt{\frac{f(a + c_a)}{2(f + c_c)}} \quad \text{Equation 3.4}$$

The set of 20 experiments for $x = 3$ was calculated from Equation 3.3 as 2^3 (factorial points) + 6 (centre points) + 2×3 (axial points). The choice of 3 factors is elaborated in section 3.3.3.

Although it is the choice of a user to assume the initial 3D model to be rotatable or orthogonal, our assumption was based upon to provide independent interaction of factors for better response signal and enhancing model quality. In case of spherical or any rotatable model, the form factor α would vary. Typically, $\alpha = \sqrt{x}$ or $\sqrt[4]{2^{(x-m)}}$ for spherical or rotatable models respectively. For full factorial design $m = 0$ [330,331].

Table 3.3: Level of significant factors tested in CCD.

Factors	Experimental value				
	$-(1 + \alpha)$	Low (-1)	Neutral	High ($+1$)	$1 + \alpha$
FeSO ₄ .7H ₂ O (mg/L)	$-(5 + \alpha)$	5	0	50	$50 + \alpha$
NaCl (mg/L)	$-(1 + \alpha)$	1	0	250	$250 + \alpha$
Time (days)	$-(\text{Min.} + \alpha)$	Min.	0	Max.	$\text{Max.} + \alpha$
Inoculum (%)	$-(\text{Min.} + \alpha)$	Min.	0	Max.	$\text{Max.} + \alpha$

3.2.7. Modified media for enhanced bioremediation efficiency

The media optimization was carried out to identify nutrient and growth factors which the potential candidates utilize in addition to the original media components. The optimized factors were added to the initial media at their optimum concentrations. The optimum time period and inoculum concentrations were used further in the experiments from here on.

3.2.8. Consortia preparation

The experiments were statistically analysed by Minitab v19.1. All the experiments were performed in the modified media which was obtained by amending the optimum nutrient components from the previous optimization experiments. The following experiments were carried out for 48 days (optimum time period of 47.25 days) at the optimized inoculum concentrations obtained from the previous optimization studies.

3.2.9. Screening of significant strains affecting biomass production in modified media

After media optimization with the respective MTCs of the HCs, the strains were checked for their significant influence for biomass production in the engineered media. To understand the contribution of the individual strains, a trial of 2 sets containing the solution of mixture of HCs were carried out. Since the MTC of all the HCs were deduced earlier, the mixture of HCs was limited only upto MTCs. A trial of 2 sets were prepared by supplementing the mixture of HCs in the modified media. Set 1 comprised of HCs of the maximum concentration where the strains could exhibit visible growth (i.e. MTC) and set 2 comprised of the minimum concentrations of HCs where visible growth was observed. The inoculum concentrations of all the isolates were varied as per PBD of experiments (Table 3.4). Optimum inoculum concentrations obtained after 2nd order media optimization were used for assigning the upper levels of PBD. The isolates that either did not have significant inoculum influence or were not used for media optimization were assigned highest level as per the concentrations

obtained from inoculum optimization experiments of the initial growth studies. Strain *Aquamicrobium* sp. GR3 did not have positive influence in either growth or media optimization studies. Hence, it was not used in this experiment.

Table 3.4: Level of isolates studied in PBD.

Factors (Inoculum concentration as % v/v)	Symbol	Experimental value	
		Low (−1)	High (+1)
<i>Aquamicrobium</i> sp. GR1 (% v/v)	A	1	4
<i>Staphylococcus</i> sp. GR2 (% v/v)	B	1	3.44
<i>Aquamicrobium</i> sp. GR4 (% v/v)	C	1	3.68
<i>Bacillus</i> sp. GE1 (% v/v)	D	1	6.37
<i>Aquamicrobium</i> sp. GE2 (% v/v)	E	1	4
<i>Bacillus</i> sp. GS1 (% v/v)	F	1	6.37

Set 1: 500 mg/L *n*-hexadecane, 50 mg/L Anthracene, 20 mg/L Phenanthrene, 1000 mg/L Benzene, 1000 mg/L Toluene, 1000 mg/L Xylene.

Set 2: 10 mg/L *n*-hexadecane, 5 mg/L Anthracene, 5 mg/L Phenanthrene, 10 mg/L Benzene, 10 mg/L Toluene, 10 mg/L Xylene.

3.2.10. Optimization of significant strains for maximum biomass production

In order to obtain the optimum response of the significant to predict the maximum biomass production for the experiment under study, the significant factors obtained from the 1st order optimization i.e. PBD were further subjected to 2nd order optimization. The significant factors nutrient factors and growth parameters obtained were further varied as per CCD of experiments. CCD was constructed by assuming a cube of 8 factorial points (f) points and selecting 6 axial points (a) with 4 and 2 points each in central points of cube (c_c) and axis (c_a) respectively. Block of 3 variables with a replicate of each was formed with a set of 20 runs and form factor α . α was obtained as 1.633 similar to as discussed previously in section 3.2.6. The significant factors were varied from the range of $-(1 + \alpha)$ to $1 + \alpha$ (Table 3.5).

To simplify the experimental setup and to accommodate almost all classes of identified organisms in consortia, the significant organisms, wherever applicable, were grouped into multiple subsets to obtain the optimum response.

Table 3.5: Level of significant factors tested in CCD.

Set	Factors	Experimental value				
		$-(1 + \alpha)$	Low (-1)	Neutral	High $(+1)$	$1 + \alpha$
1	<i>Staphylococcus</i> sp. GR2 (%)	$-(1 + \alpha)$	1	0	3.44	$3.44 + \alpha$
	<i>Bacillus</i> sp. GE1 (%)	$-(1 + \alpha)$	1	0	6.37	$6.37 + \alpha$
	<i>Aquamicrobium</i> sp. GE2 (%)	$-(1 + \alpha)$	1	0	4	$4 + \alpha$
2(i)	<i>Aquamicrobium</i> sp. GR1 (%)	$-(1 + \alpha)$	1	0	4	$4 + \alpha$
	<i>Staphylococcus</i> sp. GR2 (%)	$-(1 + \alpha)$	1	0	3.44	$3.44 + \alpha$
	<i>Bacillus</i> sp. GS1 (%)	$-(1 + \alpha)$	1	0	6.37	$6.37 + \alpha$
2(ii)	<i>Staphylococcus</i> sp. GR2 (%)	$-(1 + \alpha)$	1	0	3.44	$3.44 + \alpha$
	<i>Aquamicrobium</i> sp. GR4 (%)	$-(1 + \alpha)$	1	0	3.68	$3.68 + \alpha$
	<i>Bacillus</i> sp. GE1 (%)	$-(1 + \alpha)$	1	0	6.37	$6.37 + \alpha$

3.2.11. Statistical analysis of DoEs

Statistical analysis of the model was performed to evaluate the ANOVA. Analysis includes Fisher test (F -test), its associated probability $p(F)$ and the coefficient of determination (R^2) which measure the goodness of fit of the regression model. The response surface and contour plots of predicted responses of the model were used to assess the interaction between the significant factors. Basic statistics parameters were also analysed to predict the reproducibility and precision of the predicted model.

3.2.12. Data analysis of statistical outputs

Minitab® 19.1 software was used for designing experiments as well as for regression analysis of the experimental data obtained.

3.2.13. Mutual interaction among the significant factors and optimum responses

The mutual interactions between the significant factors obtained for different sets of experiments are depicted showing their influence in increase in biomass production.

3.2.14. Comparative assessment of biomass production and COD removal before and after optimization

Biomass measurements were carried out similar to as described in section 3.2.2. The biomass obtained before and after the optimization process at the end of growth cycle of each of the tested strains are shown. COD removal was also measured as discussed in the same

section 3.2.2. Bacterial count was compared by measuring CFU/mL using Equation 2.1 as discussed in Chapter 2.

3.3. Results and discussion

3.3.1. Screening of microorganisms

Four strains namely GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1 exhibited desirable survival and growth in *n*-hexadecane and anthracene contaminated environment, *Staphylococcus sp.* GR2, *Aquamicrobium sp.* GR4 and *Bacillus sp.* GE1 could efficiently mineralize benzene, toluene and xylene were reduced significantly by *Aquamicrobium sp.* GR1, *Staphylococcus sp.* GR2 and *Aquamicrobium sp.* GR4 and *Staphylococcus sp.* GR2 and *Aquamicrobium sp.* GR4 respectively, whereas *Bacillus sp.* GE1 could show reduction of phenanthrene. These strains were further considered for nutrient optimization at MTC of the respective HCs. As discussed before, the strains were induced by the respective HC and a desired inoculum was prepared before beginning an experiment.

3.3.2. Screening of significant factors for enhanced biomass production

Prior to optimization, impact of five nutrient factors were studied on biomass production.

In case of *n*-hexadecane, the DoE obtained for *Bacillus sp.* GS1 is shown in Table 3.6.

Table 3.6: PBD for *Bacillus sp.* GS1 in *n*-hexadecane supplemented MSM.

Run	FeSO ₄ .7H ₂ O (mg/L)	CoCl ₂ .2H ₂ O (mg/L)	CuSO ₄ .5H ₂ O (mg/L)	ZnSO ₄ .H ₂ O (mg/L)	NaCl (mg/L)	<i>n</i> - hexadecane (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	5	39	4	0.571
2	5	10	30	1	250	500	9	1	0.233
3	50	10	5	10	250	5	9	1	0.948
4	50	2	5	10	250	5	39	4	1.454
5	50	2	30	1	250	500	39	4	3.066
6	50	10	30	1	1	500	39	1	1.063
7	5	10	5	10	250	500	39	1	1.079
8	50	2	5	1	1	500	9	4	0.973
9	5	2	30	10	1	500	39	1	0.581
10	5	2	5	1	1	5	9	1	0.184
11	5	10	30	1	1	5	9	4	0.656
12	5	2	5	1	250	5	39	1	0.653
13	50	10	5	1	1	5	39	1	0.418
14	5	2	5	10	1	500	9	4	0.661
15	50	2	30	10	250	500	9	1	1.233
16	5	10	5	10	1	500	39	4	1.045
17	5	10	30	10	250	5	9	4	1.141
18	50	10	30	10	1	5	39	4	0.877
19	50	10	5	1	250	500	9	4	2.164
20	50	2	30	10	1	5	9	1	0.382

For all the other potent strains, the DoE is listed in *n*-hexadecane section in Appendices. For the strains under study, for all *Staphylococcus sp.* GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2, *Bacillus sp.* GS1, concentration of FeSO₄.7H₂O (A) and NaCl (E) had positive effect on biomass production and hence will be considered for the next optimization stage.

For *Bacillus sp.* GS1 depicted in Figure 3.1, the normalized plot, pareto chart and main effects plot shows the positive influences of mineral nutrients FeSO₄.7H₂O (A) and NaCl (E) and growth parameters *n*-hexadecane (F) and inoculum (H) on biomass production.

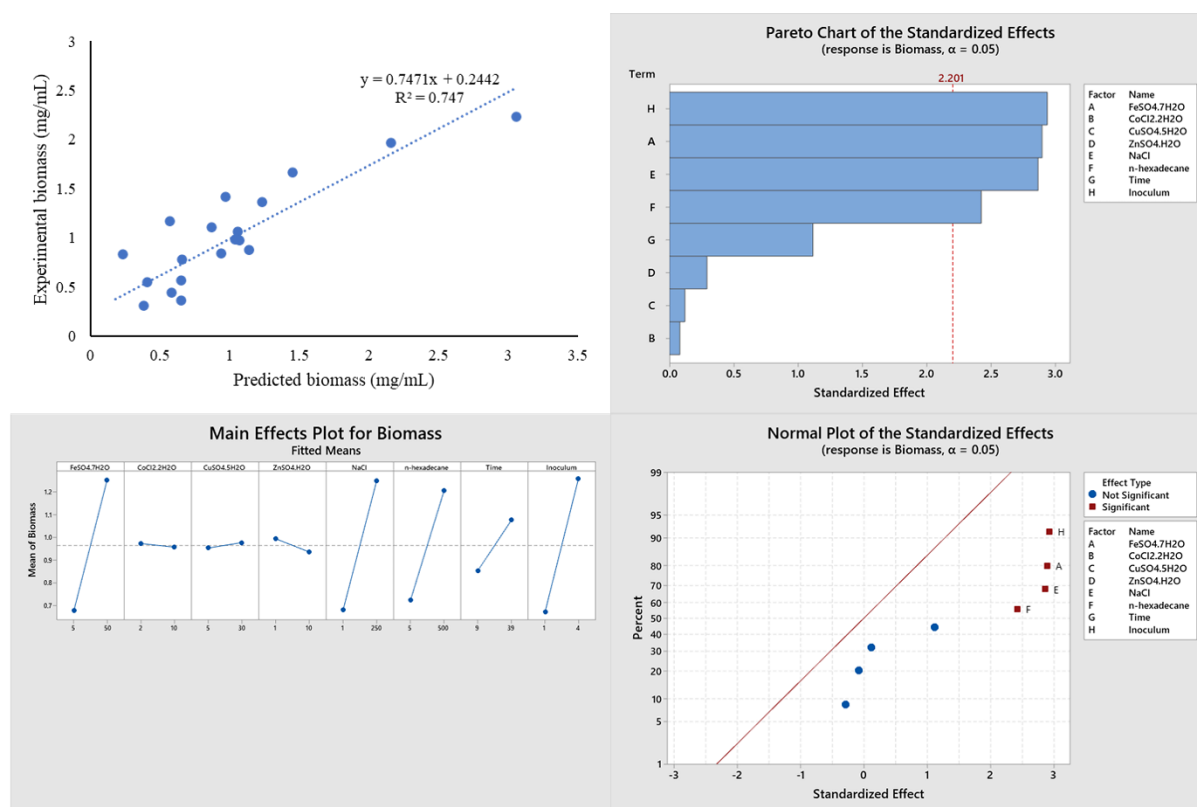


Figure 3.1: Precision, Pareto chart, main effects plot, and normal distribution of factors tested for *Bacillus sp. GS1* in *n*-hexadecane supplemented MSM as per PBD. Precision depicts the predictability of the model towards enhanced biomass production; pareto chart, main effects plot and normal distribution of factors depict the significant variables having positive effects on biomass production.

In case of anthracene, for all potent strains *Staphylococcus sp. GR2*, *Bacillus sp. GE1*, *Aquamicrobium sp. GE2* and *Bacillus sp. GS1*, the effect of factors was studied using PBD. The DoE obtained for *Bacillus sp. GE1* is listed below (Table 3.7).

Table 3.7: PBD for *Bacillus sp.* GE1 in anthracene supplemented MSM.

Run	FeSO ₄ .7H ₂ O (mg/L)	CoCl ₂ .2H ₂ O (mg/L)	CuSO ₄ .5H ₂ O (mg/L)	ZnSO ₄ .H ₂ O (mg/L)	NaCl (mg/L)	Anthracene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	10	5	10	1	50	18	4	0.333
2	50	10	30	10	1	5	18	4	0.385
3	5	10	5	10	250	50	18	1	0.311
4	5	10	30	1	1	5	3	4	0.297
5	50	10	5	1	250	50	3	4	0.555
6	50	2	30	10	1	5	3	1	0.158
7	5	2	5	1	1	5	3	1	0.112
8	5	2	5	1	250	5	18	1	0.144
9	50	10	5	10	250	5	3	1	0.269
10	50	10	5	1	1	5	18	1	0.154
11	50	2	5	10	250	5	18	4	0.361
12	5	2	30	10	1	50	18	1	0.216
13	50	10	30	1	1	50	18	1	0.317
14	5	2	5	10	1	50	3	4	0.313
15	5	10	30	1	250	50	3	1	0.198
16	5	10	30	10	250	5	3	4	0.312
17	50	2	5	1	1	50	3	4	0.35
18	50	2	30	1	250	50	18	4	0.552
19	5	2	30	1	250	5	18	4	0.336
20	50	2	30	10	250	50	3	1	0.219

For all the other potent strains, the DoE is listed in Anthracene section of Appendices. For all the strains under study, *Staphylococcus sp.* GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1, concentration of FeSO₄.7H₂O (A) and NaCl (E) had positive effect on biomass production and hence will be considered for the next optimization stage.

For *Bacillus sp.* GE1 depicted in Figure 3.2, the normalized plot, pareto chart and main effects plot shows the positive influences of mineral nutrients FeSO₄.7H₂O (A) and NaCl (E) and growth parameters anthracene (F) and inoculum (H) on biomass production.

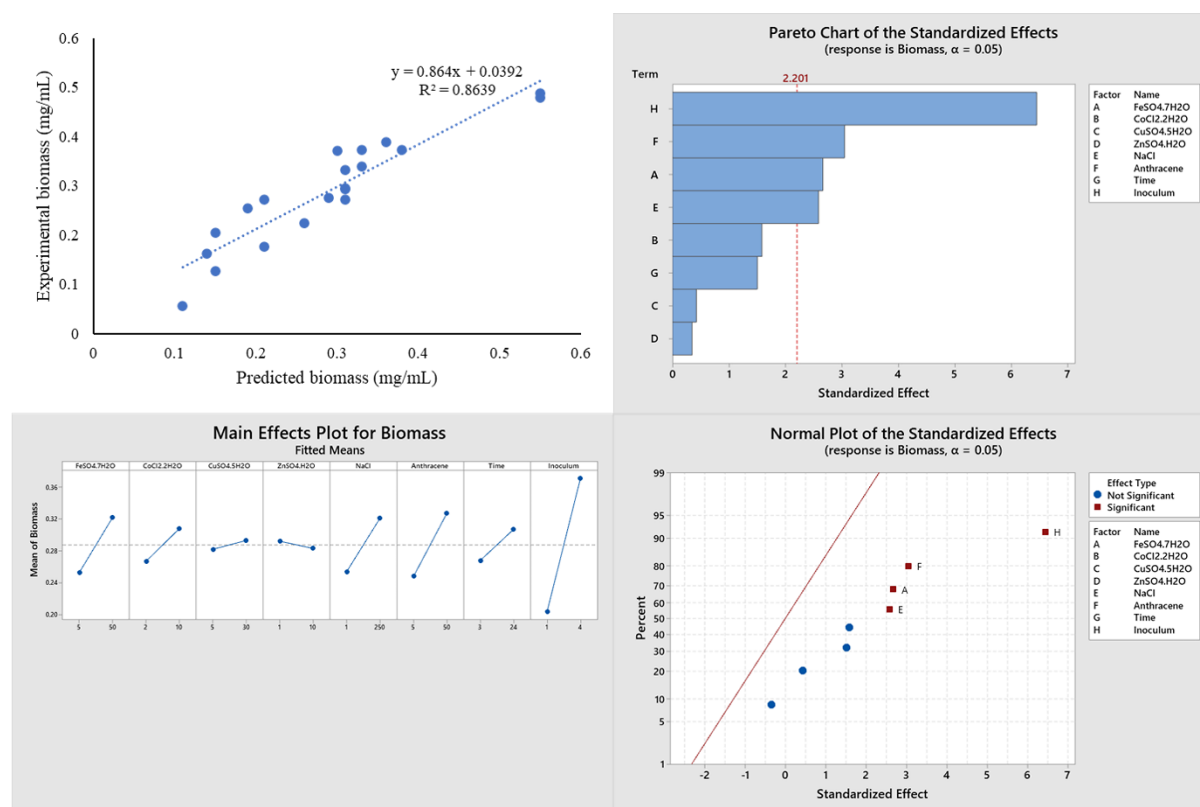


Figure 3.2: Precision, Pareto chart, main effects plot and normal distribution of factors tested for *Bacillus sp. GE1* in anthracene supplemented MSM as per PBD. Precision depicts the predictability of the model towards enhanced biomass production; pareto chart, main effects plot and normal distribution of factors depict the significant variables having positive effects on biomass production.

In a similar way, for another PAH, Phenanthrene, where only *Bacillus sp. GE1* was found to be potent, the concentration of inoculum (H) apart from mineral nutrients FeSO₄·7H₂O (A) and NaCl (E) was found to be significant. The detailed results are indicated in Appendices.

In case of benzene, the potent strains *Staphylococcus sp. GR2* and *Bacillus sp. GE1*, the experiments were conducted as per DoE in Table 3.8.

Table 3.8: PBD for *Staphylococcus sp.* GR2 in benzene supplemented MSM.

Run	FeSO ₄ .7H ₂ O (mg/L)	CoCl ₂ .2H ₂ O (mg/L)	CuSO ₄ .5H ₂ O (mg/L)	ZnSO ₄ .H ₂ O (mg/L)	NaCl (mg/L)	Benzene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	10	36	4	0.221
2	5	10	30	1	250	1000	3	1	0.166
3	50	10	5	10	250	10	3	1	0.166
4	50	2	5	10	250	10	36	4	0.241
5	50	2	30	1	250	1000	36	4	0.522
6	50	10	30	1	1	1000	36	1	0.267
7	5	10	5	10	250	1000	36	1	0.296
8	50	2	5	1	1	1000	3	4	0.176
9	5	2	30	10	1	1000	36	1	0.187
10	5	2	5	1	1	10	3	1	0.086
11	5	10	30	1	1	10	3	4	0.127
12	5	2	5	1	250	10	36	1	0.163
13	50	10	5	1	1	10	36	1	0.217
14	5	2	5	10	1	1000	3	4	0.147
15	50	2	30	10	250	1000	3	1	0.226
16	5	10	5	10	1	1000	36	4	0.186
17	5	10	30	10	250	10	3	4	0.127
18	50	10	30	10	1	10	36	4	0.152
19	50	10	5	1	250	1000	3	4	0.246
20	50	2	30	10	1	10	3	1	0.169

Normalized plot, pareto and main effects plot for *Staphylococcus sp.* GR2 illustrated in Figure 3.3 depict that concentration of mineral nutrients FeSO₄.7H₂O (A) and NaCl (E) alongwith concentration of benzene (F) and time period of incubation (G) had positive effect on biomass production as similar to the previous cases. The normalized plot, pareto and main effects plot for *Bacillus sp.* GE1 in benzene supplemented MSM are shown in Appendix Figure 24.

For other strain *Aquamicrobium sp.* GR4, the DoE and the results are shown in Benzene section of Appendices.

Similar DoE was followed for the other two aromatic HCs toluene and xylene.

Briefly, in case of toluene, for the potent strains *Aquamicrobium sp.* GR1, *Staphylococcus sp.* GR2 and *Aquamicrobium sp.* GR4, the statistical analysis of the effect of factors carried out using PBD showed that concentration of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A) and NaCl (E) had positive effect on biomass production. Moreover, concentration of toluene (F) was significant for *Staphylococcus sp.* GR2 and *Aquamicrobium sp.* GR4, time of incubation (G) was significant for *Aquamicrobium sp.* GR1, *Staphylococcus sp.* GR2 and inoculum concentration was significant for *Aquamicrobium sp.* GR1 and GR4.

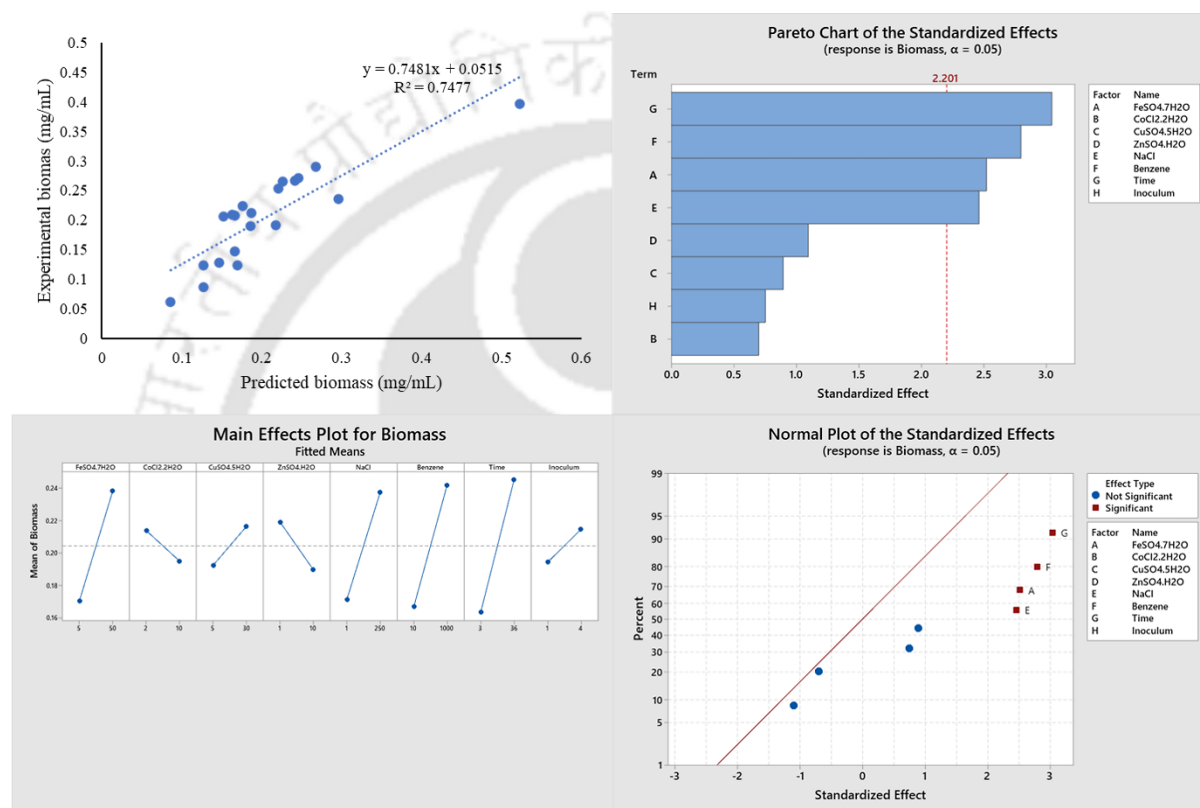


Figure 3.3: Precision, Pareto chart, main effects plot and normal distribution of factors tested for *Staphylococcus sp.* GR2 in benzene supplemented MSM as per PBD. Precision depicts the predictability of the model towards enhanced biomass production; pareto chart, main effects plot and normal distribution of factors depict the significant variables having positive effects on biomass production.

Lastly, similarly, for xylene, similar results with same combination of significant factors were obtained for strains potent strains *Staphylococcus sp.* GR2 and *Aquamicrobium sp.* GR4. All detailed results pertaining to toluene and xylene are also depicted in Toluene and Xylene sections of Appendices.

3.3.3. Optimization of significant factors for maximum biomass production

The significant factors $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl, time period and inoculum concentrations were optimized as per CCD and statistically validated further. To simplify our analysis, we have assumed the following points:

- ✓ In cases, where time period was not found significant and was not carried for 2nd order optimization, the experimental sets were incubated for the time period equal to that obtained in the initial screening (from Chapter 2) for the strains under study in the respective HC.
- ✓ In cases where HC concentration was found significant alongwith time/inoculum, the MTC values of HC (from Chapter 2) was used and was not carried forward for 2nd order optimization. This is to avoid any detrimental effect on the strains that may not be tolerant to higher concentration of the HC as could be the case of experimental sets as per CCD.

In case of *n*-hexadecane, the DoE as per CCD for one of the strains *Bacillus sp.* GS1 is shown in Table 3.9.

Table 3.9: CCD for *Bacillus sp.* GS1 in *n*-hexadecane supplemented MSM.

- Concentration of *n*-hexadecane = 500 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	125.5	2.5	4.553
2	65.34	125.5	2.5	1.194
3	27.5	125.5	2.5	4.231
4	50	1	1	0.662
5	5	250	1	0.781
6	50	1	4	1.334
7	27.5	125.5	2.5	4.29
8	27.5	125.5	2.5	4.212
9	27.5	334.883	2.5	4.281
10	5	1	1	0.37
11	50	250	1	0.99
12	27.5	125.5	5.023	2.764
13	27.5	125.5	2.5	4.41
14	-10.34	125.5	2.5	0.493
15	5	1	4	1.533
16	50	250	4	4.862
17	27.5	125.5	-0.023	0.051
18	5	250	4	1.561
19	27.5	125.5	2.5	4.514
20	27.5	-83.883	2.5	0.082

The optimum response (Table 3.10) were recorded as 3.44% inoculum, 229.13 mg/L NaCl and 36.29 FeSO₄·7H₂O. For other strains, the results are indicated in *n*-hexadecane section of Appendices.

Table 3.10: Multiple response prediction for *Bacillus sp.* GS1 in *n*-hexadecane MSM.

Variable	Inoculum	NaCl	FeSO ₄ ·7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	3.443	229.134	36.291	4.86	5.019

From the various optimum responses obtained from 2nd order optimization for all the potent strains (Appendix Table 108), the maximum values of optimum inoculum of 3.44%, time period of 41.64 days, NaCl of 246.05 mg/L and FeSO₄.7H₂O of 41.64 mg/L were chosen which could predict maximum biomass production as applicable in *n*-hexadecane supplemented MSM. The model checked statistically and is explained in the subsequent section 3.3.5.

Similarly, for anthracene, the DoE as per CCD for *Bacillus* sp. GE1 is depicted below in Table 3.11.

Table 3.11: CCD for *Bacillus* sp. GE1 in anthracene supplemented MSM.

- Concentration of anthracene = 50 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ .7H ₂ O (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	125.5	1	0.245
2	65.34	125.5	2.5	0.218
3	27.5	125.5	2.5	0.221
4	50	1	2.5	0.319
5	5	250	2.5	0.303
6	50	1	1	0.122
7	27.5	125.5	2.5	0.295
8	27.5	125.5	1	0.142
9	27.5	334.883	-0.023	0.02
10	5	1	2.5	0.283
11	50	250	1	0.187
12	27.5	125.5	2.5	0.313
13	27.5	125.5	2.5	0.357
14	-10.34	125.5	2.5	0.301
15	5	1	4	0.451
16	50	250	4	0.298
17	27.5	125.5	4	0.193
18	5	250	5.023	0.455
19	27.5	125.5	2.5	0.171
20	27.5	-83.883	4	0.356

The optimum response (Table 3.12) were recorded as 5.02% inoculum, 267.2 mg/L NaCl and 65.34 FeSO₄.7H₂O. Results for others can be found in Anthracene section of Appendices.

Table 3.12: Multiple response prediction for *Bacillus sp.* GE1 in anthracene MSM.

Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	5.023	267.204	65.34	0.43	0.456

From the various optimum responses obtained from 2nd order optimization for all the strains (Appendix Table 108), the maximum values of optimum inoculum of 6.37%, time period of 27.14 days, NaCl of 334.88 mg/L and FeSO₄.7H₂O of 65.34 mg/L may be applicable in anthracene supplemented MSM.

Similarly, for Phenanthrene, optimum inoculum of 3.68%, NaCl: 334.88 mg/L and FeSO₄.7H₂O of 65.34 mg/L may be applicable in phenanthrene supplemented MSM. The optimum response for the DoE as per CCD for *Bacillus sp.* GE1 in 20 mg/L phenanthrene is shown in Appendices.

Table 3.13 shows the DoE as per CCD for obtaining optimized response for *Staphylococcus sp.* GR2 in benzene supplemented media.

Table 3.13: CCD of *Staphylococcus sp.* GR2 in benzene supplemented MSM.

- Concentration of benzene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Time (Days)	Biomass (mg/mL)
1	27.5	125.5	47.25	0.186
2	5	250	3	0.099
3	27.5	125.5	-8.25	0.056
4	27.5	125.5	19.5	0.169
5	27.5	125.5	19.5	0.173
6	5	250	36	0.189
7	27.5	125.5	19.5	0.175
8	27.5	-83.883	19.5	0.098
9	50	1	36	0.196
10	27.5	125.5	19.5	0.176
11	50	1	3	0.097
12	65.34	125.5	19.5	0.197
13	5	1	36	0.095
14	-10.34	125.5	19.5	0.107
15	27.5	125.5	19.5	0.167
16	50	250	3	0.188
17	50	250	36	0.289
18	5	1	3	0.096
19	27.5	334.883	19.5	0.184
20	27.5	125.5	19.5	0.173

The detailed results pertaining to CCD DoE of other strains are illustrated in Benzene section of Appendices.

The optimum response for the experimental sets were noted as time period of 47.25 days, 334.88 mg/L NaCl, 65.34 mg/L FeSO₄·7H₂O (Table 3.14).

Table 3.14: Multiple response prediction for *Staphylococcus sp.* GR2 in benzene MSM.

Variable	Time	NaCl	FeSO ₄ ·7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	47.25	334.883	65.34	0.279	0.377

The maximum values of optimum inoculum based on the responses obtained for other potent strains (Appendix Table 108) are summarized as 3.68%, time period of 47.25 days, NaCl of 334.88 mg/L and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ of 65.34 mg/L may be applicable in benzene supplemented MSM.

In a similar way, for other aromatic HC Toluene, the maximum values of optimum inoculum of 3.68%, time period of 43.23 days, NaCl of 334.88 mg/L and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ of 65.34 mg/L were obtained. For Xylene, the values of optimum inoculum of 3.68%, time period of 39.2 days, NaCl of 334.88 mg/L and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ of 65.34 mg/L may be applicable in xylene supplemented MSM. The results are depicted in Appendix Table 108.

3.3.4. Statistical analysis for most significant factors for enhanced biomass production

Experimental values obtained were statistically analysed using *F*-test and *p*-test for ANOVA.

In case of *n*-hexadecane, the overall model for *Bacillus sp.* GS1 depicted $F = 4.06$. It suggested that the model(s) may be appropriate and significant and have positive effect on the biomass production. However, Model *F*-value occurred at minimal noise value of 0.01%. *p*-value (P (Probability) $> F$) was used as a tool to identify significant factors. *p*-values less than 0.05 denoted significant factors and were taken to have affected biomass production which is the response (*Y*). The R^2 value of 0.747 indicated that 56.31% of variability in biomass production could be calculated. Predicted R^2 of 0.364 shows that around 37% of this data can be predicted correctly. The coefficient of variation < 1.0 indicated that the models are predicted accurately and are reproducible [325,327]. However, several external factors might other factors not considered in this study may be affecting the biomass production also. Hence, further optimization may help to minimize any error.

ANOVA Table 3.15 shows the interactions and contributions by the individual components.

Table 3.15: ANOVA and model summary of PBD for *Bacillus sp.* GS1 in *n*-hexadecane MSM.

Analysis of variance					
Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	6.418	0.802	4.06	0.002
Linear	8	6.418	0.802	4.06	0.002
FeSO ₄ .7H ₂ O	1	1.659	1.659	8.4	0.002
CoCl ₂ .2H ₂ O	1	0.001	0.001	0.01	0.937
CuSO ₄ .5H ₂ O	1	0.003	0.003	0.01	0.906
ZnSO ₄ .H ₂ O	1	0.017	0.017	0.09	0.776
NaCl	1	1.625	1.625	8.22	0.015
<i>n</i> -hexadecane	1	1.162	1.162	5.88	0.034
Time	1	0.246	0.246	1.25	0.288
Inoculum	1	1.705	1.705	8.63	0.013
Error			11	2.173	0.198
Total			19	8.591	
Model Summary					
S	R-sq	R-sq(adj)	R-sq(pred)		
0.444	74.7%	56.31%	36.37%		

The regression equation explaining the enhanced biomass production by *Bacillus sp.* GS1 is explained in Equation 3.5:

$$\begin{aligned} \text{Biomass} = & -0.554 + 0.013 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.002 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} \\ & + 0.001 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.006 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.002 \text{ NaCl} + 0.001 \text{ } n\text{-hexadecane} \\ & + 0.007 \text{ Time} + 0.195 \text{ Inoculum} \end{aligned} \quad \text{Equation 3.5}$$

The results obtained for the other strains pertaining to PBD in *n*-hexadecane supplemented media are listed in *n*-hexadecane section of Appendices.

For anthracene, ANOVA Table 3.16 shows the interactions and contributions by the individual components. The overall model for *Bacillus sp.* GE1 depicted $F = 6.26$. It suggested that the model(s) may be appropriate and significant and have positive effect on the biomass production. p -value (p (Probability) $> F$) of 0.003 denoted significance of the factors affecting biomass production response (Y). The R^2 value of 0.863 indicated that 68.89% of variability in biomass production could be calculated. Predicted R^2 of 0.555 shows significant accuracy in

predictability of the model. The coefficient of variation indicated that the models are predicted accurately and are reproducible [325,327].

Table 3.16: ANOVA and model summary of PBD for *Bacillus sp.* GE1 in anthracene MSM.

Analysis of variance					
Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.094	0.094	6.26	0.003
Linear	8	0.094	0.094	6.26	0.003
FeSO ₄ .7H ₂ O	1	0.026	0.026	13.85	0.003
CoCl ₂ .2H ₂ O	1	0.006	0.006	3.09	0.107
CuSO ₄ .5H ₂ O	1	0.003	0.003	1.54	0.241
ZnSO ₄ .H ₂ O	1	0	0.000	0.10	0.762
NaCl	1	0.017	0.017	8.99	0.012
Anthracene	1	0.012	0.012	6.16	0.030
Time	1	0.009	0.009	4.71	0.053
Inoculum	1	0.022	0.022	11.64	0.006
Error			11	0.021	0.002
Total			19	0.114	
Model Summary					
S	R-sq	R-sq(adj)	R-sq(pred)		
0.043	86.31%	68.89%	55.47%		

The regression equation explaining the enhanced biomass production by *Bacillus sp.* GE1 is explained in equation:

$$\begin{aligned} \text{Biomass} = & 0.035 + 0.002 \text{ FeSO}_4.7\text{H}_2\text{O} - 0.004 \text{ CoCl}_2.2\text{H}_2\text{O} \\ & + 0.001 \text{ CuSO}_4.5\text{H}_2\text{O} + 0.001 \text{ ZnSO}_4.\text{H}_2\text{O} + 0.002 \text{ NaCl} + 0.001 \\ & \text{Anthracene} + 0.003 \text{ Time} + 0.022 \text{ Inoculum} \end{aligned}$$

Equation 3.6

The results obtained for the other strains pertaining to PBD in Anthracene supplemented media are listed in Anthracene section of Appendices.

The ANOVA and model summary for screening out significant factors for *Bacillus sp.* GE1 in phenanthrene supplemented media (Appendix Table 51) suggested and adjusted R^2 of 0.84 and $p(>F) = 0$ was contributing significantly towards biomass production. The R^2 was

0.936, indicating that the model was predicted accurately and is reproducible. Hence, no separate factors are required to be incorporated, and the model can be used for further optimization studies.

For benzene, ANOVA Table 3.17 shows the interactions and contributions by the individual components.

Table 3.17: ANOVA and model summary of PBD for *Staphylococcus sp.* GR2 in benzene MSM.

Analysis of variance					
Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.117	0.015	4.07	0.017
Linear	8	0.117	0.015	4.07	0.017
FeSO ₄ ·7H ₂ O	1	0.023	0.023	6.36	0.028
CoCl ₂ ·2H ₂ O	1	0.002	0.002	0.49	0.498
CuSO ₄ ·5H ₂ O	1	0.003	0.003	0.8	0.39
ZnSO ₄ ·H ₂ O	1	0.004	0.004	1.2	0.296
NaCl	1	0.022	0.022	6.07	0.032
Benzene		0.028	0.028	7.83	0.017
Time		0.033	0.033	9.27	0.011
Inoculum		0.002	0.002	0.57	0.467
Error			11	0.039	0.004
Total			19	0.157	
Model Summary					
S	R-sq	R-sq(adj)	R-sq(pred)		
0.06	74.77%	56.42%	37.59%		

The overall model for *Staphylococcus sp.* GR2 depicted $F = 4.07$. It suggested that the model(s) may be appropriate and significant and have positive effect on the biomass production. p -value ($p > F$) of 0.017 denoted significance of the factors affecting biomass production (Y). The R^2 value of 0.748 indicated that 56.42% of variability in biomass production could be calculated. Predicted R^2 shows around 37.6% of the values could be predicted correctly. The coefficient of variation was < 1.0 which indicated that the models are predicted accurately and are reproducible [325,327].

The regression equation explaining the enhanced biomass production by *Staphylococcus sp.* GR2 is explained in Equation 3.7:

$$\begin{aligned} \text{Biomass} = & 0.042 + 0.002 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.002 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} + 0.001 \\ & \text{CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.003 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.000 \text{ Benzene} + 0.002 \\ & \text{Time} + 0.007 \text{ Inoculum} \end{aligned} \quad \text{Equation 3.7}$$

The results obtained for *Aquamicrobium sp.* GR4 and *Bacillus sp.* GE1 pertaining to PBD in benzene supplemented media are listed in Benzene section of Appendices.

Similarly, for other aromatic HCs Toluene and Xylene (Appendices), the significant factors were statistically consistent and they can be relied upon the predictability of biomass production. The significant may also be used for further optimization studies. The significant factors of the strains are also supportive of the existing reports suggesting the importance of salt presence for better performance towards hydrocarbon removal [300,303,307].

For each of the PBD outputs, the discrepancies in low coefficient of determination may be a result of multiple factors such as underlying complexity of the microbial growth, linear interpretation of a non-linear process, influence of other variables that inherently affect biomass production etc. Lower adjusted R^2 and predicted R^2 might signify poor fit, and the models might not describe biomass production accurately. The cause of overfitting could be attributed to the complexity of the microbial growth and nutrient utilization that can further result in poor generalization. However, while optimizing a complex media, the mechanism of microbial response and their possible limitations in HC environments may not be cumulatively explained. Also, it can be argued that only R^2 or poor fit does not necessarily fully describe the reliability of a model. Since PBD is one of the simplest forms of screening out significant factors, statistical analyses such as coefficient of variation and p -value ($p > F$) also accounts to explain significant difference of response when the variables are added for simulation. Hence, alongwith the whole model characteristics, we also relied on the contribution of each of individual factors and understanding their respective statistical significances for deciding whether these variables could create a notable deviation towards obtaining enhanced biomass at the F -value ($p > F$). Accurate and precise measurements may enable reduced noise generation, using alternative procedures such as cross-validation, bootstrapping, or splitting the data into training and testing sets can provide a better estimate of the predictive power of a model and help in identifying potential issues [323,327,328,330–336].

3.3.5. Statistical analysis of optimized factors for maximum biomass production

At MTC of *n*-hexadecane, optimization of *Bacillus* sp. GS1 exhibited satisfactory statistical values (Table 3.18). Basic statistical parameters like mean, coefficient of variation, and standard deviation (SD) were analysed to predict the model efficiency.

From Table 3.19, the coefficient of variation of 0.704 suggested that the optimised response was accurate, and the results are reproducible. Overall, the SD and mean values were 1.238 and 1.758 respectively. These values obtained were within $p \leq 0.05$ suggesting minimal residual values and statistically different. The R^2 value was 0.92 and the adjusted value indicated that 87.46% of variability in biomass production could be calculated. Predicted R^2 of 0.447 distant to the range of adjusted value of 0.885 suggesting that the model only predicts 44.66% of the values accurately, however, the corresponding optimum response suggested a predicted biomass value within closer range from the actual experimental target value.

The regression equation governing 2nd order interaction between the variables is shown below as Equation 3.8:

$$\begin{aligned} \text{Biomass} = & 0.103 + 0.025 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.002 \text{ NaCl} \\ & + 0.654 \text{ Inoculum} - 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - \\ & 0.000 \text{ NaCl} * \text{NaCl} - 0.114 \text{ Inoculum} * \text{Inoculum} \\ & + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.01 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Inoculum} \\ & + 0.002 \text{ NaCl} * \text{Inoculum} \end{aligned}$$

Equation 3.8

Table 3.18: ANOVA of CCD for *Bacillus sp.* GS1 in *n*-hexadecane supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	26.767	2.974	12.74	0
Linear	3	18.597	6.199	26.55	0
FeSO ₄ .7H ₂ O	1	2.175	2.175	9.32	0.012
NaCl	1	5.943	5.943	25.46	0.001
Inoculum	1	10.479	10.479	44.89	0
Square	3	4.87	4.87	6.95	0.008
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	3.127	3.127	13.39	0.004
NaCl*NaCl	1	1.651	1.651	7.07	0.024
Inoculum*Inoculum	1	0.953	0.953	4.08	0.071
2-Way Interaction	3	3.301	3.301	4.71	0.027
FeSO ₄ .7H ₂ O*NaCl	1	1.462	1.462	6.26	0.031
FeSO ₄ .7H ₂ O*Inoculum	1	0.845	0.845	3.62	0.086
NaCl*Inoculum	1	0.994	0.994	4.26	0.066
Error	10	2.335	0.234		
Lack-of-Fit	5	2.182	0.436	14.3	0.006
Pure Error	5	0.153	0.031		
Total	19	29.102			

Table 3.19: Model summary of CCD for *Bacillus sp.* GS1 in *n*-hexadecane MSM.

Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	1.238	1.758	0.704	0.92	0.875	0.447

Similarly, statistical analysis pertaining to maximum biomass production for all the other strains were also analysed. The detailed results are indicated in *n*-hexadecane section of Appendices.

At MTC of anthracene, predicted response of maximum biomass for *Bacillus sp.* GE1 were analysed as per ANOVA in Table 3.20.

Table 3.20: ANOVA of CCD for *Bacillus sp.* GE1 in anthracene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.196	0.0218	11.89	0
Linear	3	0.168	0.056	30.64	0
Inoculum	1	0.128	0.128	70	0
NaCl	1	0.022	0.022	11.97	0.006
FeSO ₄ .7H ₂ O	1	0.018	0.018	9.94	0.01
Square	3	0.022	0.022	4.08	0.039
Inoculum*Inoculum	1	0.006	0.006	3.1	0.109
NaCl*NaCl	1	0.018	0.018	10.06	0.01
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.04	0.851
2-Way Interaction	3	0.005	0.005	0.95	0.455
Inoculum*NaCl	1	0.003	0.003	1.75	0.216
Inoculum*FeSO ₄ .7H ₂ O	1	0.002	0.002	0.98	0.345
NaCl*FeSO ₄ .7H ₂ O	1	0	0	0.11	0.748
Error	10	0.018	0.002		
Lack-of-Fit	5	0.018	0.004	25.8	0.001
Pure Error	5	0.001	0		
Total	19	0.214			

The regression equation for biomass production as predicted by CCD for *Bacillus sp.* GE1 in anthracene supplemented media is presented in Equation 3.9:

$$\begin{aligned} \text{Biomass} = & 0.027 + 0.083 \text{ Inoculum} + 0.001 \text{ NaCl} + 0.000 \text{ FeSO}_4.7\text{H}_2\text{O} - \\ & 0.009 \text{ Inoculum*Inoculum} - 0.000 \text{ NaCl*NaCl} - 0.000 \\ & \text{FeSO}_4.7\text{H}_2\text{O*FeSO}_4.7\text{H}_2\text{O} + 0.000 \text{ Inoculum*NaCl} + 0.000 \\ & \text{Inoculum*FeSO}_4.7\text{H}_2\text{O} + 0.000 \text{ NaCl*FeSO}_4.7\text{H}_2\text{O} \end{aligned}$$

Equation 3.9

From Table 3.21, the coefficient of variation of 0.411 suggested that the optimised response was highly accurate, and the results are reproducible.

Table 3.21: Model summary of CCD for *Bacillus sp.* GE1 in anthracene MSM.

Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	0.106	0.259	0.411	0.915	0.838	0.469

Overall, the SD and mean values were 0.106 and 0.259 respectively. Moreover, the values were within $p \leq 0.05$ suggesting correlation among factors and were statistically different. The adjusted R^2 value indicated that 83.76% of variability in biomass production could be calculated. A predicted R^2 of 0.469 suggested a lower model predictability; however, the corresponding optimum response suggested a predicted biomass within 14% of the actual experimental target value suggesting that the model could be applied to describe maximum biomass production in the 3D space [323,326].

For other strains checked for optimum factors to obtain maximum biomass production, the detailed results are shown in Anthracene section of Appendices.

Similarly, for Phenanthrene (Appendices), at MTC, the coefficient of variation was listed as 0.337. The values suggested that the optimised response was close to accurate, and the results are reproducible. The SD was recorded as 0.0383 whereas the mean value was obtained as 0.115. The model was within $p \leq 0.05$ and hence, was statistically different. Moreover, the model had minimal residual and satisfactory correlation among factors were observed. The R^2 value of 86.05% indicated significant prediction in variability in biomass production. Optimum inoculum of 3.68%, NaCl: 334.88 mg/L and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ of 65.34 mg/L suggested a predicted biomass value of 0.267 mg/mL which was 27% distant from the actual experimental target value.

For MTC of benzene, the experimental was carried out for *Staphylococcus sp.* GR2 at 1000 mg/L as per CCD and the optimum response obtained as shown Table 3.22.

Table 3.22: ANOVA of CCD for *Staphylococcus sp.* GR2 in benzene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.053	0.006	19.82	0
Linear	3	0.046	0.015	51.9	0
FeSO ₄ .7H ₂ O	1	0.014	0.014	48.02	0
NaCl	1	0.013	0.013	44.45	0
Time	1	0.019	0.019	63.23	0
Square	3	0.003	0.003	3.57	0.055
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.4	0.543
NaCl*NaCl	1	0.001	0.001	2.2	0.168
Time*Time	1	0.003	0.003	9.23	0.012
2-Way Interaction	3	0.004	0.004	3.98	0.042
FeSO ₄ .7H ₂ O*NaCl	1	0.001	0.001	3.17	0.105
FeSO ₄ .7H ₂ O*Time	1	0.002	0.002	5.16	0.046
NaCl*Time	1	0.001	0.001	3.62	0.086
Error	10	0.003	0		
Lack-of-Fit	5	0.003	0.001	48.05	0
Pure Error	5	0	0.000		
Total	19	0.056			

The biomass production was described as per the following regression Equation 3.10:

$$\begin{aligned}
 \text{Biomass} = & 0.073 + 0.001 \text{ FeSO}_4.7\text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.002 \text{ Time} - \\
 & 0.000 \text{ FeSO}_4.7\text{H}_2\text{O} * \text{FeSO}_4.7\text{H}_2\text{O} - 0.000 \text{ NaCl} * \text{NaCl} - \\
 & 0.000 \text{ Time} * \text{Time} + 0.000 \text{ FeSO}_4.7\text{H}_2\text{O} * \text{NaCl} \\
 & + 0.000 \text{ FeSO}_4.7\text{H}_2\text{O} * \text{Time} + 0.000 \text{ NaCl} * \text{Time}
 \end{aligned}
 \tag{Equation 3.10}$$

The model predicted the coefficient of variation of 0.35 in Table 3.23, suggesting that the optimised response was close to accurate, and the results are reproducible.

Table 3.23: Model summary of CCD for *Staphylococcus sp.* GR2 in benzene MSM.

Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	0.054	0.156	0.35	0.947	0.9	0.577

Overall, the SD and mean values were in the range 0.054 and 0.156 respectively which were in agreement within $p \leq 0.05$. Hence, the data sets were statistically different, and factors were correlated with each other. The R^2 value of 0.947 indicated that 89.91% of variability in biomass production could be calculated. Predicted R^2 of 0.577 suggests that the model may have close resemblance, and the corresponding optimum response suggested a predicted biomass value within 17% from the actual experimental target value.

Similarly, for the other strains in benzene (Benzene section of Appendices) and other aromatic HCs Toluene and Xylene, statistical analysis showed that the optimum response could be satisfactory for predicting maximum biomass production in a system contaminated with the respective HCs. The detailed results are shown in Appendices.

In a typical mathematical design, it is always desired to have significant model fit and the lack-of-fit to be insignificant. In each of our cases, the ANOVA tables indicated significant lack-of-fit that was undesirable, and the model might not accurately define interactions among the factors and their responses. This could be attributed to the limited number of variables tested in our study and there are additional inherent factors that might influence biomass production. Moreover, since, refinery waste contains variable composition with multiple factors that could be untenable, the dynamics of the whole system might become tedious to demonstrate through quadratic model. Also, HC utilization is a complex phenomenon, and other external disturbances affect microbial performances, that may result in disruptive results in terms of biomass production [332,336,337]. To address this, the experiments were carried out multiple times, and the best possible outcomes were reported. However, it might be desirable to validate with higher order models or alternate factorial designs to obtain superior outcomes.

3.3.6. Modified media components for enhanced bioremediation efficiency

From the 2nd order optimization studies, 65.34 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 334.88 mg/L were added to original media components 200 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg/L K_2HPO_4 and 100 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. pH of the original media was set at 6.75 ± 0.25 . The same was further used

as after addition of new components together, no detrimental effect among microbial growth was observed.

3.3.7. Consortia preparation

3.3.7.1. Screening of significant strains affecting biomass production in modified media

At the end of 48 days, each experimental set was analysed for biomass production. The significant strains obtained from the 1st order optimization was further subjected to 2nd order optimization to identify optimum inoculum concentrations for the mixed consortia.

While optimizing the optimum inoculum combinations, it was assumed that the strains whose optimum inoculum was not obtained while conducting media optimization, the optimum inoculum obtained during initial screening (Chapter 2, section 2.3.9) was used for creating the DoE using PBD and CCD. For example, since inoculum concentrations of *Aquamicrobium sp.* GR2 and *Aquamicrobium sp.* GE2 were not significant previously, the lower and upper range of their inoculum optimization was used as 1% and 4% respectively, which was obtained in Chapter 2.

For set 1, screening of significant strains was carried as per the sets described in Table 3.24.

Table 3.24: PBD for strains in Set 1 mixture of HCs.

Run	GR1	GR2	GR4	GE1	GE2	GS1	Biomass (mg/mL)
1	4	3.44	1	1	4	6.37	2.251
2	4	3.44	3.68	6.37	1	1	2.354
3	4	3.44	1	1	1	1	1.563
4	1	1	3.68	1	4	1	1.431
5	1	1	1	6.37	1	6.37	1.647
6	1	3.44	1	6.37	1	6.37	2.569
7	1	1	1	1	4	1	1.292
8	1	3.44	1	6.37	4	6.37	3.453
9	4	1	1	6.37	4	1	2.766
10	4	3.44	3.68	1	1	6.37	1.378
11	4	1	3.68	6.37	1	1	1.773
12	4	1	3.68	6.37	4	6.37	1.568
13	1	3.44	3.68	1	4	6.37	1.448
14	1	1	1	1	1	1	0.098
15	4	1	3.68	1	4	6.37	1.371
16	1	3.44	3.68	6.37	4	1	3.469
17	4	3.44	1	6.37	4	1	3.393
18	4	1	1	1	1	6.37	0.104
19	1	1	3.68	6.37	1	6.37	1.703
20	1	3.44	3.68	1	1	1	1.449

Staphylococcus sp. GR2 (B), *Bacillus sp.* GE1 (D) and *Aquamicrobium sp.* GE2 (E) showed positive effects when the isolates were inoculated at highest (MTC) concentration of mixture of HCs. The positive effects are indicated in the normalized plot, pareto chart and main effects plot in Figure 3.4.

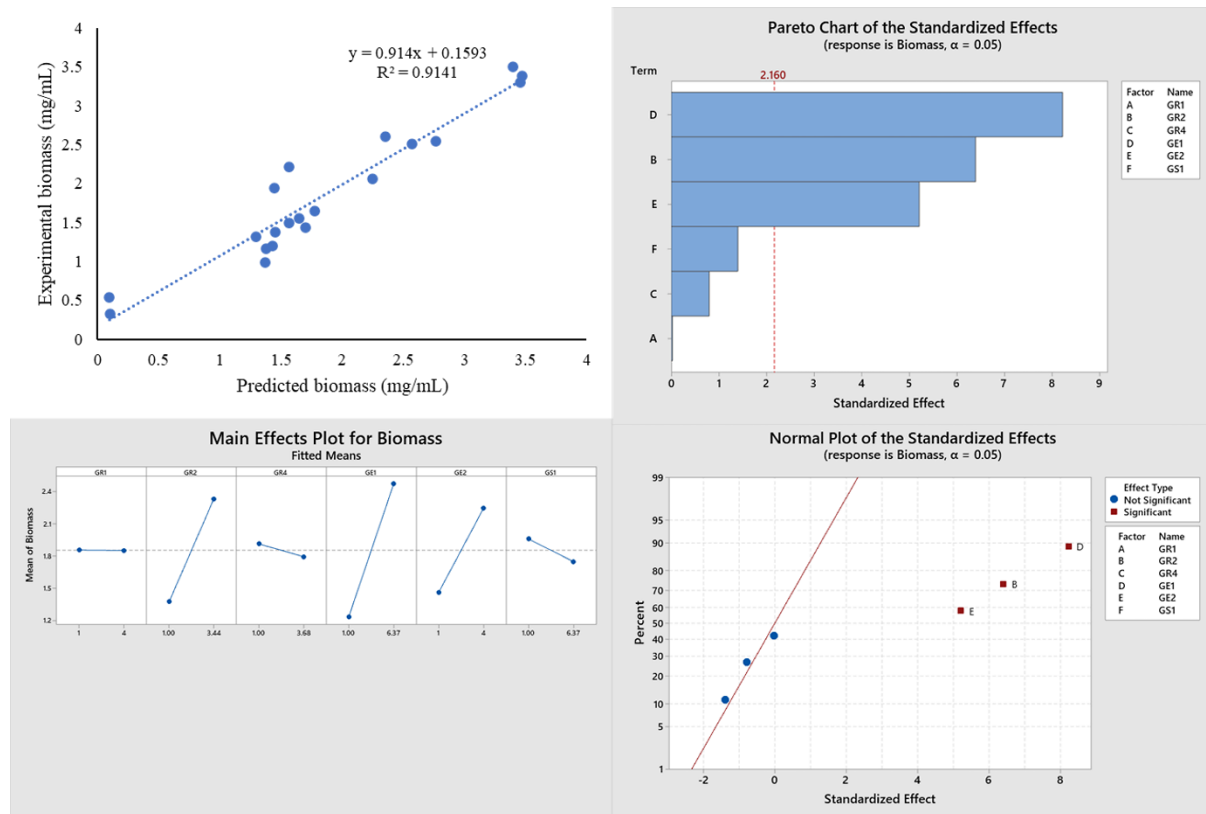


Figure 3.4: Precision, Pareto chart, main effects plot, and normal distribution of factors tested for strains in Set 1 mixture MSM. Precision depicts the predictability of the model towards enhanced biomass production; pareto chart, main effects plot and normal distribution of factors depict the significant variables having positive effects on biomass production.

The biomass product for 1st order optimization is described in Equation 3.11 as:

$$\begin{aligned} \text{Biomass} = & -0.261 - 0.001 \text{ GR1} + 0.392 \text{ GR2} - 0.045 \text{ GR4} + 0.229 \text{ GE1} \\ & + 0.26 \text{ GE2} - 0.039 \text{ GS1} \end{aligned} \quad \text{Equation 3.11}$$

Similarly, for Set 2: 1st order Plackett-Burman Design screening, the results are shown in Appendices.

3.3.7.2. Optimization of significant strains for maximum biomass production

The optimum factors were obtained after experimentation of the significant strains as per CCD. For set 1, the design of experiments obtained are shown in Table 3.25.

Table 3.25: CCD for strains in Set 1 mixture of HCs.

- Set 1: 500 mg/L *n*-hexadecane, 50 mg/L Anthracene, 20 mg/L Phenanthrene, 1000 mg/L Benzene, 1000 mg/L Toluene, 1000 mg/L Xylene
- Design: CCD ($\alpha = 1.633$)

Run	GR2	GE1	GE2	Biomass (mg/mL)
1	2.22	3.685	2.5	0.558
2	2.22	3.685	2.5	0.537
3	3.44	1	4	0.538
4	1	1	1	0.026
5	3.44	6.37	1	0.777
6	2.22	3.685	2.5	0.618
7	1	6.37	1	0.481
8	1	6.37	4	0.654
9	2.22	3.685	2.5	0.607
10	3.44	6.37	4	0.758
11	1	1	4	0.372
12	3.44	1	1	0.481
13	2.22	8.07	2.5	1.097
14	2.22	3.685	4.95	1.137
15	2.22	3.685	2.5	0.654
16	2.22	-0.7	2.5	0.218
17	4.212	3.685	2.5	0.66
18	0.228	3.685	2.5	0.618
19	2.22	3.685	0.051	0.296
20	2.22	3.685	2.5	0.372

The optimum response obtained after completing each set of experiment as per CCD for Set 1 is shown in Table 3.26.

Table 3.26: Multiple response prediction of strains from CCD in Set 1 mixture HCs.

Variable	GR1	GE1	GE2	Biomass fit	
				Target	Predict
Optimized setting	3.82	7.02	3.844	0.96	1.056

From the various optimum responses obtained from 2nd order optimization for all the significant strains, the optimum inoculums of 3.82% *Staphylococcus sp.* GR2, 7.02% *Bacillus sp.* GE1 and 3.84% *Aquamicrobium sp.* GE2 may be applicable for higher concentration mixture of HCs (upto MTC) supplemented in MSM. The model obtained was further checked statistically.

Similarly, for Set 2: 2nd order Central Composite Design optimization, the results are detailed in Appendices.

3.3.8. Statistical analysis for DoEs of consortia preparation

3.3.8.1. Analysis of significant strains for enhanced biomass production

For set 1, the ANOVA Table 3.27 is shown below:

Table 3.27: ANOVA and model summary of strains for PBD in Set 1 mixture of HCs.

Analysis of variance					
Source	DF	Adj SS	Adj MS	F-value	p-value
Model	6	15.5	2.583	23.05	0
Linear	6	15.5	2.583	23.05	0
GR1	1	0	0	0.00	0.98
GR2	1	4.583	4.583	40.9	0
GR4	1	0.071	0.071	0.63	0.44
GE1	1	7.577	7.577	67.62	0
GE2	1	3.045	3.045	27.18	0
GS1	1	0.22	0.22	1.96	0.185
Error	13			1.457	0.112
Total	19			16.952	
Model Summary					
S	R-sq	R-sq(adj)	R-sq(pred)		
0.031	91.41%	87.44%	79.66%		

The model exhibited *F-value* of 23.05. It suggested that the model is appropriate and significant and have positive effect on the biomass production. However, Model *F-value* occurred at minimal noise value of 0.01%. *p-value* ($p > F$) was used as a tool to identify significant factors. *p-value* less than 0.05 denoted significant factors and were taken to have affected biomass production (*Y*). Predicted R^2 of 0.797 was within 13% to the actual value of 0.914 and adjusted value of 0.838. The model was predicted accurately and is reproducible. No

separate factors were required to be incorporated, and the model was used for further optimization studies [323,325].

Similarly, statistical analysis was carried out for Set 2: 1st order Plackett-Burman Design screening also. The detailed results are shown in Appendix Table 99.

As discussed in section 3.3.4, exploring alternative model structures, such as polynomial regression, or ensemble methods, in addition to data splitting and augmentation to retain linearity, can sometimes provide better fits for complex data.

3.3.8.2. Analysis for optimized strains for maximum biomass production

For set 1, predicted response of maximum biomass were analysed using basic statistical measures and ANOVA presented in Table 3.28 and Table 3.29 respectively.

Table 3.28: Model summary of CCD for strains in Set 1 mixture of HCs.

Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	0.184	0.444	0.413	0.96	0.916	0.54

The biomass production is described as a regression model in Equation 3.12 as follows:

$$\begin{aligned} \text{Biomass} = & 0.265 - 0.096 \text{ GR2} + 0.008 \text{ GE1} - 0.055 \text{ GE2} \\ & + 0.032 \text{ GR2*GR2} + 0.007 \text{ GE1*GE1} + 0.013 \text{ GE2*GE2} - \\ & 0.002 \text{ GR2*GE1} + 0.014 \text{ GR2*GE2} + 0.001 \text{ GE1*GE2} \end{aligned} \quad \text{Equation 3.12}$$

The coefficient of variation for set 1 obtained as 0.415 suggested that the optimised response was close to accurate, and the results are reproducible. The SD was recorded as 0.1836 whereas the mean value was obtained as 0.444. The model was within $p \leq 0.05$ suggesting the model was statistically different, having minimal residual and satisfactory correlation among factors. The R^2 value of 0.96 indicated that 91.58% of variability in biomass production could be calculated. Predicted R^2 of 0.54 is 41% distant to the range of adjusted value of 0.916, suggesting that the model for set 1 may have close resemblance, however, the corresponding optimum inoculum of 3.82% *Staphylococcus sp.* GR2, 7.02% *Bacillus sp.* GE1 and 3.84% *Aquamicrobium sp.* GE2 suggested a predicted biomass value of 1.056 mg/mL which was 9% distant from the actual experimental target value.

Table 3.29: ANOVA of CCD for strains in Set 1 mixture of HCs.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	10	0.615	0.061	21.66	0
Blocks	1	0.001	0.001	0.22	0.649
Linear	3	0.54	0.18	63.43	0
GR2	1	0.108	0.108	37.95	0
GE1	1	0.365	0.365	128.58	0
GE2	1	0.067	0.067	23.75	0.001
Square	3	0.068	0.023	8.02	0.007
GR2*GR2	1	0.029	0.029	10.25	0.011
GE1*GE1	1	0.036	0.036	12.85	0.006
GE2*GE2	1	0.012	0.012	4.10	0.074
2-Way Interaction	3	0.006	0.002	0.68	0.585
GR2*GE1	1	0	0	0.07	0.797
GR2*GE2	1	0.005	0.005	1.91	0.201
GE1*GE2	1	0	0	0.07	0.797
Error	9	0.026	0.003		
Lack-of-Fit	5	0.025	0.005	61.8	0.001
Pure Error	4	0	0		
Total	19	0.641			

The results for Set 2: 2nd order Central Composite Design optimization were similarly analysed. The optimized factors were in statistical agreement and hence, the combinations could well be used for practical experiments (Appendices).

Similar to as discussed in section 3.3.5, the ANOVA tables indicate significant lack-of-fit, and the model might not accurately define interactions among the factors and their responses and repeated experiments were carried to obtain the best possible outcomes.

3.3.9. Mutual interaction between the significant factors and optimum response

The interaction between the factors on biomass concentration were studied by constructing contour plot (graphical representation of regression model) and surface plot. The

shape of the contour plots and surface plots were studied to identify whether the independent variables that are mutually interacting are significant or not.

Figure 3.5 depicts the contour plots and surface plots to optimize response in CCD model for isolate *Bacillus sp.* GS1 at MTC of *n*-hexadecane. Each contour plots and surface plots showed shapes which agreed to response well within the boundary level suggesting the validity for consideration of CCD as second order tool for design of experiments. The mutual interactions of the other strains are shown in *n*-hexadecane section of Appendices.

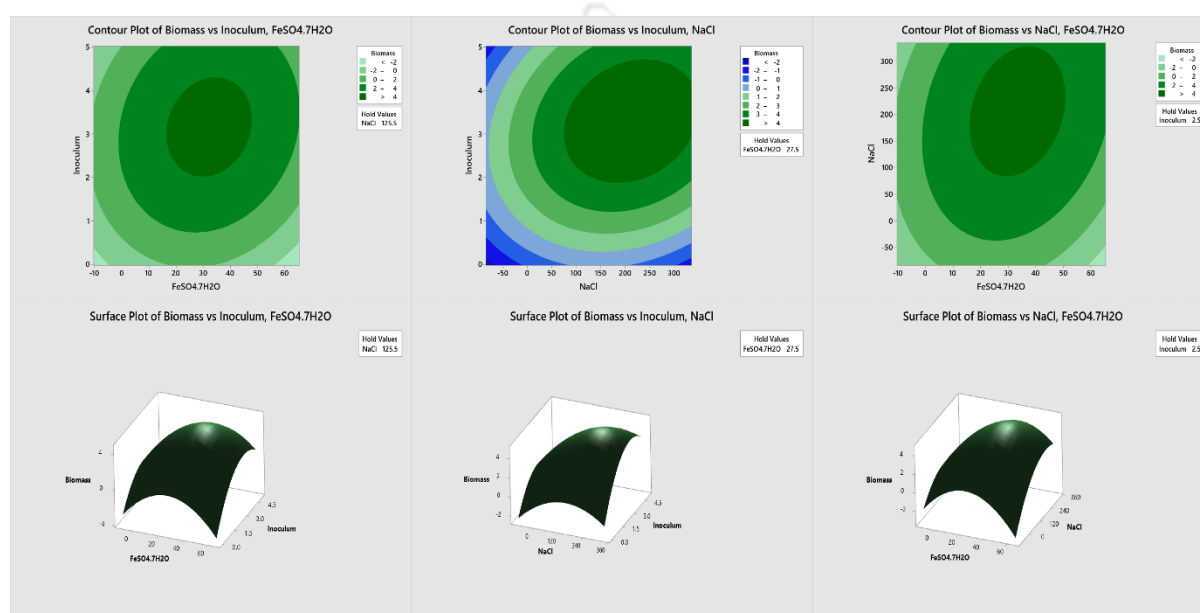


Figure 3.5: Contour and surface plots of biomass at MTC of *n*-hexadecane for *Bacillus sp.* GS1. The dark green spot in the contour plot and bright white spot on the surface plot corresponds to the optimum conditions of 3.44% inoculum (v/v), 229.13 mg/L NaCl and 36.29 mg/L FeSO₄.7H₂O where a maximum biomass of 5.02 mg/mL could be predicted.

Similarly, the contour plots and surface plots for one each of the potent strains at the MTCs of anthracene (Figure 3.6), benzene (Figure 3.7) are represented. The same for the rest of the strains for Anthracene, Benzene and all of Toluene, Xylene and Phenanthrene are represented in Appendices.

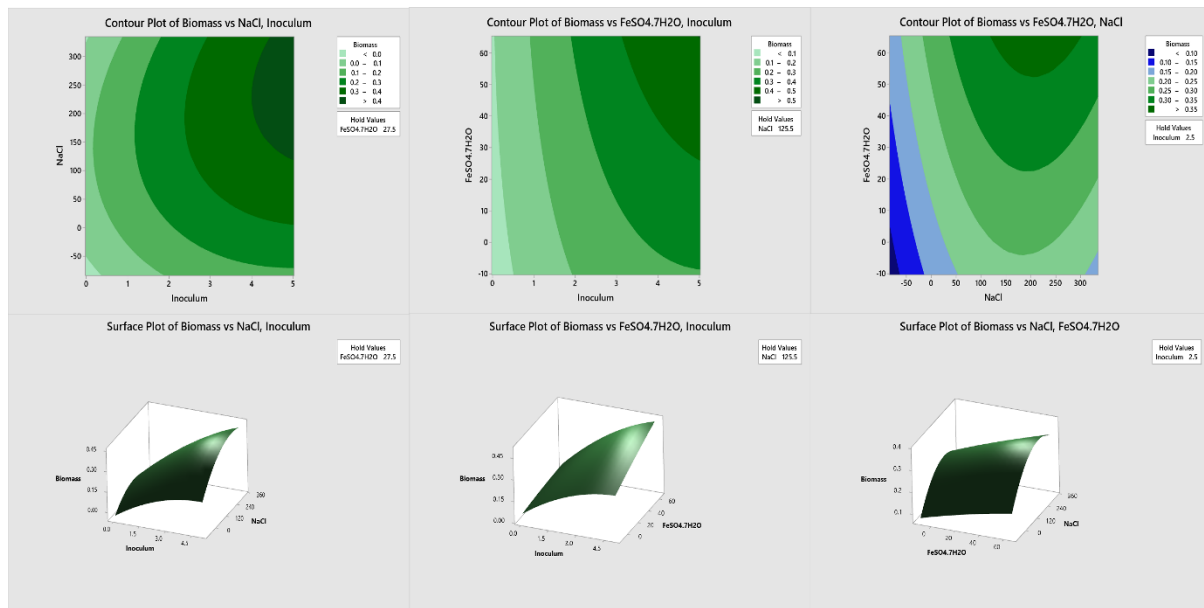


Figure 3.6: Contour and surface plots of biomass at MTC of anthracene for *Bacillus sp. GE1*. The dark green spot in the contour plot and bright white spot on the surface plot corresponds to the optimum conditions of 5.02% inoculum (v/v), 267.2 mg/L NaCl and 65.34 mg/L FeSO4.7H₂O where a maximum biomass of 0.46 mg/mL could be predicted.

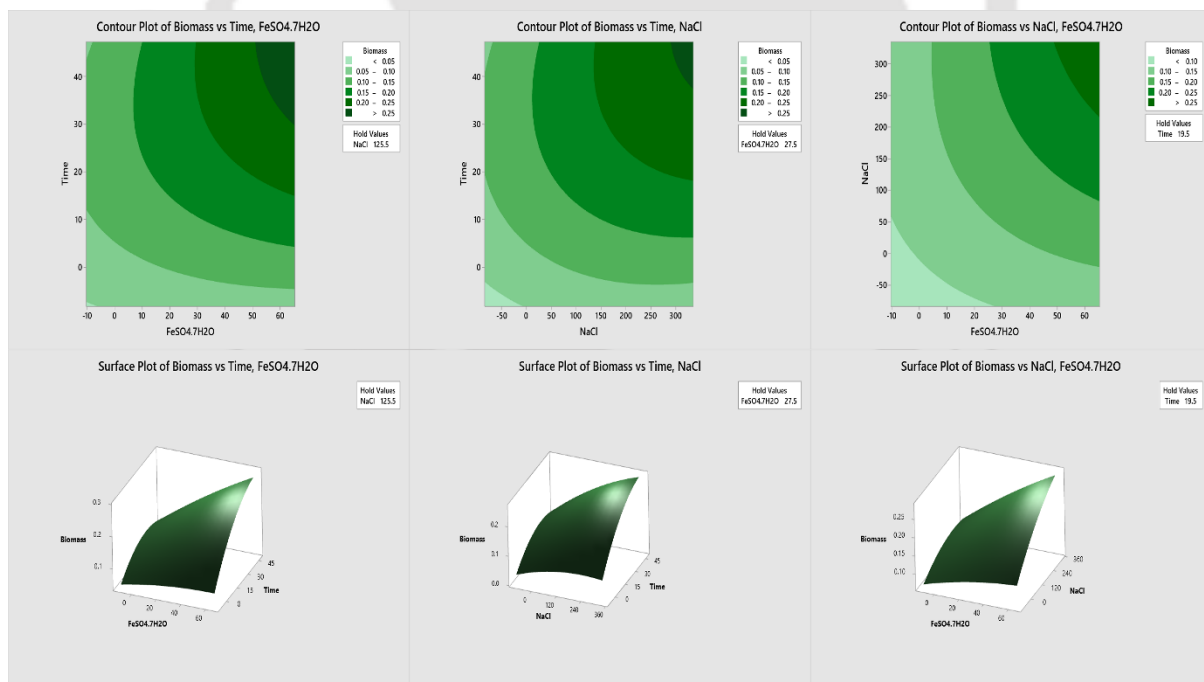


Figure 3.7: Contour and surface plots of biomass at MTC of benzene for *Staphylococcus sp. GR2*. The dark green spot in the contour plot and bright white spot on the surface plot corresponds to the optimum conditions of 47.25 days of incubation, 334.88 mg/L NaCl and 65.34 mg/L FeSO4.7H₂O where a maximum biomass of 0.38 mg/mL could be predicted.

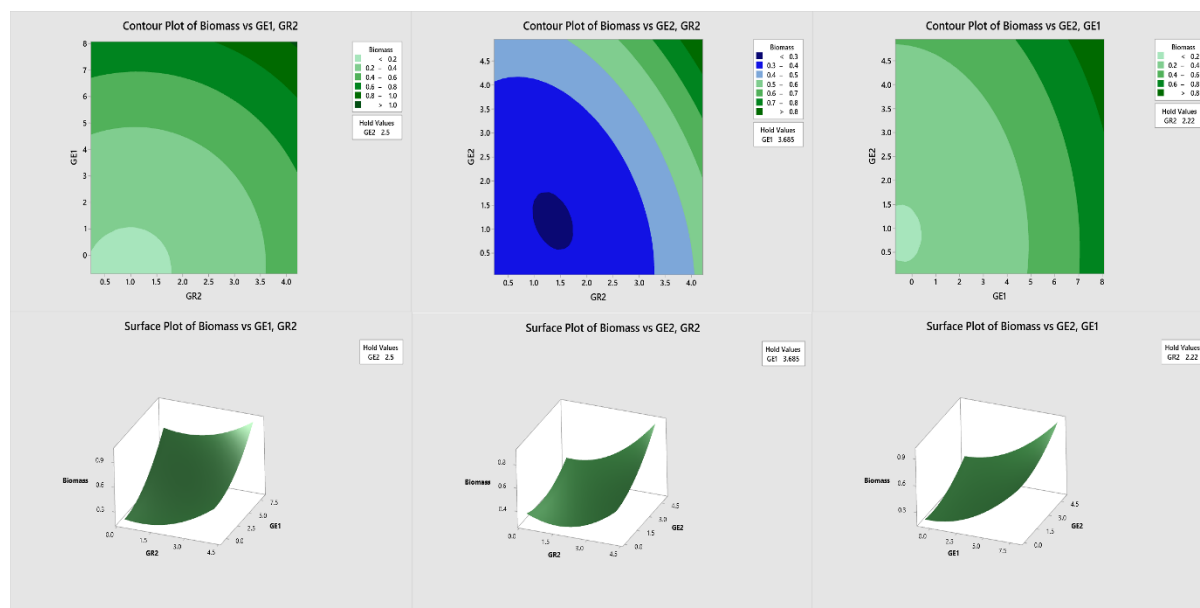


Figure 3.8: Contour and surface plots of biomass at MTC for Set 1 mixture of HCs. The dark green spot in the contour plot and bright spot on the surface plot corresponds to the optimum inoculum conditions (v/v) of 3.82% GR1, 7.02% GE1 and 3.84% GE2 where a maximum biomass of 1.06 mg/mL could be predicted.

The mutual interactions among the significant strains of consortia design are indicated in Figure 3.8. For the interactions between strains obtained from Set 2: 2nd order Central Composite Design optimization, the detailed results are shown in Appendices.

3.3.10. Biomass and COD removal comparison for assessing growth in optimized media

The biomass concentration was measured at the end of growth cycle of each of the potent strains after optimization. Similar to initial screening discussed in Chapter 2, section 2.2.10, the strains were incubated in the MSM supplemented individually with the different HCs.

In *n*-hexadecane, the maximum biomass of 5.06 mg/mL was observed for *Staphylococcus sp.* GR2. The increase in biomass concentration, bacterial count and COD removal is depicted in Figure 3.9:

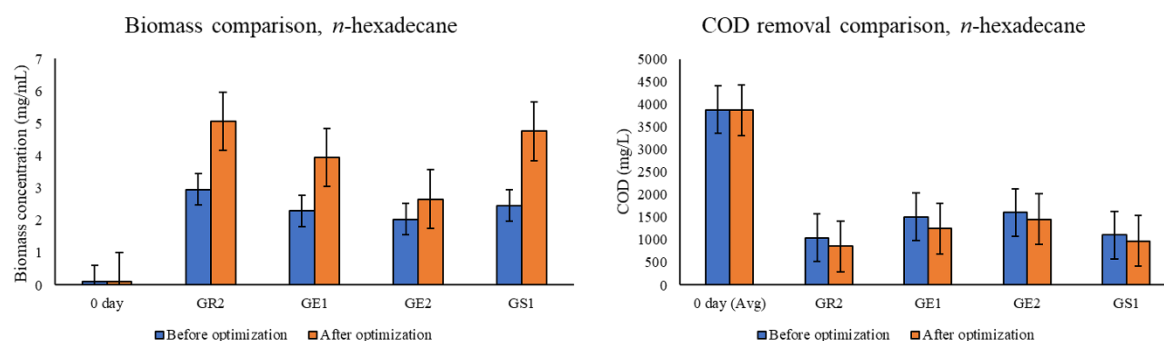


Figure 3.9: Comparison of biomass growth and COD removal of strains before and after statistical optimization for *n*-hexadecane. Biomass concentration increased significantly after optimization. A maximum biomass of 5.06 mg/mL was obtained alongside increased COD removal of ~78% by *Staphylococcus sp.* GR2. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Similarly, for anthracene, the maximum biomass of *Bacillus sp.* GE1 also increased to 0.45 mg/mL as shown in Figure 3.10.

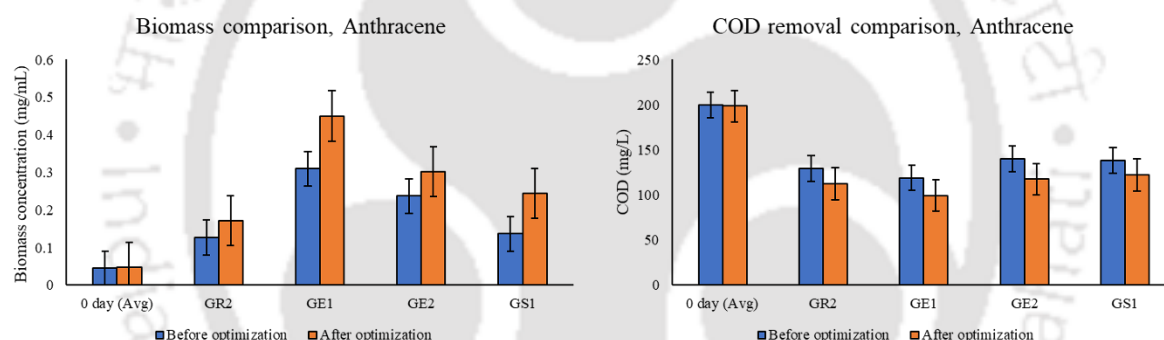


Figure 3.10: Comparison of biomass growth and COD removal of strains before and after statistical optimization for anthracene. Biomass concentration increased significantly after optimization. A maximum biomass of 0.45 mg/mL was obtained alongside increased COD removal of >50% by *Bacillus sp.* GE1. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Similarly for benzene, maximum biomass after optimization was observed as 0.289 mg/mL for *Bacillus sp.* GE1. COD removal of 0 day is depicted as 10 times less the actual value to accommodate the COD values obtained after the incubation process for the strains within the same axis. The results are depicted in Figure 3.11.

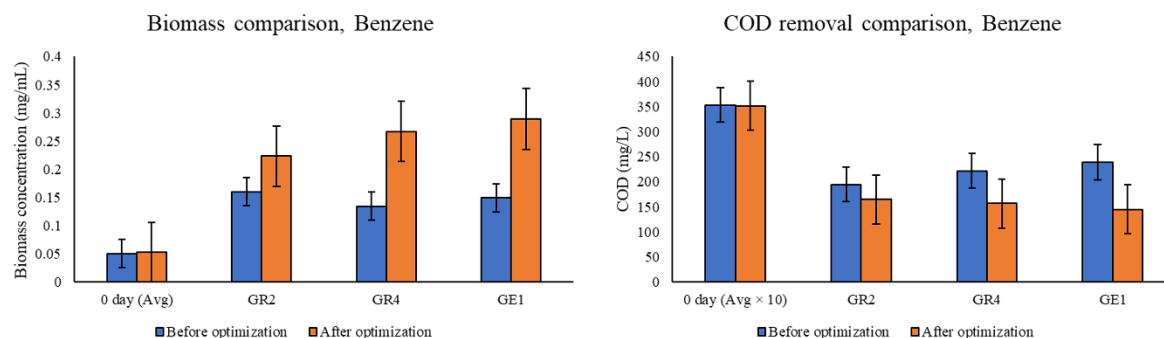


Figure 3.11: Comparison of biomass growth and COD removal of strains before and after statistical optimization for benzene. Biomass concentration increased significantly after optimization. A maximum biomass of 0.289 mg/mL was obtained alongside increased COD removal of ~98% by *Bacillus sp.* GE1. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The depiction of change in biomass concentration and COD removal for other pollutants Phenanthrene, Toluene and Xylene are depicted as Biomass and COD removal comparison for assessing enhanced growth in optimized media in Appendices. The viability of the biomass obtained in each of the cases were validated by enumerating CFU/mL as indicated in Appendix Figure 42.

3.3.11. Optimum consortia for enhanced bioremediation efficiency

From the 2nd order optimization studies, combination 1 (%v/v) of 3.82% *Staphylococcus sp.* GR2, 7.02% *Bacillus sp.* GE1, 3.84% *Aquamicrobium sp.* GE2 was tentatively designated as **C1**, combination 2 (%v/v) of 4.24% *Aquamicrobium sp.* GR1, 3.89% *Staphylococcus sp.* GR2, 6.11% *Bacillus sp.* GS1 was marked as **C2** and combination 3 (%v/v) of 3.51% *Staphylococcus sp.* GR2, 4% *Aquamicrobium sp.* GR4, 6.31% *Bacillus sp.* GE1 was named as **C3**. Further, unless mentioned; C1, C2, C3 has been respectively used for conducting the experiments.

3.4. Summary

In this chapter, we tried to devise an optimum condition best suited for the microbial strains for their growth and survival. Addition of external micronutrient factors $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl proved to be better for their survival and extensive research was put to obtain the composition that could best mimic a waste environment. Further, biomass production was taken as an indicative measure to understand the increased adaptability into such toxic waste mixture. A summary of optimum factors obtained under laboratory-controlled HC supplemented media is placed in Appendix Table 108 for the reader's reference. The optimum media obtained by

series of 1st and 2nd order optimization was used to deduce the optimum inoculum concentrations of different groups of microbial populations (consortia). On account of higher growth and removal efficiency, the optimized combinations could be expected to perform enhanced bioremediation in addition to delivering maximum biomass concentration.

It is possible that additional factors not studied could have cumulative effect on biomass production and are required to be studied. Also, lower R^2 might signify poor fit. It is worthy to understand that optimizing a complex system may result in erroneous depiction on the mechanism of biomass production considering the limitations posed by a toxic HC contaminated environment. Hence, instead of solely relying on the fit characteristics, we also relied on statistical studies to explain the simulated condition and deciding whether significant difference in biomass production was observed as per p -value ($p > F$) at their F -value. Although significant lack-of-fit was observed, the optimum responses of screened factors from the CCD experiments suggested reliability of obtaining maximum experimental biomass and accurate prediction of same, that was the aim of the study.

Analysis of biomass characteristics and enzyme activities for insight into the bioremediation of waste sludge

4.1. Background

Enzymes play a crucial role in the treatment of refinery waste sludge. They are produced by microorganisms and have great metabolic catalytic activity capable of reducing complex toxic compounds into less harmful ones. These enzymes specifically digest organic matter, dividing up the organic contaminants into manageable components. There are several classes of enzymes involved in this process, including protease, amylase, lipase, cellulase, urease, and xylanases. These enzymes are produced by microorganisms present in the sludge and are responsible for the degradation of complex organic matter such as polysaccharides, proteins, and lipids.

In the context of degradation of alkane and PAHs, several enzymes come into play. Alkane mono-oxygenases initiate the degradation of short (C_5 - C_{15}) and long-chain alkanes ($>C_{15}$). The *alkB/M* is rubredoxin-dependent, while *almA* and *ladA* are flavin-dependent mono-oxygenases. For PAHs degradation, different enzymes like mono-oxygenases, PAH ring hydroxylating dioxygenase such as catechol 1,2-dioxygenase, and lipases are involved in the transformation/degradation of PAHs like phenanthrene, pyrene, anthracene, fluorene, etc. Other enzymes involved include lignin peroxidase, manganese peroxidase, laccase, cytochrome P450, and epoxide hydrolase [210,216,221,229,233,238]. The use of these enzymes for waste treatment is environmentally friendly and sustainable. It is a promising approach to manage waste effectively while protecting environmental and public health.

The biomass generated after the treatment of refinery waste sludge has significant potential for conversion into biofuels and other value-added products. The efficacy of this biomass as a future source of fuel or value-added products depends on the composition, that include carbohydrates, lipids, proteins, and other organic compounds [252,258]. The fate of the biomass post-treatment can be diverse. It can be converted into biofuels through various processes such as gasification, combustion, pyrolysis, enzymatic hydrolysis routes, and fermentation. For instance, thermochemical processes like pyrolysis are promising because they operate at a relatively lower temperature of up to 500 °C, compared to gasification, which operates at 800-1300 °C. The integration of hydrothermal and biochemical routes is promising for the circular economy [252,254].

To determine whether the biomass is suitable for future use as fuel or for the production of value-added products, it is essential to analyse its characteristics. This includes determining

the carbohydrate content, lipid content, protein content etc. Spectrophotometric determination of carbohydrates has also been used vigorously by various investigators and is an important biochemical test to analyse C-deposition. Estimating lipid deposition is critical for the production of quality biodiesel. Determination of C-chain length and the degree of saturation is key to ensuring high-quality biodiesel that delivers optimum performance during engine combustion. Protein content can also be determined using existing spectrophotometric methods using Bradford's reagent or Lowry method. Various other methods such as the Kjeldahl method, Duma's method, or the Biuret method have also resulted in obtaining precise outcomes [253,338–340]. In conclusion, the biomass generated from refinery waste sludge treatment holds significant potential for future use as a source of fuel or value-added products. However, it is crucial to analyse the characteristics thoroughly to ensure its suitability for these applications.

In this chapter we primarily focus on estimating lipid content through gravimetric method, carbohydrate content and protein content through their respective spectrophotometric methods to understand the possibility of the obtained microbial biomass for further applications. The enzyme assays pertaining to alkane, aromatic and PAHs metabolism have been studied to gain insight into the utilization of these components by the respective microbial strains as their substrate.

4.2. Materials and methods

Solvents and chemicals

All the solvents, chemicals and reagents were collectively procured from Merck Lifesciences Pvt. Ltd., Kolkata, Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru and HiMedia Laboratories, Mumbai. Chloroform, methanol, phenol, 2-methoxymethanol and sulfuric acid were purchased from Merck. Media components such as $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl were collectively purchased from HiMedia and Merck. Reagents related to enzyme assays such as NADH, FAD were purchased from HiMedia. BSA standard, Bradford reagent, CHAPS, substrates *n*-hexadecane, *n*-pentadecane, naphthalene, anthracene, phenanthrene and xylene were purchased from Sigma. Catechol, benzene, toluene and cyclohexane was purchased from Merck. Micropipettes were collectively obtained from Thermo Fisher Scientific, India as Finnpiquette F2 grade of ranges (μL) 0.2-10, 0.2-20, 20-100, 20-200, 10-1000 and Eppendorf India, Chennai as Research Plus edition. Plasticwares and

glassware including filtration assembly for biomass estimation were used from Tarsons, India and Borosil India respectively. Water bath and autoclave was used from Equitron Medica Pvt. Ltd., India. Bacterial incubator was obtained from Scigenics Biotech, Chennai, India, Biosafety Cabinet Class II from Cleanair, India and pH meter was used from Hanna Instruments, India. Whatman qualitative filter papers (No. 1; pore size 0.7 μm ; 125 mm diameter) was procured from Cytiva Lifesciences, India. Syringe filters (sterile; 25 mm diameter, 0.2 μm pore size) for buffer sterilization were obtained from Axiva Sicheem Biotech, India. DispoVan syringes obtained from local pharmacy were used for better precision.

4.2.1. Estimation of total lipids

Total lipids from the harvested biomass were estimated using Folch method [339]. Briefly, biomass obtained from growth of the respective bacterial cells were separated by centrifuging the cultures (Eppendorf, 5810R, Germany) at 7000 rpm for 10 minutes. The supernatant was discarded, and the residual pellet was allowed to dry up in a hot air oven (Equitron Medica Private Limited, India). To the dried cells, biphasic solvent mixture of Chloroform:Methanol was added in the ratio of 2:1 (v/v). 0.8% (v/v) water also added at the end to solubilize other waste soluble biomolecules. The biomass was then homogenized to obtain a uniform solution, and the mixture was vortexed for 30 minutes to provide maximum agitation and mixing in order to attain maximum phase separation. The resultant mixture was allowed to stand for 10 minutes and the bottom organic layer containing lipid molecules was carefully removed to a pre-weighed sterile centrifuge tube. The organic layer was then evaporated out for 24-36 hours and lipid concentration were calculated gravimetrically as per the following Equation 4.1:

$$\text{Lipid content (mg/mL)} = \frac{W_2 - W_1}{\text{Volume of sample (mL)}} \times 1000 \quad \text{Equation 4.1}$$

Where,

W_2 = Weight after solvent evaporation (grams);

W_1 = Initial weight of centrifuge tube (grams);

1000 = Conversion factor from “grams” to “milligrams”

4.2.2. Estimation of total carbohydrates

Total carbohydrates were estimated as per the spectrophotometric method using phenol and sulfuric acid as described by DuBois et al., 1956 [338]. The estimation was based on the principle that dehydration of carbohydrates by strong acid such as concentrated sulfuric acid produced furfural derivatives which on further reaction with phenol produces characteristic yellow-gold colour. This colour was detected at 490 nm and was used to estimate the carbohydrate content by interpolating the standard curve obtained by observing the optical densities under similar reaction conditions for known concentrations of glucose. Briefly, 2 mL of the biomass solution obtained at the end of each growth experiments were mixed with freshly prepared 5% phenol solution in a test tube. 5 mL of concentrated sulfuric acid was rapidly added to initiate the reaction. After allowing the reaction to occur for 10 minutes, the test tubes were vortexed for 30 seconds and were further allowed to stand for 20 minutes for colour development at room temperature. OD was measured at 490 nm (OD₄₉₀) and the resulting concentrations for the respective biomass samples were calculated from the standard curve prepared (Appendix Figure 43).

4.2.3. Preparation of cell free extracts

The biomass obtained after growth of respective bacterial cells individually and in consortia in MSM supplemented with HCs *n*-hexadecane, anthracene, phenanthrene, benzene, toluene and xylene were used to prepare cell free extracts. Cell free extracts were prepared in different buffers separately in order to meet the necessary conditions for total protein estimation and enzyme assays. For example, phosphate buffer (pH = 7.4) was suitable for assay of alkane hydroxylase as described by Medić et al., 2020 [166], whereas phosphate buffer (pH = 8.0) was reported to be suitable for assay of catechol dioxygenases [238]. In a similar way, biomass obtained post application of consortia into sludge sample (as explained in Chapter 5) was also used for preparation of cell free extracts in different buffers for total protein estimation and enzyme assay.

The culture broth in each case (individual strains or consortia) obtained from growth in different HCs were centrifuged at 7000 rpm for 10 minutes at 4 °C. The extracellular supernatant was separated and stored at –20 °C for further experimentation. The residual pellets were suspended in the required buffer. For ease of explanation, we use 1X phosphate buffer

saline (PBS, pH = 7.4) as the buffer here. However, this buffer was replaced as per the necessary experimentation requirements as was clarified earlier.

After suspending the pellets in PBS, the mixture was sonicated using an ultrasonic sonicator (Vibra Cell, VCX-500, SONICS, USA) at 33% amplitude for 10 minutes by maintaining pulse for 2 seconds and sonication rest for 5 seconds. The mixture was further ultracentrifuged at $12000 \times g$ using high speed centrifuge (Z32 HK, HERMLE, Germany) for 20 minutes to settle down cell debris. The pellets were discarded and the resultant supernatant containing the intracellular crude protein extract was stored at $-20\text{ }^{\circ}\text{C}$ for experimentation.

4.2.4. Estimation of total protein

Total protein was estimated from the cell free extracts obtained above by method as described by Bradford, 1976. The estimation was carried out based on the principle that the acidic solution of Coomassie Brilliant Blue G-250 dye bind to protein forming a stable blue coloured protein-dye complex which could be detected spectrophotometrically at 595 nm [340]. In this process, commercially available Bradford reagent (Sigma, B6916-500ML) was used for the estimation as subsequently the manual was followed as described by the principal supplier. Standard curves using known protein concentrations were made separately in different buffers that might be applicable for different assays by using bovine serum albumin (BSA) procured from Sigma (Catalogue number A1470-25G). Briefly, 5 μL of protein samples were pipetted into 96-well plates and 250 μL Bradford reagent was added. The colour was allowed to develop, and OD were measured at 595 nm (OD_{595}) using microplate spectrophotometer (MultiSkan Sky High, Thermo Fisher Scientific, India). Unknown protein concentrations were calculated from the regression equation obtained from the standard curve (Appendix Figure 44).

4.2.5. Enzyme assay of alkane hydroxylase (*alkB*)

Alkane hydroxylase (*alkB*) identified as EC 1.14.15.3 is an oxidoreductase class of enzyme. Enzyme assay was carried out to understand the ability of cells to metabolise alkane to primary alcohol (Figure 4.1). The intracellular hydroxylase enzyme accepts straight chain aliphatic HCs as its substrate and converts into alkanol by regulating the first step of alkane metabolism at the juncture of cell membrane. During conversion to alcohol electrons are supplied by oxidizing NADH to NAD^+ and subsequently the O_2 molecule is transferred to 1'

position of alkane to form alkan-1-ol. This product is introduced into the matrix and is fed into the central metabolic pathway after a series of catalytic conversions [210,341,342].

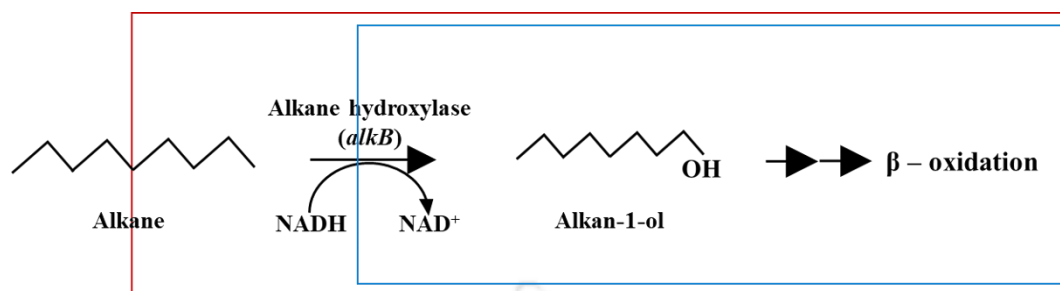


Figure 4.1: Mechanism of alkane catalysis by *alkB*. Alkane is broken down by membrane bound *alkB* as the first step of its metabolism into alkane-1-ol. Subsequently, alkanol is further catalyzed and introduced to central metabolic pathway.

alkB assay was carried out as per the method described in Medić et al., 2020 [166]. Briefly, harvested cells were re-suspended in 2 mL 20 mM sodium phosphate buffer (pH = 7.4). The reaction mixture of 1 mL consisted of 0.15% CHAPS buffer in addition of 20 mM sodium phosphate buffer (pH = 7.4), 0.1 mM NADH, 10 μ L of 1% *n*-hexadecane solution dissolved in 80% DMSO and 50 μ L of crude extract. Deionized distilled water (DDW) was added with required proportion to adjust the total reaction volume to 1 mL. The enzyme activity was calculated by monitoring the decrease in OD of NADH using spectrophotometer (Schimadzu, UV-2600, Tokyo, Japan) at 340 nm (OD₃₄₀). Similar set up without addition of NADH and crude extracts separately were used as blanks. The molar extinction coefficient of 6220 M⁻¹ cm⁻¹ was used to determine concentration of residual NADH at different intervals of time. One unit of enzyme activity was defined as the μ mol of NADH consumed per minute of reaction time per unit volume of reaction. Specific activity was determined as one unit of enzyme activity per unit amount of total protein in the crude extract.

4.2.6. Enzyme assay of naphthalene dioxygenase (*ndo*)

NDO (gene encoding the enzyme is *ndo*) is another oxidoreductase class of enzyme identified as EC 1.14.12.12. The assay was carried out to understand the efficiency of the strains to catalyse the first step of the metabolism of symmetric PAHs such as naphthalene, anthracene etc. The final product of this reaction has been reported to be naphthalene 1,2-dihydroxydiol (Figure 4.2). The mechanism of the catalysis is similar to that explained in case of *alkB* where NADH is the electron donor and subsequently O₂ is transferred to 1' and 2' position. The reaction is forwardly driven by a cascade of enzymatic reactions to ultimately

form catechol after which the aromatic ring is cleaved to further introduce the cascade into the central metabolic pathway [343,344].

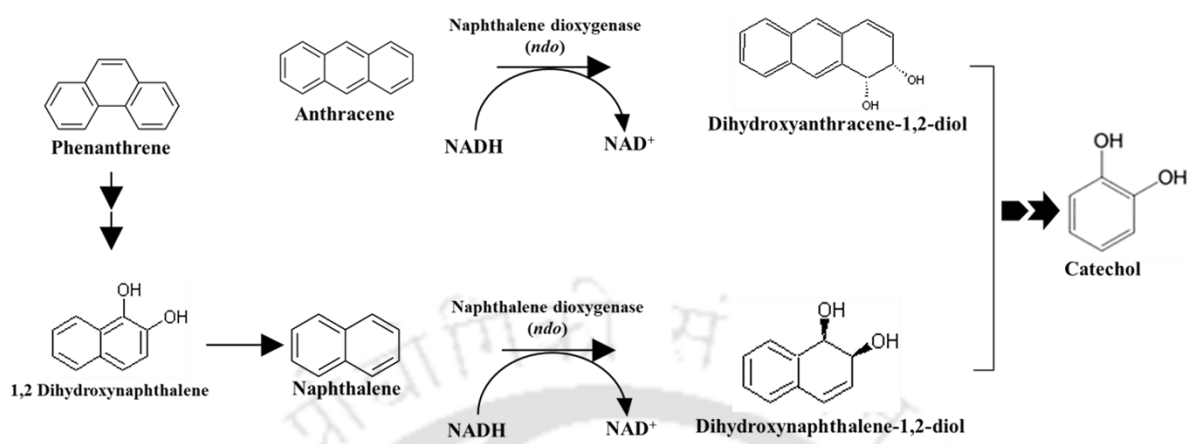


Figure 4.2: Catalytic mechanism of NDO on naphthalene and other PAHs. Naphthalene, anthracene and other PAHs where oxygen is introduced in the 1,2 positions by NDO, 1,2 dihydrodiol is formed, and is the first step of metabolism.

The enzymatic reactions were monitored by decrease in the OD₃₄₀ of NADH as per the spectrophotometric method described in Pozdnyakova-Filatova et al., 2020 [345]. Briefly, harvested cells were re-suspended in 5 mL 20 mM sodium phosphate buffer (pH = 7.5). Instead of pH = 6.5, our studies suggested pH = 7.5 was the optimum pH for this reaction which was in agreement to the method described in Ensley et al., 1982 [346]. Also, to be synergistic to other enzymatic assays carried out in the chapter, the enzyme amount was added as volume instead of adjusting to the concentration of enzyme. This step was modified to reduce wastage of cell free supernatant and reaction volume for consistent determination and understanding. The reaction mixture of 1 mL hence, consisted of 50 mM phosphate buffer (pH = 7.5), 2 mM NADH and 50 μ L of crude extract. As also reported in Larkin et al., 1999; the optimum NADH concentration for enzyme activity varied and added concentration of FAD also substantially aided to enhanced *ndo* activity [347]. In our study also, all the strains showed significant increase in *ndo* activity when 2 mM NADH (instead of 0.5 mM NADH) and 5 μ M FAD was added [346,347]. DDW was added in required proportion to adjust the total reaction volume to 1 mL. The reaction was initiated by addition of 10 mM naphthalene solution dissolved in 2-methoxymethanol. The molar extinction coefficient of 6220 M⁻¹ cm⁻¹ was used to determine concentration of residual NADH at different intervals of time. Specific activity was defined as μ mol of NADH consumed in one minute per unit amount of protein in the crude extract.

4.2.7. Enzyme assay of catechol dioxygenase (CDO)

Catechol dioxygenases catalyse catechol where the single aromatic rings are cleaved to form products. This step is deemed significant towards aromatic and PAHs metabolism as the aromatic ring is cleaved after this step.

As depicted in Figure 4.3, the formation of end products depends on the *ortho* cleavage which is facilitated by catechol 1,2 dioxygenase (EC 1.13.11.1) (*catA*) or *meta* cleavage by catechol 2,3 dioxygenase (EC 1.13.11.2) (*catE*). *Ortho* cleavage results in generation of *cis, cis*-muconic acid which involves multiples steps before being inducted into Tricarboxylic acid (TCA) cycle whereas the end product of *meta* cleavage is 2-hydroxymuconic semialdehyde and is more energy expensive since lesser number of steps are required for its conversion and can be introduced to TCA cycle more easily [238,341].

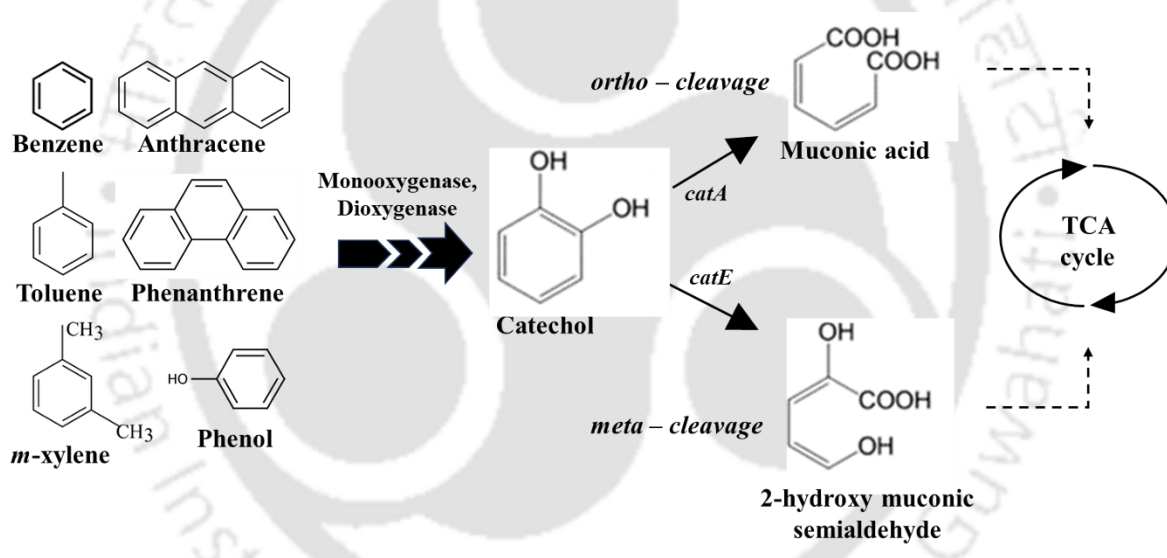


Figure 4.3: Mechanism of aromatic and PAHs catalysis by CDO. Catechol, an essential intermediate in the breakdown of aromatic and PAHs, is broken down by CDO either at 1,2 position (*ortho*) or 2,3 position (*meta*) by forming muconic acid or 2-hydroxy semialdehyde. The products are then introduced into TCA cycle.

CDO assay was performed according to the method described by Setlhare et al., 2019 [238]. For *catA*, the harvested cells were re-suspended in 5 mL 50 mM sodium phosphate buffer (pH = 7.0). Instead of pH = 8.0, our studies suggested pH = 7.0 was the optimum pH for this reaction [348]. The reaction mixture of 1 mL hence, 50 mM phosphate buffer (pH = 7.0) and 100 μ L of crude extract. DDW was added as required to adjust the total reaction volume to 1 mL. The reaction was initiated by addition of 10 mM catechol. OD was measured at 260 nm (OD_{260}) for monitoring the formation of *cis, cis*-muconic acid. The molar extinction

coefficient of $16800 \text{ M}^{-1} \text{ cm}^{-1}$ was used to determine concentration of *cis, cis*-muconic acid at different intervals of time. Enzyme activity was defined as nmol of *cis, cis*-muconic acid formed per minute per unit volume of reaction. Specific activity was expressed as enzyme activity per unit concentration of total protein in the crude extract.

For *catE*, the harvested cells were re-suspended in 5 mL 50 mM sodium phosphate buffer (pH = 7.5). Instead of pH = 8.0, pH = 7.5 was found to be the optimum pH for this reaction [349]. The reaction mixture of 1 mL contained 50 mM phosphate buffer (pH = 7.5) and 100 μL of crude extract. DDW was added as required to adjust the total reaction volume to 1 mL. The reaction was initiated by addition of 10 mM catechol. OD was measured at 375 nm (OD_{375}) for monitoring the formation of 2-hydroxymuconic acid semialdehyde. The molar extinction coefficient of $14700 \text{ M}^{-1} \text{ cm}^{-1}$ was used to determine concentration of 2-hydroxymuconic acid semialdehyde at different intervals of time. Enzyme activity was expressed as nmol of 2-hydroxymuconic acid semialdehyde formed per minute per unit mg of protein.

4.2.8. Optimum temperature and pH for different enzyme reactions

The optimum temperature was determined by incubating the reaction mixtures at different temperatures ranging 25-60 °C for 10 minutes. Enzyme assays were performed separately for alkane hydroxylase, naphthalene dioxygenase and catechol dioxygenase as per the set up discussed earlier. The activities at each temperature were re-calculated relative to the highest activity that is expected to occur at the optimum temperature and were further depicted as percentage activity relative to the maximum value.

The optimum pH was determined by incubating reaction mixtures in citrate phosphate buffer (pH = 4.0-6.0), sodium phosphate buffer (pH = 7.0) and Tris-phosphate buffer (pH = 8.0-10.0) for 10 minutes. Enzyme assays were performed as per the methods described above. Similar to above, the results are illustrated as percentage activity where the maximum activity at the optimum pH was assumed 100%.

4.2.9. Substrate specificity of crude complex for different enzyme assays

Substrate specificity was determined by analysing the change in enzyme activity when the reaction mixtures were assayed under different substrates. The results were depicted as

percentage of enzyme activity obtained for different substrates with respect to (w.r.t) the enzyme activity obtained for actual substrate.

For *alkB*, the enzyme assay was carried out in 1% *n*-pentadecane and 1% cyclohexane. Both *n*-pentadecane and cyclohexane were dissolved in 80% DMSO. Percentage change in enzyme activity when *n*-pentadecane and cyclohexane were used as substrates w.r.t activity using *n*-hexadecane as the substrate was analysed for *alkB* specificity.

For *ndo*, the enzyme assay was carried out separately in 10 mM anthracene and phenanthrene dissolved in 2-methoxymethanol. Activities under these substrate conditions relative to activity using naphthalene as the substrate were studied and compared for *ndo* substrate specificity.

For catechol dioxygenases (both *catA* and *catE*), substrate specificity was checked using 10 mM benzene, toluene, xylene and phenol as substrates. The activities were compared to that obtained using catechol as the substrate and were illustrated relative to the respective values for understanding their substrate specificity.

4.2.10. Stability of the crude enzymes at optimum temperature and pH

To check for the disintegration of the enzymes and the maximum duration the crude complex could be used for studies, enzyme assay was performed at every 1-hour interval by withdrawing the respective amount of crude extract using the methods discussed earlier for the respective enzymes. In all cases, while no reaction set ups were prepared, the crude extracts were carefully stored at 4 °C to retain maximum activity of the enzymes and protein content. Similar to substrate specificity, the activities obtained after each hour of experimentation was reported in terms of percentage change in activities w.r.t to the activities obtained for all of *alkB*, *ndo*, *catA* and *catE* after the first experiment performed after withdrawing the crude protein from –20 °C storage.

4.2.11. Determination of enzyme kinetic parameters

The kinetic parameters were determined by measuring the enzyme activity of each enzyme over a range of concentration of substrates. The enzyme activities obtained for each concentration were then illustrated as per the Michaelis-Menten equation Equation 4.2 by plotting the of rate of substrate catalysis (*V*) and concentration of substrate (*[S]*). Kinetic

parameters K_m and V_{max} were determined by fitting the enzyme activities for each concentration into the non-regression Michaelis-Menten equation using Minitab v19.1.

$$V = \frac{V_{max} [S]}{K_m + [S]} \quad \text{Equation 4.2}$$

Where,

V = Rate of reaction (enzyme activity, U mg^{-1})

$[S]$ = Concentration of substrate (μM , mM etc.)

V_{max} = Maximum reaction rate (U mg^{-1})

K_m = Michaelis-Menten constant (μM , mM etc.)

alkB activity was performed using *n*-hexadecane as the substrate on concentration ranging from 1-5% dissolved in 80% DMSO. To ease our calculations, the concentrations were converted to their respective molarities and hence, the kinetic studies were conducted within 0.034-0.17 M *n*-hexadecane. *ndo* activities were performed on 1-10 mM naphthalene and the kinetic parameters were determined. Similarly, *catA* and *catE* activities were experimented on 10-500 μM of catechol.

4.3. Results and discussion

4.3.1. Estimation of total lipids

Lipid content was measured twice during experiment: start (0 day) and end (onset of decay phase). The lipid profile of all the strains at different HC concentrations are shown in Figure 4.4.

For *n*-hexadecane (Figure 4.4(a)), *Aquamicrobium* sp. GR1, GR3 and GR4 showed negligible lipid deposition at the end of their stationary phases. This was due to lesser biomass production at 500 mg/L *n*-hexadecane as these strains were not found tolerant after 10 mg/L *n*-hexadecane. The detailed discussion about the extent of tolerance in different class of HCs have already been made in Chapter 2.

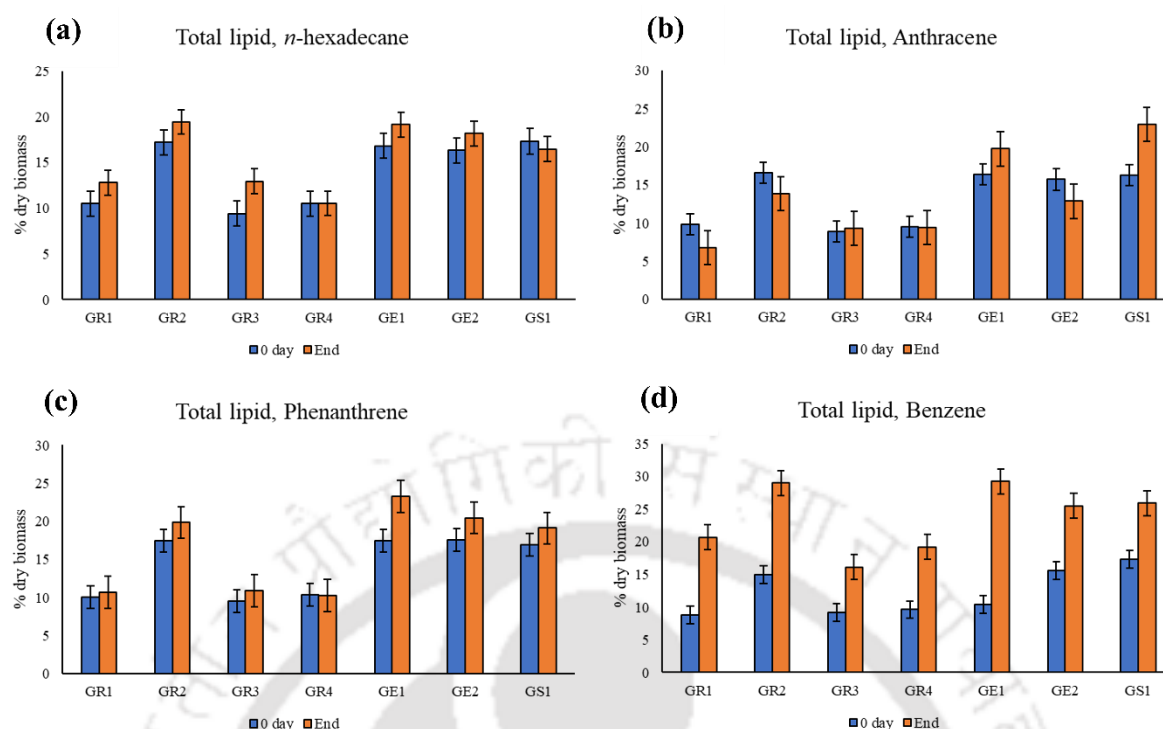


Figure 4.4: Total lipid content from biomass obtained after incubation in (a) *n*-hexadecane; (b) anthracene; (c) phenanthrene; and (d) benzene supplemented MSM. For most isolates, the lipid content increased after incubation in different HCs. Increase in lipid content is attributed to the tendency of the microbes to efficiently accept HCs as their nutrient. At instances, where the lipid content is depicted lower, it could be attributed to the higher biomass concentration and that the overall percentage share was less. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The maximum lipid content was seen in *Staphylococcus sp.* GR2 as 0.057 mg/mL which was ~19.4% of the biomass value (dry weight). The lipid accumulation at the end of the experimentation was in the order *Staphylococcus sp.* GR2 > *Bacillus sp.* GS1 > *Bacillus sp.* GE1 > *Aquamicrobium sp.* GE2 > *Aquamicrobium sp.* GR1 > *Aquamicrobium sp.* GR3 > *Aquamicrobium sp.* GR4. Overall, the lipid concentrations were found to be in the range of ~10-20% of dry biomass concentration.

For anthracene (Figure 4.4(b)), *Aquamicrobium sp.* GR1, GR3 and GR4 showed very less lipid deposition at the end of their stationary phases similar to the trend observed in case of *n*-hexadecane. This was due to lesser biomass production at 50 mg/L anthracene as these strains were not found tolerant after 5 mg/L anthracene. The maximum lipid content was seen in *Bacillus sp.* GE1 as 0.074 mg/mL which was ~25% of the dry biomass value. Lipid accumulation of ~20% for *Bacillus sp.* GS1 and *Aquamicrobium sp.* GE2 at 50 mg/L anthracene showed the potency of survival of strains at concentrations higher than their tolerance value (20 mg/L) and generating good quality biomass at the same time. The lipid

accumulation at the end of the experimentation was found more pronounced than in the mid of the experiment. Overall, the lipid concentrations were found to be in the range of ~10-25% of dry biomass concentration.

In case of phenanthrene (Figure 4.4(c)), no strains could exhibit significant lipid deposition. This was due to lesser tolerance at 20 mg/L phenanthrene except *Bacillus sp.* GE1 which showed the maximum lipid content as 0.01 mg/mL. Whereas, overall, the lipid concentrations were found to be in the range of ~10-25% of dry biomass concentration, the next best lipid concentration was observed for *Aquamicrobium sp.* GE2, which was only 7 µg/mL.

For aromatic HC benzene (Figure 4.4(d)), the lipid accumulation at the end of the experimentation was in the order *Staphylococcus sp.* GR2 = *Bacillus sp.* GE1 > *Bacillus sp.* GS1 = *Aquamicrobium sp.* GR1 > *Aquamicrobium sp.* GE2 > *Aquamicrobium sp.* GR4 > *Aquamicrobium sp.* GR3. Lipid accumulation in the range of 16-26% of dry biomass was observed with the highest and lowest value being 0.045 mg/mL and 0.021 mg/mL for *Staphylococcus sp.* GR2 and *Aquamicrobium sp.* GR3 respectively.

Similarly, lipid content obtained from biomass of toluene/xylene supplemented media were also analysed. The detailed results are shown in Appendix Figure 45.

4.3.2. Estimation of total carbohydrates

Similar to lipid content, carbohydrate content was also measured at 2 stages of experiment: start (start of lag phase), and end (end of stationary phase). The carbohydrate content of all the strains at different HC concentrations are shown in Figure 4.5.

For *n*-hexadecane (Figure 4.5(a)), similar to lipid profiles, *Aquamicrobium sp.* GR1, GR3 and GR4 showed lesser carbohydrate at the end of their growth cycles. This is also due to lower biomass concentration at 500 mg/L *n*-hexadecane as these strains were not tolerant at the said concentration. The maximum carbohydrate deposition of ~16.6% of biomass dry weight was seen in *Bacillus sp.* GS1 during the mid phase, however, the same was reduced to a low of ~12-14% at the end. The carbohydrate content of *Staphylococcus sp.* GR2 and *Bacillus sp.* GE1 was similar ($\sim 0.032 \pm 0.002$ mg/mL) to *Bacillus sp.* GS1 at the end of the experiment. Decrease in carbohydrate value of *Bacillus sp.* GS1 could be due to more N deposition during the stationary phase and lesser C deposition. The carbohydrate content at the end of the

experimentation was in the order *Bacillus sp.* GS1 > *Bacillus sp.* GE1 > *Staphylococcus sp.* GR2 > *Aquamicrobium sp.* GE2 > *Aquamicrobium sp.* GR1 > *Aquamicrobium sp.* GR3 > *Aquamicrobium sp.* GR4. Overall, the carbohydrate concentrations were found to be in the range of ~11-17% of dry biomass concentration.

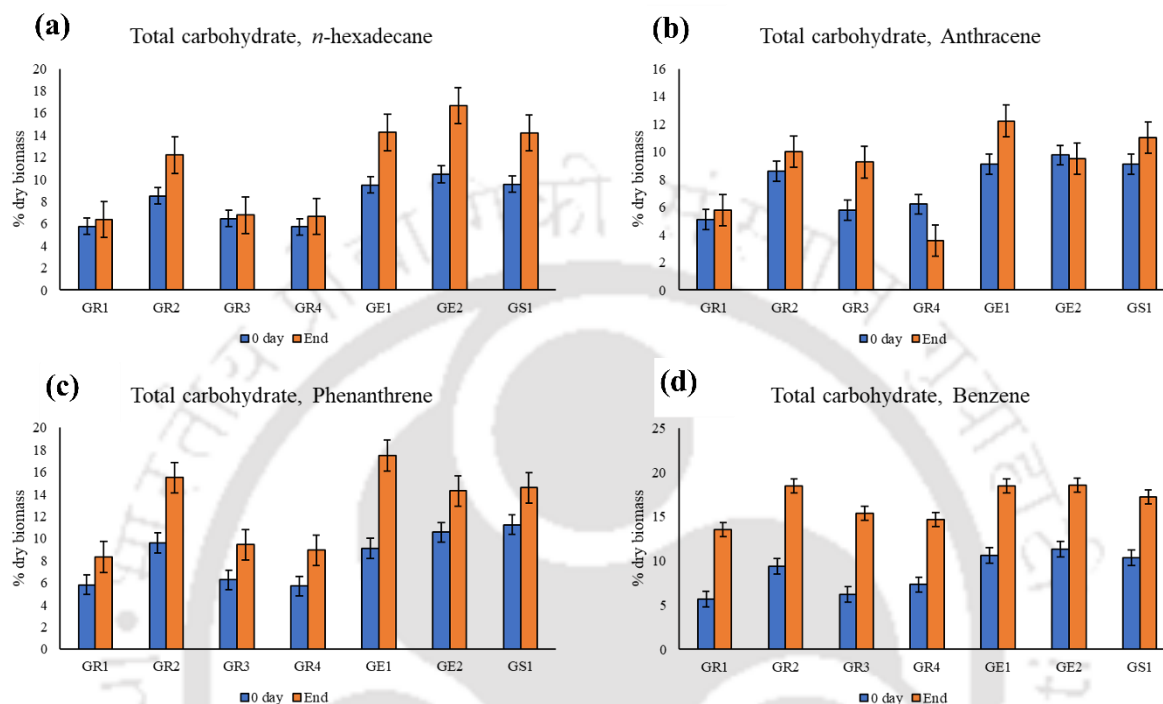


Figure 4.5: Total carbohydrate content from biomass obtained after incubation in (a) *n*-hexadecane; (b) anthracene; (c) phenanthrene; and (d) benzene supplemented MSM. For most isolates, the carbohydrate content increased after incubation in different HCs. Increase in carbohydrate content is attributed to the increased deposition of C on biomass during the metabolism of HCs. Error bars indicate the standard deviation (±SD) of three experimental replicates.

For anthracene (Figure 4.5(b)), *Aquamicrobium sp.* GR1, GR3 and GR4 showed lesser carbohydrate at the end of their growth cycles. The maximum carbohydrate deposition of ~15% of biomass dry weight was seen in *Bacillus sp.* GE1, *Bacillus sp.* GS1 and *Aquamicrobium sp.* GE2 till the end of experiment. The highest carbohydrate content was seen in *Bacillus sp.* GE1 0.044 mg/mL whereas the lowest value was observed for *Aquamicrobium sp.* GR3 (5 µg/mL) at the end of the experiment. Significant increment in carbohydrate was due to continuous increase in biomass value. Due to sluggish growth in 50 mg/L anthracene, *Bacillus sp.* GE1 attained higher biomass towards the end of the experiment. In general, the carbohydrate concentrations were found to be in the range of ~11-15% of dry weight of biomass.

In case of phenanthrene (Figure 4.5(c)), similar observations were seen as in case of lipid accumulation. The highest carbohydrate accumulation was only 7 µg/mL for *Bacillus sp.* GE1. This is also on the account of lower biomass concentration due to lower tolerance of strains in 20 mg/L phenanthrene.

In case of aromatic HC benzene (Figure 4.5(d)), the highest carbohydrate content was observed in *Bacillus sp.* GE1 as 0.028 mg/mL whereas the lowest value was observed for *Aquamicrobium sp.* GR4. Overall, ~14-19% of dry biomass weight contributed to carbohydrate deposition. Similarly, the results for toluene/xylene are depicted in Appendix Figure 46. For all of benzene/toluene/xylene, the carbohydrate content did not show sharp rise which could be due to the onset of C-repressing environment in the culture media from the early stationary phase on account of intense competition and substrate exhaustion.

4.3.3. Estimation of total protein

The protein content was similarly measured at 2 stages of experiment as described above. The protein content of all the strains at different HC concentrations are shown in Figure 4.6.

For *n*-hexadecane (Figure 4.6(a)) *Aquamicrobium sp.* GR1, GR3 and GR4 exhibited lower protein content at the end of their growth cycles as was also the case with lipid and carbohydrate accumulation. The maximum protein content of ~21.4% of dry biomass weight was seen in *Bacillus sp.* GS1 at the end of the experiment. Increase in carbohydrate value of *Bacillus sp.* GS1, *Staphylococcus sp.* GR2 and *Bacillus sp.* GE1 was due to more N deposition after the onset of the stationary phase. At this phase nutrients in the form of C-sources are assumed to be limiting. The protein content at the end of the experimentation was in the same order as was seen in case of carbohydrate deposition. Overall, the protein was secreted in the range of ~12-22% of dry biomass concentration which was higher than that seen in cases of lipid and carbohydrate.

For anthracene (Figure 4.6(b)), the maximum protein content of ~21% of dry biomass weight was seen in *Bacillus sp.* GE1 at the end of the experiment. Similar to the case of *n*-hexadecane, increase in carbohydrate value of all the strains was due to increased N deposition after the onset of the stationary phase. The highest protein content at the end of the experimentation was observed in *Bacillus sp.* GE1 similar to the trend in lipid and carbohydrate

values when subjected to 50 mg/L anthracene. Whereas the protein content was found in the range of ~11-22% of dry biomass weight, *Aquamicrobium sp.* GR1, GR3 and GR4 exhibited lower protein content at the end of their growth cycles due to lower tolerance in anthracene.

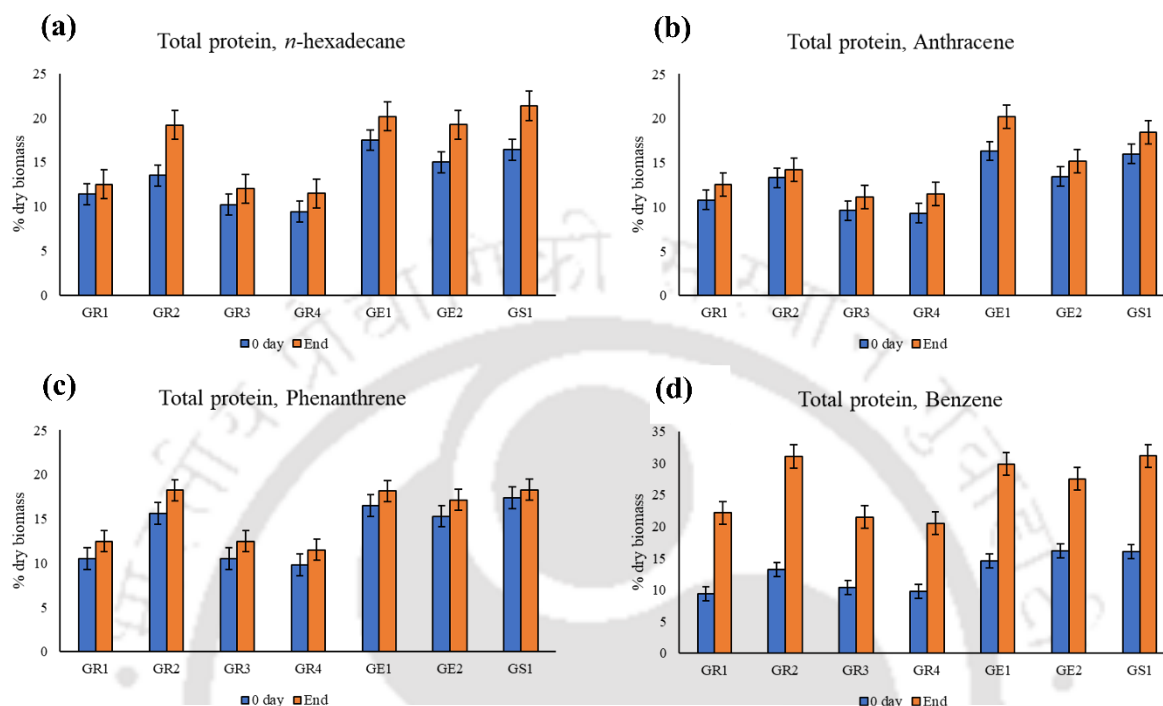


Figure 4.6: Total protein content from biomass obtained after incubation in (a) *n*-hexadecane; (b) anthracene; (c) phenanthrene; and (d) benzene supplemented MSM. For most isolates, the protein content increased after incubation in different HCs. Increase in protein content alongside lipid and carbohydrate is attributed to N deposition on biomass. This typically happened during the stationary phase of incubation. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For phenanthrene (Figure 4.6(c)), in addition to the results obtained for lipid and carbohydrate deposition, lower protein content was seen with *Bacillus sp.* GE1 depicting the highest value of 0.013 mg/mL. Although, significant increase in N accumulation could be seen after the stationary phase was attained by the strains, lower protein content was due to lower biomass values by the strains in 20 mg/L phenanthrene.

In case of benzene (Figure 4.6(d)), almost similar trend was observed as in case of lipid accumulation. Whereas the protein concentration was identical in *Bacillus sp.* GE1 and *Staphylococcus sp.* GR2, the highest protein content was observed in *Staphylococcus sp.* GR2 as 0.046 mg/mL and the lowest was observed in *Aquamicrobium sp.* GR3. Significant increase in protein concentration was also seen which was due to increased N deposition and decreased nutrients in the form of C-source.

Similarly, for toluene, overall accumulation of protein in the range of 14-25% showed the generation of biomass quality aiding in the mineralization of 1000 mg/L toluene. For xylene, protein content confirmed the adept characteristics of biomass obtained in xylene supplemented MSM (Appendix Figure 47).

In a typical microbial treatment of refinery waste, significant lipid, carbohydrate and protein are expected. Our results also agree to the studies conducted by other investigators [350,351].

4.3.4. Comparison of biomass characteristics after statistical optimization

Biomass obtained before and after optimization were already compared of their enhanced values in Chapter 3, section 3.3.10. It was also seen that enhanced biomass production of the selected best strains also aided enhanced biodegradation efficiency of the respective HCs. In this section, comparison of the biomass characteristics namely lipid accumulation, carbohydrate deposition and protein content were carried out to understand the biomass quality that was obtained after amending the media with additional micronutrient factors (FeSO₄ and NaCl) alongwith tweaking certain growth parameters (time of incubation, inoculum concentration). As has already been discussed in Chapter 3 section 3.2.5, since the tolerance values were already known, the concentration of HCs if and wherever found significant were not chosen for 2nd order optimization through CCD to obtain optimized response.

The comparison of biomass characteristics of the potent strains in different HCs are depicted in Figure 4.7.

In case of *n*-hexadecane (Figure 4.7(a)), the highest lipid content, carbohydrate concentration and protein content were observed as 0.11 mg/mL, 0.028 mg/mL and 0.09 mg/mL respectively for *Staphylococcus sp.* GR2. Lipid content increased to a high of 21.1% of biomass dry weight whereas carbohydrate and protein content also increased to 31.1% and 20.5% respectively thus indicating enhanced biomass quality. The absolute carbohydrate concentrations seemed less when compared to that before statistical media optimization, however the percentage share of the biomass concentration increased. The decreased value of carbohydrate was also due to very high biomass concentration obtained after optimization, that ultimately aided to lower values even after contributing to higher percentage of the total

biomass content. In other cases, lipid content and protein content showed upward trend further depicting increased biomass quality and viability.

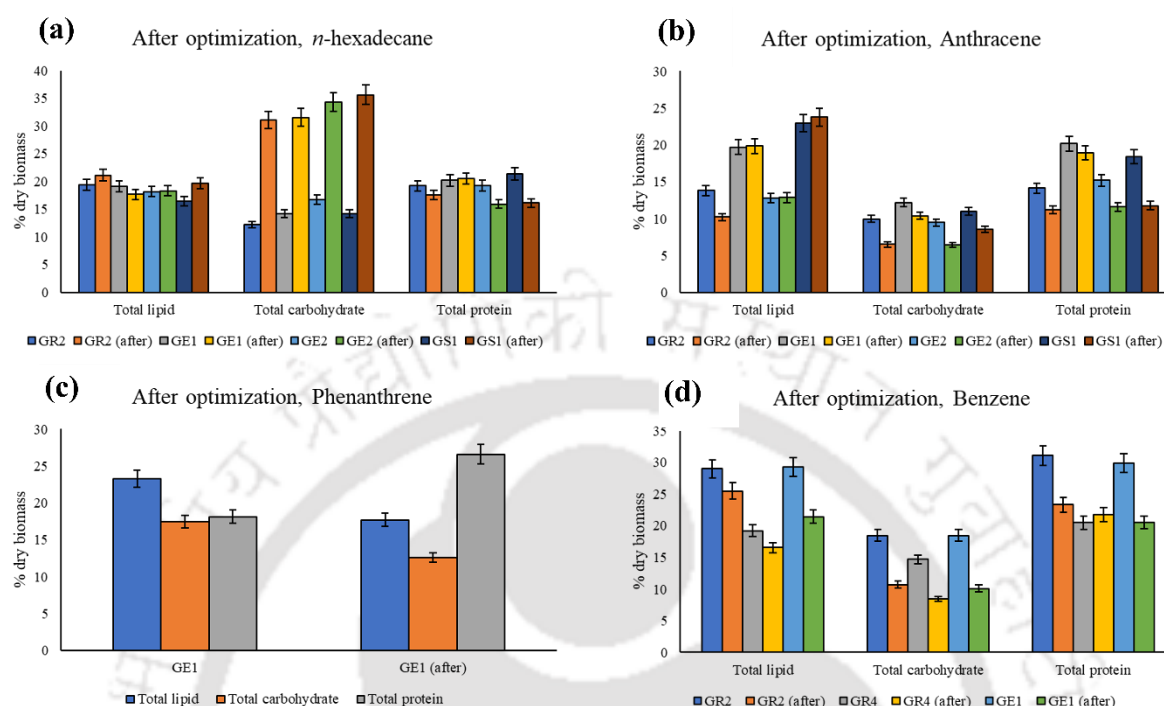


Figure 4.7: Comparison of total lipid, carbohydrate and protein content from biomass of selected strains obtained after statistical optimization in (a) *n*-hexadecane; (b) anthracene; (c) phenanthrene; and (d) benzene supplemented modified MSM. Biomass quality was greatly improved for the isolates under study after the optimum factors were introduced. At instances, where the lipid, carbohydrate and protein content are depicted lower, it could be attributed to the higher biomass concentration after optimization and that the overall percentage share was less. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For PAH anthracene (Figure 4.7(b)), where the highest biomass concentration was obtained for *Bacillus sp.* GE1, also depicted highest biomass characteristics as 0.09 mg/mL lipid content, 0.045 mg/mL carbohydrate content and 0.085 mg/mL protein concentration. Percentage contribution of lipid, carbohydrate protein remained almost the same, however, enhanced biomass concentration contributed mostly to enhanced values of lipid, carbohydrate and protein.

Similarly, for phenanthrene (Figure 4.7(c)), where *Bacillus sp.* GE1 was the only strain that showed higher tolerance at 20 mg/L, the lipid content, carbohydrate content and protein content increased to 0.014 mg/mL, 0.01 mg/mL and 0.021 mg/mL respectively.

For aromatic HC benzene (Figure 4.7(d)), lipid content, carbohydrate content and protein content were identical for the potent strains *Staphylococcus sp.* GR2, *Aquamicrobium*

sp. GR4 and *Bacillus sp.* GE1. The average values of lipid, carbohydrate and protein ranged within 0.056 ± 0.007 mg/mL, 0.024 ± 0.005 mg/mL and 0.052 ± 0.007 mg/mL respectively. Decreased percentage share of carbohydrate content from as high as 18.5% to only 8.5% shows gradual delimitation of C and gradual deposition of N which could also be seen from the protein concentration. The lipid and protein percentage of dry biomass remained almost the same inferring that the biomass may be suitable for value addition.

Similarly, the results for toluene and xylene are illustrated in Appendix Figure 48. All such studies are suggestive of the enhanced biomass composition rendering efficient treatment quality [350,351].

For each class of pollutants and each isolate, MTC was determined in Chapter 1. Except for the pollutant phenanthrene and for *Aquamicrobium sp.* GR3 strain, satisfactory tolerance was depicted by other strains in different HCs. The lipid, carbohydrate and protein content depict the ability of the microbes to secrete extracellular biomolecules that are indicative to the efficiency of substrate utilization and bioremediation. Hence, even if the substrates were toxic to some strains, experiments were carried out to derive out a comparative assessment to understand the efficacy of the strains for bioremediation applications and the scope of processing the generated biomass for further valorisation.

4.3.5. Optimum temperature and pH for different enzyme reactions

The optimum temperature, pH was analysed based on the highest enzyme activity obtained when the reactions were subjected to incubation as per the methods discussed earlier. Temperature and pH were varied by incubating the reactions for 30 minutes for all the enzyme reactions.

For *alkB*, the activity at various temperatures and pH are shown in Figure 4.8. All the strains showed the best enzyme activity within the temperature range of 35-40 °C (Figure 4.8(a)). Whereas *Bacillus sp.* GE1 and GS1 showed almost identical activities at temperature 35-40 °C, the same for *Staphylococcus sp.* GR2 dipped slightly from 35 °C to 40 °C. The highest average activities were obtained within this temperature range with the maximum activity being in case of *Staphylococcus sp.* GR2 was 102.95 ± 1.33 U mg⁻¹. On the other hand, maximum activities for the other strains *Aquamicrobium sp.* GR1, GR3 and GR4 were in the range of 30-35 °C.

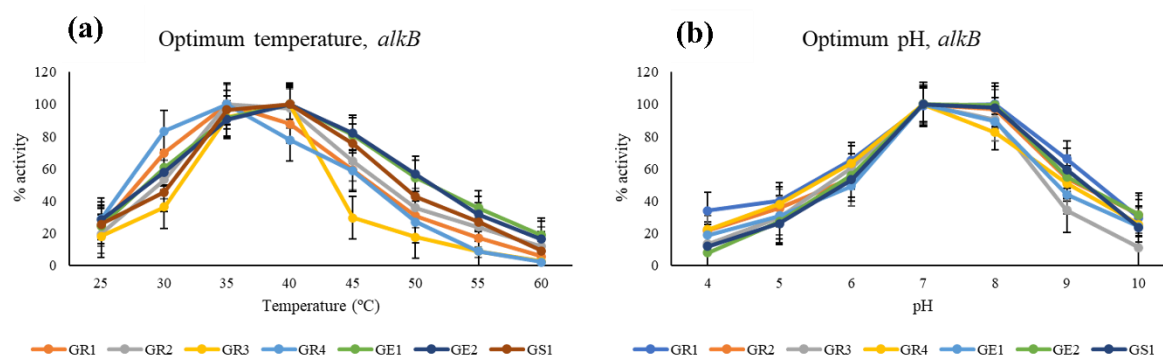


Figure 4.8: (a) Temperature; and (b) pH optimization for *alkB* assay. Based on identical activities observed at ranges 35–40 °C and pH 7.0–8.0, average values of 37.5 °C and 7.5 were calculated as optimum. To be consistent with literature, optimum pH 7.4 was considered. Error bars indicate the standard deviation (±SD) of three experimental replicates.

Whereas almost 45–60% activities were retained by *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1 and *Aquamicrobium sp.* GE2, to be consistent with all the strains and their reaction conditions, the average value of 37.5 °C was calculated and subsequently, 37 °C was used as the optimum temperature. At this temperature, almost 77–90% activities were also reported for the *Aquamicrobium sp.* GR1, GR3 and GR4. The results also agree the studies that are conducted by other investigators pertaining to *alkB* assay [310,322].

Similarly, for optimum pH (Figure 4.8(b)), the activities of *Bacillus sp.* GS1 and *Staphylococcus sp.* GR2 were found to be the highest as $100.35 \pm 1.38 \text{ U mg}^{-1}$ at pH ranging from 7.0–8.0. All the other strains individually showed 100% maximum activity at this pH. Since the change in activities in this range was not so pronounced for all the strains, the average value of 7.5 was calculated as the pH where the highest enzyme activity could be expected. At lower pH of 4.0–6.0 or higher range of 8.0–10.0, the activities of the enzymes showed significant dip to not consider those necessary to consider for further optimization studies. However, to be consistent with literature and keeping in mind the further optimization of reaction time, pH of 7.4 was chosen as the optimum pH [166].

For *ndo*, the activity at various temperatures and pH are shown in Figure 4.9. All the strains showed the best enzyme activity within the temperature range of 30–35 °C except *Aquamicrobium sp.* GE2 whose maximum activity was more pronounced at 30 °C. All these were used as reference 100% activity to analyse the trend at other temperatures (Figure 4.9(a)). Below or above this temperature range, the activities were greatly reduced to as low as 30% and the crude complex disintegrated depicted undesirable results. To attain a constant value of temperature and further simplify our approach towards pH optimization, the average value of

32.5 °C was subsequently used as the optimum temperature. Furthermore, for optimum pH (Figure 4.9(b)), the activities of the respective strains were found to be the highest pH ranging from 7.0 to 8.0. Since the change in activity in this range was not so pronounced for all the strains, the average value of 7.5 was chosen as the optimum pH where the highest enzyme activity could be expected. Apart from this range the activities were greatly reduced with the recorded activities reduced to a maximum of 76% for *Staphylococcus sp.* GR2 at pH 9.0 followed by ~81% by *Aquamicrobium sp.* GE2 at pH 6.0 [343,345].

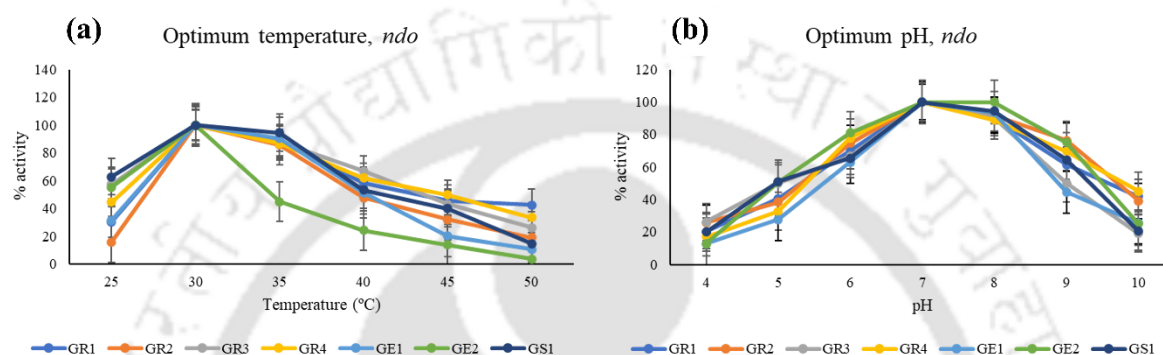


Figure 4.9: (a) Temperature; and (b) pH optimization for *ndo* assay. Except *Aquamicrobium sp.* GE2, all others showed identical activities within 30-35 °C. However, to maintain uniformity, an average of 32.5 °C was taken as optimum temperature. Similarly, on account of similar activities within pH 7.0-8.0, 7.5 was chosen as optimum. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For *catA*, the activity at various temperatures and pH are shown in Figure 4.10. All the strains showed the best enzyme activity at 30 °C (Figure 4.10(a)). All the strains except *Aquamicrobium sp.* GR3 exhibited almost identical activities within the range of 10.5-16 U mg^{-1} with the highest being by *Aquamicrobium sp.* GR4 as 15.7 U mg^{-1} . Hence, 30 °C was used as the optimum temperature. The activities were significantly reduced to at least almost 50% with the next best retained activity recorded at 35 °C for *Aquamicrobium sp.* GR4 as 52.44%. Similarly, for optimum pH (Figure 4.10(b)), the highest activity of *Aquamicrobium sp.* GR4 as 16.33 U mg^{-1} at pH 7.0 followed by *Aquamicrobium sp.* GR1 (14.55 U mg^{-1}) with catechol as the substrate indicated optimum pH as 7.0. Overall, except *Aquamicrobium sp.* GR3, all other strains showed significant activities at the optimized conditions and hence, the same could be used for further studies [238].

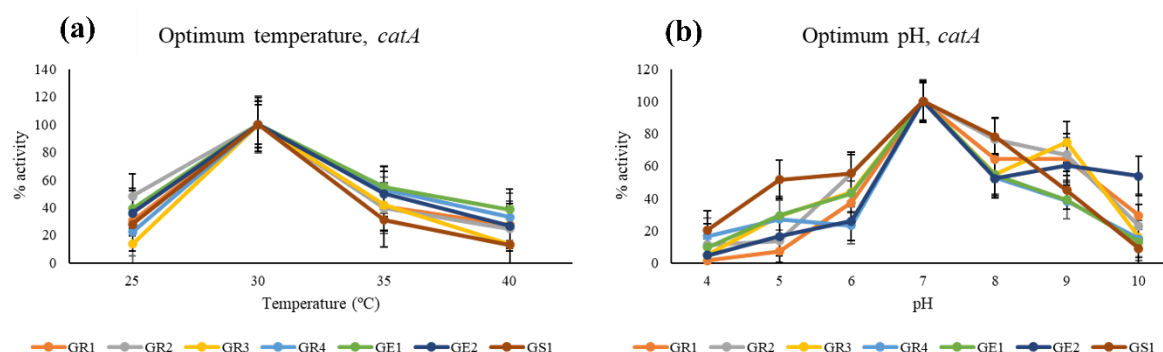


Figure 4.10: (a) Temperature; (b) pH optimization for *catA* assay. Maximum activities for all the isolates were observed at 30 °C and pH 7.0. Hence, optimum temperature and pH were taken as 30 °C and 7.0 respectively. Error bars indicate the standard deviation (±SD) of three experimental replicates.

For *catE*, the activity at various temperatures and pH are shown in Figure 4.11. All the strains showed the best enzyme activity at 30 °C (Figure 4.11(a)). All the strains except *Bacillus* sp. GE1 and GS1 exhibited almost identical activities within the range of 2.5-4.5 U mg⁻¹ with the highest being by *Aquamicrobium* sp. GR4 as 4.49 U mg⁻¹. However, the other two *Bacillus* strains exhibited significantly higher activities as 21.31 U mg⁻¹ and 19.58 U mg⁻¹ for GE1 and GS1 respectively. Similar to the case of *catA*, the activities were greatly reduced when the reaction mixture was allowed to incubate at any other temperature. The higher rate of catechol catalysis showed prevalence of 2,3 dioxygenase activity over 1,2 dioxygenases by *Bacillus* sp. GE1 and GS1 and hence, more affinity towards *meta* cleavage of the aromatic ring. Thus, 30 °C was used as the optimum temperature. For optimum pH (Figure 4.11(b)), the highest activity of all the strains was observed in the pH range of 7.0 and 8.0. *Bacillus* sp. GE1 and GS1 exhibited the maximum average activity of 20.59 ± 1.36 U mg⁻¹ amongst all and the average value of 7.5 was considered as the optimum pH [310,349].

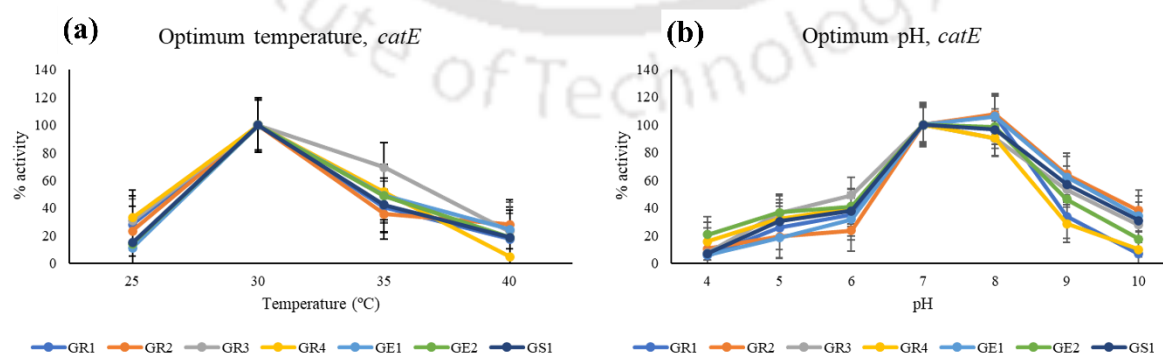


Figure 4.11: (a) Temperature; and pH optimization for *catE* assay. Maximum activities for all the isolates were observed at 30 °C. Hence, optimum temperature was taken as 30 °C. On account of similar activities within 7.0-8.0, an average of 7.5 was chosen as the optimum pH for *catE* assay. Error bars indicate the standard deviation (±SD) of three experimental replicates.

4.3.6. Alkane hydroxylase assay (*alkB*)

Alkane hydroxylase assay was performed as per the reaction conditions at their respective optimum temperature, pH and time period of incubation. The enzymatic activity was compared with the extracellular supernatant which was used as the positive control as these enzymes are not commercially available. The comparative assessment of *alkB* assay of different strains using *n*-hexadecane as the substrate has been shown in Figure 4.12.

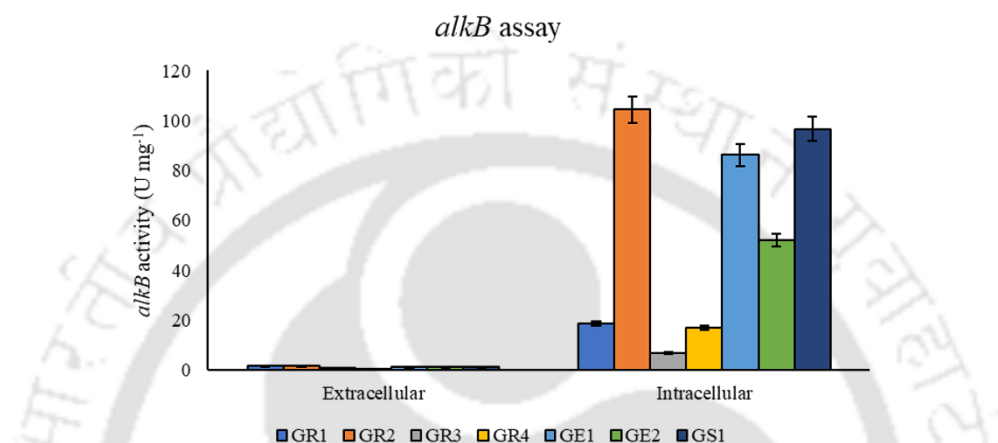


Figure 4.12: *alkB* assay from crude extract of the isolates. The significantly high activities (~ 100 U mg⁻¹) of *Staphylococcus* sp. GR2, *Aquamicrobium* sp. GE2 and *Bacillus* sp. GE1/GS1 is attributed to the higher tolerance and their ability to efficiently metabolize *n*-hexadecane. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Since *Aquamicrobium* sp. GR1, GR3 and GR4 aided to lower metabolism (Chapter 2), their enzyme activities were also found to be lesser as compared to the other strains. The maximum activity was seen in *Staphylococcus* sp. GR2 as 104.35 ± 0.97 U mg⁻¹ whereas the lowest was observed in case of *Aquamicrobium* sp. GR3 as 7.79 ± 0.55 U mg⁻¹. On comparison, the activities were found higher than the positive control, depicting that alkane hydroxylase enzyme existed as an integral protein [352].

The *alkB* assay carried out on the extracellular positive control were mostly in the range of $\sim 1.5 \pm 0.2$ U mg⁻¹ except *Aquamicrobium* sp. GR4 whose extracellular activity was recorded as 0.39 ± 0.09 U mg⁻¹. Minimal activity in the positive control could be related to transfer of alkane molecules towards the cell membrane of the respective strains. Moreover, enhanced activity within the intracellular cell extract was suggestive that the enzyme acted on alkane as the first step of metabolism and the reaction was driven towards formation of alcohol by oxidation of the terminal C [166,310,352]. Overall, the order of *alkB* activity was found as

Staphylococcus sp. GR2 > *Bacillus sp.* GS1 > *Bacillus sp.* GE1 > *Aquamicrobium sp.* GE2 > *Aquamicrobium sp.* GR4 > *Aquamicrobium sp.* GR1 > *Aquamicrobium sp.* GR3.

4.3.7. Naphthalene dioxygenase assay (*ndo*)

ndo activities (Figure 4.13) were comparable for all the strains except *Bacillus sp.* GE1 which exhibited highest activity as 6.92 ± 0.89 U mg⁻¹ in metabolism of naphthalene. *ndo* activities of all other strains ranged within 1-3 U mg⁻¹ except *Aquamicrobium sp.* GR3, the activity of which was found to be the least as 0.42 ± 0.02 U mg⁻¹. The activities could also justify the lower tolerance towards PAHs such as anthracene and phenanthrene (Chapter 2) except for *Bacillus sp.* GE1, however, comparatively, *ndo* activities were higher than that in the extracellular positive control signifying that naphthalene was metabolised inside the cell [236,345].

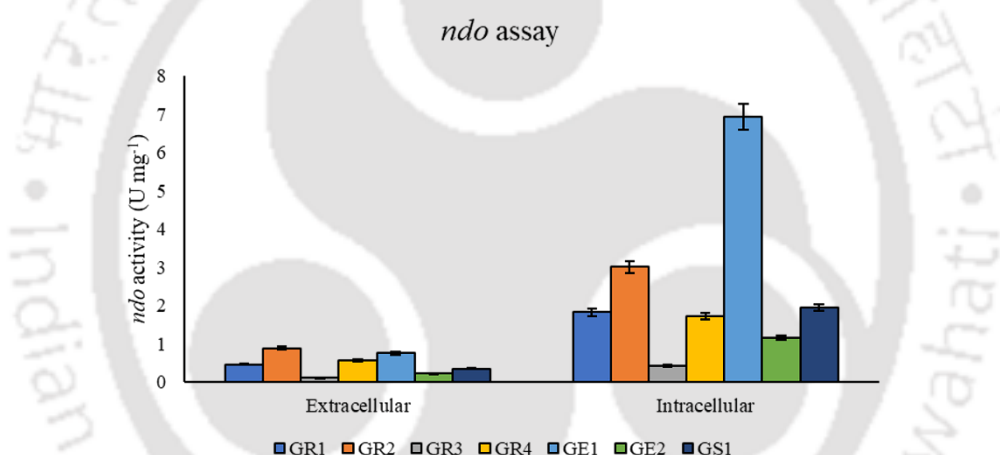


Figure 4.13: *ndo* assay from crude extract of the isolates. The activity was maximum for *Bacillus sp.* GE1 (~7 U mg⁻¹). Reduced activity of others was suggestive of lower tolerance. Amongst all, *Bacillus sp.* GE1 showed greater capacity to initialize the metabolism of naphthalene, anthracene and other PAHs. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

4.3.8. Catechol dioxygenase (CDO) assay

The enzyme activities pertaining to catechol 1,2 dioxygenase (*catA*) and catechol 2,3 dioxygenase (*catE*) are shown in Figure 4.14(a) and Figure 4.14(b) respectively.

catA activities (Figure 4.14(a)) were comparable for all the strains except *Aquamicrobium sp.* GR3 which did not show significant efficiency in metabolism of catechol through *ortho* cleavage. The highest activity was however, noticed for *Aquamicrobium sp.* GR1 and GR4 as 13.92 ± 1.04 U mg⁻¹ and 13.7 ± 0.97 U mg⁻¹ respectively. *catA* activities were

prominently higher in comparison to the extracellular positive control indicating that the enzyme existed purely within the membrane and cytoplasm [238,353].

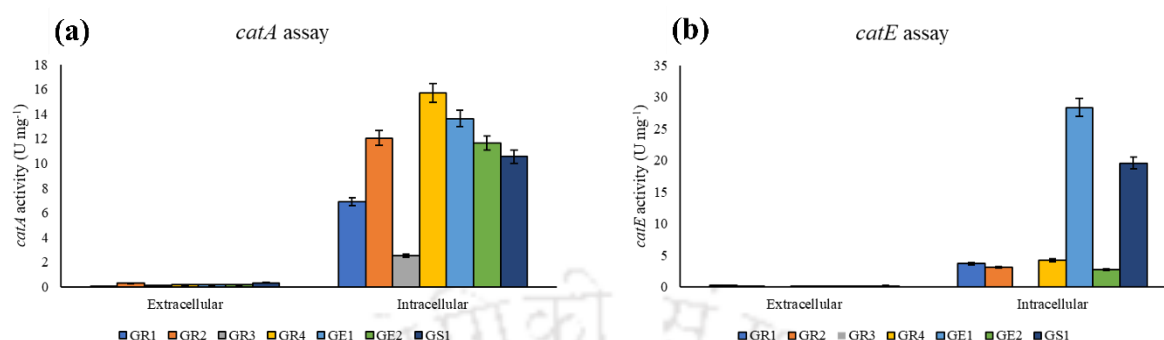


Figure 4.14: (a) *catA*; and (b) *catE* assays from crude extract of the isolates. Whereas all the isolates except *Aquamicrobium sp.* GR3 showed significant *catA* activity, only *Bacillus sp.* GE1/GS1 could exhibit satisfactory *catE* activity. Although excellent catechol breakdown is signified, *Bacillus sp.* GE1/GS1 showed preference of breakdown through meta pathway. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

catE activities (Figure 4.14(b)) were comparable for all the strains except *Bacillus sp.* GE1 and GS1 which exhibited significantly higher efficiency in metabolism of catechol through *meta* cleavage. Their corresponding activities were calculated as $26.02 \pm 1.25 \text{ U mg}^{-1}$ and $19.58 \pm 1.05 \text{ U mg}^{-1}$ respectively. *catE* activities were comparatively higher than that in the extracellular positive control signifying the metabolism of catechol occurred inside the cell membrane [166,310,322].

4.3.9. Storage stability of crude extracts

The storage stability of the crude extracts was checked at their respective optimum temperature and pH by regularly monitoring the enzyme activity (Figure 4.15).

For *alkB* (Figure 4.15(a)), a residual activity of 2% could be seen upon incubation upto 360 minutes of storage by all the strains. The enzymes however, started disintegrating after 120 minutes and by the end of 180 minutes, more than 50% of the activity was lost. The maximum enzyme activity of *Staphylococcus sp.* GR2 dipped from 64.24 U mg^{-1} to 49.38 U mg^{-1} thereby retaining only 47% of activity during this tenure of 60 minutes. Hence, all the experiments were carried out within this time interval after preparing the crude extracts of the respective strains.

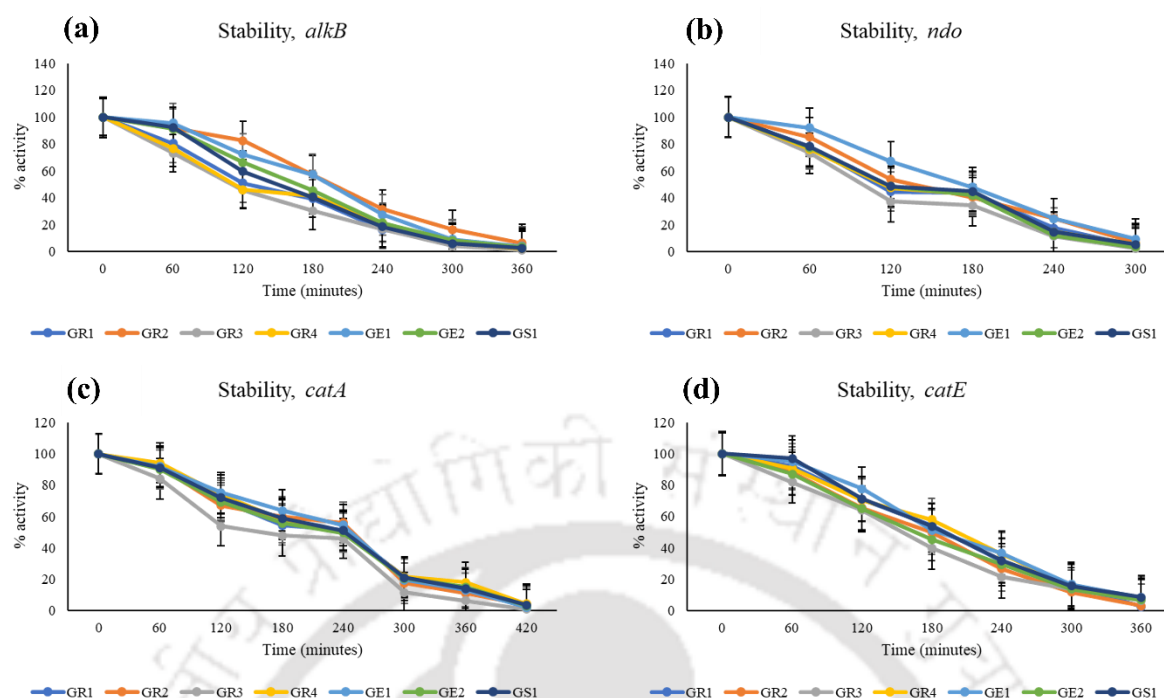


Figure 4.15: Storage stability of crude extract for (a) *alkB*; (b) *ndo*; (c) *catA*; and (d) *catE* assays. The activities of enzymes in the crude extract decreased on exposure to ambient conditions. It was deduced that *alkB*, *ndo*, *catA* and *catE* assays should be conducted within 180, 120, 240 and 180 minutes respectively, where atleast 50% of the activities were retained after preparation of the respective crude extracts. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For *ndo* (Figure 4.15(b)), the enzyme could only retain 50-60% of activity after 120 minutes of storage. Until 120 minutes where atleast 75% of the activity was assumed to have been retained, all the experiments were carried out within this time after the crude extracts were prepared for the respective strains.

For *catA* (Figure 4.15(c)), a residual activity could be seen upon incubation upto 420 minutes of storage where atleast 2% of the activity was found to be retained by all the strains. The enzymes, however, became unstable after 240 minutes where upto ~50% of the activity was lost. The enzyme activity was drastically reduced between 240-300 minutes, and all the experiments were carried out within this time after preparing the crude extracts of the respective strains.

For *catE* (Figure 4.15(d)), the enzyme was found to be stable upto 180 minutes of storage where atleast 50% of the activity was found to be retained by all the strains except *Aquamicrobium* sp. GR3. The enzyme became unstable after 180 minutes where only a maximum of ~37% of the activity was retained by *Bacillus* sp. GE1. The activity was

drastically reduced between 180-240 minutes, and all the experiments were carried out within this time after preparing the crude extracts of the respective strains.

4.3.10. Substrate specificity of crude complex for different enzyme assays

Specificity to substrate were analysed by changing the substrate for the enzymes and analysing the change in enzyme activity (Figure 4.16).

For *alkB* (Figure 4.16(a)), the enzyme was observed to have more affinity towards straight chain aliphatic alkanes as the substrate. On changing the substrate to *n*-pentadecane (C_{15}) from *n*-hexadecane (C_{16}), most of the strains showed increase in enzyme activity to as high as 139.65 U mg^{-1} for *Staphylococcus sp.* GR2 compared to 104.22 U mg^{-1} obtained using *n*-hexadecane as the substrate. In contrary, replacing *n*-hexadecane with cyclohexane, which was a cyclic aliphatic alkane (C_6), significant loss in enzymatic activity was noted. Whereas the maximum activity was still depicted by *Staphylococcus sp.* GR2 as 74.52 U mg^{-1} , the loss in enzymatic activity accounted for $\sim 28.5\%$. However, *alkB* was found to be able to metabolise straight chain aliphatic compound with smaller chains degraded with more efficiency.

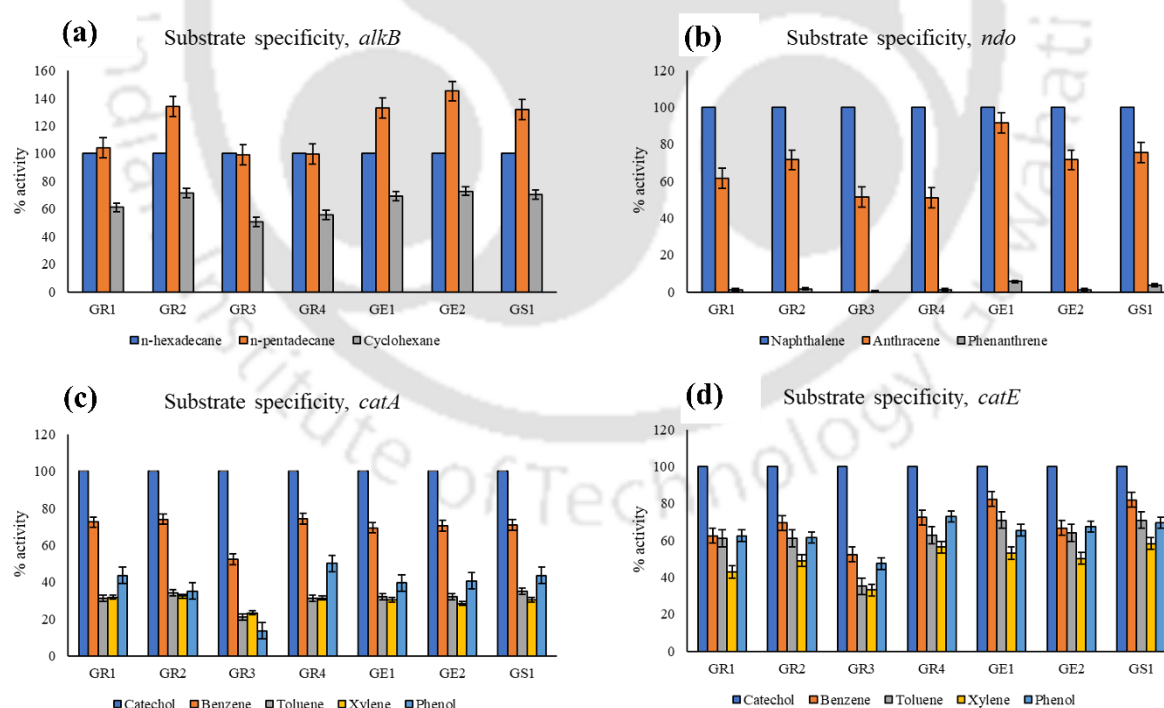


Figure 4.16: Substrate specificity of crude extract for (a) *alkB*; (b) *ndo*; (c) *catA*; and (d) *catE* assays. The activities of enzymes in the crude extract decreased upon incubation with with non-specific substrates. *alkB* depicted higher specificity towards metabolizing straight chain *n*-alkanes, *ndo* showed tremendous specificity in naphthalene and anthracene metabolism. Furthermore, *catA* and *catE* activities were relatively specific to using catechol as the substrate. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For *ndo* (Figure 4.16(b)), the crude enzymes had more affinity towards naphthalene as the substrate. Significant loss in enzyme activity was observed for all the strains when the substrate was replaced with either anthracene or phenanthrene. In both the cases, then maximum loss of activity was noticed for *Aquamicrobium sp.* GR3 for changing the substrate to anthracene and phenanthrene as ~46% and upto 100% respectively. The maximum activities were observed for *Bacillus sp.* GE1 as 6.34 U mg⁻¹ and 0.39 U mg⁻¹ respectively after setting up the reaction with anthracene and phenanthrene, thus, signifying the inability to oxidize 1,2 positions of phenanthrene. However, anthracene could be catalysed slower than naphthalene due to presence of an additional aromatic ring.

For *catA* (Figure 4.16(c)), affinity towards catechol as the substrate was more prominent among all the strains. Apart from catechol, around 70-75% enzyme activity was observed for all the strains when the substrate was replaced with benzene. Compared to benzene, toluene, xylene or phenol only contributed to a maximum retention of 31-50% by *Aquamicrobium sp.* GR4. The studies showed that *ortho* cleavage could be possible in benzene, however, not feasible in toluene, xylene and phenol due to presence of bulky functional groups such as methyl (–CH₃) and hydroxyl (–OH) groups.

For *catE* (Figure 4.16(d)), the crude enzymes had more affinity towards catechol as the substrate. In addition to catechol, significant enzyme activity was observed for all the strains when the substrate was replaced with either benzene, toluene, xylene or phenol. Consequently, maximum activity retention was seen for *Bacillus sp.* GE1 as 82.4%, 71%, 53% and 65.5% respectively with benzene, toluene, xylene or phenol as the substrate as compared to the activity obtained with catechol as the substrate. The studies showed the ease of *meta* cleavage by *Bacillus sp.* GE1 compared to the other strains.

4.3.11. Estimation of kinetic parameters

Kinetic parameters were estimated by fitting the experimental data into Michaelis-Menten kinetic equation.

For *alkB* (Figure 4.17) no substrate inhibition was noted and the kinetic parameters obtained (Table 4.1) showed significantly satisfactory fit for Michaelis-Menten equation. The kinetic parameters obtained by performing non-linear regression of the actual Michaelis-Menten kinetics indicated that all the non-linear fit were obtained as desired ($R^2 > 0.96$).

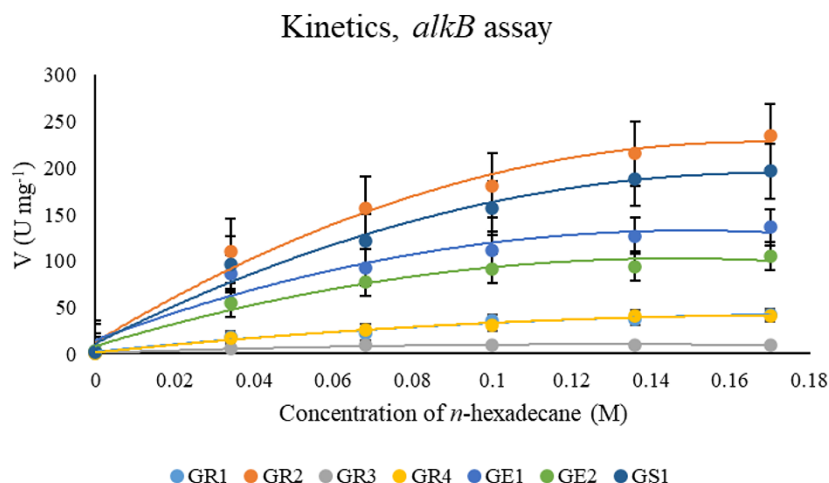


Figure 4.17: Enzyme kinetics of crude protein in terms of *alkB* assay. The isolates depicted satisfactory metabolism of *n*-hexadecane through *alkB* in terms of Michaelis-Menten's enzyme kinetics. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The maximum rate of catalysis (V_{max}) per unit amount of crude protein was found for *Bacillus* sp. GS1 as 316.16 U mg^{-1} . The corresponding Michaelis-Menten constant (K_m) was 0.097 M which is $\sim 2.84\%$ *n*-hexadecane dissolved in 80% DMSO.

Table 4.1: Enzyme kinetic parameters for *alkB* assay.

Parameters	Michaelis-Menten kinetics, <i>alkB</i>						
	GR1	GR2	GR3	GR4	GE1	GE2	GS1
K_m (M)	0.12	0.066	0.03	0.117	0.035	0.048	0.097
V_{max} (U mg^{-1})	72.45	307.89	11.9	71.05	154.6	132.32	316.16
R^2	0.968	0.99	0.987	0.986	0.963	0.997	0.954

Overall, the kinetic parameters suggested that *Staphylococcus* sp. GR2, *Bacillus* sp. GE1, *Aquamicrobium* sp. GE2 and *Bacillus* sp. GS1 possessed the necessary enzymes for catalysing alkanes and hence, have potency towards bioremediation of waste predominantly contaminated with alkanes.

For *ndo* (Figure 4.18), no substrate inhibition was noted, the kinetic parameters obtained showed significantly satisfactory fit for Michaelis-Menten equation (Table 4.2).

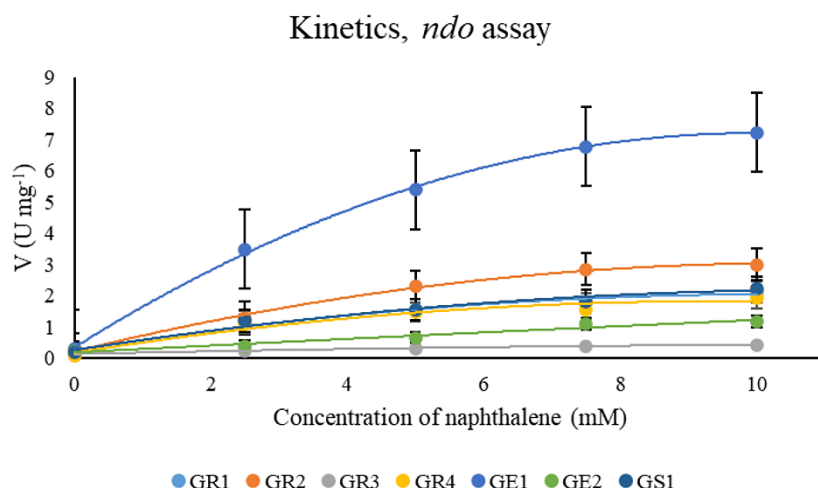


Figure 4.18: Enzyme kinetics of crude protein in terms of *ndo* assay. The isolates depicted satisfactory metabolism of anthracene through *ndo* in terms of Michaelis-Menten's enzyme kinetics. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The kinetic parameters obtained by fitting non-linear Michaelis-Menten equation was accurate and were considered for understanding *ndo* kinetics. Whereas, all the non-linear fit were obtained almost as desired ($R^2 > 0.94$), the maximum rate of catalysis (V_{max}) was found for *Bacillus sp.* GE1 as 11.52 U mg^{-1} . The corresponding Michaelis-Menten constant (K_m) was 5.63 mM naphthalene.

Table 4.2: Enzyme kinetic parameters for *ndo* assay.

Parameters	Michaelis-Menten kinetics, <i>ndo</i>						
	GR1	GR2	GR3	GR4	GE1	GE2	GS1
K_m (mM)	3.8	6.88	4.01	3.45	5.63	21	4.58
V_{max} (U mg^{-1})	2.8	5.24	0.59	2.39	11.52	3.27	3.11
R^2	0.989	0.987	0.955	0.987	0.997	0.943	0.986

Overall, the kinetic parameters suggested that *ndo* of *Bacillus sp.* GE1 possessed higher catalytic efficiency towards naphthalene breakdown and hence, may be more effective than the others for bioremediation of HC waste contaminated with PAHs.

For *catA* (Figure 4.19), except *Aquamicrobium sp.* GR3, no substrate inhibition was noted for others and the kinetic parameters obtained from Michaelis-Menten equation showed

higher precision (Table 4.3). The values obtained by fitting non-linear Michaelis-Menten equation were reliable and were considered for analysing *catA* kinetics.

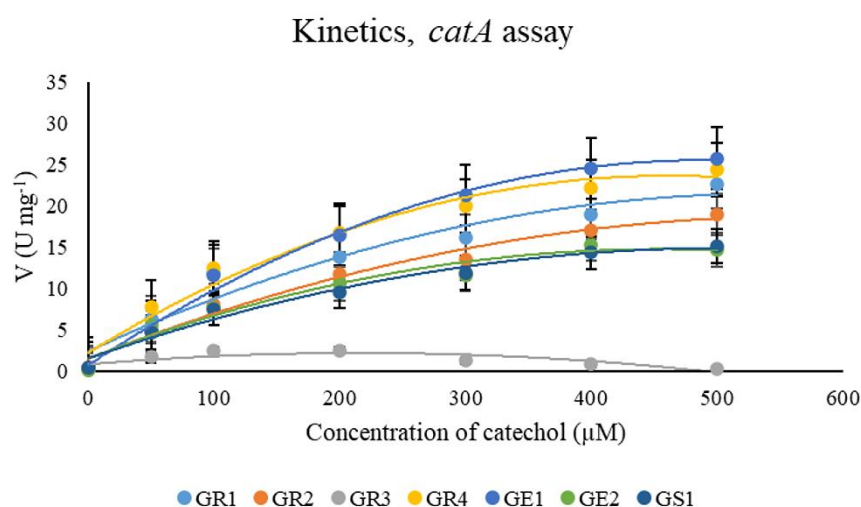


Figure 4.19: Enzyme kinetics of crude protein in terms of *catA* assay. Except *Aquamicrobium sp.* GR3, all other isolates depicted satisfactory metabolism of catechol through *catA* in terms of Michaelis-Menten's enzyme kinetics. The strains could metabolize catechol through *ortho* pathway. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Whereas, all the non-linear fit were obtained almost as desired ($R^2 > 0.95$), the maximum rate of catalysis (V_{max}) was comparable for *Aquamicrobium sp.* GR4 and *Bacillus sp.* GE1 as 35.65 U mg^{-1} and 38.3 U mg^{-1} respectively. The corresponding Michaelis-Menten constants (K_m) were 221.85 mM and 299.58 mM catechol respectively. The kinetic parameters suggested that except *Aquamicrobium sp.* GR3, all other could metabolise catechol efficiently by cleaving the benzene ring at *ortho* position as soon as it is formed from aromatic or PAHs.

Table 4.3: Enzyme kinetic parameters for *catA* assay.

Parameters	Michaelis-Menten kinetics, <i>catA</i>						
	GR1	GR2	GR3	GR4	GE1	GE2	GS1
K_m (μM)	176.04	275.09	-18.21	221.85	299.58	150.1	195.51
V_{max} (U mg^{-1})	28.06	28.37	1.3	35.65	38.3	19.24	20.64
R^2	0.971	0.992	0.382	0.953	0.964	0.978	0.989

For *catE*, except *Bacillus sp.* GE1 and GS1, all others showed substrate inhibition, and the kinetic parameters obtained from Michaelis-Menten equation showed lesser precision except the above two *Bacillus sp.* strains (Table 4.4).

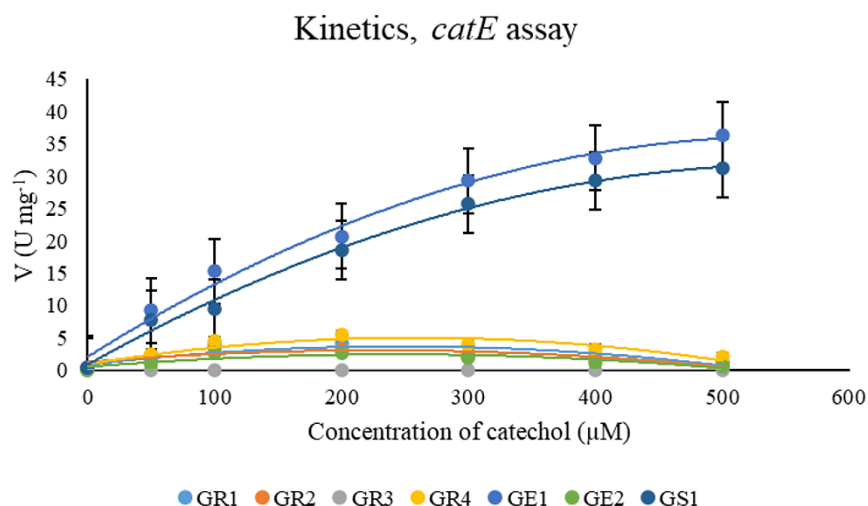


Figure 4.20: Enzyme kinetics of crude protein in terms of *catE* assay. Except *Bacillus sp.* GE1/GS1, all other isolates couldn't depict satisfactory metabolism of catechol through *catE* in terms of Michaelis-Menten's enzyme kinetics. *Bacillus sp.* GE1/GS1 preferred metabolism of catechol through *meta* pathway more efficiently compared to *ortho* pathway. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Whereas, their non-linear fit was obtained as desired ($R^2 > 0.98$), comparable $V_{max} \sim 59.3$ U mg^{-1} and the corresponding Michaelis-Menten constants (K_m) as 324.99 mM and 424.81 mM catechol respectively suggested their efficiency to cleave catechol through *meta* pathway. The non-linear fit towards the original kinetic equation was deemed more accurate and precise and the values obtained were reliable.

Table 4.4: Enzyme kinetic parameters for *catE* assay.

Parameters	Michaelis-Menten kinetics, <i>catE</i>						
	GR1	GR2	GR3	GR4	GE1	GE2	GS1
K_m (μM)	2.81	-11.65	-12.98	7.57	324.99	2.57	424.81
V_{max} (U mg^{-1})	2.67	2.12	0.08	3.81	59.33	1.7	59.27
R^2	0.393	0.438	0.023	0.552	0.98	0.361	0.991

Overall, the kinetic parameters suggested that *Bacillus sp.* GE1 and GS1 are more equipped to catalysing catechol as soon as it is formed from the breakdown of aromatic or PAHs through the energy expensive pathway and can readily convert to 2-hydroxymuconic acid semialdehyde.

For both *catA* and *catE* assays, negative K_m values were suggestive of poor fit and expresses the inability of the enzymes to use catechol as substrate. Although poor fit quality does not determine inhibition, experimental results were suggestive of the inability of the enzyme to access the substrate may have resulted in poor metabolism and final product formation. Whereas no significant increase in activities was observed after 100 μM catechol, steady increment in activity was seen from 0-100 μM . It reduced significantly at >200 μM catechol and dipped at even higher concentrations. Hence, Michaelis-Menten's mechanism could not be explained properly in the form of a rectangular hyperbola [354]. In case of *Aquamicrobium sp.* GR3, the strain already had the least tolerance, and PAHs being the most difficult to metabolize resulted in diminished *catA* activity. As a result, the Michaelis-Menten's fit also resulted in poor fit characteristics. Similarly, in case of *catE*, except *Bacillus sp.* GE1 and GS1, all others expressed the inability to break catechol through the energy expensive *meta* pathway, and hence, their activities started to decrease after 100 μM catechol.

It is already known that HCs offer low solubility. To avoid such limitations, the assay protocols clearly define the experimental conditions where *n*-hexadecane was dissolved in 80% DMSO and naphthalene was dissolved in 2-methoxymethanol to facilitate their dissolution in the aqueous reaction mixture. Owing to the tolerances obtained from Chapter 1, it may be assumed that certain strains, especially *Aquamicrobium sp.* GR3 could lack the capacity of metabolising the HC substrates into products. Moreover, since crude enzyme complex in each of the cases were used to analyse the presence of these enzymes, it can be concluded, inefficient enzymatic conversion of substrates for some strains resulted in increased toxicity and vice-versa [166,238,345,354].

This study showed that biodegradation of refinery sludge components could be achieved by a diverse group of bacteria having enzymes operating at constant kinetics that is generally very slow at environmentally relevant concentrations. To observe the presence of such enzymes, direct study of the crude enzyme cocktail provides insight into their activity and functioning, and enzyme kinetics of the crude protein is helpful [355,356].

4.4. Summary

Biomass quality is a true indicative of the efficiency of the microbial cells to accept the toxic HC and their derivatives as their source of nutrient. These components are readily accepted by the cells and are metabolized by their respective machineries. The process helps

in reducing the pollution load and eliminating toxicity from the environment. Microbes have always been reported as well versed with the necessary mechanisms that could convert the harmful contaminants into a lower toxic form. Moreover, various reports have also been published claiming the obtained microbial biomass could also be excellent source for further value addition. In this chapter, we have tried to provide such insight pertaining to estimating the biochemical properties of a typical microbial biomass. A summary of salient finding is provided in Appendix Table 109. The absolute concentrations before and after statistical optimization is also depicted in Appendix Table 110 and Appendix Table 111 respectively. Lipid, carbohydrate and protein contents supported the tendency of these strains to metabolize the HCs. Higher deposition of these biomolecules on the cell membrane exhibited the extent of HCs metabolism and that some of the strains showed greater tolerance and their potency to survive in such harsh environmental conditions. Presence of enzymes related alkane, aromatic and PAHs breakdown were also suggestive of the upregulation of the corresponding genes that are actively participating in conversion of these contaminants into further non-toxic forms. Enzyme assay and their kinetics suggested their possible role in the breakdown of HCs and most of the strains showed satisfactory fit for Michaelis-Menten kinetics explaining their strength of existence within HCs contaminated site. The parameters and experimental analysis further confirmed the applicability of the strains for efficient an enhanced bioremediation of a typical refinery waste contaminated site.

5

Application of designed consortia in bioremediation of waste sludge

5.1. Background

The importance of the indigenous microbial population and their division of tasks in a group has been extensively manipulated in order to obtain a better understanding of their applicability in the bioremediation of refinery waste. Moreover, amending the pollutant mixture with the optimum C:N:P ratio greatly enhances the adaptability of the microbes in such a complex mixture. In addition, the complexity of the process of HC metabolism and engineering for field application involves understanding the principles of stoichiometry, thermodynamics, and ecological chemistry [110,137,147,173,178].

In general, the C:N:P ratio in ecological systems is determined by the balance between the biochemical demands of organisms and the supply of these elements in the environment. This balance is influenced by factors such as nutrient availability, metabolic rates, and environmental conditions. The efficiency of nutrient use and yield can affect this balance. For example, efficiency <100% could signify that not all of the available nutrients are being utilized for growth and reproduction. This could result in a higher demand for certain nutrients, altering the optimal C:N:P ratio. Similarly, biomass yield is often considered a thumb rule to determine the ability of microbes to avail the available nutrients in order to retain their necessary machineries that could be required for higher rate of metabolism. Further, low yield is also suggestive that the system is not in a state of equilibrium [145,171,172]. This could be due to factors such as nutrient limitation or changes in environmental conditions.

Hence, in order to maintain the perfect balance, obtaining an optimum C:N:P ratio under these conditions are helpful, and the need to consider these factors and potentially develop a strategic process that could describe the dynamics of HC utilization. This would likely involve the applications of various engineering domains and also an understanding of the biological and physical processes that govern nutrient cycling in ecological systems to achieve our desired target result. In this chapter, we primarily depict the application of consortia to better understand the HC uptake. In the final parts of the chapter, we studied the application of these microbial groups and analysed their dynamics in terms of nutrient addition in the form of N and P, biomass growth and characteristics, enzyme activities and reduction in toxicity in terms of COD alongside N and P utilization.

5.2. Materials and methods

Reagents and Chemicals

All the solvents, chemicals and reagents were collectively procured from Merck Lifesciences Pvt. Ltd., Kolkata, Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru and HiMedia Laboratories, Mumbai. Pure HCs compounds anthracene, *n*-hexadecane, phenanthrene and xylene were procured from Sigma Aldrich. Benzene and toluene, potassium dichromate ($K_2Cr_2O_7$), sulfuric acid (conc. H_2SO_4), mercuric sulfate ($HgSO_4$), silver sulfate (Ag_2SO_4), stannous chloride ($SnCl_2$), and catechol was all obtained from Merck. Media components K_2HPO_4 , $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and NaCl were obtained collectively from HiMedia and Merck. Solvents chloroform, methanol and phenol were procured from Merck. CHAPS was purchased from Sigma Aldrich. Ammonium molybdate, brucine sulfate, sulfanilic acid, NADH, FAD, $NaNO_3$ and KH_2PO_4 was also procured from HiMedia. Micropipettes used during the experiments were Finnpiptette F2 (Thermo Fisher Scientific, India) of ranges (μL) 0.2-20 μL , 20-100, 20-200, 10-1000 and Research Plus edition (Eppendorf India). All the plasticwares including filtration unit for biomass estimation were obtained from Tarsons India whereas the glassware was procured from Borosil India. Water bath (refrigerated circulating), hot air oven and autoclave was procured from Equitron Medica Pvt. Ltd., India. Biosafety cabinet class II was obtained from Cleanair, India. Weighing balance was used from Sartorius, India (Model: Quintix). Bacterial incubator was obtained from Scigenics Biotech, Chennai, India, and pH meter was used from Hanna Instruments, India. Whatman qualitative filter papers (No. 1; pore size 0.7 μm ; 125 mm diameter) was procured from Cytiva Lifesciences, India. Syringe filters (sterile; 25 mm diameter, 0.2 μm pore size) for buffer sterilization were obtained from Axiva Sicheem Biotech, India. DispoVan syringes obtained from local pharmacy were used for better precision.

5.2.1. Shake flask growth study in hydrocarbon(s) (HCs) in amended mMSM by optimum consortia for enhanced degradation

Batch study was carried out to check the growth profile of the obtained consortia at maximum HC concentrations supplemented in the amended media as discussed in Chapter 3 section 3.3.6 alongwith the optimized inoculum combinations obtained from the above study in terms of maximum biomass the combinations could produce in HC supplemented modified MSM (mMSM).

In this phase, the combinations were inoculated for all of *n*-hexadecane, anthracene, phenanthrene, benzene, toluene and xylene. The growth profile was measured as OD₆₀₀ using UV-Vis Spectrophotometer (UV-2600, Shimadzu) till the death phase was observed (similarly as discussed during presenting Chapter 2). CFU/mL was also calculated to validate the presence of viable cells in HC supplemented mMSM (pH = 6.75 ± 0.25). Biomass concentrations were also measured to obtain the profile of biomass change at each interval of time period of incubation.

5.2.2. COD removal of hydrocarbon(s) (HCs)

COD removal for each of the HCs were checked as per the spectrophotometric method described in Chapter 2 section 2.2.12. Briefly, glucose was used to prepared as standard. Stock glucose solution was prepared by dissolving 100 mg glucose in reagent water and diluting upto final volume of 100 mL. 1 L = 1.067 mg COD. Standard potassium dichromate digestion mixture was prepared by dissolving 0.0347 M K₂Cr₂O₇, 16.7 mL H₂SO₄ and 3.3 g HgSO₄ in reagent water. Final volume was adjusted to 100 mL. Catalyst solution was prepared by dissolving 5 g Ag₂SO₄ in 500 mL H₂SO₄. Briefly, to 2.5 mL sample, 1.5 mL of digestion mixture and 3.5 mL catalyst solution was added in COD vial and was heated for 55 minutes (2 hours for sludge) at 150 °C. After cooling and precipitating down the mixture, OD was measured at 600 nm. COD was measured by interpolating the standard curve.

5.2.3. Microbial biomass growth and hydrocarbon (HC) utilization kinetics to analyse bioremediation of hydrocarbon(s) (HCs)

Biomass concentrations obtained on dry cell weight basis during the log phase was used to estimate the specific growth rate (μ) as per Equation 2.2. Kinetic models were fit into Monod's model/ Andrew's model as per Equation 2.3 and Equation 2.4 respectively.

The effect of initial concentration of HCs was analysed by fitting the experimental data to first order, second order and logarithmic kinetic models as per Equation 5.1, Equation 5.2, and Equation 5.3 respectively.

$$\text{First order, } S = S_0 e^{(-kt)} \quad \text{Equation 5.1}$$

$$\text{Second order, } \frac{1}{S} - \frac{1}{S_0} = kt \quad \text{Equation 5.2}$$

$$\text{Logarithmic, } S = S_0 + k \ln t \quad \text{Equation 5.3}$$

Half-life was measured using either of Equation 5.5 or Equation 5.6 depending on best-fit for 1st order or 2nd order/logarithmic. HC utilization was further analysed through Equation 5.7, Equation 5.8 and Equation 5.9.

Biomass yield (Y_B) was calculated as per Equation 5.4.

$$Y_B = \frac{X - X_0}{S_0 - S} \quad \text{Equation 5.4}$$

Where,

μ_m = Maximum specific growth rate (time⁻¹),

S_0 = Initial substrate concentration (g/L),

S = Substrate concentration at time, t (g/L),

K_s = Half-saturation constant (g/L),

K_i = Substrate Inhibition constant (g/L),

X_0 = Initial biomass concentration (mg/mL), and;

X = Biomass concentration at time t (mg/mL).

$$\text{Half-life for 1}^{\text{st}} \text{ order, } t_{1/2} = \frac{0.693}{k} \quad \text{Equation 5.5}$$

$$\text{Half-life for 2}^{\text{nd}} \text{ order/logarithmic, } t_{1/2} = \frac{S_0}{2k} / t_{1/2} = e^{-\frac{S_0}{2k}} \quad \text{Equation 5.6}$$

$$q = -\frac{1}{X} \frac{dS}{dt} \quad \text{Equation 5.7}$$

$$\text{Monod's, } q = \frac{q_{max}S}{K_s + S} \quad \text{Equation 5.8}$$

$$\text{Haldane's } q = \frac{q_{max}S}{K_s + S + \frac{S^2}{K_i}} \quad \text{Equation 5.9}$$

Where,

q_{max} = Maximum hydrocarbon (HC) utilization rate (time⁻¹), and;

k = Reaction rate constant (time⁻¹ for 1st order, (mg/L)⁻¹ time⁻¹ for 2nd order).

All the parameters were calculated using Minitab v19.1 using least square method. The calculations were done by minimizing the sum of squares (SSE) between the experimental and predicted values.

5.2.4. Experimentation with added nutrients N and P for accelerated biodegradation

N and P amendment in addition of micronutrients has been described as an efficient option to attain higher treatment efficiency [171,357]. Depending on the initial COD, the C:N:P ratio may be in the range of 100:5:1 to as high as 500:5:1 [171]. In this phase we studied the effect of C:N:P ratio of 100:5:1 and 100:10:1 in COD removal, and biomass production (Table 5.1). A waste mixture of 500 mg/L *n*-hexadecane, 50 mg/L anthracene, 20 mg/L phenanthrene, 1000 mg/L each of benzene, toluene and xylene was added and the total volume of the waste was adjusted to 150 mL by addition of mMSM ($\text{pH} = 6.75 \pm 0.25$) into 250 mL Erlenmeyer flask. The COD was adjusted to ~ 11500 mg/L and additionally N and P were added as per the following table to fulfil the ratio of 100:5:1 and 100:10:1 C:N:P.

Table 5.1: N and P amendment for C:N:P experimentation.

C:N:P	N and P required	Amendment
100:5:1	~ 575 mg/L N; ~ 115 mg/L P	~ 3.5 g/L NaNO_3 ; ~ 350 mg/L KH_2PO_4
100:10:1	~ 1150 mg/L N; ~ 115 mg/L P	~ 7 g/L NaNO_3 ; ~ 350 mg/L KH_2PO_4

For example, for C:N:P 100:5:1, for an input COD of ~ 11500 mg/L, 575 mg/L N was fulfilled by adding ~ 3.5 g/L of NaNO_3 whereas 115 mg/L P was fulfilled by required quantity of KH_2PO_4 . Since, 200 mg/L K_2HPO_4 was already present in the media, that contributed to ~ 35.6 mg/L P, additional 79.4 mg/L P was fulfilled by addition of ~ 350 mg/L KH_2PO_4 was to adjust the ratio as C:N = 100:5 and C:P = 100:1. Similarly, the calculations for the other ratio were made.

N was estimated as NO_3^- and P was estimated as PO_4^{3-} as per methods explained elsewhere [38,274,275]. All the N and P in the solution was initially allowed to completely oxidize to NO_3^- and PO_4^{3-} by application of a strong oxidizing agent potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) and heating the mixture at high temperature in using a hot plate magnetic stirrer [276,277,358]. Phosphate (PO_4^{3-}) was estimated by adding stannous chloride (SnCl_2) as reducing agent whereas nitrate (NO_3^-) was estimated using brucine-sulfanilic acid digestion method [274,275]. Standard curves for NO_3^- (Appendix Figure 49) and PO_4^{3-} (Appendix Figure 50) are illustrated in Appendices. Fractional increase in biomass growth and CFU/mL was calculated as per Equation 5.10 whereas % removal of COD, N and P were calculated as per Equation 5.11. Three consortia were subjected to inoculation in the HC mixture as explained above alongwith the addition of relevant amount N and P divided into 4 different sets such as (i) without N and P, (ii) with only N, (iii) with only P and (iv) with both N and P.

An uninoculated abiotic control was incubated to check the effect of natural attenuation, if any. A separate inoculated control without the HC mixture was checked to observe the potency of the consortia in a nutrient limiting environment. All these sets were incubated at 35 °C and 170 rpm till the death phase was observed. Alongwith NO_3^- and PO_4^{3-} , the estimation of which has been explained above, COD removal was also measured at the end of the stationary as per the method described in section 5.2.2.

$$\text{Fractional increase in growth} = \frac{X - X_0}{X_0} \quad \text{Equation 5.10}$$

Where,

X_0 = Initial biomass concentration (mg/mL) or cell count (CFU/mL);

X = Final biomass concentration (mg/mL) or cell count (CFU/mL).

$$\% \text{ Removal} = \frac{I - I_0}{I_0} \times 100 \quad \text{Equation 5.11}$$

Where,

I_0 = Initial COD/N/P concentration (mg/L);

I = Final COD/N/P concentration (mg/L).

5.2.5. Deduction of optimum C:N:P for efficient biodegradation

The optimum C:N:P was chosen based on the comparative studies carried out in the synthetic mixture as described earlier. The best C:N:P was selected by analysing the combination of nutrient addition alongwith the maximum COD removal and fractional increase in biomass growth.

5.2.6. Trial study of optimum C:N:P on diluted sludge sample to check the treatment efficiency of the designed consortia

The experimentation for checking whether the optimum consortia could be applied to the waste sludge sample alongwith amending the system with their respective optimum C:N:Ps, was carried out by first conducting a trial run. In the trial study, the designed consortia were checked for their efficiency in sludge sample with the optimum C:N:P ratio, the exact values of which are depicted in Table 5.2 and discussed in this section, keeping the input COD as ~11500 mg/L as was the case previously while studying the synthetic mixture. A portion of the

sludge sample was diluted using mMSM ($\text{pH} = 6.75 \pm 0.25$) to upto a volume of 25 mL to adjust the incoming COD to ~ 11500 mg/L theoretically.

Table 5.2: N and P amendment for trial experimentation in diluted sludge.

Input COD: ~ 11500 mg/L			
Consortia	C:N:P	N and P required	Amendment
C1	100:10:1	~ 1131 mg/L N; ~ 113 mg/L P	~ 4.16 g/L NaNO_3 ; ~ 483.63 mg/L KH_2PO_4
C2	100:5:1	~ 565.5 mg/L N; ~ 57 mg/L P	~ 0.73 g/L NaNO_3 ; ~ 236 mg/L KH_2PO_4
C3	100:10:1	~ 1131 mg/L N; ~ 113 mg/L P	~ 4.16 g/L NaNO_3 ; ~ 483.63 mg/L KH_2PO_4

The experimental value of this mixture was obtained as 11309.14 ± 76.51 mg/L. C:N and C:P were further calculated as per the experimental COD suggesting that N and P were required in the amounts mentioned in the following Table 5.2 to fulfil the ratio of 100:5 C:N for C2, 100:10 C:N for C1 and C3 and 100:1 C:P. The C:N:P values obtained are discussed in detail in the subsequent section 5.3.9.

For example, to fulfil the optimum ratio of 100:10:1 for C1, 1131 mg/L N was fulfilled by adding ~ 4.16 g/L of NaNO_3 whereas 113 mg/L P was fulfilled by adding ~ 483.63 mg/L KH_2PO_4 . The calculations were carried out assuming there is continuous distribution of components and an input N of 445.4 mg/L and 8.447 mg/L of P-PO_4^{3-} is fed into the system alongwith the diluted sludge. P-PO_4^{3-} present in mMSM while dilution was also eliminated while making the P amendment. In order to eliminate loss due to sticking on the walls of the flask during rotation, the sludge sample diluted in mMSM ($\text{pH} = 6.75 \pm 0.25$) upto COD of ~ 11500 mg/L was incubated statically in different sample containers properly wrapped with parafilm to remove any external interventions due to various factors that were out of control. The volume of the mixture was maintained to 25 mL. N and P were added accordingly after carefully calculating and considering the amount of N and P already fed in the mixture alongwith the mMSM and the sludge sample itself. An uninoculated control was also incubated alongwith the experimental sets at 33 ± 2 °C for a maximum of 48 days, which was obtained as the optimum time to attain maximum biomass production in the new engineered mMSM. COD removal, N and P removal were depicted as % removal as per equation 3.12 and biomass growth and cell viability were depicted as fractional increase in growth as per equation 3.11. COD removal, N as NO_3^- and P as PO_4^{3-} was estimated as per the optimized and standard methods. Biomass concentration was calculated of dry cell weight (d.c.w) basis gravimetrically

whereas CFU/mL was deduced using standard plate count method on 0 day and after 24 days (mid of experiment) and 48 days i.e. at the end of the experiments.

5.2.7. Application of optimum C:N:P on the sludge sample to check the treatment efficiency of the designed consortia

The experimentation on undiluted sludge sample was similar to the one described above except the N and P amendments were recalculated as per the input COD of 46017 mg/L. The experimental COD of this sample at the time of initiating this experiment was 42314.91 ± 308.41 mg/L. Hence, this value was considered as the 0-day value with the adjusted SD. The N and P amendments for the sludge sample are shown in the Table 5.3 below:

Table 5.3: N and P amendment for experimentation of sludge sample.

Input COD: ~46017 mg/L			
Consortia	C:N:P	N and P required	Amendment
C1	100:10:1	~4231.5 mg/L N; ~423 mg/L P	~23 g/L NaNO ₃ ; ~1.84 g/L KH ₂ PO ₄
C2	100:5:1	~2116 mg/L N; ~211.5 mg/L P	~10 g/L NaNO ₃ ; ~0.92 g/L KH ₂ PO ₄
C3	100:10:1	~4231.5 mg/L N; ~423 mg/L P	~23 g/L NaNO ₃ ; ~1.84 g/L KH ₂ PO ₄

To understand an instance of the calculation done for C1 with C:N:P 100:10:1, the components of the incoming feed of undiluted waste sludge was subtracted. For example, Total N of 445.4 mg/L was subtracted from the required N of 4231.5 mg/L N and the rest of N was adjusted by adding ~23 g/L NaNO₃ to fulfil the C:N ration of 100:10. Similarly, 8.447 mg/L PO₄³⁻ which contributed to approximately 2.76 mg/L P was also subtracted from the required 423.2 mg/L P and simultaneously ~1.84 g/L KH₂PO₄ was added to adjust the ratio of C:P as 100:1. Similarly, the calculations were established for the other combinations.

The inoculum, for each of the members in different consortia C1, C2 and C3 were first grown for 18 hours by dislodging a loopful number of cells from the master streak plate. The cells from these cultures were mixed in appropriate fractions (% v/v) to obtain the optimized combinations of C1, C2 and C3. The total volume was adjusted to 25 mL using mMSM (pH = 6.75 ± 0.25). These combinations were further grown into the mMSM which was obtained after a series of optimization studies in Chapter 3. The mMSM culture was allowed to grow in 100 mL Erlenmeyer flasks for 3 days to obtain the best quality inoculum. These inoculums were then added separately to each container containing 25 mL of waste sludge of input COD 46017 mg/L (experimental value: 42314.91 mg/L) alongwith the necessary N and P in the form of NaNO₃ and KH₂PO₄. The experiment was statically allowed to stand for 48 days with change

in COD, NO_3^- -N and PO_4^{3-} -P noted down on 0th day (day 0), 24th day (mid of experiment) and 48th day (end of experiment). Biomass concentration and bacterial count was described as fractional increase in growth whereas COD, NO_3^- and PO_4^{3-} was illustrated as % removal/utilization.

5.2.8. Analysis of biomass characteristics at the end of incubation of each set of experiments

Biomass characteristics in terms of lipid, carbohydrate and protein contents were analysed as per the methods described in Chapter 4. All of the characteristics are represented as % of dry biomass as was considered in previously to ease our understanding.

In brief, lipid content was estimated gravimetrically as per Folch method. Pellet fraction obtained after centrifugation of biomass obtained were dried, extracted using biphasic solvent mixture of Chloroform: Methanol was added in the ratio of 1:2 (v/v) and further homogenised for 30 minutes to provide sufficient agitation and mixing. After solubilizing soluble components using 0.8% (v/v) water, the lipid content was measured gravimetrically by carefully separating out and evaporating the bottom organic layer of the resultant mixture [339].

Carbohydrate content was estimated as per DuBois et al., 1956. Briefly, 2 mL of the biomass from culture medium were mixed with freshly prepared 5% phenol solution and 5 mL of concentrated sulfuric acid in a test tube. After allowing the reaction to occur for 10 minutes, the test tubes were vortexed for 30 seconds and was further allowed to stand for 20 minutes for colour development at room temperature. OD_{490} was measured and the unknown concentrations were obtained from the standard curve prepared [338].

Protein content was determined using the standard method described by Bradford, 1976. 5 μL of protein samples were incubated with 250 μL Bradford reagent was added and the colour was allowed to develop for 5 minutes. OD_{595} and unknown protein concentrations were calculated from the standard curve [340].

5.2.9. Enzyme assays from crude enzyme complex after the end of incubation of sets of experiments

Enzyme assays of alkane hydroxylase (*alkB*), naphthalene dioxygenase (*ndo*), catechol 1,2 dioxygenase (*catA*) and catechol 2,3 dioxygenase (*catE*) were performed as per the methods

described in Chapter 4. To accommodate and compare the presence and activity of enzymes, the results were described as fractional increase in enzyme activity as per the following Equation 5.12 (similar to Equation 5.10).

$$\text{Fractional increase in enzyme activity} = \frac{A - A_0}{A_0} \quad \text{Equation 5.12}$$

Where,

A = Enzyme activity per unit amount of crude protein after incubation (U mg^{-1}); and

A_0 = Enzyme activity per unit amount of crude protein during initiating of experiment (U mg^{-1}).

alkB assay was carried out in a reaction mixture of 1 mL consisting of 0.15% CHAPS buffer, 20 mM sodium phosphate buffer ($\text{pH} = 7.4$), 0.1 mM NADH and 50 μL of crude extract. The reaction was initiated by adding 10 μL of 1% *n*-hexadecane solution dissolved in 80% DMSO and OD_{340} was measured to visualize NADH utilization. Enzyme activity was calculated as μmol of NADH utilized per minute per unit amount of crude protein [166].

ndo activity was carried out in a reaction mixture of 1 mL consisting of 50 mM sodium phosphate buffer ($\text{pH} = 7.5$), 2 mM NADH, 5 μM FAD and 50 μL of crude extract. The reaction was initiated by addition of 10 mM naphthalene solution dissolved in 2-methoxymethanol. OD_{340} was measured and enzyme activity was calculated as μmol of NADH consumed per minute per unit amount of crude complex [343,345–347].

catA and *catE* assays were performed in 1 mL reaction mixture containing 50 mM sodium phosphate buffer ($\text{pH} = 7.0$ and 7.5 respectively) and 100 μL crude extract. The reaction was initiated by adding 10 mM catechol. *catA* activity was analysed by monitoring OD_{260} . One unit of *catA* activity was defined as nmol of *cis, cis*-muconic acid formed per minute per unit crude protein, whereas for *catE*, OD_{375} was measured, and activity was calculated as nmol of 2-hydroxymuconic acid semialdehyde formed per minute per unit amount of protein [238,348,349].

5.3. Results and discussion

5.3.1. Shake flask study in hydrocarbon(s) (HCs) in amended media by optimized consortia for enhanced bioremediation

The growth profile in terms of biomass concentration and CFU/mL for C1, C2 and C3 at 500 mg/L *n*-hexadecane is depicted in Figure 5.1.

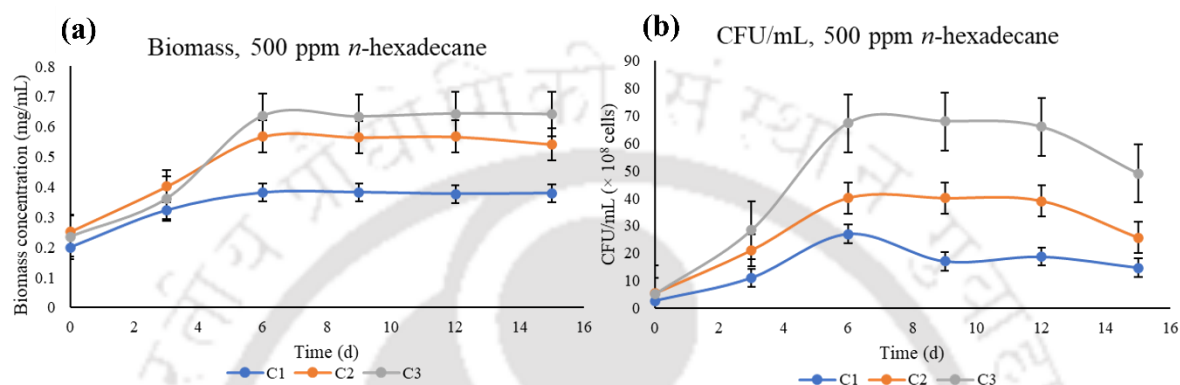


Figure 5.1: (a) Biomass concentration; and (b) CFU/mL of consortia in 500 mg/L *n*-hexadecane mMSM. All the derived combinations C1, C2 and C3 depicted significant biomass concentration which were validated by the presence of active viable cells through CFU/mL. Compared to individual isolates, the time period of incubation was greatly reduced on application of the optimized consortia. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For 500 mg/L *n*-hexadecane (high concentration), survival times were in the order C3 < C1 < C2. The maximum survival time (18 days) required to utilize 500 mg/L *n*-hexadecane was also significantly lower in case of the optimized consortia compared to 33 days which was at the minimum required by *Aquamicrobium sp.* GE2 assuming at least 75% COD removal is attained. The highest bacterial count was observed in case of C3 ($\sim 6.77 \times 10^9$ CFU/mL) and the least was observed in case of C1 (1.87×10^9 CFU/mL). The highest biomass concentration was simultaneously obtained as 0.643 mg/mL for C3.

Similarly, biomass concentration, and CFU/mL for C1, C2 and C3 at 50 mg/L of anthracene, and 1000 mg/L benzene are depicted in Figure 5.2, and Figure 5.3, respectively. For 20 mg/L phenanthrene and 1000 mg/L each of toluene and xylene, the same is depicted in Chapter 5: Application of designed consortia for bioremediation of waste sludge section of Appendices.

For 50 mg/L anthracene (Figure 5.2), survival times were also in the order $C3 < C2 < C1$. The maximum survival time (10 days) required to utilize 50 mg/L anthracene was also significantly lower in case of the optimized consortia compared to 24 days which was at the minimum required by *Aquamicrobium sp.* GR4 assuming atleast 50% COD removal is attained. The highest bacterial count was observed in case of C3 ($\sim 2 \times 10^9$ CFU/mL) and the least was observed in case of C1 ($\sim 10^9$ CFU/mL). The highest biomass concentration was simultaneously obtained as 0.447 mg/mL for C3. The consortia were found slightly better adapted as compared to individual strains in case of phenanthrene supplemented mMSM also (Appendix Figure 51).

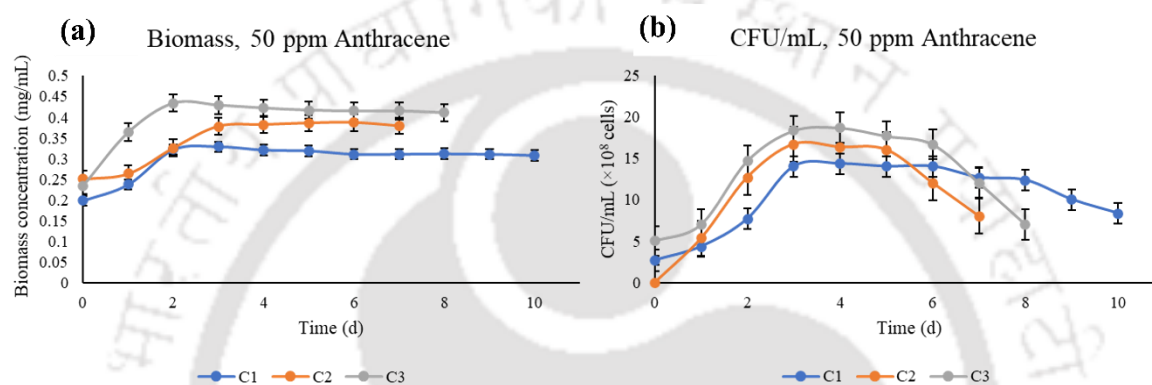


Figure 5.2: (a) Biomass concentration; and (b) CFU/mL of consortia in 50 mg/L anthracene mMSM. All the derived combinations C1, C2 and C3 depicted significant biomass concentration which were validated by the presence of active viable cells through CFU/mL. Compared to individual isolates, the time period of incubation was greatly reduced on application of the optimized consortia. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For 1000 mg/L benzene (Figure 5.3), survival times were also in the order $C2 < C3 < C1$. The maximum survival time (6 days) required to utilize 1000 mg/L benzene was also significantly lower as compared to 24 days which was at the minimum required by *Aquamicrobium sp.* GE2 assuming atleast 75% COD removal is attained.

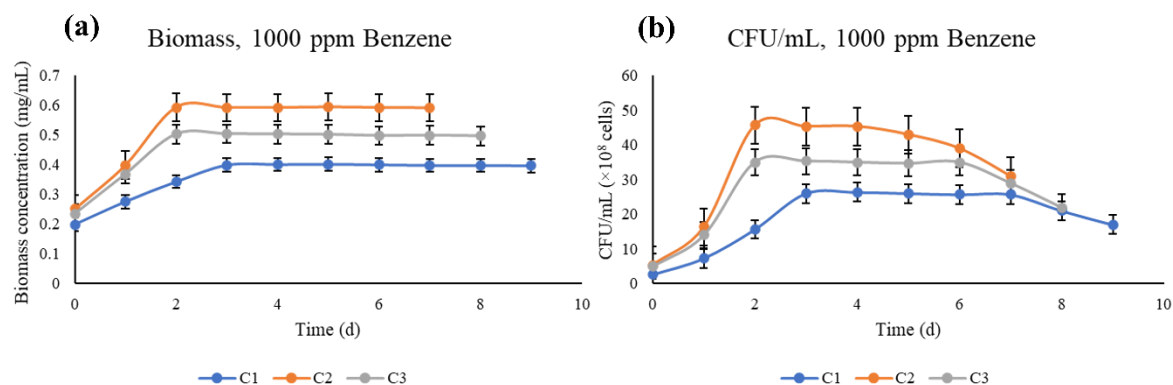


Figure 5.3: (a) Biomass concentration; and (b) CFU/mL of consortia in 1000 mg/L benzene mMSM. All the derived combinations C1, C2 and C3 depicted significant biomass concentration which were validated by the presence of active viable cells through CFU/mL. Compared to individual isolates, the time period of incubation was greatly reduced on application of the optimized consortia. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

The highest bacterial count was observed in case of C2 ($\sim 5 \times 10^9$ CFU/mL) and the least was observed in case of C1 (2×10^9 CFU/mL). The highest biomass concentration was simultaneously obtained as 0.612 mg/mL for C2. Similar to benzene, significantly higher biomass concentration and bacterial count was observed in case of toluene and xylene when the consortia were incubated in these HCs supplemented in mMSM (Appendix Figure 52 and Appendix Figure 53).

5.3.2. COD removal of hydrocarbon(s) (HCs)

COD removal at the end of stationary phase for each combination was studied.

In case of *n*-hexadecane, more than 50% of the COD is removed at the end of 6 days for each of C1, C2 and C3. A lag was observed for around 2 days before the log phase started. The highest removal at the end of stationary was obtained for C3 as 78%. Figure 5.4 depicts COD removal at both low and high concentrations.

The removal observed was also comparable to different studies. However, we report here the removal in terms of COD at higher concentration and the time period of incubation was lower [319,322,352].

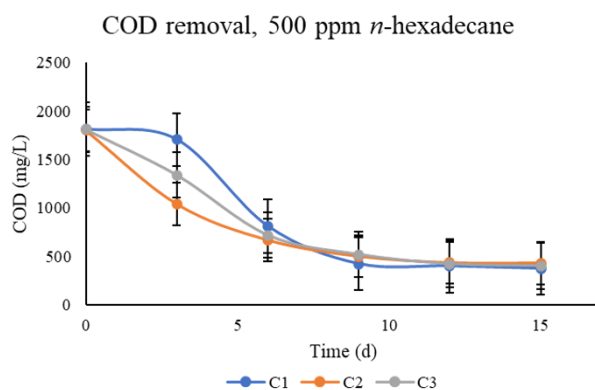


Figure 5.4: COD removal in 500 mg/L *n*-hexadecane by consortia. All the derived combinations C1, C2 and C3 depicted significantly higher COD removal. Compared to individual isolates, the maximum COD removal increased on application of the optimized consortia. The highest COD removal was depicted by C1 as ~79%. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For 50 mg/L anthracene, COD removal (Figure 5.5(a)) was studied at the end of stationary phase for each combination. Almost 50% of the COD is removed at the end of 6 days for each of C1, C2 and C3. A lag was observed for 1 day before the log phase started. The highest removal at the end of stationary was obtained for C3 as ~55%.

For 20 mg/L phenanthrene (Figure 5.5(b)), maximum COD removal of 23.59% was obtained for C2. A lag of 1 day was observed for all the combinations before the log phased was attained. However, the combinations could not show significant reduction of COD in phenanthrene supplemented media and hence, other external factors may need to be altered.

For both anthracene and phenanthrene, the removal was lower than reported by some of the investigators, however, overall reduction in COD was more in consortia as compared to individual strains in our study [311,317].

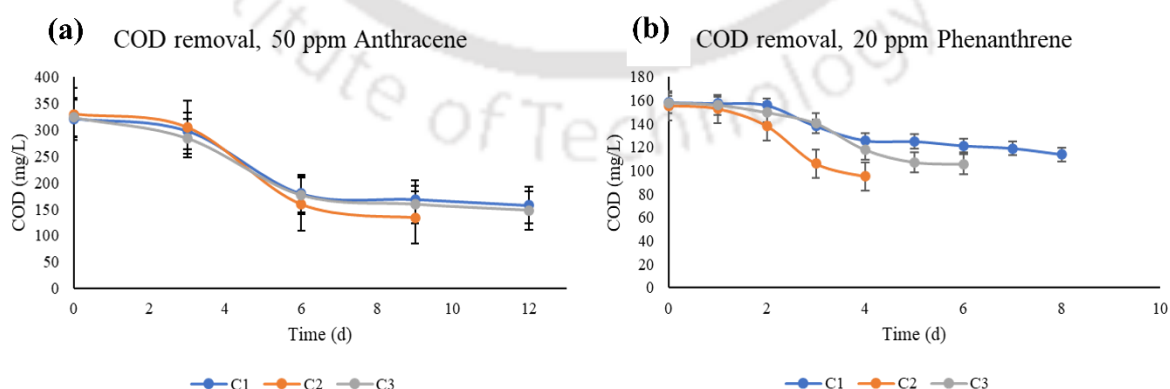


Figure 5.5: COD removal in (a) 50 mg/L anthracene; and (b) 20 mg/L phenanthrene by consortia. All the derived combinations C1, C2 and C3 depicted significantly higher COD removal. Compared to individual isolates, the maximum COD removal increased on application of the optimized consortia. The highest COD removal was depicted by C3 as ~55% in case of anthracene; and by C2 as ~35% in case of phenanthrene. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For 1000 mg/L benzene (Figure 5.6(a)), almost 95% of COD is utilized by the end of 4 days. The COD removal of all the combinations were ~97%. A lag of 1 day was observed for all the combinations before the log phased was attained. The combinations showed significant reduction of COD in benzene supplemented media signifying that C1, C2 and C3 are more helpful during initial phases of treatment as benzene is emitted out first of all the HCs present in the waste environment. Similarly, the consortia could also be reliable for toluene (Figure 5.6(b)) and xylene (Appendix Figure 54) [166,297].

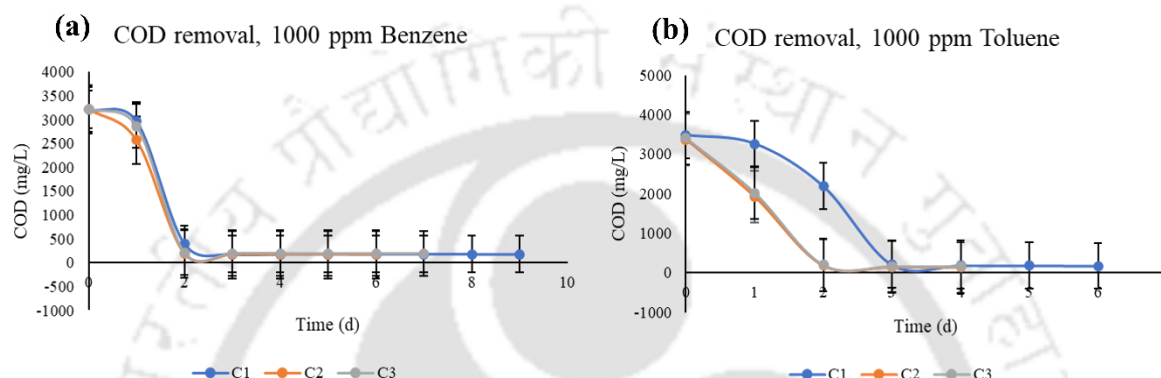


Figure 5.6: COD removal in 1000 mg/L of (a) benzene; and (b) toluene by consortia. All the derived combinations C1, C2 and C3 depicted significantly higher COD removal. Compared to individual isolates, the maximum COD removal increased on application of the optimized consortia. The highest COD removal was depicted by all of C1, C2 and C3 as ~97-98% in case of benzene, toluene and xylene. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

A comparative table of enhanced removal efficiency of the consortia under supplementation of different HCs in mMSM is depicted in Table 5.4:

Table 5.4: Comparison of maximum COD removal in different HCs between individual isolates and optimum consortia.

Pollutant(s)/ Hydrocarbon(s) (HCs)	Maximum removal (%)	
	Individual isolates	Consortia
<i>n</i> -hexadecane	~67% by GR2	~79% by C1
Anthracene	~35% by GE1	~55% by C3
Phenanthrene	~17% by GE1	~29% by C2
Benzene	~93% by GR2, GR4	~97% by C1, C2 and C3
Toluene	~94% by GR2, GR4, GE1	~98% by C1, C2 and C3
Xylene	~94% by GR2, GR4	~98% by C1, C2 and C2

5.3.3. Microbial biomass growth and hydrocarbon (HC) utilization kinetics to analyse bioremediation of hydrocarbon(s) (HCs)

The kinetics of microbial growth and HC removal were analysed as described in section 5.2.3.

For 500 mg/L *n*-hexadecane (Table 5.5), all the combinations showed best growth for logarithmic kinetics. The maximum reaction rate was calculated as 0.143 d⁻¹ for C3. However, specific growth rate was higher for C2 (0.127 d⁻¹) than C1 and C3 which further infers preference of C3 over other combinations in case wastes dominated by high concentration of *n*-hexadecane or alkane derived compounds. Higher specific growth rate values during log phases of C1 (0.116 h⁻¹), C2 (0.125 h⁻¹) and C3 (listed above) than the maximum value obtained during initial screening (0.059 d⁻¹ for *Staphylococcus* sp. GR2) suggested the potential of the obtained consortia for application in waste treatment having significant amount of alkanes/ paraffins present in it. This is further confirmed by the obtained biokinetic parameters for HC utilization rate. No substrate inhibition was depicted by C1, C2 and C3. The least error of fit was obtained for C1 ($R^2 = 0.99$) for C2, whereas C1 and C3 showed best-fit with R^2 of 0.762 and 0.988 respectively. Half-lives were calculated as 5.73 days, 5.87 days and 5.5 days for C1, C2 and C3 respectively. Yield coefficients were obtained as 0.13 mg biomass/g COD, 0.289 mg biomass/g COD and 0.233 mg biomass/g COD for C1, C2 and C3 respectively. Biomass growth showed best-fit in Andrew's kinetic model ($R^2 > 0.9$). Except C2, for which μ_{max} was very high (273 d⁻¹), C1 and C3 depicted values of 76.86 d⁻¹ and 11.038 d⁻¹ respectively. The corresponding K_s values were 570.81 g/L and 102.83 g/L for C1 and C3 respectively. The maximum utilization rate was calculated as 0.63 mg COD/mg biomass d⁻¹ for C2 at initial COD concentration of 1812.6 mg/L. Biokinetic parameters values of C2 obtained as $q_{max} = 818$ g COD/g biomass d⁻¹ and $K_s = 1710$ g/L suggested reliability and efficiency of C2 to significantly mineralize high concentration of *n*-hexadecane dominant waste.

For 50 mg/L of anthracene (Table 5.6), the best-fit for logarithmic biomass growth was obtained for C1, C2 and C3. Reaction rate constant was further calculated as 0.096 mg/L d⁻¹, 0.064 mg/L d⁻¹ and 0.044 mg/L d⁻¹ respectively. The corresponding doubling time of 7.92 days for C1, 51.42 days for C2 and 208.51 days for C3 suggested that C1 utilizes anthracene much faster than C1 and C3 and may be preferred during the initial phases of treatment in case of waste dominated by anthracene or other anthracene derived components. Yield coefficients were obtained as 2 mg biomass/g COD, 1 mg biomass/g COD and 0.9 mg biomass/g COD for

C1, C2 and C3 respectively. Specific growth rate for C1, C2 and C3 were also calculated 0.189 d⁻¹, 0.243 d⁻¹ and 0.133 d⁻¹ respectively which was also significantly higher from the maximum value obtained (0.069 d⁻¹ for *Bacillus sp.* GE1). Biomass growth also showed fit in Andrew's model ($R^2 > 0.7$) for all the combinations C1, C2 and C3. μ_{max} values of 145.85 d⁻¹ for C1 and 7678.45 d⁻¹ for C2 indicated higher rate of biomass growth in anthracene supplemented media. The corresponding K_s obtained for C1 were obtained as 198.64 g/L, whereas for C2, K_s was obtained as 8317.35 g/L. Least K_s value of 9.83 g/L for C3 depicts the maximum affinity towards growth in anthracene media as compared to C1 and C2. Further, HC utilization rate results suggested that all the combinations showed best-fit for Haldane's model. Half-lives were calculated as 48.42 days, 17.29 days and 7.57 days for C1, C2 and C3 respectively. HC utilization rate was similar for C1 and C3 (~80 mg/L d⁻¹). The maximum anthracene utilization rate was observed as 225.94 mg COD/g biomass d⁻¹ for C2 at initial COD concentration of 321.6 mg/L. Maximum biokinetic parameters values obtained as $q_{max} = 1654.17$ g COD/g biomass d⁻¹ and $K_s = 2755.68$ g/L suggested higher rate of COD utilization, although lower affinity of C2 over C1 and C3. Hence, C1 may be preferred to biotransform anthracene dominant waste into lower toxic form. Other the other hand, similar analysis of the kinetic parameters (Appendix Table 113) suggested C2 could be preferred for treatment of waste contaminated prevalently with phenanthrene and its derivatives.

For 1000 mg/L of benzene (Table 5.7), the best-fit for logarithmic biomass growth was obtained for C2 and C3 ($R^2 = 0.87$) whereas C1 showed best-fit for exponential biomass growth. Reaction rate constants were further calculated as 0.184 d⁻¹, 0.187 mg/L d⁻¹ and 0.131 mg/L d⁻¹ for C1, C2 and C3 respectively. The corresponding doubling time of 3.77 days for C1, 1.34 days for C2 and 5.99 days for C3 suggested that all of these can be preferred during the initial phases of treatment in case of waste dominated by benzene or its derived components. Yield coefficients were obtained as 67 mg biomass/g COD, 112 mg biomass/g COD and 89 mg biomass/g COD for C1, C2 and C3 respectively. Specific growth rate for C1, C2 and C3 were also calculated 0.101 d⁻¹, 0.193 d⁻¹ and 0.178 d⁻¹ respectively which was also significantly higher from the maximum value obtained (0.057 d⁻¹ for *Staphylococcus sp.* GR2). Biomass growth also showed fit in Andrew's model ($R^2 > 0.8$) for all the combinations C1, C2 and C3. μ_{max} values of 3.39 d⁻¹ for C2 and 3.8 d⁻¹ for C3 indicated higher rate of biomass growth in benzene supplemented media. The corresponding K_s obtained for C2 were obtained as 20.43 g/L, whereas for C3, K_s was obtained as 20.34 g/L. Least K_s value of 7.77 g/L for C1 also depicted the maximum affinity towards growth in benzene media alongwith C2 and C3.

Further, HC utilization rate results suggested that all the combinations showed best-fit for Haldane's model. Half-lives were calculated as 0.49 days, 0.51 days and 0.52 days for C1, C2 and C3 respectively. HC utilization rates were calculated as 1.41 d^{-1} for C1, 1.33 d^{-1} for C2 and 1.36 d^{-1} for C3. The maximum benzene utilization rate was observed as $1.9 \text{ g COD/g biomass d}^{-1}$ for C1 at initial COD concentration of 3.22 g/L . Maximum biokinetic parameters values obtained as $q_{max} = 286.29 \text{ g COD/g biomass d}^{-1}$ and $K_s = 15.33 \text{ g/L}$ suggested higher rate of COD utilization and significant affinity of C3 towards benzene removal. Similar, studies were also carried out for toluene and xylene, and it could be possible that C1, C2 and C3 all may be preferred to mineralize aromatic HC contaminated waste (Appendix Table 114 and Appendix Table 115).

Higher yield and significant lowering of half-life in each for C1, C2 and C3 suggested higher biomass growth, faster COD utilization and the kinetic studies provide a better perspective of the reliability of the consortia for bioremediation of refinery waste sludge [313,322,359].

Table 5.5: Biomass growth and HC utilization kinetics of consortia in 500 mg/L *n*-hexadecane mMSM.

Biomass growth: Logarithmic growth ($R^2 = 0.91-0.95$), Andrew's kinetic model for C1, Monod's kinetic model for C2 and C3 Hydrocarbon (HC) utilization: Exponential COD removal ($R^2 = 0.92-0.98$), Monod's kinetic model for C1, C2 and C3													
Consortia	Biomass growth						Hydrocarbon (HC) utilization rate						Yield (Y_B) (g/g)
	k (d ⁻¹)	t_d (d)	μ_{max} (d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	k (d ⁻¹)	$t_{1/2}$ (d)	q_{max} (g/g d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	
C1	0.082	8.45	76.86	570.81	-	0.98	0.121	5.73	39.5	32.302	10.56	0.762	0.13
C2	0.117	5.92	273	2280	-	0.99	0.118	5.87	818	1710	-	0.99	0.289
C3	0.145	4.85	11.038	102.83	-	0.99	0.126	5.5	315	6.65	-	0.988	0.233

Table 5.6: Biomass growth and HC utilization kinetics of consortia in 50 mg/L anthracene mMSM.

Biomass growth: Logarithmic growth ($R^2 = 0.72-0.94$), Andrew's kinetic model for C1, C2 and C3 Hydrocarbon (HC) utilization: Logarithmic COD removal ($R^2 = 0.9-0.97$), Haldane's kinetic model for C1, C2 and C3													
Consortia	Biomass growth						Hydrocarbon (HC) utilization rate						Yield (Y_B) (g/g)
	k (d ⁻¹)	t_d (d)	μ_{max} (d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	k (d ⁻¹)	$t_{1/2}$ (d)	q_{max} (g/g d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	
C1	0.096	7.92	145.85	198.64	1.59	0.78	82.82	48.2	1.05	2.55	20.09	0.96	2
C2	0.064	51.42	7678.45	8317.35	63.2	0.8	57.93	17.29	1654.17	2755.68	11594.63	0.9	1
C3	0.044	208.51	6.09	9.83	14.66	0.79	79.98	7.57	0.266	60.47	166.66	0.84	0.9

Table 5.7: Biomass growth and HC utilization kinetics of consortia in 1000 mg/L benzene mMSM.

Biomass growth: Exponential growth ($R^2 = 0.99$) for C1, Logarithmic growth ($R^2 = 0.86, 0.87$), Andrew's kinetic model for C1, C2 and C3 Hydrocarbon (HC) utilization: Exponential COD removal ($R^2 = 0.96-0.99$) for C1, C2 and C3, Haldane's kinetic model for C1, C2 and C3													
Consortia	Biomass growth						Hydrocarbon (HC) utilization rate						Yield (Y_B) (g/g)
	k (d ⁻¹)	t_d (d)	μ_{max} (d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	k (d ⁻¹)	$t_{1/2}$ (d)	q_{max} (g/g d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	
C1	0.184	3.77	0.422	386.9	7.77	0.93	1.41	0.49	33.71	2.84	390.65	0.92	0.067
C2	0.187	1.34	3.39	20.43	15.1	0.8	1.33	0.52	154.57	12.1	28.84	0.78	0.112
C3	0.131	5.99	3.8	20.34	13.01	0.78	1.36	0.51	286.29	15.33	12.32	0.94	0.089

5.3.4. Biomass characteristics after growth study of consortia in hydrocarbon(s) (HCs)

Biomass characteristics in terms of total lipid, carbohydrate and protein were determined during three phases similar to the process explained in Chapter 4. The results are illustrated in Figure 5.7 below. The absolute concentrations can be found in Appendix Table 116.

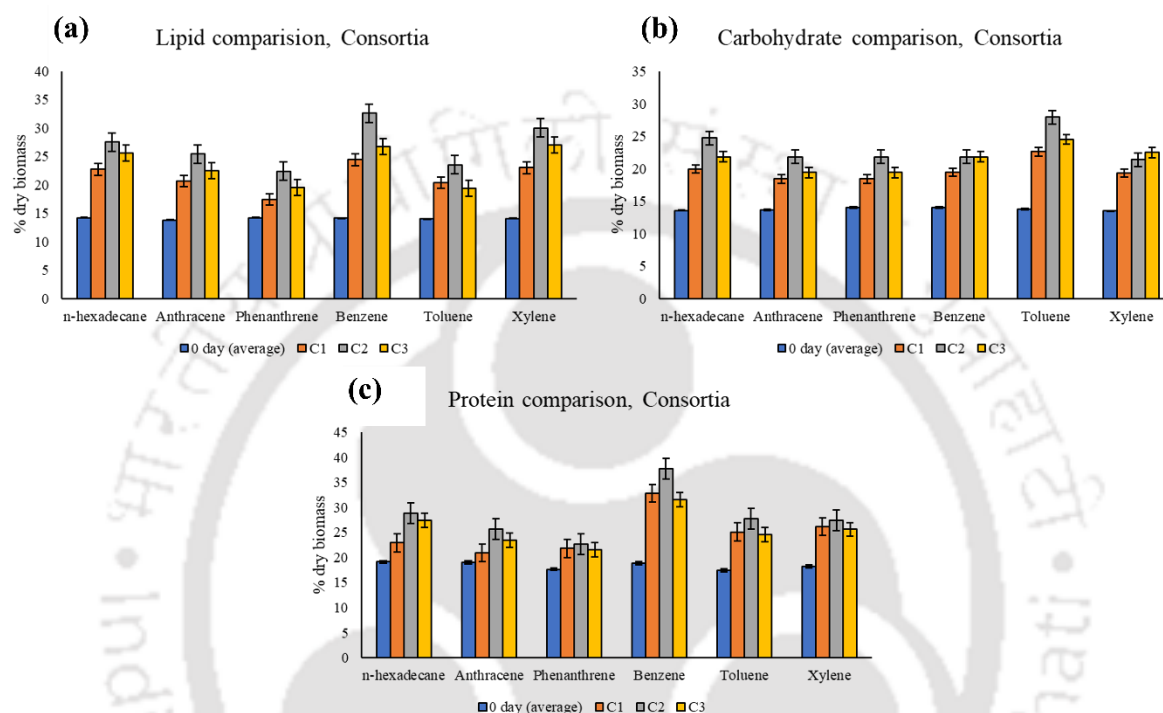


Figure 5.7: (a) Lipid; (b) carbohydrate; and (c) protein content of biomass of consortia after incubation in different class of HCs. All the derived combinations C1, C2 and C3 depicted significantly higher biomass quality. Compared to individual isolates, the lipid, carbohydrate and protein contents increased on application of the optimized consortia. The maximum of ~30-35% of the biomass was found to have deposition of lipid, carbohydrate and protein. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For *n*-hexadecane, each of total lipid, carbohydrate and protein increased gradually till the end of the experiment. The maximum of those values was obtained for C2. As compared to the biomass characteristics explained in Chapter 3, the biomass showed enhanced quality when mixed for experimentation as consortia in their respective optimum concentrations in *n*-hexadecane supplemented mMSM. Lipid, carbohydrate and protein depicted increased maximum share of 25.6%, 21.8% and 27.4% of dry biomass respectively for C3 as compared to 19.4%, 16.6% and 21.4% for *Staphylococcus* sp. GR2 and *Bacillus* sp. GS1 respectively.

For anthracene, the maximum lipid, carbohydrate and protein concentrations were identical for both C2 and C3 as 0.095 mg/mL, 0.08 mg/mL and 0.097 mg/mL respectively. Maximum lipid, share of 25.6% was similar to *Bacillus sp.* GE1 (25%) whereas carbohydrate and protein share of 25.7% and 21.8% of dry biomass respectively for C2 increased as compared to 15%, 16.6% and 21% for *Bacillus sp.* GE1 respectively when the optimum consortia were incubated in 50 mg/L anthracene supplemented mMSM. Similarly, for phenanthrene, the maximum lipid, carbohydrate and protein shares were obtained for C2 were higher than that obtained *Bacillus sp.* GE1 (Chapter 4) when the optimum consortia were incubated in 20 mg/L phenanthrene supplemented media.

For benzene, the maximum biomass characteristics was observed for C2 as 0.193 mg/mL total lipid, 0.129 mg/mL total carbohydrate and 0.223 mg/mL total protein which constituted 32.57% 21.76% and 37.66% respectively of dry biomass weight. The respective parameters showed increased values as compared to the maximum values obtained for *Staphylococcus sp.* GR2 and *Bacillus sp.* GE1 when they were individually subjected to growth in benzene supplemented minimal media. Similarly, for toluene/xylene, enhanced values suggested the efficiency of the mixed consortia to mineralize 1000 mg/L toluene/xylene supplemented in minimal media.

As also discussed in Chapter 4, biomass composition provides an insight into the capability of the microbes to utilize refinery waste components [350,351].

5.3.5. Enzyme assays after growth study of consortia in hydrocarbon(s) (HCs)

alkB assay was carried out on the crude extracted from the biomass at the end of the experiment and was compared with the activity that was recorded during the initiation of the experiment. The maximum activity increased to 48.6 U mg⁻¹ for C2 whereas the same for C3 was also comparable as 42.79 U mg⁻¹. As shown in Figure 5.8 The fractional increase in the activities for C1 and C3 to ~30 folds and that of C2 to ~31 folds suggested that *alkB* was upregulated when the combinations were provided with *n*-hexadecane as the substrate and was actively participating in the metabolism of alkane to alkanol.

ndo assays were carried out using naphthalene as the substrate. Fractional increase in activities ranging from 15-17.5 folds collectively for C1, C2 and C3 suggested slower metabolism of PAHs. However, the gene was active and expected to catalyse naphthalene to 1,2 dihydrodiol.

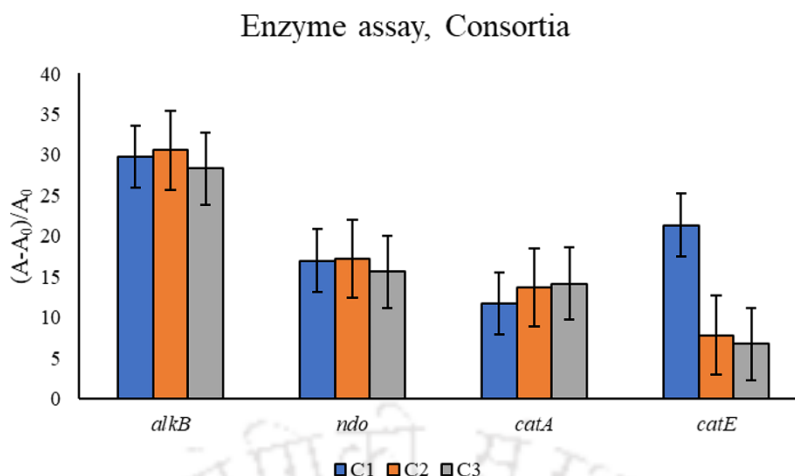


Figure 5.8: Fractional increase in enzyme activities for consortia. All the derived combinations C1, C2 and C3 depicted significantly higher enzyme activities. Compared to individual isolates, the enzyme activities increased on application of the optimized consortia. All of *alkB*, *ndo*, *catA* and *catE* showed a collective increase in activity of 20-30 folds. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

In case of *catA* and *catE*, both of which used catechol as the substrate, *catA* activity was more pronounced in C2 and C3 whereas *catE* was more active in case of C1. The highest *catA* activity was seen in C2 as 2.06 U mg^{-1} , however, comparable fractional increase of ~ 13.71 and ~ 14.2 folds for C2 and C3 suggested that C2 (and C3) preferred catechol metabolism through *ortho* pathway whereas for C1, whose *catE* activity was fractionally increased to 21.36 folds during the incubation, catechol metabolism initiation occurred by cleaving at the *meta* position.

The enzyme activities suggested the enhanced capability of mineralization of different waste components that might be present in a typical refinery waste sludge [166,238,310,345].

5.3.6. Experimentation with added N and P for accelerated biodegradation

The growth profile and removal profile pertaining to C:N:P utilization for ratio 100:5:1 and 100:10:1 of C1, C2 and C3 is depicted in Figure 5.9, Figure 5.10 and Figure 5.11 respectively. The consortia could survive and use the HC mixture as their source of nutrient and survival.

For C1 (Figure 5.9), COD removal, P utilization and N utilization has been illustrated as % removal/utilization whereas growth in terms of biomass and CFU/mL has been illustrated as fraction of growth using the formula in Equation 5.10. Whereas the COD removal was comparable with or without additional amendment of N and P, however, maximum COD

removal alongwith N and P utilization was observed in case where both the components were applied in the applicable ratio. COD removal, N utilization and P utilization with addition of both N and P were found to be maximum at ratio 100:10:1 and were calculated as 94.53%, 81.37% and 82.42% respectively. Around 25% of P utilization in terms of phosphate could also be seen for C1 where neither N nor P were added. This was due to a minimal amount of P which was already present in mMSM. P utilization was much faster when N was also added with it. Hence, in case where only N was added, around 95% and 89% of the P was consumed when C:N ratio was supplied as 100:5 and 100:10 respectively suggesting only N alongwith 200 mg/L of K_2HPO_4 could be limiting and hence, both N and P are required to be added in proper ratio to attain higher treatment efficiency. NO_3^- and PO_4^{3-} at the end of treatment of 24 days for C:N:P 100:10:1 was found to be 1.17 g/L and 73.51 mg/L respectively. Biomass growth increased to almost 12.5 folds to a maximum value of 1.94 mg/mL when both N and P were amended as compared to around 4.85 folds when neither N nor P were added. The same could also be validated through cell viability (CFU/mL), where the addition of both N and P resulted in 36 folds more cells upto 9.8×10^9 CFU/mL.

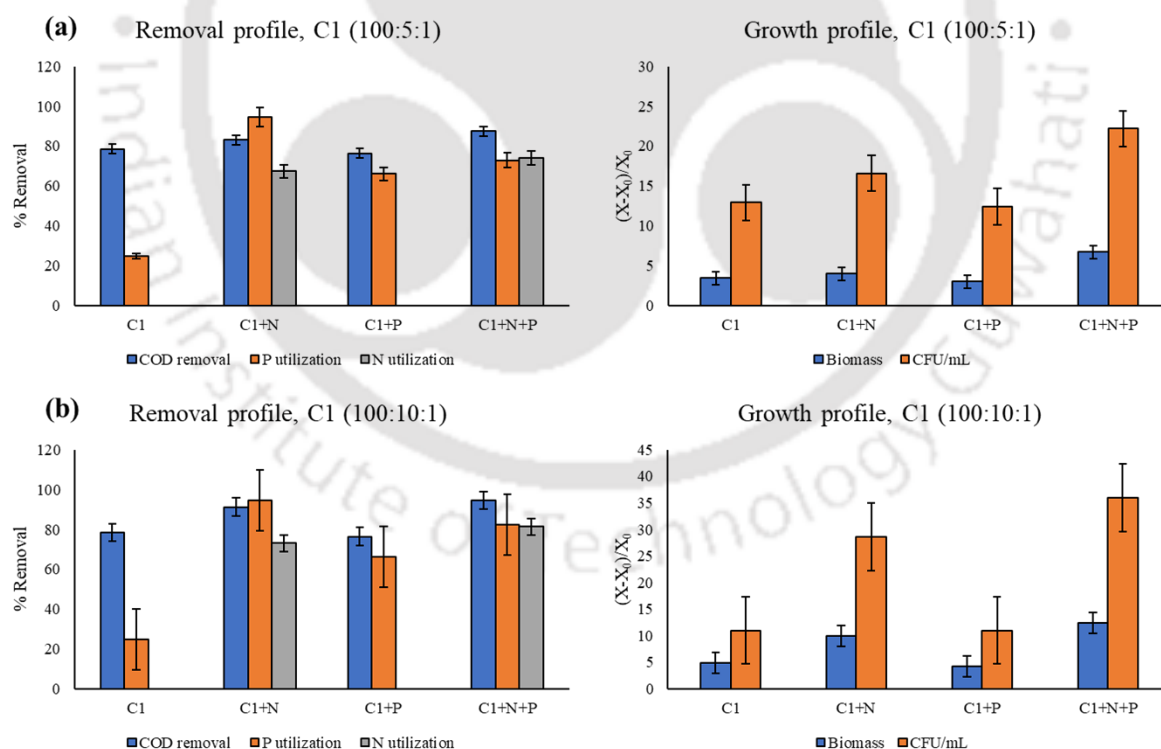


Figure 5.9: Removal profile and growth profile of C1 on addition of N and P as (a) C:N:P = 100:5:1; and (b) C:N:P = 100:10:1. COD removal, N and P utilization was the highest when both N and P were added. On account of higher biomass concentration, signified by fractional increase in biomass and bacterial count, the optimum C:N:P was chosen as 100:10:1 for C1. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

COD removal on addition of N and P was best illustrated as exponential removal with COD utilization of $0.889 \text{ g COD/g biomass d}^{-1}$, rate constant as 0.463 d^{-1} and half-life of 1.32 days. Yield coefficient of $\sim 0.084 \text{ g biomass/g COD}$ was also 3.4 times more than that obtained without addition of N and P. Hence, N and P addition in the ratio of 100:10 and 100:1 respectively helped in accelerating biodegradation of the waste type by C1.

For C2 (Figure 5.10), similar to C1, COD removal was comparable with or without additional amendment of N and P, however, maximum COD removal alongwith N and P utilization was observed in case where both the components were applied in the ratio 100:5:1. COD removal, N removal and P removal with addition of both N and P were calculated as 92.44%, 85.1% and 80.4% respectively. Around 33% of P utilization in terms of phosphate could also be seen for C2 as in the case with C1 where neither N nor P were added. This was also due to a minimal amount of P which was already present in mMSM. Also, in case where only N was added, around 97% of the P was consumed suggesting only N alongwith 200 mg/L of K_2HPO_4 was limiting as was the case with C1 and hence, both N and P are required to be added in the ratio 100:5:1 to attain better COD removal.

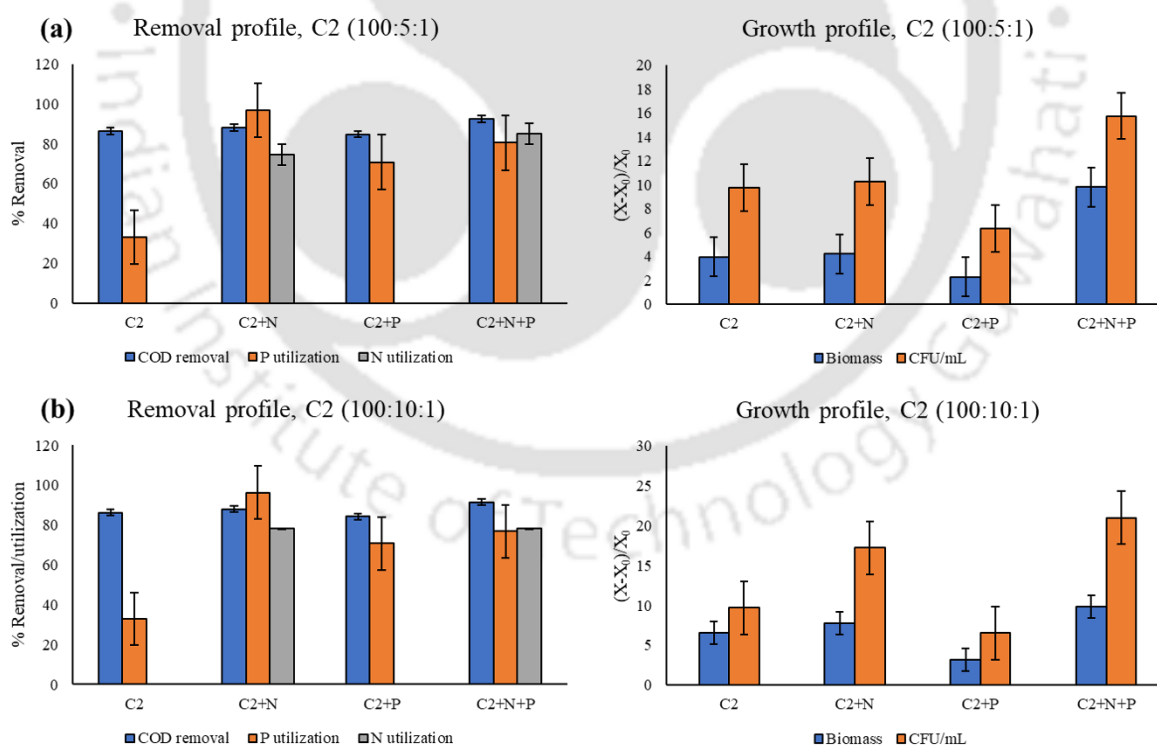


Figure 5.10: Removal profile and growth profile of C2 on addition of N and P as (a) C:N:P = 100:5:1; and (b) C:N:P = 100:10:1. COD removal, N and P utilization was the highest when both N and P were added. On account of higher biomass concentration, signified by fractional increase in biomass and bacterial count, the optimum C:N:P was chosen as 100:5:1 for C2. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Biomass growth increased to almost 10 folds when both N and P were amended as compared to around 3.95 when neither N nor P were added. The same was also validated by deducing CFU/mL, where the addition of both N and P resulted in 16 folds more cells. N in the form of NO_3^- , P in the form of PO_4^{3-} , biomass concentration and CFU/mL at the end of treatment were calculated as 0.483 g/L, 91.76 mg/L, 1.84 mg/mL and 8.92×10^9 cells respectively.

COD removal on addition of N and P was best understood as 1st order exponential removal with COD utilization of 0.879 g COD/g biomass d⁻¹, rate constant as 0.21 d⁻¹ and half-life of 3.3 days. Yield coefficient of ~0.088 g biomass/g COD was also 2.5 time more than that obtained without addition of N and P. Hence, N and P addition in the ratio of 100:5 and 100:1 respectively could be applied for treatment of such type of waste mixture by C2.

The residual COD, N, P alongwith the corresponding biomass concentration and bacterial count is mentioned in Appendix Table 120.

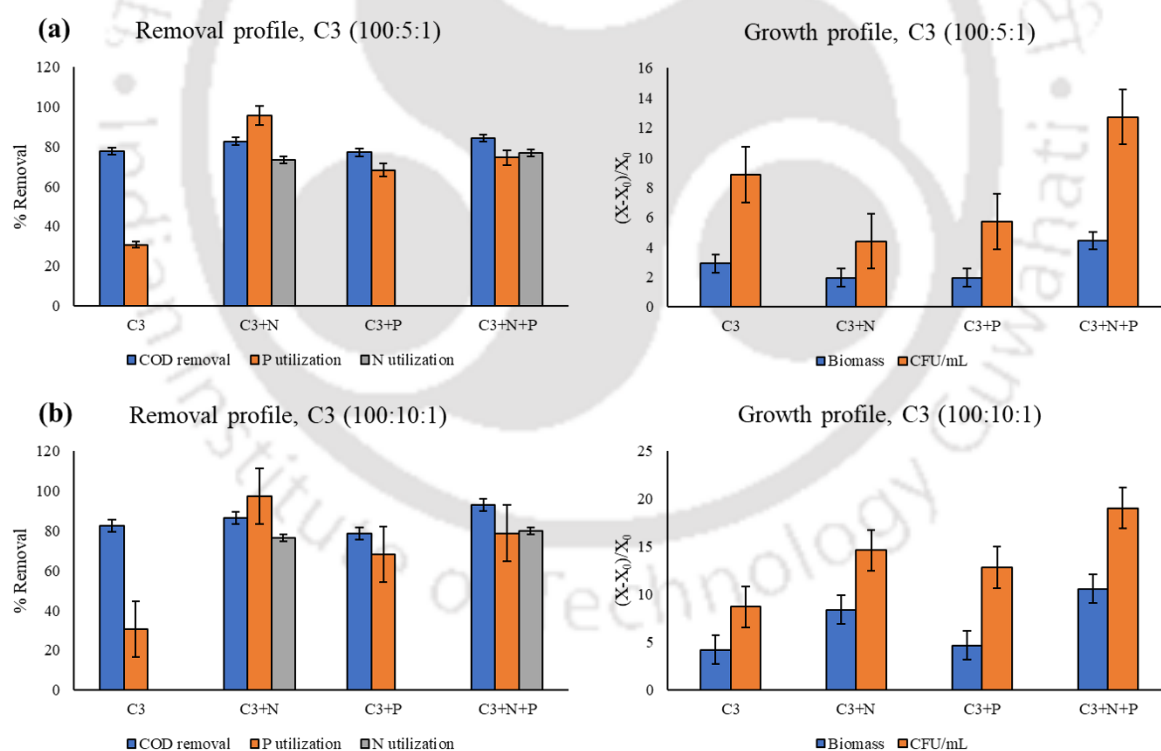


Figure 5.11: Removal profile and growth profile of C3 on addition of N and P as (a) C:N:P = 100:5:1; and (b) C:N:P = 100:10:1. COD removal, N and P utilization was the highest when both N and P were added. On account of higher biomass concentration, signified by fractional increase in biomass and bacterial count, the optimum C:N:P was chosen as 100:10:1 for C3. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For C3 (Figure 5.11), similar to C1, N and P addition in the ratio of 100:10 and 100:1 respectively was observed to be efficient for treatment of such waste using C3. A detailed summary of N and P addition for C:N:P=100:5:1 (Appendix Table 119) and C:N:P=100:10:1 (Appendix Table 121) is depicted in Appendices. For all of C1, C2 and C3, effect of nutrient addition favoured acceleration COD removal in addition to N and P utilization and hence N and P should be incorporated to obtain better bioremediation efficiency [147,183,195].

5.3.7. Biomass characteristics after N and P addition

Biomass quality was significantly improved after N and P addition as depicted below (Figure 5.12). The absolute concentration is mentioned in Appendix Table 118.

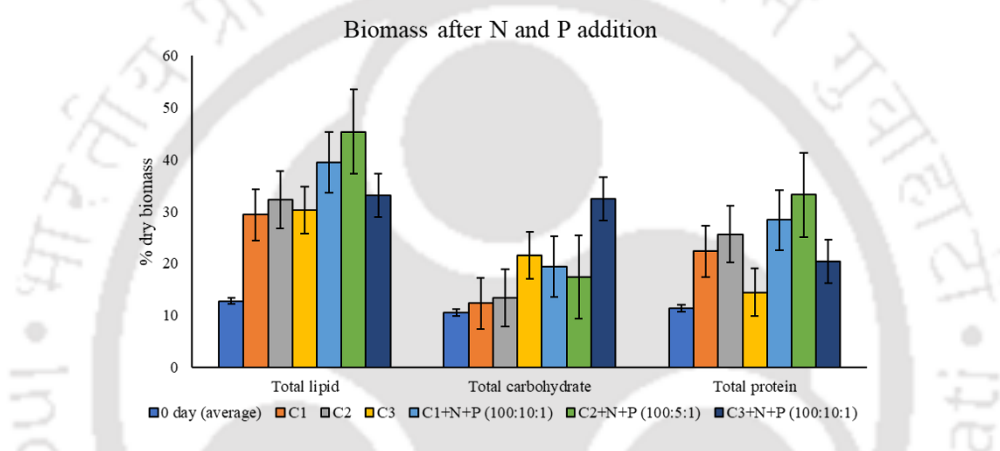


Figure 5.12: Biomass characteristics after N and P addition. All the derived combinations C1, C2 and C3 depicted significantly higher biomass quality on addition of both N and P. The increased lipid, carbohydrate and protein contents were indicative of the efficiency of consortia to utilize the HCs as their nutrient source. The maximum of ~45% lipid, and ~35% each of carbohydrate and protein deposition was found. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For C1, which showed maximum biomass production when both N and P were amended in the ratio 100:10:1, the lipid content, carbohydrate and protein concentrations were obtained as 39.44%, 28.33% and 19.33% of the total biomass dry weight. These shares were higher as compared to when N and P were not added.

For C2, maximum biomass concentration was seen when the culture was amended as C:N:P of 100:5:1. Similar to C1, the lipid content, carbohydrate content and protein content were also higher than that obtained when no additional N and P were added. The respective values were calculated as 45.32% lipid, 17.36% carbohydrate and 33.17% protein.

For C3, at C:N:P 100:10:1, the highest biomass characteristics were obtained as 33.12% lipid content, 32.44% carbohydrate content and 20.37% protein content.

5.3.8. Enzyme assay after N and P addition

The enzyme activities also increased after N and P addition (Figure 5.13). This could be suggestive of the enhanced bioavailability of the contaminants as substrate. Excess N and P has depicted to facilitate increased nutrient availability that aided to enhanced production of microbial biomass.

alkB activity increased by 44.2, 63.23 and 54.66 folds for the crude protein obtained from biomass of C1, C2 and C3 respectively when the culture was added with C:N:P ratios 100:10:1 for C1 and C3 and 100:5:1 for C2 as compared to when no N and P were added. The maximum activity of 92.5 U mg⁻¹ for C2 after N and P addition was 1.9 times higher than the activity obtained without N and P. This was comparable with the value of 104.35 U mg⁻¹ obtained for *Staphylococcus sp.* GR2 in Chapter 4 section 4.3.6. Among the others, C1 and C3 also showed 1.66- and 1.81-times higher values respectively on N and P amendment.

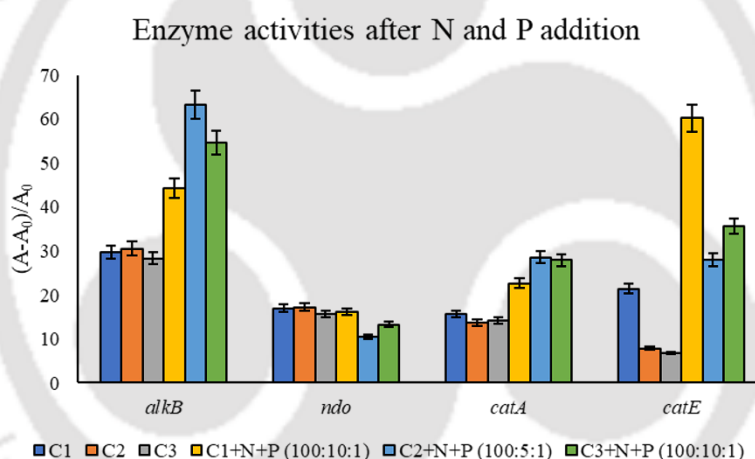


Figure 5.13: Fractional change in enzyme activities after N and P addition. All the derived combinations C1, C2 and C3 depicted significantly higher enzyme activities on addition of both N and P as compared to that when N and P were not present. The enzyme activities increased of *alkB*, *ndo*, *catA* and *catE* and showed a collective increase in fractional activity of 40-70 folds compared to 20-30 folds on N and P addition. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

ndo activities were also enhanced when the microbial cells were facilitated with C:N:P ratio where biomass content were the highest. The *ndo* activity 2.86 U mg⁻¹ were comparable to that of *Staphylococcus sp.* GR2 (3 U mg⁻¹) in Chapter 4 section 4.3.7, however, the maximum value of 6.93 U mg⁻¹ by *Bacillus sp.* GE1 was not attained which could signify the slower metabolism of PAHs in the mixture. Similar to the case of *alkB*, activities of C1, C2 and C3 were greatly raised collectively by 1.7-1.8 times for all the combinations on N and P addition.

Maximum *catA* activity increased for C2 by almost 29 folds for C2 whereas the same for *catE* was increased to as high as 60 folds for C1. The maximum *catA* and *catE* activity of 7.99 U mg⁻¹ for C2 and 11.63 U mg⁻¹ for C1 were lesser than the maximum values obtained for individual strains in Chapter 4 section 4.3.8.

A detailed summary of biomass characteristics and enzyme activities obtained by C1, C2 and C3 after N and P addition is depicted in Appendix Table 117.

5.3.9. Deduction of optimum C:N:P for efficient biodegradation

The detailed removal profile and growth for all the C:N:P ratios 100:5:1 and 100:10:1 are illustrated in Appendix Table 119 and Appendix Table 121. It is evident that biomass growth and COD removal were increasing when N and P were added. While C2 showed the best fractional increase in biomass growth and COD removal in case of C:N:P 100:5:1, C1 and C3 exhibited maximum characteristics at C:N:P 100:10:1. Hence, accordingly, optimum ratio were chosen as 100:10:1 for C1 and C3 whereas, 100:5:1 for C2.

5.3.10. Trial study of optimum C:N:P on diluted sludge sample to investigate the treatment efficiency of the designed consortia

The growth profile and removal profile pertaining to application of the designed consortia in ~11500 mg/L COD sludge are discussed.

In Figure 5.14, COD removal for all the combinations increased when N and P were added. The maximum COD removal alongwith N and P utilization was observed in case of C2 where both N and P were applied in the ratio 100:5:1. COD removal was calculated as 74.12%. N utilization and P utilization were noted as 75.62% and 72.4% respectively. Least value of final NO₃⁻-N and PO₄³⁻-P were estimated as 0.682 g/L and 0.109 g/L respectively for C2 where both N and were added. For other combinations C1 and C3, N and P utilization were also ~5% less than the values obtained for C2, however, COD removal were low by 10%. Biomass growth and CFU/mL of C2 increased to ~7.42 folds and ~17.48 folds respectively on amendment with both N and P. At the end of 48 days, biomass and CFU/mL for C2 were estimated as 1.55 mg/mL and 8 × 10⁹ cells respectively. For C1 and C3, the biomass growth and bacterial count were also significantly increased.

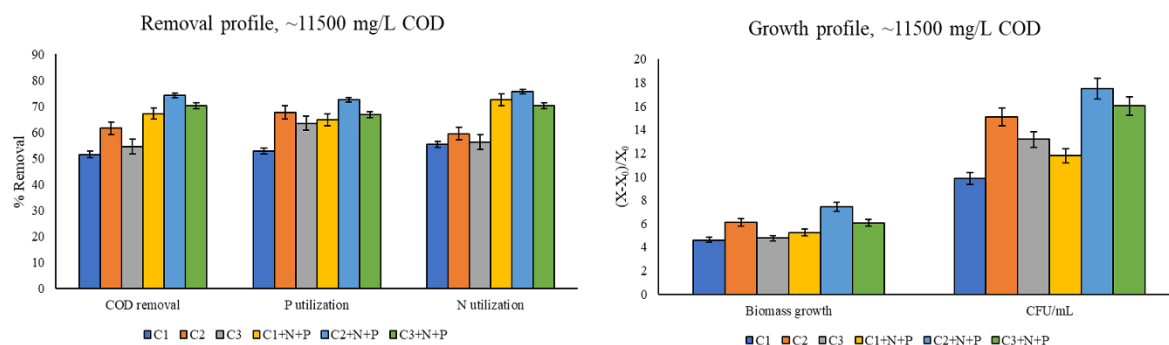


Figure 5.14: Removal profile and growth profile of designed consortia on the waste sludge with addition of C:N:P= 100:10:1 for C1 and C3; 100:5:1 for C2. COD removal, N and P utilization was the significantly higher when both N and P were added. Higher viability in microbial growth was also observed where biomass concentration increased to ~16-18 folds. On account of higher biomass concentration, signified by fractional increase in biomass and bacterial count, the addition of N and P in terms of respective optimum C:N:P were deemed beneficial. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

COD removal after adding N and P was depicted as exponential removal with maximum COD utilization of 258.7 mg COD/g biomass d^{-1} depicted by C2. Least half-life and maximum rate of utilization was observed for C2 with N and P as 1.55 days and 0.676 d^{-1} for COD utilization respectively. Biomass growth and COD utilization, with N and P suggested the importance of addition of external nutrient to facilitate more bioavailability of the pollutants as well better degradation efficiency. This could also be validated from the calculated values of specific growth rate (μ) and COD utilization (q). q values increased when the sludge sample was amended with N and P. The doubling time observed was also in the order $\text{C2} < \text{C3} < \text{C1}$. Lower values of doubling time was observed as compared to the values seen during experimentation of C:N:P optimization. This is due to the fact the incubation time was quite high upto 48 days and the cells were allowed to grow for prolonged period of time. Moreover, as the sets were incubated under static condition, the cells acclimatised slowly under the condition where supply of oxygen was lesser as compared to its availability when rotation was provided and at the end, slower growing strains contributed to higher growth and biomass content. Hence, after addition of N and P, the combinations did show enhanced growth aiding in more acceleration in biodegradation. The effect of sluggish growth and prolonged incubation time could be seen in the yield coefficient (Y_B). Whereas the COD removal was less in case no N and P were added, higher biomass value per unit higher utilization of COD eventually lowered the yield at the end. The maximum yield coefficient was observed for C2 as 0.137 g biomass/g COD when it was supplied with N and P. Lastly, it could be inferred that these

combinations are applicable and have high potency for application in HC waste of the said composition [183,198,313].

5.3.11. Biomass characteristics after trial experiment

Whereas biomass concentrations increased gradually during the experimentation period, the qualities were enhanced when amended with their respective optimum C:N:P ratios (Figure 5.15). The absolute concentrations are mentioned in Appendix Table 122.

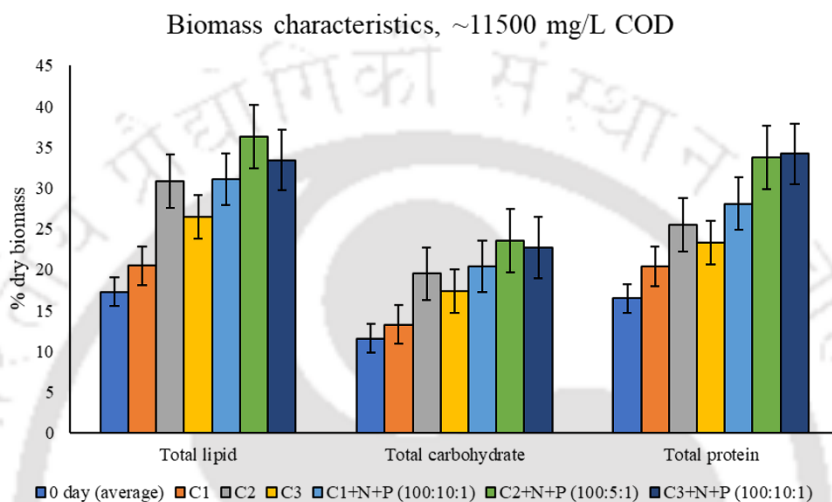


Figure 5.15: Biomass characteristics after trial experiment with diluted sludge. The optimum combinations C1, C2 and C3 depicted significantly higher biomass quality on addition of both N and P. The enhanced lipid, carbohydrate and protein contents were indicative of the efficiency of consortia breaking down HC components by utilizing them as their nutrient source. The maximum lipid, carbohydrate and protein deposition was found for C2 as ~25-35% when both N and P were externally added. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For C1 amended with N and P in the ratio 100:10:1, lipid, carbohydrate and protein concentrations were obtained as 0.357 mg/mL, 0.234 mg/mL and 0.327 mg/mL respectively. These values were having higher share as compared to the experimental values conducted without N and P. The lipid content increased from 22.4% to 31.08% of dry biomass when the sludge was facilitated with additional N and P. Similarly, carbohydrate increased from 13.55% to 20.36% and protein concentration from 20.37% to 28.44%.

Similarly, for C2, increased lipid, carbohydrate and protein shares were observed as 35.14%, 23.62% and 33.77% respectively. The lipid content increased from 0.32 mg/mL to 0.545 mg/mL, carbohydrate content increased from 0.233 mg/mL to 0.366 mg/mL and protein content increased from 0.247 mg/mL to 0.523 mg/mL when the sludge with 11500 mg/L COD was amended with additional N and P in the ratio of 100:5:1.

For C3, on addition of C:N:P 100:10:1, similar to C1 and C2, enhanced lipid, carbohydrate and protein concentrations were obtained as 0.434 mg/mL, 0.295 mg/mL and 0.445 mg/mL respectively, thus comprising of 33.4%, 22.7% and 34.2% dry biomass respectively. These values were higher as compared to when N and P were not added for experimentation.

5.3.12. Enzyme assay after trial experiment

Enzyme activities were found to be higher in cases when N and P were added separately (Figure 5.16). Gradual increase in enzyme activities during the course of the experiments were suggestive of their respective activities and that the strains could metabolize contaminants as their source of nutrient. Nutrient bioavailability was more pronounced on addition of N and P the subsequently the enzymes were also upregulated.

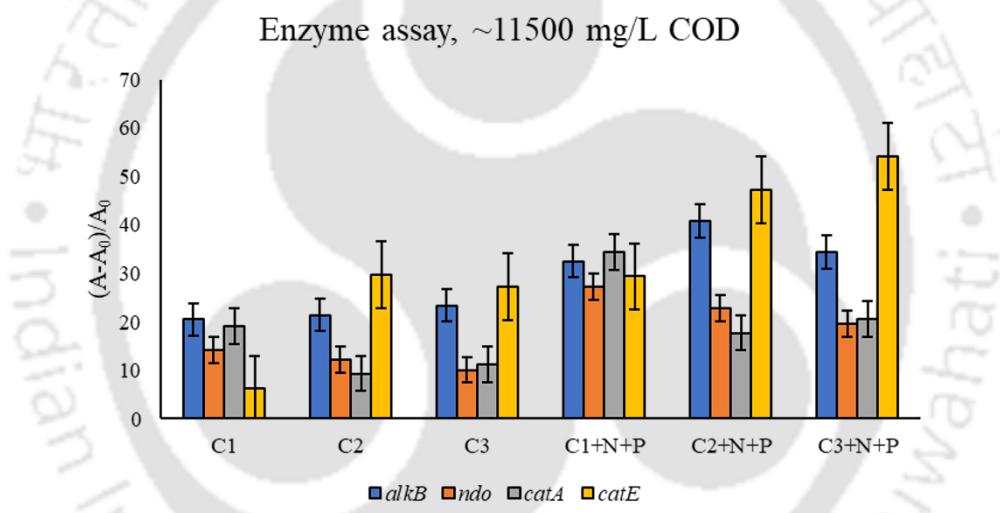


Figure 5.16: Fractional change in enzyme activities after trial experiment in diluted sludge. The optimum combinations C1, C2 and C3 depicted significantly higher enzyme activities on addition of both N and P as compared to that when N and P were not present. The enzyme activities of *alkB*, *ndo*, *catA* and *catE* increased collectively to 30-50 folds on N and P addition. C1 contributed to maximum increase in *ndo* and *catA* activities, *alkB* activity was found the highest in C2, whereas *catE* activity was maximum in C3, all when N and P were externally added. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

For *alkB*, the maximum activity was seen both in cases of C2 with or without N and P. Fractional increase in activities 21.38 to 40.8 corresponded to increase in enzyme activity of 45.66 U mg⁻¹ to 85.27 U mg⁻¹ for C2. For C1 and C3 the activities increased by 1.55 and 1.45 times respectively after N and P addition in their optimum proportion.

For *ndo*, the maximum fractional increase in activity was recorded for C1 as 27.14 folds. However, maximum activity after N and P addition was noted for C2 as 5.22 U mg⁻¹.

Similar to *alkB*, the activities increased on addition of N and P. C1, C2 and C3 showed at least 1.79 times increase in *ndo* activity.

catA activities were comparable before N and P addition which were further calculated as $\sim 4.35 \text{ U mg}^{-1}$. For each of C1, C2 and C3 the enzyme activities increased to 1.76-1.8 times suggesting effective metabolism of aromatic as well as PAHs. The maximum fractional increase of *catA* activity was calculated as 34.43 folds for C1 followed by C3 and C2.

For *catE*, C3 showed the maximum fractional increase as 54.05 folds, followed by C2. The activity after N and P addition, however, was maximum for C3 as 12.11 U mg^{-1} citing the potential of the consortium to metabolise catechol through *meta* pathway. *catE* activity increased by ~ 4.5 times from 1.36 U mg^{-1} to 5.77 U mg^{-1} for C1, suggesting all of C1, C2 and C3 could be actively involved driving the metabolism of PAHs and aromatic HCs towards forward direction.

The detailed values of kinetic parameters, biomass characteristics and enzyme activities obtained for the trial experiment are shown in Appendix Table 123 and Appendix Table 124.

5.3.13. Application of optimum C:N:P on sludge waste to study treatment efficiency of the designed consortia

The growth profile and removal profile pertaining to application of the designed consortia in 46017 mg/L COD sludge are discussed.

In Figure 5.17, COD removal for all the combinations increased when N and P were added as was also observed during the trial experiment. The maximum COD removal was observed for C2 indicating 68.7% COD was consumed on batch incubation for 48 days. The maximum values were observed for all C1, C2 and C3 when both N and P were applied in their respective C:N:P ratio as compared to values where no N and P were amended.

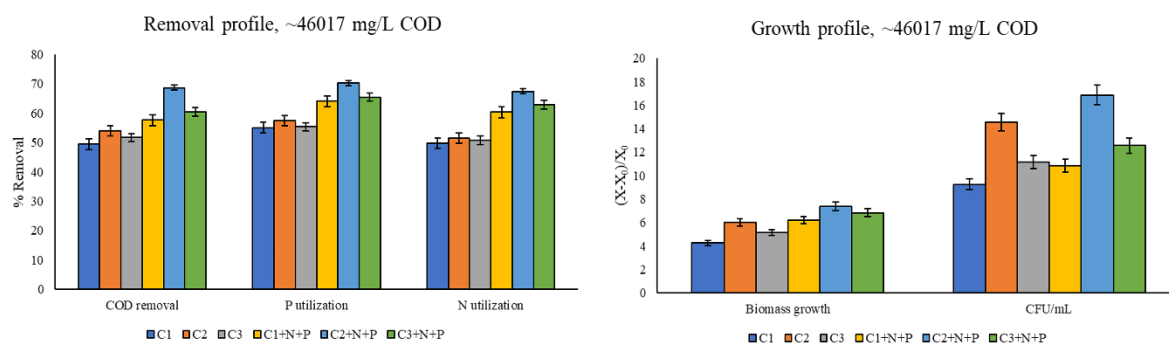


Figure 5.17: Removal profile and growth profile of consortia on the waste sludge with addition of C:N:P = 100:10:1 for C1 and C3 and 100:5:1 for C2. COD removal, N and P utilization was the significantly higher when both N and P were added. Higher viability in microbial growth was also observed where biomass concentration increased to ~16-18 folds. On account of higher biomass concentration, signified by fractional increase in biomass and bacterial count, the addition of N and P in terms of respective optimum C:N:P were deemed beneficial. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

C1 and C3 also accounted for 57.65% and 60.4% COD reduction respectively. N utilization and P utilization were in the order $C2 > C3 > C1$ and the maximum values for C2 were noted as 67.48% and 70.24% respectively. Least value of final NO_3^- -N and PO_4^{3-} -P at the end of experiment were estimated as 3.5 g/L and 252.9 mg/L respectively for C2 where both N and P were added. For other combinations C1 and C3, N utilization was comparable whereas P utilization was ~5% distant for C1 and C3 than the values obtained for C2. However, due to amendment of higher N and P, residual nutrient values of ~8.5 g/L NO_3^- and ~600 mg/L PO_4^{3-} collectively for C1 and C3 were observed in addition to residual COD of ≥ 13 g/L collectively for all of C1, C2 and C3. The same were carefully decontaminated before discarding after the tenure of experimentation to avoid microbial contamination due to nutrient availability. The decreasing trend in N-utilization, COD removal was attributed to the higher input pollution load. High toxicity in the waste sample contributed to delayed response and hence %COD removal was less as compared to the trial experiment which was conducted at ~4 times diluted sludge sample. Fractional increase in biomass growth and CFU/mL of C2 were also higher as ~7.35 folds and ~16.86 folds respectively on amendment with both N and P. At the end of 48 days, biomass and CFU/mL for C2 indicated maximum values, and were estimated as 1.536 mg/mL and 7.73×10^9 cells respectively. The enhanced biomass and bacterial is supportive of the fact that these combinations are highly acclimatised to growth, survival and utilization of complex waste components as their source of energy. COD removal after adding N and P was demonstrated as exponential removal with maximum COD utilization of 217.8 mg COD/g biomass d^{-1} depicted by C2. Least half-life and maximum rate of utilization was evidently also

observed for C2 with N and P as 1.99 days and 0.572 d^{-1} for COD utilization. Collectively, %COD removal, N and P utilization of all of C1, C2 and C3 attributed to enhanced HC intake by the strains in the respective combinations and their metabolism was strongly contributing to COD reduction of the sample.

Hence, during the application of the designed combinations into the waste sludge sample, biomass growth and COD utilization alongwith N and P addition suggested that supplementation of optimized formulations may allow more bioavailability of the nutrients which the microbes could feed upon for their survival. Consequently, better mineralization as well higher degradation efficiency could be observed. This could also be validated from the calculated values of specific growth rate (μ) and COD utilization (q). Whereas q values were lower when the sludge sample was undiluted, however amendment with N and P did facilitate enhanced COD utilization. The doubling time observed was also in the order $C2 < C3 < C1$. Higher values of doubling time were observed due to slower growth in the highly toxic sludge waste as compared to the values that were seen during trial run of diluted sludge. Moreover, due to higher incubation time of upto 48 days and the cells were allowed to grow for prolonged period of time, the toxicity of the waste inhibited the growth of the strains under static condition. However, despite of slower growth, strains contributed to cell numbers and biomass content due to their prolonged exposure. High incubation time and addition of N and P could also illustrate higher efficiency in biodegradation. The lower values of yield coefficient (Y_B) compared to the trial experiment was also due to higher input COD. The maximum yield coefficient was observed for C2 as $0.118 \text{ g biomass/g COD}$ when it was supplied with N and P which was lower than $0.129 \text{ g biomass/g COD}$ when none of N and P were separately added. A summary of COD removal, N and P utilization and biomass growth is depicted in Table 5.8. Finally, the strains have higher potential for HC degradation and can be applied at their optimized combinations and nutrient formulations to obtain the best removal [147,196,198,313].

Table 5.8: Summary of COD, N and P utilization and biomass growth for undiluted waste sludge.

Input COD: 46017 mg/L, C1: 100:10:1; C2: 100:5:1; and C3: 100:10:1												
COD removal, N and P utilization and biomass growth summary												
Consortia	COD removal				N and P utilization			Biomass growth				
	q (mg/g.d)	k (d ⁻¹)	$t_{1/2}$ (d)	R^2	% removal	% N	% P	μ (d ⁻¹)	k (d ⁻¹)	t_d (d)	R^2	Fractional increase
C1+N+P	176.9	0.488	2.73	0.99	57.65	60.38	64.02	0.027	0.82	2.16	0.95	6.19
C2+N+P	217.8	0.572	1.99	0.99	68.7	64.08	70.24	0.03	1.04	1.49	0.95	7.35
C3+N+P	182.33	0.556	2.37	0.97	60.4	62.9	65.5	0.029	0.92	1.82	0.97	6.84

Table 5.9: Summary of biomass characteristics and enzyme activities for undiluted waste sludge.

Input COD: 46017 mg/L, C1: 100:10:1; C2: 100:5:1; and C3: 100:10:1								
Biomass characteristics and enzyme assay								
Consortia	Yield (Y_B) (g/gCOD)	Characteristics (% dry biomass)			Enzyme activity (U mg ⁻¹)			
		Carbohydrate content	Protein content	Lipid content	<i>alkB</i>	<i>ndo</i>	<i>catA</i>	<i>catE</i>
C1+N+P	0.103	25.93	15.22	18.37	42.66	2.03	5.24	4.16
C2+N+P	0.118	33.77	19.42	22.93	65.27	3.22	6.23	4.33
C3+N+P	0.109	26.38	16.29	23.54	65.5	2.34	5.99	4.11

5.3.14. Biomass characteristics after experimentation on waste sludge

Similar to the trial experiment, biomass quality increased during the experimentation period, and subsequently, addition of N and P aided in enhanced biomass qualities Figure 5.18.

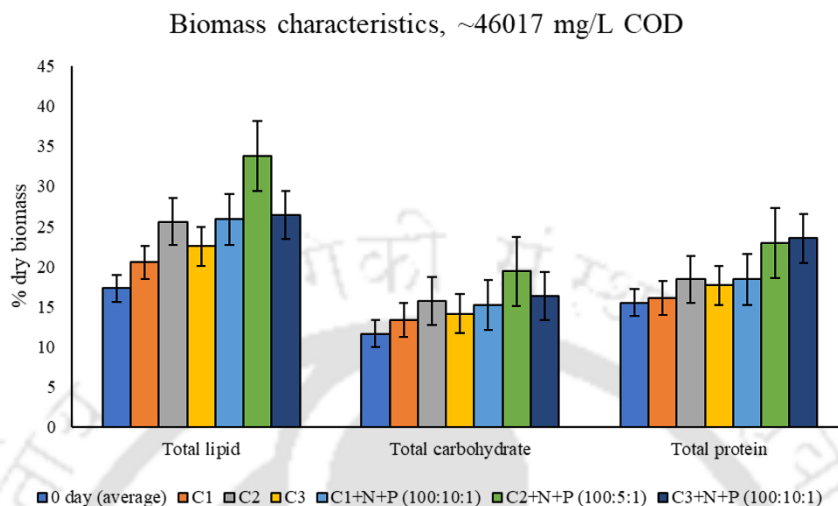


Figure 5.18: Biomass characteristics after incubation in undiluted waste sludge. The optimum combinations C1, C2 and C3 depicted significantly higher biomass quality on addition of both N and P. The enhanced lipid, carbohydrate and protein contents were indicative of the efficiency of consortia breaking down HC components by utilizing them as their nutrient source. The maximum lipid and carbohydrate deposition was found for C2 as ~20-35% when both N and P were externally added. The maximum protein deposition was found for C3 in presence of N and P as ~25%. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

From Table 5.9, the lipid and carbohydrate concentrations were in the order $C2 > C3 > C1$, whereas for protein content, C3 was slightly higher than C2. The maximum % content of dry biomass was obtained as 33.77% (for C2), 19.42% (for C2) and 23.54% (for C3) respectively. The absolute concentrations corresponding to these percentages increased from 0.265 mg/mL to 0.519 mg/mL lipid (For C2), 0.178 mg/mL to 0.298 mg/mL carbohydrate (for C2), and 0.224 mg/mL to 0.34 mg/mL protein (for C3) when N and P were added in the respective proportions (Appendix Table 122). Similarly, on addition of N and P for C1 and C3, increased lipid shares were found to be 25.93% and 26.38% respectively. Carbohydrate shares were also recorded as $> 15\%$ whereas protein content of C1 and C2 were 18.37% and 22.9% of dry biomass respectively. The biomass characteristics were also indicative of the potential of the strains to utilize the available nutrients in the waste. The lipid, carbohydrate and protein deposition also suggested their affinity towards HC degradation [350,351].

5.3.15. Enzyme assay after experimentation on waste sludge

Similar to the trial experiment, enzyme activities increased during the experimentation tenure and were found to be higher in cases when N and P at their optimum ratio (Figure 5.19).

Although comparable maximum *alkB* activities seen for C2 and C3 with N and P addition as 65.3 U mg^{-1} and 33 U mg^{-1} , fractional increase was found to be higher for C3 as 34.6 folds. C1 and C3 also exhibited significant *alkB* and hence, were suggestive of pronounced alkane metabolism in the waste [166,238,310]. A summary of the enzyme activities is placed in Table 5.9.

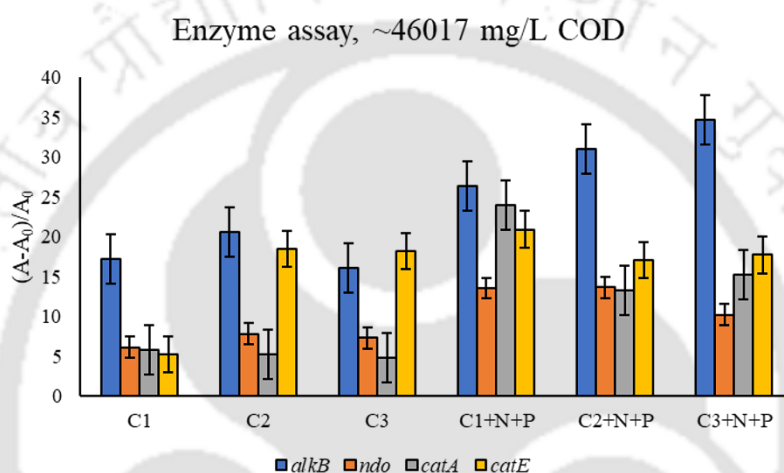


Figure 5.19: Fractional increase in enzyme activities after incubation in undiluted waste sludge. The optimum combinations C1, C2 and C3 depicted significantly higher enzyme activities on addition of both N and P as compared to that when N and P were not present. The enzyme activities of *alkB*, *ndo*, *catA* and *catE* increased collectively to 25-35 folds on N and P addition. C3 contributed to maximum increase in *alkB* activity, *ndo* activity was found the highest in C2, whereas *catA* and *catE* activities were maximum in C1, all when N and P were externally added. Error bars indicate the standard deviation ($\pm$ SD) of three experimental replicates.

Similarly, maximum *ndo* activity was observed for C2 as 3.23 U mg^{-1} , however, comparable fractional increase for C1 and C2 as 13.5 folds and 13.63 folds respectively suggested that naphthalene and other PAHs metabolism in C1 and C2 might be more prominent. *catA* and *catE* activities increased significantly in case of C1 as 23.95 folds and 20.9 folds respectively, however, identical enzyme activities for all C1, C2 and C3 after N and P addition was suggestive of utilization of catechol. *catA* activities were in the range $5.2\text{--}6.2 \text{ U mg}^{-1}$ whereas *catE* showed activities as $4.1\text{--}4.3 \text{ U mg}^{-1}$ which were indicative of formation of catechol from metabolisms of aromatic HCs and PAHs. Significant activities illustrated the efficiency of the strains to metabolize different class of HCs that could be present in the waste.

The advantages of microbial consortia and their application in bioremediation of HC is well documented. Also, necessary formulation of nutrient amendment alongwith optimizing growth parameters are pivotal to create a near perfect mimic of the waste environment and provide support for microbial sustenance. Enhancing the bioavailability of the pollutants thereby result in microbial breakdown and reduction in the toxicity of the contaminated site. Some investigators have reported 84-99% of degradation efficiency after 5 days of incubation suggesting the potential of the microbes to participate in HC metabolism [183,196,198]. Compared to our incubation time of 48 days, Shankar et al., 2014 reported the application of a microbial consortia consisting of *Pseudomonas sp.* and *Bacillus sp.* and attained 100% reclamation of oil-contaminated soil within 30 days of incubation [195]. On the other hand, Sarkar et al., 2020 has also demonstrated a nitrate reducing consortia to obtain ~86% degradation after 30 days of incubation [147]. Elsewhere, 53% of degradation was reported after incubating an immobilized consortia on zeolite for 100 days [197] which was lower compared to our COD removal of 70%. Hence, biodegradation is not only the function of time, but also of the initial pollution load received for treatment. It is also important to understand that due to variable composition of sludge, site specific designs are more aligned to offer desirable and efficient results.

5.4. Summary

The optimized consortia obtained after series of optimization processes in Chapter 3 were employed to observe their efficiency in different classes of HCs before applying into the real sample. The efficiency of the removal was highly increased, and the time period of incubation was significantly lower for consortia as compared to that obtained in case of individual isolates in Chapter 2. Simultaneously, the biomass concentration was greatly enhanced, and biomass growth kinetics confirmed the improved performance. Moreover, the utilization kinetics showed significant potential of the optimized consortia to accept HCs present in refinery waste as their source of nutrients thereby enabling their potency for application for bioremediation. Higher biomass quality in terms of enhanced lipid, carbohydrate and protein contents and presence of active enzymes such as *alkB*, *ndo*, *catA* and *catE* in the crude protein were suggestive of superior activities and faster HC metabolism by the designed consortia. Moreover, addition of nutrients in the form of N and P accelerated the COD utilization as well as biomass production in addition to increment in enzyme activities and improved biomass quality. COD removal was greatly enhanced alongside N and P

utilization and was suggestive of the ability of microbes present in each consortium to participate in accelerated reduction in pollution level. Optimum C:N:P from the study of 100:10:1 for C1 and C3; and 100:5:1 for C2 were employed in the real sample to check for potential consortia application and assess the bioremediation efficiency. Almost identical observations were obtained for both diluted as well as undiluted sludge sample and ~70% of COD removal alongwith similar percentage of N and P utilization depicting the applicability of the designed optimized microbial population. Alongside, the increased biomass qualities were also suggestive of HC utilization and presence of the necessary enzymes pertaining to metabolism of aliphatic, aromatic and PAHs were indicative of constant upregulation thereby signifying their active participation in breakdown of complex HC derivatives. Overall, the proposed consortia satisfactorily exhibited higher adaptability and enhanced capability for bioremediation of refinery waste sludge.



6

Chapter 6. **Conclusion and future perspectives**

6.1. Thesis overview

Through the research gaps that were identified, we tried to demonstrate a detailed overview of the efficiency of bioremediation process through this work. Whereas, various *in situ* systems are somehow criticised for the extended treatment time, a disadvantage that have been tried to replace with other *ex situ* systems, the ability of indigenous microbes to acquaint better and survive on the toxic waste components would be the greatest merit if they are facilitated with some additional nutrient and micronutrient components. We also tried to review the best possible gaps to identify points where oily sludge contaminations were regularly neglected, we also attempted to take firm note of the challenges of implementation of the bioremediation process at a particular site. Moreover, we tried to comprehensively study the dynamics of each of the of isolated strains to understand the mechanism of their action on different class of pollutants that were expected to be present in the waste sample. We also tried to establish a simpler design of optimized minimal media and microbial consortia based on statistical approach and analysing full factorial and 3D CCD to an optimum level that could help in biodegradation of a complex waste system such as refinery waste oily sludge by formulating factors that could help maximizing biomass concentrations. We also tried to visualize the biomass quality that were obtained post incubation of strains in HC supplemented minimal media. It was expected that the biomass quality too would improve upon statistical optimization with increase in biomass content. Activities of some key enzymes have been reported extensively as reference in terms of the metabolic pathways that the strains could resort to. Similar, to the biomass quality, enzyme activities were also apprehended to increase on increase in biomass concentration after statistical optimization. Biomass quality and enzyme activities would give us an insight on their efficacy for future value addition and specific metabolic pathways respectively. Lastly, we tried to facilitate the contaminated sample with an optimum nutrient conditions in terms of N) and P in the proportion C:N:P to make the waste components more bioavailable. A very small lab scale mesocosm was set up to mimic the tank bottom sludge condition containing the waste sludge, optimum microbial consortia alongwith the optimum C:N:P. The results obtained significantly forced the issue of implementation of biostimulation strategy over bioaugmentation as it has already been discussed in detail in the previous chapters about how these indigenous microorganisms can expertly acclimatise and survive on these harsh conditions. In this chapter, we summarise the minute details that comprehensively conclude this work.

6.1.1. Chapter 1: Introduction and literature review

Chapter 1 highlighted the importance of an environment friendly clean-up strategy required for eliminating the effect of toxic contaminants such as refinery waste oily sludge. Several persistent xenobiotic components have been reportedly causing extensive damage to soil and water due to issues related to their handling and disposal. Whereas it was also noted that petroleum was an important fossil fuel, and that the current world demands in terms of energy production and consumption relies heavily on petroleum as an efficient source, the non-renewability of fossil fuel does put an alarm to look for alternatives in our near future. Moreover, the increased anthropogenic activities and climate changes have also posed challenges to opt for sustainable solutions.

Due to higher complexity in composition of the crude oil, petroleum refineries are set up to convert it into simpler components which are beneficial to mankind. It has been reviewed that most of crude petroleum is converted to gasoline, LPG and fuel oils whereas only around 10% are converted to form plastics, resins, asphaltenes etc. In addition to these products, the refineries generate enormous volume of wastes in the form of oily sludges and controlling these to negatively affect the environment had been the toughest challenge. Whereas the composition of refinery wastes vary location wise, typically of such wastes contain moisture fraction, HC fraction and bottom sediments. It has also been found that these wastes are generally characterised by higher contents of suspended solids (SS), TPH, COD, BOD, phenolic compounds etc. Some adverse effects of crude oil spillage and accidents have been well documented in the history with examples being from The Gulf War, Kuwait, 1991, Deep Horizon spill, Mexico, 2010, *Chitra* oil spill, Mumbai, 2010 and Ennore oil spill, Chennai, 2017, Tobago oil spill, 2024 etc.

Physico-chemical techniques have been extensively used for treatment of such kind of contamination. Each of the techniques such as excavation, air sparging, *in situ* chemical degradation etc. have several advantages and disadvantages, however, *in situ* bioremediation, despite the time effectiveness have emerged as an appropriate strategy. In this technique, indigenous microbes are believed to be more acclimatised to similar waste conditions and it has been proven through various studies about their ability to accept the waste components as their sole C-source for survival. Microbes are believed to ingest these components and either degrade to a non-toxic form before excreting to the environment through a process known as

biotransformation or completely mineralize into CO₂ and H₂O through the process known as biomineralization.

Literature review was carried out to understand mostly the generation of oil refinery waste sludge, different ways to execute biological treatment and the phenomenon associated with microbes to form a group (consortia) in order to facilitate enhanced bioremediation efficiency. Primary sources of waste discharge were found out as water generating from the desalter, condenser, crude tank (storm water), cooling tower, sewage tank etc. whereas, sludge generation has been mostly seen from the effluent treatment plants of sewage and desalting towers. Sludges often formed as tank bottom sludge or in the dissolved air flotation (DAF) tanks are directly applied for field applications after dewatering owing to the characteristics within environmental regulations. Some of the field applications include composting, landfilling, AD etc. On the other hand, oily sludges have shown greater promises in resource recovery and several techniques such as pyrolysis, solvent extraction, gasification, adsorption are being used for value addition and biofuel generation.

Despite biological treatment being considered a clean and green technology, several challenges related to long treatment time, poor treatment quality, higher land requirement and ensuring extensive regulatory measures in terms of hazardous waste treatment and disposal cause various organizations to resort to age old physico-chemical techniques. Moreover, poor bioavailability has been investigated as a potent issue of attaining treatment efficiencies lesser than desired. To combat such challenges, aspects of enhancing microbial biomass have been studied and research showed that addition of external nutrients (biostimulation) or exogenous microorganisms (bioaugmentation) have greatly improved treatment proficiency. As mentioned earlier, *in situ* bioremediation is preferred over *ex situ* bioremediation as microbes added externally might not be fully potent to survive in the complex waste system. However, addition of nutrients in the form of N) and P have facilitated higher biomass formation and hence, in turn higher COD removal, TPH removal etc.

Microbes are considered appropriate for applications in treatment of HC waste because of their versatility in adapting to sudden changes in environmental conditions. Whereas, individual microbes can metabolize different class of pollutants separately, application of these microbes in their necessary proportions together might be advantageous as it can reduce the treatment time severely. The concept of microbial consortia hence relies on application of different genus or species of microbes in complex waste together to attain higher biomass and

accelerated biodegradation efficiency. Various reports have been reviewed to better understand the importance of biological treatment as microbial consortia and it has been found out that the efficiency of biomass quality obtained post treatment are greatly improved. Microbial consortia design can either be by means of understanding interactions among the species or the population or both. Rarely, statistical approaches have been used to design optimum microbial consortia despite simplicity, and we have tried to work upon this gap into extensive detail. Moreover, despite advantages, the disadvantages lie in late adaptation owing to a complex environment and competition among the members which might affect overall biomass production and hence, substantial insights related to metabolism and varying parameters of a process are required to design the best and optimum consortia.

Microbes contain a vast network in their system and could metabolize various nutrient sources through their respective pathways to facilitate growth. The breakdown of nutrients in the form of C-source or N-source etc. into simpler components are carried out through a cascade of reactions constituting the overall pathway. Enzymes are protein molecules that catalyses the reactions in each step and such set of enzymes are generally found in a microbial species capable of metabolizing different class of substrates. Enzymes contain active sites specific to substrates and convert them to products which become substrates for the next class of enzymes. In addition to the readily reported lipases, proteases, catalases, oxidases classes of enzymes, oxidoreductases such as alkane hydroxylase (*alkB*), naphthalene 1,2-dioxygenase (*ndo*), catechol 1,2-dioxygenase (*catA*) and catechol 2,3-dioxygenase (*catE*) are some of the important enzymes that collectively catalyses aliphatic, aromatic and PAHs. Alkanes are converted to primary alcohol by *alkB* as the first step of metabolism whereas PAHs such as naphthalene and anthracene are initially accepted by *ndo*. Both these enzymes are energy intensive and use NADH as the electron donor. *alkB* is an integral protein consisting of subunits as monooxygenase and rubredoxin centre facilitating intake and transport of alkanes through the cell membrane into the cytoplasm, *ndo* is an heterohexamer capable of catalysing larger molecules such as PAHs. On the other hand, *catA* and *catE* act on catechol. This step is an essential step as the benzene ring in catechol obtained through series of biochemical reactions of metabolism of aromatic and PAHs is cleaved through *ortho* or *meta* pathways. Catechol dioxygenases work as homotetramer and is quite evidently able to accept smaller molecules such as benzene, toluene, phenol or catechol. Hence, understanding activities of these enzymes is an indicative of the metabolic proficiency of these microbes aiding bioremediation process.

Apart from the enzymes, biomass quality has also been seen as an indicative of an efficient bioremediation process. The biomass obtained after nutrient addition studies were significantly high and possessed inherent microbial characteristics. Biomass quality in terms of lipid content, carbohydrate content and protein content have added advantages to sustainability, metabolic flexibility and can serve as a good feedstock for biofuel generation and value addition.

6.1.2. Chapter 2: Screening, isolation and identification of indigenous microorganism(s) from the waste sludge

In Chapter 2, the experimental works were initiated after the sample was collected from the effluent treatment plant of IOCL, Guwahati refinery. The physical texture of sample was sticky and dark green in colour with characteristic odour of a typical crude oil contaminated semi-solid mass. The sample contained relatively high COD (46.02 g/L), TSS (9.81 g/L), TPH (~27.4%), metal concentration (3.1 mg/L Cr), anion concentration (12.89 mg/L NO_3^- , 31.65 mg/L NO_2^- , 8.447 mg/L PO_4^{3-} , 198 mg/L SO_4^{2-} and 306 mg/L Cl^-) and the pH (6.67) was also relatively acidic confirming highly hostile environment. ORP of -152 mV confirmed reducing environment and conductivity of 2.4 S/m showed availability of free ions in the sample. Water content was also 56.8% (wet sludge) which was similar to a typical refinery sludge.

A total of 7 strains were isolated using enrichment method based on their colony morphology. Initially they were named as GR1, GR2, GR3, GR4, GE1, GE2 and GS1 in the order of their isolation. The gram characteristics were checked, and it was found out that except GR2, GE1 and GS1 all others were gram negative. On 16S rRNA identification, it was revealed that GR1, GR3, GR4 and GE2 had their closes resemblance to *Aquamicrobium sp.*, GR2 had the closest relative as *Staphylococcus sp.* while GE1 and GS1 showed characteristics of multiple *Bacillus* strains. Subsequently, they were renamed as *Aquamicrobium sp.* GR1, *Staphylococcus sp.* GR2, *Aquamicrobium sp.* GR3, *Aquamicrobium sp.* GR4, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1 and were used in recording and reporting observations thereafter.

The strains were further checked for their growth characteristics in HC contaminated environment. The results suggested that the strains had the potential to survive in conditions where C-source was limiting. The survival time observed in NB medium was 4 days with specific growth rate obtained as high as 0.071 h^{-1} . The optimum temperature of growth of all the isolated strains were 35 °C (except *Aquamicrobium sp.* GR3, whose optimum temperature

was found out to be 30 °C) and optimum pH for *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1 was obtained as 7, whereas for others, optimum pH was 6.5. To maintain uniformity, an average optimum temperature of 33 ± 2 °C and pH 6.75 ± 0.25 was derived for experimentation.

The strains were also checked for their tolerance to certain classes of heavy metals. Collectively, the strains showed tolerance at 50 mg/L of salts Cu, Co, Fe, Zn, Pb, Ni and Cr. The isolates also exhibited higher sustenance in saline conditions of 250 mg/L NaCl.

Further, when *n*-hexadecane were supplemented as the sole source of C, the survival time increased from an average of 15 days to as high as 39 days. Microbial count (CFU/mL), biomass concentration and COD removal validated that strains *Staphylococcus sp.* GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1 could use *n*-hexadecane as their C-source more efficiently than the others with their maximum tolerance concentrations (MTC) as 500 mg/L, 250 mg/L, 250 mg/L and 500 mg/L *n*-hexadecane respectively. An optimum inoculum of 4% (v/v) was found optimum and suitable for experimentation in *n*-hexadecane supplemented media as it provided the CFU in the range 10^8 cells/mL that was in range of the total bacterial count initially obtained for the sludge. The specific growth rate increased for these strains at relatively higher concentrations than that compared without any C-source which suggested that these strains were efficient enough to survive in HC contaminated environment, if provided proper nutrients. The highest specific growth rate of *Staphylococcus sp.* GR2 (0.029 d^{-1}) without *n*-hexadecane increased to almost 2.66 times (0.078 d^{-1}) when HC was supplemented in MSM. The lowest specific growth rate for *Aquamicrobium sp.* GR3 (0.01 d^{-1}) also increased to almost 2.3 times when HC was supplemented in MSM. Kinetic parameters obtained by studying the growth of these strains suggested very slow growth in mineral salt conditions. The μ_m and K_s were in the range $0.003\text{--}0.094 \text{ d}^{-1}$ and $0.4\text{--}92 \text{ mg/L}$ respectively.

Further, for anthracene, when HC was supplemented as the sole source of C, the survival time increased from an average of 15 days to as high as 21 days. *Staphylococcus sp.* GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1 showed higher potential to utilize anthracene as their C-source more efficiently than the others with the maximum biomass concentration as 0.31 mg/mL for *Bacillus sp.* GE1. MTC values were obtained as 50 mg/L for *Bacillus sp.* GE1, 20 mg/L for *Aquamicrobium sp.* GR1 and GE2, and *Bacillus sp.* GS1, 10 mg/L for *Aquamicrobium sp.* GR3 and GR4. The specific growth rates also increased suggesting that these strains were efficient enough to survive in anthracene contaminated

environment. 4% inoculum for different HC concentrations provided CFU of 10^8 cells/mL which was in agreement to the total bacterial count initially obtained for the sludge, and hence was considered as optimum inoculum. Kinetic parameters obtained by studying the growth of these strains suggested very slow growth in mineral salt conditions. The μ_m and K_s were in the range 0.02-0.09 d⁻¹ and 1-15 mg/L respectively. Except *Bacillus sp.* GE1, growth of all others was best described as Andrew's kinetics. Considerable COD removal confirmed their potency for bioremediation of waste with abundant anthracene and its derivatives.

Similarly, growth of these strains was also analysed in phenanthrene supplemented media.

For aromatic HCs benzene, toluene and xylene, the survival time increased from an average of 15 days to as high as 33 days. Microbial count (CFU/mL), biomass concentration and COD removal validated that all the strains could use benzene as their C-source with their MTC as 1000 mg/L benzene. Similarly, for toluene and xylene, MTC was found to be 1000 mg/L each for all the strains. An optimum inoculum of 4% (v/v) was found optimum and suitable for experimentation in benzene and toluene supplemented media, whereas 3% was optimum for xylene supplemented media. The specific growth rate increased for these strains when supplemented with 1000 mg/L benzene, toluene or xylene. Overall, the strains showed potential in utilizing BTX as their C-source.

Refinery waste sludge may vary in their composition of contents as per the location they are formed. The inherent characteristics however support the presence of living organisms, primarily microorganisms. Since, refinery sludge is primarily composed of HCs, especially paraffins consisting straight chain HCs, most of them are accepted by microbes as their C-source, in addition to the other nutrients and micronutrients, thus creating an excellent habitat for their growth and survival. Various classes of microorganisms could be harboured from such contaminated sites, although *Pseudomonas* and *Bacillus* dominate others on account of their increased versatility in reducing toxicity posed by HC pollution. In addition, presence of anaerobic microbes also suggests the extensive diversity of microbiota a typical refinery waste can possess, and harnessing their potential would tremendously help in devising optimum cost-effective strategies to attain higher bioremediation efficiency.

6.1.3. Chapter 3: Statistical optimization for designing optimum consortia for the remediation of waste sludge

In Chapter 3, statistical optimization of different variables paved us ways to media engineering, which could enhance the growth as well as degradation kinetics. Some of the strains indicating maximum growth and removal were screened for further optimization studies by amending the MSM with more nutrients and micronutrient for enhancing the growth rate as well as the biomass concentration. *Staphylococcus sp.* GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1 were selected for optimizing *n*-hexadecane and anthracene contaminated media, whereas *Staphylococcus sp.* GR2, *Aquamicrobium sp.* GR4, *Bacillus sp.* GE1 and *Bacillus sp.* GS1 were constitutively selected for optimization of benzene, toluene and xylene. All the experiments were carried out in Minitab v19.1.

During 1st order PBD, for all of the respective potent strains, at MTC of *n*-hexadecane, anthracene and benzene, concentration of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl, concentration of HC, time period of incubation and inoculum concentration were mostly significant and had positive effects on biomass production ($p < 0.05$). The significant factors were analysed statistically and were found to be consistent at 95% confidence level. The significant factors were further taken forward towards 2nd order CCD to obtain the optimized response of the significant factors in each case with an objective to maximize biomass production. In cases where HC concentration was found significant, the same was optimized to keep the tolerance of all the strains consistent for all the experimentations carried in the subsequent chapter(s).

During CCD, for *n*-hexadecane supplemented MSM, it was observed that the optimum response was obtained as 3.44% inoculum concentration, 36.29 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 229.13 mg/L NaCl for one of the potent strains *Bacillus sp.* GS1. Overall, based on the optimized responses for other potent strains, the maximum of those for the significant parameters were constituted together as 41.64 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 267.21 mg/L NaCl, time period of 41.64 days and 3.44% inoculum concentrations as the optimum conditions to obtain a uniform media.

Similarly, for anthracene, on altering the media using CCD DoE, optimized responses were constituted as 65.34 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 334.88 mg/L NaCl, time period of 27.11 days and 6.37% inoculum based on overall responses obtained for all the strains.

In case of phenanthrene, where *Bacillus sp.* GE1 was the only potent strain, optimum values of 65.34 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 334.88 mg/L NaCl and 3.68% inoculum were obtained.

For benzene, statistical analysis showed the optimum values as 65.34 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 334.88 mg/L NaCl and 47.25 days of incubation for *Staphylococcus sp.* GR2. To constitute uniformity, 3.68% of optimum inoculum obtained as optimum setting for *Aquamicrobium sp.* GR4 was also included in the list.

In a similar way, overall optimum values for toluene and xylene supplemented DoEs were identical except optimum time was obtained as 35.18 days and 39.2 days respectively.

As had been for individual HCs, while designing an optimum uniform media which could be solely used for all the experiments hereafter, the initial media containing 200 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg/L K_2HPO_4 and 100 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was amended with $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl with their maximum values of optimum response among the pollutants. These values were identified as 65.34 mg/L and 334.88 mg/L respectively. Further, maximum time period of 47.25 days was rounded up as 48 days. The biomass values were checked for all the potent strains after 48 days of incubation in different class of HCs and the same were compared with the values obtained before optimization.

For *n*-hexadecane, the maximum biomass value was obtained as 2.96 g/L for *Staphylococcus sp.* GR2 which after optimization increased to 5.06 g/L. Bacterial count increased for all the potent strains too and increased COD removal was also observed.

For anthracene, maximum biomass for *Bacillus sp.* GE1 increased to 0.45 g/L with increase in bacterial count and COD removal. Biomass concentration in case of phenanthrene for the same strain increased to 0.079 g/L after optimization.

For benzene, maximum biomass for *Staphylococcus sp.* GR2 of 0.16 g/L increased to 0.263 g/L whereas overall, *Bacillus sp.* GE1 showed highest biomass value as 0.289 g/L after optimization. Biomass values in case of toluene and xylene, similarly, increased to 0.265 g/L and 0.273 g/L respectively for the strains *Aquamicrobium sp.* GR1 and GR4. In all the cases of BTX, increased bacterial count and COD removal was also observed.

Microbial consortia designing was also carried out following similar statistical optimization process using this new media with incubation carried out for 48 days. The experiment was conducted at two stages to better understand the effect of different HC concentrations in the new media with added components. Set 1 consisted of all the HCs mixed together at higher concentrations such as 500 mg/L *n*-hexadecane, 50 mg/L anthracene, 20

mg/L phenanthrene, 1000 mg/L each of benzene, toluene and xylene. Set 2 contained HCs in lowest concentrations as 10 mg/L *n*-hexadecane, 5 mg/L anthracene, 5 mg/L phenanthrene, 10 mg/L each of benzene, toluene and xylene.

During PBD, to screen out the most significant strain factors having positive influence on biomass production, *Staphylococcus* sp. GR2, *Bacillus* sp. GE1 and *Aquamicrobium* sp. GE2 were having significant effect on biomass production for Set 1, whereas, for set 2, all the strains except *Aquamicrobium* sp. GR3 and *Bacillus* sp. GE1 were found to be significantly contributing to enhanced biomass production ($p < 0.05$).

2nd order optimization of the significant strains yielded 3 possible combinations and were tentatively named as C1 (%v/v) = 3.82% *Staphylococcus* sp. GR2, 7.02% *Bacillus* sp. GE1, 3.84% *Aquamicrobium* sp. GE2, C2 (%v/v) = 4.24% *Aquamicrobium* sp. GR1, 3.89% *Staphylococcus* sp. GR2, 6.11% *Bacillus* sp. GS1 and C3 (%v/v) = 3.51% *Staphylococcus* sp. GR2, 4% *Aquamicrobium* sp. GR4, 6.31% *Bacillus* sp. GE1. Statistical analysis was performed in each and every set to understand the accuracy and predictability of the models generated.

Statistical tools and DoEs are helpful not only in delivering quicker results with intricate analysis to support the observations, but also in reducing time required for carrying out experiments that are otherwise vast and laborious. Randomized designs generated through already defined algorithms is advantageous in constructing quality experimental setups and protocols that can greatly minimize resource wastage and cost. Mathematical modelling-based studies validated by statistical parameters provides significant justification and enhances the discussion and inferences drawn out to explain a complex process. Several models ranging from linear to higher order polynomial regressions allow the users to choose to best explain their objectives and the researchers to investigate the dynamics of any phenomena to the minutes details, thereby creating simplified depiction of results for the ease of the readers. The only disadvantage that may lie is the application of a computerized software and the user might need pre-requisite knowledge pertaining to statistics and the applications to analyse the design outputs.

6.1.4. Chapter 4: Analysis of biomass characteristics and enzyme activities for insight into the bioremediation of waste sludge

In Chapter 4, we limited our scope only towards understanding the biomass and the characteristics by performing simple biochemical estimations in terms of lipid content,

carbohydrate content and protein content. Moreover, enzyme activities of certain key enzymes such as *alkB*, *ndo*, *catA* and *catE* were also carried out to get an insight into the metabolism of the class of HCs that are typically present in a refinery waste sludge. Enzyme assays were performed based on the different methodologies described elsewhere, lipid estimation was carried out using Folch method [339], carbohydrate estimation by phenol disulfonic acid method described by DuBois et al., 1956 [338] and protein estimation by the method described by Bradford, 1976 [340].

For the biomass obtained from *n*-hexadecane experimentation without media optimization, all of lipid, carbohydrate and protein contents were found higher in *Staphylococcus sp.* GR2, *Bacillus sp.* GE1, *Aquamicrobium sp.* GE2 and *Bacillus sp.* GS1 as compared to the others. This is due to higher tolerance of these strains compared to the others. Lipid and protein contents at the end of the experiments were in the range of 19-22% of dry biomass whereas carbohydrate constituted 12-16%. The maximum lipid and protein concentrations were obtained as 0.05 mg/mL for *Staphylococcus sp.* GR2, carbohydrate concentration was the highest in *Bacillus sp.* GS1 as 0.034 mg/mL. After media optimization, since, the biomass concentration increased, it was also expected that the biomass quality would also increase. The maximum lipid and protein content of *Staphylococcus sp.* GR2 did increase to 0.11 mg/mL and 0.09 mg/mL respectively, their percentage share was lesser, and percentage of protein share decreased for all the potent strains except *Bacillus sp.* GE1, whose values were identical. Similarly, lipid share after optimization was identical for *Aquamicrobium sp.* GE2 and decreased slightly for *Bacillus sp.* GE1. On the other hand, carbohydrate content was identical in both *Staphylococcus sp.* GR2 and *Bacillus sp.* GS1, it decreased from the maximum value that was obtained before media optimization. This could also be attributed to the increase in biomass concentrations after optimization, and that the percentage share was lesser.

For the biomass obtained from one of the PAHs anthracene experimentations without media optimization, all of lipid, carbohydrate and protein contents were found higher in *Bacillus sp.* GE1 as compared to the others. This is also due to higher tolerance compared to the others. Although, *Bacillus sp.* GS1 did not owe higher biomass concentration, it showed promises of having good quality biomass on incubating with anthracene media, further validated by the fact that lipid content was ~23% of dry biomass which was the highest among all. Overall, lipid and protein contents of *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and *Aquamicrobium sp.* GE2 were in the range of 18-20% of dry biomass whereas carbohydrate

constituted 9-12%. The other strains *Aquamicrobium sp.* GR1, GR3 and GR4 also showed significant shares and most of their carbohydrate and protein shares showed upward trend towards the end of experiment, their final overall concentration was significantly lesser. The maximum lipid, carbohydrate and protein concentrations were all obtained for *Bacillus sp.* GE1 as 0.06 mg/mL, 0.038 mg/mL and 0.055 mg/mL respectively. After media optimization, the maximum lipid, carbohydrate and protein content of *Bacillus sp.* GE1 did increase to 0.09 mg/mL, 0.047 mg/mL and 0.086 mg/mL respectively, their percentage share decreased for both protein and carbohydrate. On the other hand, except *Staphylococcus sp.* GR2, lipid content was identical in both *Aquamicrobium sp.* GE2 and increased slightly in *Bacillus sp.* GE1 and GS1. This could also be attributed to the increase in biomass concentrations after optimization, and that the percentage share was lesser.

Biomass obtained from phenanthrene experimentation before and after optimization were similarly recorded and analysed.

For the biomass obtained from benzene experimentation without media optimization, whereas all the strains were tolerant to 1000 mg/L benzene, lipid, carbohydrate and protein contents were found comparatively higher in *Staphylococcus sp.* GR2 and *Bacillus sp.* GE1. Lipid, carbohydrate and protein contents at the end of the experiments were in the range of 16-31%, 14-18% and 20-30% of dry biomass which were significantly higher than that obtained for the previous compounds. The maximum lipid, carbohydrate and protein concentrations were comparable for *Staphylococcus sp.* GR2 and *Bacillus sp.* GE1 and the values were obtained as 0.045 mg/mL, 0.028 mg/mL and 0.46 mg/mL respectively. After media optimization, the lipid, carbohydrate and protein content increased for all the strains that were subjected to statistical optimization experiments however, their percentage share in terms of lipid and carbohydrate decreased. However, contrary of the trend, the carbohydrate content of *Staphylococcus sp.* GR2 decreased. On the other hand, protein share also decreased, it showed slight increase for *Aquamicrobium sp.* GR4. Overall, maximum lipid, carbohydrate and protein content obtained from the biomass after optimization experiments were listed as 0.062 mg/mL, 0.029 mg/mL and 0.059 mg/mL respectively for *Bacillus sp.* GE1.

Similarly, for toluene and xylene supplemented MSM, biomass quality greatly improved after optimization on account of increase in biomass concentration.

alkB assay was carried out to understand whether the first step of alkane metabolism and subsequent transport of alkane inside the cell was accomplished. The optimum conditions were found out as 37 °C incubation temperature and pH of 7.4 except for *Aquamicrobium sp.* GR1, GR3 and GR4 whose optimum reaction times were 20 minutes. Whereas the maximum activities were almost comparable for *Staphylococcus sp.* GR2, *Bacillus sp.* GE1 and GS1, the activities of *Aquamicrobium sp.* GR1, GR3 and GR4 were lower owing to the lesser efficiency of metabolising *n*-hexadecane as the substrate. The maximum activity found out for *Staphylococcus sp.* GR2 was 104.35 U mg⁻¹. Michaelis-Menten kinetics suggested the strains have potential in *n*-hexadecane intake with the maximum V_{max} obtained as 316.16 U mg⁻¹ for *Bacillus sp.* GS1. *alkB* activity increased for all the strains when *n*-pentadecane (C₁₅) was introduced as substrate, however, decreased by atleast 28% on using a cyclic alkane such as cyclohexane as the substrate. The enzyme complex was found stable and *alkB* assay could be conducted satisfactorily upto 120 minutes of preparation and storage.

ndo assay was performed to check whether PAHs such as naphthalene and anthracene are converted to 1,2-dihydroxydiol as the first step of their metabolism inside the cell. The optimum conditions were found out as 32.5 °C incubation temperature and pH of 7.5 for all. The maximum activity was found out for *Bacillus sp.* GE1 was 6.93 U mg⁻¹. Michaelis-Menten kinetics suggested that *Bacillus sp.* GE1 was more capable among the strains with maximum V_{max} obtained as 11.52 U mg⁻¹. *ndo* showed comparable specificities as it could use anthracene with almost equal efficiency as naphthalene. Adding phenanthrene as the substrate reduced activities to upto 100% citing the inability of *ndo* to accept phenanthrene as the substrate. This is due to difference in aromatic ring orientation on phenanthrene as compared to anthracene. Hence, phenanthrene is converted through a series of reactions to 1,2-dihydroxynaphthalene and then converted to naphthalene which is then accepted by *ndo*. Also, it was indicative that the other enzymes in the crude complex were unable to convert phenanthrene readily. The enzyme complex was found stable for 120 minutes where a maximum of 73% of the activity was retained by *Bacillus sp.* GE1.

catA assay was carried out to check for the conversion of catechol to *cis*, *cis*-muconic acid through *ortho* pathway. The optimum conditions were found out as 30 °C incubation temperature, pH of 7.0 and reaction time of 30 minutes. Whereas activity of *Aquamicrobium sp.* GR4 was maximum as 15.7 U mg⁻¹, all others except *Aquamicrobium sp.* GR3 also showed significantly high activities. Michaelis-Menten kinetics suggested comparable maximum V_{max}

as 35.65 U mg^{-1} and 38.3 U mg^{-1} for *Aquamicrobium sp.* GR4 and *Bacillus sp.* GE1 respectively. The corresponding K_m values were $221.85 \text{ }\mu\text{M}$ and $299.58 \text{ }\mu\text{M}$ catechol respectively showing significant affinity towards catechol at 1,2-position. *catA* showed higher specificity towards using catechol as its substrate, however, around 70% of the activities were retained on using benzene as the substrate. *catA* activities using toluene and xylene as substrate decreased by 65% whereas, using phenol, it increased slightly and a maximum of 50% activity was regained by *Aquamicrobium sp.* GR4. The enzyme complex was stable for 180 minutes with 55-65% of retention in activities observed collectively.

catE assay was carried out to check for the conversion of catechol to 2-hydroxysemialdehyde through *meta* pathway. The optimum conditions were found out as 30°C incubation temperature, pH of 7.5 and reaction time of 20 minutes. The maximum activity was observed for *Bacillus sp.* GE1 as 28.33 U mg^{-1} . Michaelis-Menten kinetics suggested substrate inhibition by all others except *Bacillus sp.* GE1 and GS1 having comparable maximum V_{max} as 59.3 U mg^{-1} . The corresponding K_m values however differed as $324.99 \text{ }\mu\text{M}$ catechol for *Bacillus sp.* GE1 and $424.81 \text{ }\mu\text{M}$ catechol for *Bacillus sp.* GS1 showing *Bacillus sp.* GE1 was slightly more affined towards catechol breakdown at 2,3-position. *catE* showed significant activity as $>70\%$ on using benzene as the substrate. *catE* activities diminished by atleast 30% and 45% using toluene and xylene as substrate, whereas, using phenol, comparable activities of $13.7 \pm 0.2 \text{ U mg}^{-1}$ were observed for both *Bacillus sp.* GE1 and GS1 which was $\sim 66\%$ of the activity when catechol was used as the substrate. Hence, *catE* might be satisfactorily depicting higher activity by acting upon catechol through *meta* pathway. The enzyme complex was stable for 180 minutes with $> 50\%$ of retention in activities observed collectively.

Microbes are well versed with network of machineries that are helpful in sequential degradation of toxic pollutants that are present in refinery sludge waste. Most HCs are utilized by microorganisms as their C-source and are metabolised by generating cascade of enzymatic reactions. Enzymes possess higher stability and specificity towards substrate recognition and each HC has its own breakdown pathway, suggesting the complexity of tasks that may arise to attain significant HC metabolism. Microbes also possess the capability of secreting extracellular biomolecules in the form of lipid, carbohydrate and protein deposition. Simple analysis of these concentration of biomolecules would indicate the affinity of microorganisms towards HC utilization and their tendency towards enzymatic conversion of pollutants. Overall,

studying biomass quality and enzyme kinetics greatly enhances the understanding microbial action on biotransformation of refinery components.

6.1.5. Chapter 5: Application of design consortia for bioremediation of waste sludge

In Chapter 5, these optimum consortia were checked in the original sludge sample, However, before proceeding with the actual application, the optimum media and the consortia were applied to check their growth profile maximum tolerance concentrations of all the HCs namely *n*-hexadecane, anthracene, phenanthrene, benzene, toluene and xylene supplemented in the amended media. Lastly, the specific growth rate of the designed consortia increased rapidly compared to the maximum values obtained for the individual strains separately in NB and modified mMSM. Similarly, COD removal was also higher for the HCs as compared to the maximum values obtained in Chapter 2.

For *n*-hexadecane, at 500 mg/L, the biomass growth was modelled using Monod's and Andrew's kinetic models and *n*-hexadecane utilization was modelled using Monod's and Haldane's kinetic models. The maximum biomass concentration was obtained as 0.635 mg/mL for C3. Moreover, the Andrew's model showed best-fit for biomass growth for C1 whereas Monod's kinetics was observed as best-fit for C2 and C3 with the maximum μ_{max} of 273 d⁻¹ for C2. *n*-hexadecane utilization rate was also fitted in Monod's model with maximum q_{max} of 818 g COD/g biomass d⁻¹. Whereas, other combinations also showed satisfactory results, the combinations were well adept to growth and survival in waste conditions abundant with *n*-hexadecane or other short or longer chain alkanes.

For 50 mg/L anthracene, biomass growth showed the best fit for Andrew's kinetics, whereas anthracene utilization also showed best fit for Haldane's kinetics. The maximum biomass concentration was obtained as 0.452 mg/mL for C3. The maximum μ_{max} of 76.78 d⁻¹ and q_{max} of 27.55 g COD/g biomass d⁻¹ for C2 suggested that the combination had potency towards growth and survival in waste contaminated predominantly with PAHs. Similarly, for 20 mg/L phenanthrene, Andrew's kinetics for biomass growth and Haldane's kinetics for phenanthrene utilization was obtained for all C1, C2 and C3. Based on the characteristics, C2 showed better adaptivity than C1 and C3 in phenanthrene supplemented optimum MSM.

For benzene, toluene and xylene, all of whose experiments were carried out at 1000 mg/L each, C2 was shown to have maximum biomass concentration in each case as 0.609 mg/mL, 0.587 mg/mL and 0.511 mg/mL respectively. Despite showing excellent growth, the

biomass growth and HC utilization were best described by Andrew's and Haldane's kinetic model respectively. The kinetic parameters were suggestive of the fact that the optimum consortia were potent in adapting to waste conditions where contamination of aromatic components are more pronounced.

The biomass qualities were also studied. In all the cases, the lipid content, carbohydrate content and protein content increased at the end of the experiments. Whereas, in all the cases, the highest share of the characteristics was shown the C2, the highest increase in lipid and protein content was observed in case of mMSM supplemented with benzene as 32.57% and 37.66% dry biomass respectively, whereas carbohydrate content was the highest for mMSM supplemented with toluene as 27.56% dry biomass.

The enzymes tested for their activities were also checked from biomass obtained by incubating the consortia C1, C2 and C3 in each of the substrates such as *n*-hexadecane for *alkB* assay, naphthalene for *ndo* assay and catechol for *catA* and *catE* assay. C2 was seen to have the maximum fractional increase in activity for *alkB* and *ndo* whereas increase in activities for *catA* and *catE* activities were found highest for C3 and C1 respectively. The consortia showed significant evidence of their activities that could be indicative that biomass generated from these experiments posed efficient quality and that the combinations were able to mineralize the aliphatic, aromatics and PAHs proficiently. Increase in *alkB* activities were within 28-31 folds collectively for C1, C2 and C3, where maximum increase activity of C2 was seen as 30.56 folds. Similarly, highest increase in *ndo* activity for C2 was observed as 17.22 folds. *catA* activities were within 11-14 folds for all the consortia, however, significant difference in activities of *catE* was observed where C1 exhibited highest increase in activity of 21.36 folds compared to C2 and C3 whose increase in activities were calculated as ~6-8 folds.

Further nutrient amendment in the form of C:N:P, experiments was carried out in a culture containing the mixture of MTC of all the HCs under study supplemented in mMSM. N and P addition were preferred to provide the microbial consortia with the best habitat to accept the waste as their C-source for their survival. C:N:P of 100:5:1 and 100:10:1 were added in necessary proportions to adjust the respective ratios. Whereas, COD removal was still comparable, addition of N and P did affect biomass production positively. The higher trends fractional increase in growth was observed when both N and P were added to the mixture. The highest COD removal, N utilization, P utilization and fractional increase in growth was observed in the mixture incubated with C2 when N and P were added in the ratio 100:5:1

whereas the same for C1 and C3 was seen when C:N:P was 100:10:1. With ~92% COD removal and ~82% N and P utilization for C1, biomass concentration increased by ~12.5 folds. Similarly, for C3, ~80% N and P utilization, ~92.5% COD removal was seen, contributing to an increased biomass production of 10 folds. For C2, ~80-85% N and P utilization was observed alongwith ~95% COD removal which contributed to an increased biomass value of 21 folds.

Biomass characteristics were also studied for the biomass obtained from the C:N:P experimentation. Lipid and protein content shoot up to as high as 45% and 33% dry biomass respectively in case of C2 where the maximum biomass was found in case of C:N:P whereas carbohydrate content was the highest in case of C3 incubated in C:N:P 100:10:1. The lipid, carbohydrate and protein concentrations for C1 where biomass concentration was maximum at C:N:P = 100:10:1 were 39.44%, 19.33% and 28.33% respectively of dry biomass.

Enzyme activities were also greatly enhanced after C:N:P addition. For *alkB* and *catA*, the order of activity was noticed as C2+N+P > C3+N+P > C1+N+P. The highest increase in *alkB* activity was recorded as 63.24 folds for C2. The highest increase in no activity as 16.09 folds were noted for C1. *catA* activity increased to the highest as ~28.5 folds for C2 followed by 27.86 folds for C3. Contrary to the above trend, *catE* activity increased to a high in C1 as 60.21 folds. The lowest value was noted for C2 as 28 folds.

Based on the experimental values obtained from C:N:P experimentation, the optimum C:N:P were used as 100:10:1 for C1, 100:5:1 for C2 and 100:10:1 for C3.

The optimum C:N:P ratio obtained were used for application of the consortia in the sludge sample. The application experiments were carried out as a mesocosm firstly with the sample diluted to 11500 mg/L COD in mMSM as all the previous experimentations were carried out in the mixture of HCs having similar COD value. The similar experiments were then carried out with undiluted sludge sample.

For the trial experiment at COD 11500 mg/L, as was evident, the microbial count and biomass concentrations were higher when the sample was amended with N and P into their respective proportions for C1, C2 and C3. The highest fractional increase in biomass concentration and microbial count of 7.45 folds and 17.48 folds, respectively, were suggestive of the excellent survivability and enhanced bioavailability. More than 50% of COD was consumed within 24 days of incubation with the final removal obtained as 72.3%, 82.4% and

78.6% and 74.3% respectively for C1, C2 and C3 respectively. More than 65% of N and P utilization was seen collectively in all C1, C2 and C3 with the highest being >70% for C2. Biomass characteristics were also greatly enhanced with lipid, carbohydrate and protein concentrations showing increasing trend on N and P addition. The higher shares of biomass included collectively 31-35% lipid, 20-23% carbohydrate and 28-34% protein based on dry biomass. *alkB*, *ndo*, *catA* and *catE* were prominently active in the biomass obtained confirming the continuous metabolism of aliphatic, aromatic and PAHs. The highest fractional increase in activities were noted collectively for all C1, C2 and C3 when amended with their optimum C:N:P indicated that the optimized combinations of microbes were adept to metabolism of HCs in the present waste sludge.

Similarly, for undiluted sludge of COD 46017 mg/L, the highest fractional increase in biomass concentration and microbial count of 7.35 and 16.86 folds were comparable with the trial experiment and also were suggestive of the excellent survivability and enhanced bioavailability. Almost 50% of COD was consumed within 24 days of incubation with the final removal obtained as 57-69% collectively for C1, C2 and C3. These values were lower than the trial experiment and it could be due to enhanced toxicity and contamination. Compared to the trial, ~65% of N utilization whereas P utilization was higher as 74-80% collectively in all C1, C2 and C3. Biomass characteristics were also greatly enhanced, however, lipid, carbohydrate and protein shares of biomass included were lower as compared to the trial experiment. Collectively 25-29% lipid, 15-18% carbohydrate and 18-23% protein based on dry biomass were obtained. *alkB*, *ndo*, *catA* and *catE* activities were also pronounced confirming the breakdown and metabolism of the respective classes of HCs. The highest increase in *alkB* activities was noted for C1, whereas C2 signified the highest for *ndo* activities. C1 depicted highest increase for both *catA* and *catE* activities when amended with the optimum N and P. The experimentation suggested that the designed consortia were highly potent and applicable for bioremediation of waste sludge.

Consortia designing is pivotal for application of microbial population for bioremediation of refinery waste. The efficacy of microbes and their advantages related to their acclimatisation and utilization potential has been well known. Strategies related to harnessing indigenous microorganisms and employing them at their optimum proportion to attain maximum clean up efficiency has been studied vigorously. Statistical optimization-based consortia design has been demonstrated to uplift the microbial performance by virtue of

providing adequate initial inoculum at their optimal percentage to accord for faster acclimatisation and accelerated HC utilization. Addition of nutrients in terms of N and P also facilitates abundant conditions to support microbial growth and enhances the bioavailability of the toxic HCs as C-source. Since bioremediation is considered a cost-effective process to mitigate challenges posed by refinery waste, it is also worthy to choose simpler and quicker experimental design strategies to reduce cost, resources and labour.

6.2. Future perspectives

The treatment of a typical refinery waste sludge is generally affected by the complexity of such kind of waste systems and their variations depending on their occurrence and location. Thus, attaining a holistic approach in terms of designing microbial proportions and additional nutrient conditions is challenging and several other aspects of treatment are required to be studies that might not have been accomplished in this work. However, we believe the solutions derived will help understand the treatment mechanisms in their different dimensions. Moreover, we also took care of the fact to comprehensively study the preliminary characteristics of microbes, waste conditions and necessary approaches to help reduce toxicity.

Microbes and their interactions in a pollutant medium were only evaluated through aerobic incubation. However, it will be interesting to check for the microorganisms and their response to different pollutants using anaerobic mode of incubation. With the modern-day advent of -omics and the available online tools, it would be helpful to understand the diversity of microbial community in a waste system into their extended details. Whereas we tried to present a simpler spectrophotometric method for COD determination of the respective HCs, understanding their extent of degradation and analyzing the intermediate products through chromatographic techniques would further clarify the biomineralization efficiency of these strains.

In case of media optimization, where two additional components were further added, vigorous studies are required to be carried out to search for any additional factors in terms of nutrient or growth parameters that might have better influence and can aid to more biomass production and COD removal. On the same note, biomass production itself might be a tedious output and better alternative responses to accelerate the optimization process might be preferred. Moreover, one-to-one interaction among the significant strains within the HC mixtures were not carried out, that is further expected to provide us with deeper insights into

the application of microbial consortia in wastes obtained from different locations and different characteristics.

Biomass characteristics indicated their quality obtained after incubation in aerobic conditions. Similarly, obtaining anaerobic biomass would provide us with better comparison of the quality and aid to more efficient application. In addition to lipid, carbohydrate and protein contents, it would be interesting to estimate the other components that make up the biomass composition and decide whether these characteristics are adequate to use the biomass for further value addition and resource recovery. Enzyme assays were checked only on the crude complex, however, purification of these enzymes and checking their respective activity would enhance our understanding of catalytic potential in presence of the respective substrates, and the mechanisms of mineralization, especially the intermediates that might remain in the mixture for a very short period of time. Lastly, application of -omics platforms would facilitate better inference about upregulation and downregulation of these genes that is otherwise not conclusive through analyzing enzyme activity only.

Apart from batch mode of operation, a similar set of experiments with similar initial feed of COD and microbial proportions in fed batch and mixed operations would allow us to redefine our treatment strategy and also recalculate the incubation/retention time. Hence, a change in treatment efficiency could also be expected. Similarly, biomass characteristics and enzyme activities are expected to be altered. Lastly, strategies related to scaling up the experiments to pilot levels would be challenging and effective methodologies for implementing the same are required to be found out.



Appendices

Appendices

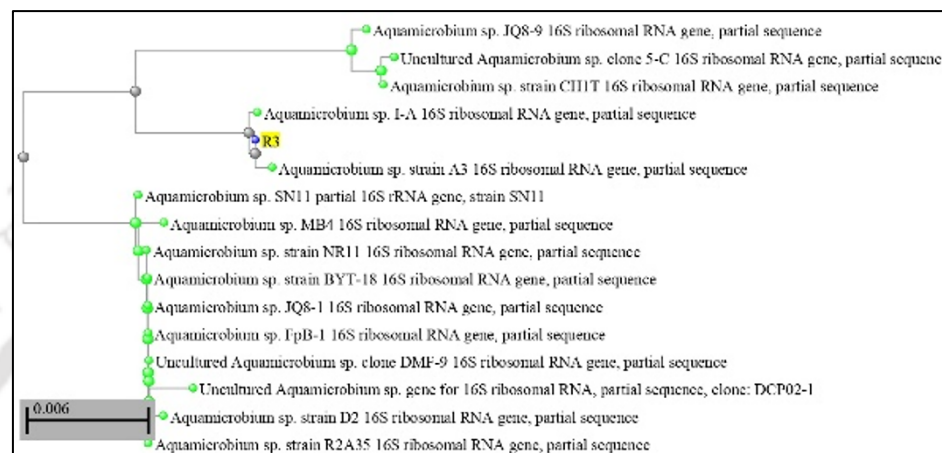
➤ Chapter 2: Screening, isolation and identification of indigenous microorganism(s) from waste sludge

▪ Strains characteristics

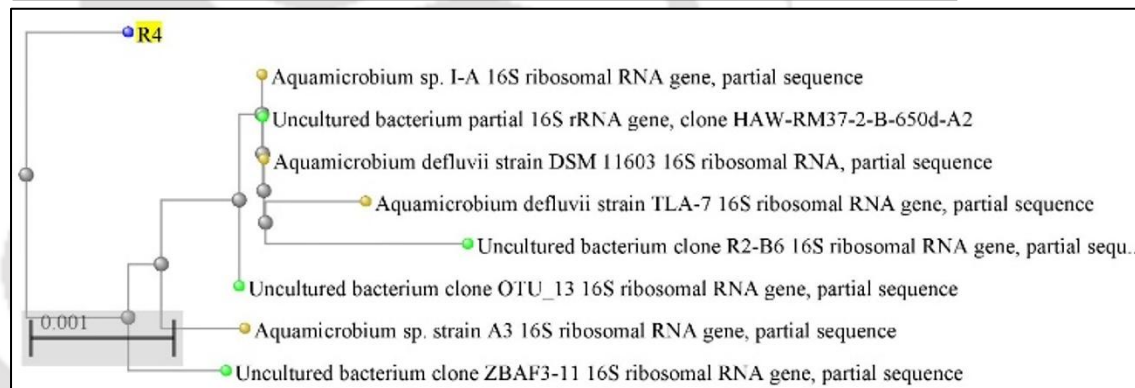
Appendix Table 1: Characteristics of identified strains.

Isolate	Identification	Size (bp)	Phylogenetic tree
GR1	<i>Aquamicrobium</i> sp.	1400	
GR2	<i>Staphylococcus</i> sp.	1369	

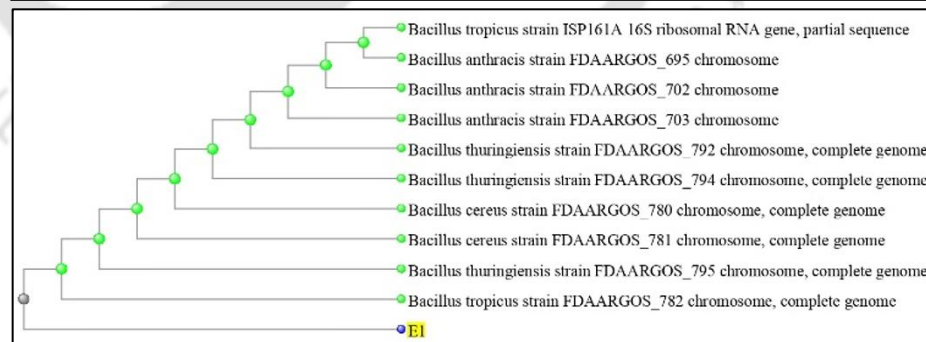
GR3 *Aquamicrobium* sp. 1396



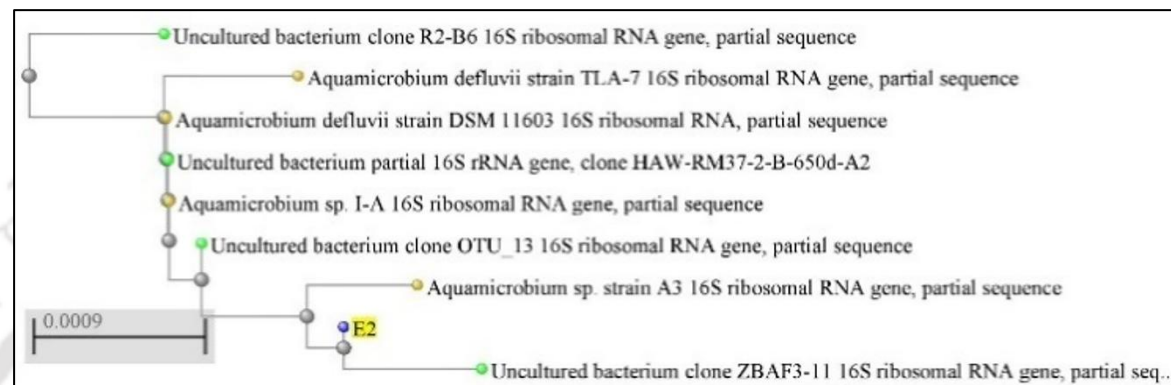
GR4 *Aquamicrobium* sp. 1420



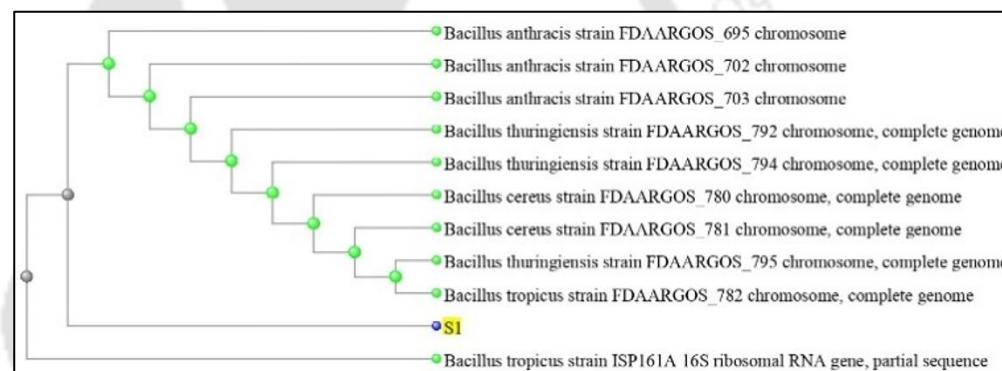
GE1 *Bacillus* sp. 1440



GE2 *Aquamicrobium sp.* 1420



GS1 *Bacillus sp.* 1431



▪ Tolerance to heavy metals and salinity

Appendix Table 2: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Fe in FeSO₄.7H₂O.

Concentration of FeSO ₄ .7H ₂ O	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.055	0.069	0.028	0.045	0.087	0.078	0.068
20	0.098	0.104	0.038	0.102	0.102	0.099	0.109
30	0.128	0.163	0.077	0.144	0.157	0.104	0.157
40	0.144	0.187	0.096	0.199	0.244	0.144	0.213
50	0.204	0.277	0.117	0.238	0.348	0.179	0.311
60	0.089	0.097	0.066	0.058	0.094	0.087	0.097
70	0.087	0.093	0.046	0.044	0.096	0.088	0.078
80	0.066	0.044	0.031	0.051	0.064	0.094	0.083
90	0.04	0.058	0.034	0.036	0.068	0.089	0.066
100	0.036	0.045	0.011	0.037	0.048	0.073	0.068

Appendix Table 3: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Cu in CuSO₄.5H₂O.

Concentration of CuSO ₄ .5H ₂ O	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.127	0.186	0.058	0.068	0.188	0.137	0.172
20	0.179	0.226	0.094	0.126	0.349	0.208	0.276
30	0.208	0.253	0.099	0.137	0.344	0.258	0.299
40	0.167	0.193	0.074	0.125	0.322	0.173	0.238
50	0.156	0.182	0.066	0.129	0.322	0.157	0.231
60	0.084	0.098	0.067	0.094	0.099	0.097	0.098
70	0.076	0.099	0.033	0.044	0.067	0.097	0.055
80	0.079	0.091	0.042	0.047	0.064	0.078	0.074
90	0.066	0.094	0.037	0.038	0.054	0.089	0.058
100	0.058	0.089	0.033	0.049	0.051	0.062	0.06

Appendix Table 4: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Ni in NiSO₄.6H₂O.

Concentration of NiSO ₄ .6H ₂ O	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.045	0.06	0.026	0.034	0.061	0.06	0.055
20	0.058	0.061	0.033	0.033	0.064	0.058	0.049
30	0.088	0.073	0.037	0.051	0.059	0.055	0.057
40	0.073	0.088	0.041	0.048	0.056	0.049	0.066
50	0.054	0.077	0.038	0.047	0.055	0.053	0.061
60	0.044	0.058	0.039	0.044	0.055	0.055	0.063
70	0.039	0.054	0.035	0.044	0.049	0.047	0.058
80	0.033	0.053	0.033	0.032	0.051	0.043	0.055
90	0.034	0.044	0.034	0.037	0.052	0.044	0.058
100	0.04	0.044	0.029	0.031	0.056	0.051	0.051

Appendix Table 5: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Zn in ZnSO₄.H₂O.

Concentration of ZnSO ₄ .H ₂ O	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.03	0.051	0.021	0.033	0.052	0.052	0.051
20	0.159	0.231	0.106	0.094	0.269	0.188	0.201
30	0.144	0.205	0.097	0.125	0.24	0.167	0.182
40	0.136	0.188	0.094	0.119	0.227	0.156	0.153
50	0.117	0.169	0.097	0.117	0.215	0.155	0.144
60	0.112	0.163	0.083	0.116	0.209	0.148	0.133
70	0.088	0.098	0.085	0.088	0.099	0.098	0.094
80	0.057	0.097	0.079	0.069	0.095	0.097	0.096
90	0.063	0.087	0.075	0.074	0.098	0.078	0.085
100	0.055	0.093	0.055	0.045	0.068	0.079	0.083

Appendix Table 6: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Co in CoCl₂.2H₂O.

Concentration of CoCl ₂ .2H ₂ O	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.136	0.286	0.094	0.155	0.327	0.215	0.299
20	0.119	0.279	0.087	0.143	0.301	0.203	0.291
30	0.107	0.266	0.079	0.135	0.288	0.191	0.288
40	0.113	0.256	0.073	0.123	0.275	0.182	0.281
50	0.102	0.246	0.067	0.118	0.264	0.174	0.277
60	0.084	0.096	0.058	0.098	0.1	0.097	0.101
70	0.076	0.092	0.049	0.094	0.092	0.094	0.103
80	0.068	0.084	0.043	0.086	0.05	0.084	0.066
90	0.055	0.075	0.035	0.077	0.067	0.078	0.053
100	0.032	0.053	0.029	0.059	0.053	0.066	0.062

Appendix Table 7: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Pb in Pb(NO₃)₂.

Concentration of Pb(NO ₃) ₂	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.031	0.099	0.03	0.056	0.074	0.052	0.072
20	0.057	0.123	0.045	0.072	0.093	0.057	0.203
30	0.051	0.152	0.054	0.087	0.114	0.052	0.185
40	0.052	0.144	0.059	0.077	0.136	0.055	0.164
50	0.044	0.149	0.059	0.081	0.187	0.061	0.209
60	0.043	0.099	0.055	0.077	0.102	0.058	0.104
70	0.045	0.098	0.056	0.068	0.099	0.055	0.086
80	0.044	0.101	0.047	0.064	0.086	0.059	0.077
90	0.034	0.088	0.033	0.049	0.078	0.057	0.063
100	0.032	0.068	0.027	0.035	0.063	0.053	0.053

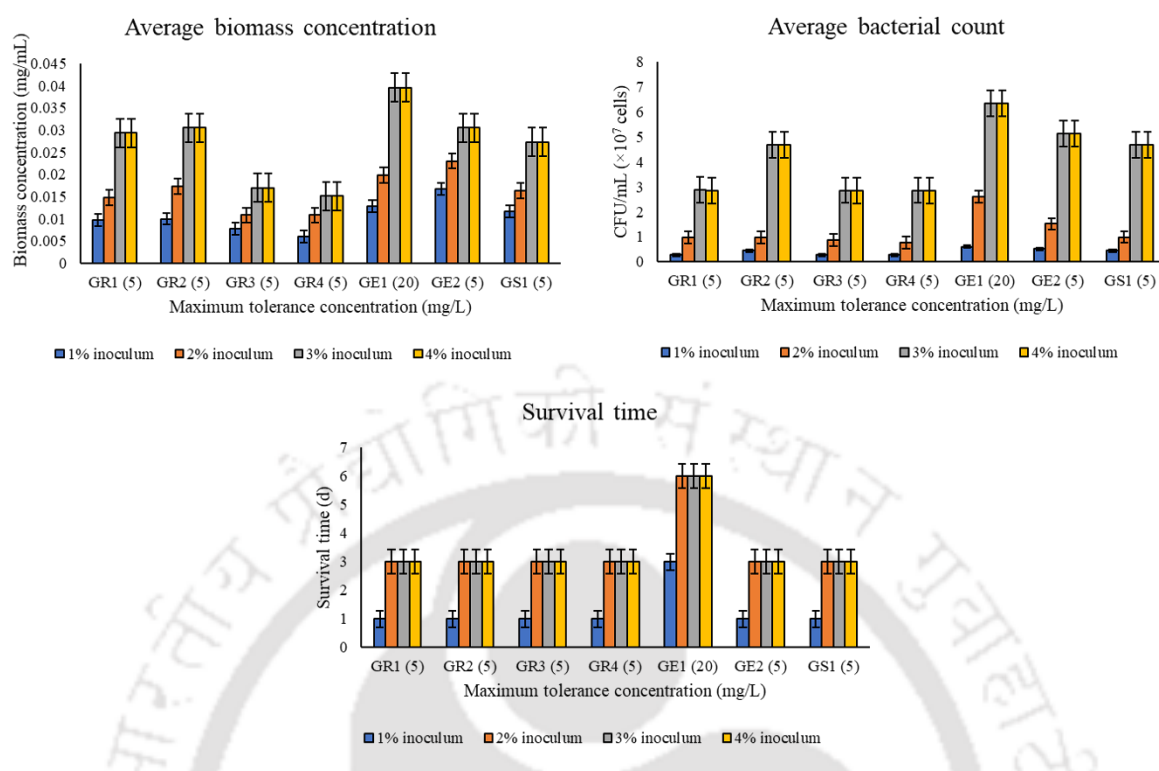
Appendix Table 8: Tolerance of isolates in terms of OD₆₀₀ for heavy metal Cr in CrO₃.

Concentration of CrO ₃	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
10	0.129	0.099	0.047	0.041	0.144	0.136	0.096
20	0.122	0.105	0.042	0.058	0.208	0.118	0.124
30	0.119	0.137	0.045	0.054	0.267	0.108	0.157
40	0.117	0.175	0.043	0.049	0.241	0.098	0.178
50	0.112	0.163	0.038	0.044	0.229	0.084	0.21
60	0.1	0.099	0.037	0.038	0.101	0.048	0.084
70	0.099	0.098	0.029	0.041	0.057	0.056	0.074
80	0.058	0.085	0.026	0.037	0.064	0.055	0.067
90	0.047	0.078	0.024	0.028	0.073	0.073	0.071
100	0.036	0.064	0.025	0.031	0.053	0.049	0.064

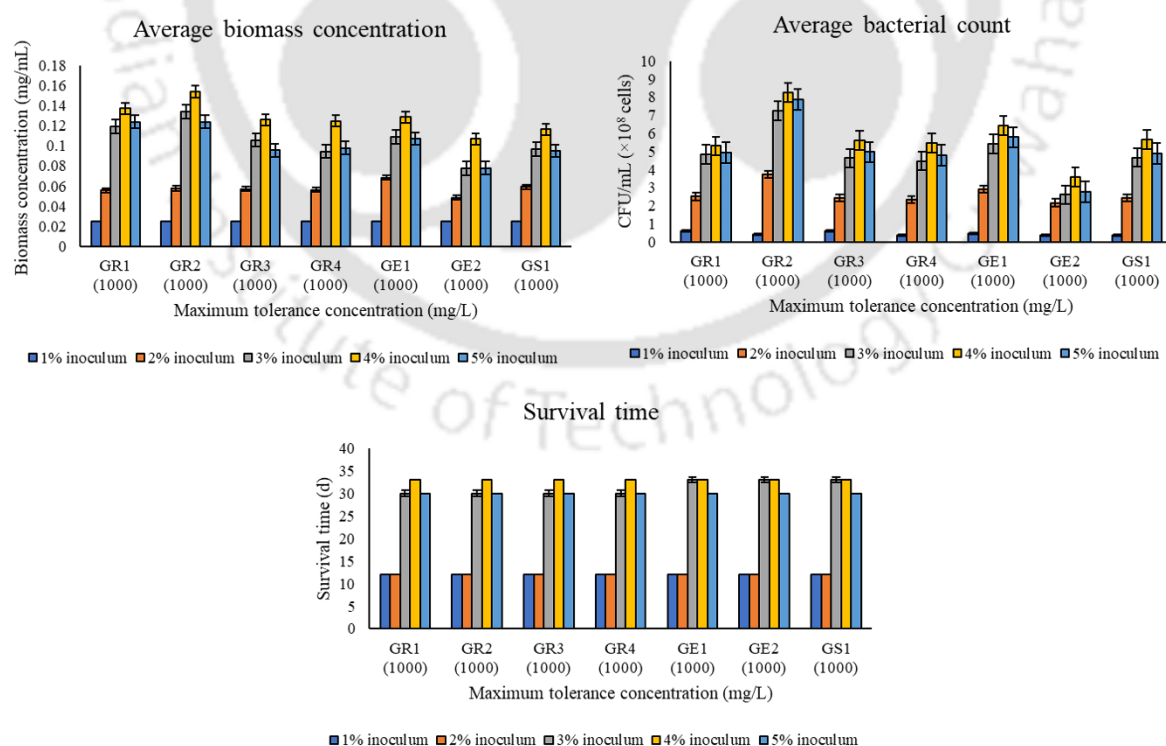
Appendix Table 9: Tolerance of isolates in terms of OD₆₀₀ for salinity in NaCl.

Concentration of NaCl	GR1	GR2	GR3	GR4	GE1	GE2	GS1
0	0.03	0.051	0.021	0.033	0.052	0.052	0.051
50	0.045	0.102	0.033	0.095	0.135	0.127	0.117
100	0.073	0.156	0.068	0.125	0.168	0.176	0.155
150	0.104	0.211	0.091	0.154	0.244	0.214	0.199
200	0.122	0.254	0.109	0.183	0.363	0.246	0.227
250	0.151	0.307	0.119	0.208	0.452	0.318	0.266
300	0.087	0.098	0.091	0.095	0.11	0.086	0.099
350	0.079	0.067	0.085	0.085	0.104	0.085	0.103
400	0.055	0.057	0.066	0.077	0.103	0.093	0.074
450	0.042	0.051	0.042	0.079	0.097	0.074	0.079
500	0.032	0.053	0.028	0.055	0.074	0.057	0.061

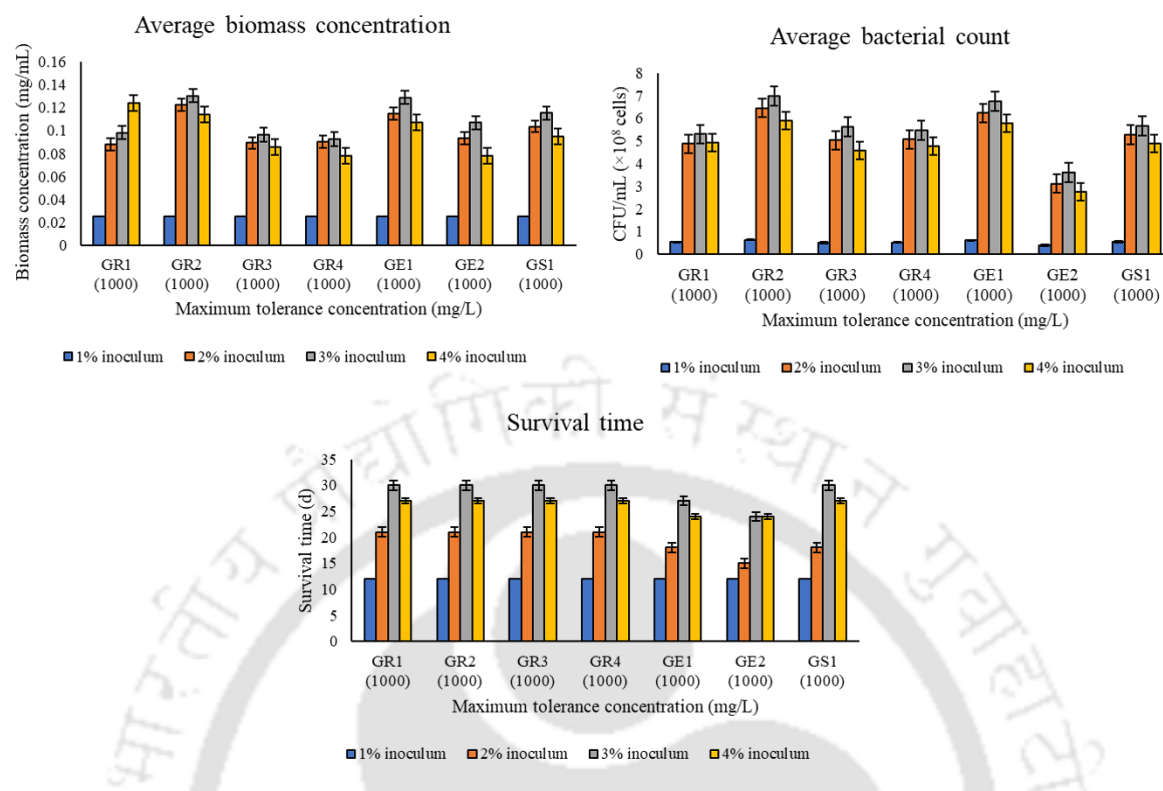
■ Inoculum preparation and optimization



Appendix Figure 1: Inoculum optimization of strains in phenanthrene supplemented MSM.

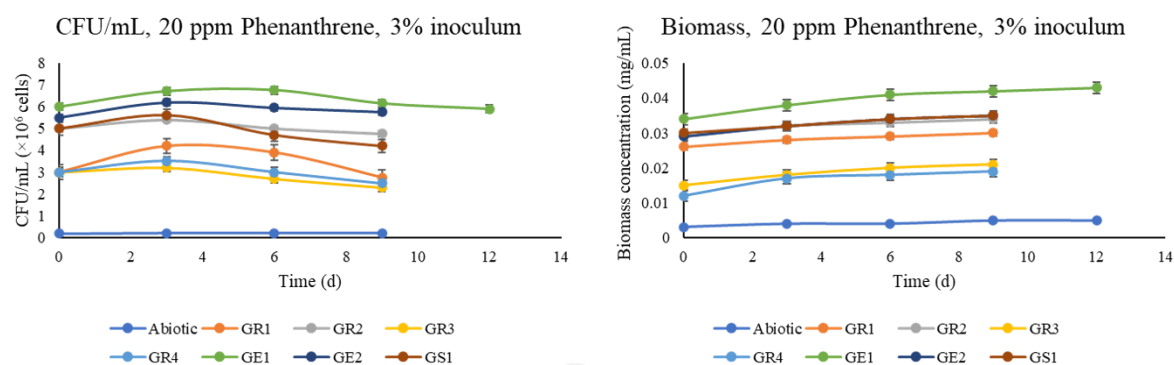


Appendix Figure 2: Inoculum optimization of strains in toluene supplemented MSM.

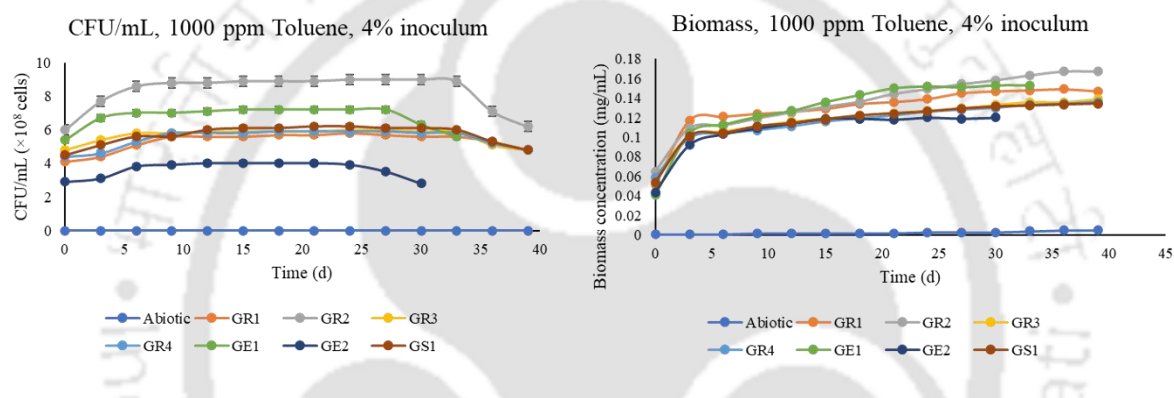


Appendix Figure 3: Inoculum optimization of strains in xylene supplemented MSM.

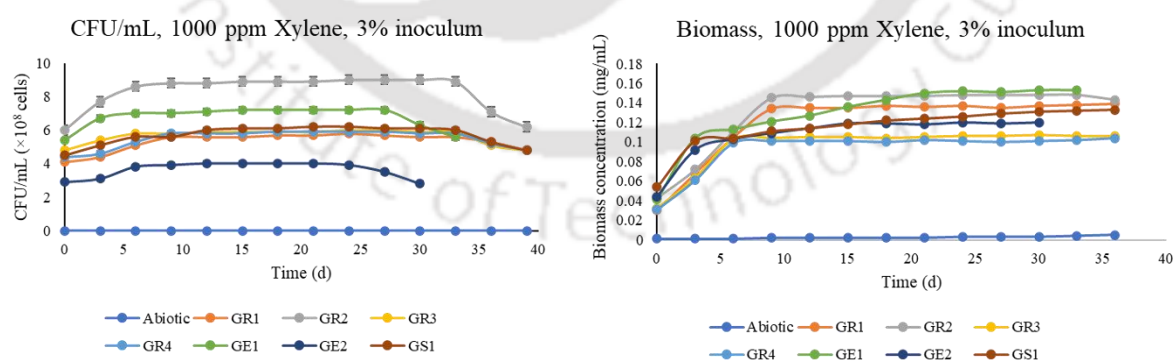
■ Shake flask growth study of isolated strains in different class of hydrocarbon(s) (HCs)



Appendix Figure 4: Growth characteristics of isolated strains in phenanthrene supplemented MSM.



Appendix Figure 5: Growth characteristics of isolated strains in toluene supplemented MSM.



Appendix Figure 6: Growth characteristics of isolated strains in xylene supplemented MSM.

▪ Kinetic parameters of biomass growth of isolated strains

Appendix Table 10: Biomass growth kinetics of isolated strains in phenanthrene supplemented MSM.

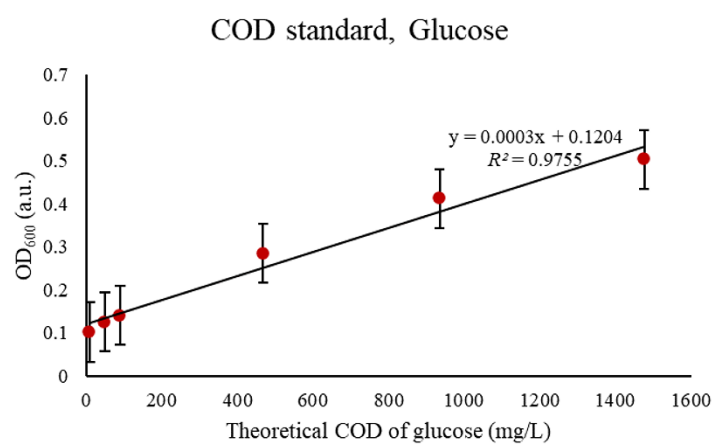
Pollutant: Phenanthrene, Andrew's model							
Strains/ parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
μ_m (d ⁻¹)	0.019	0.023	0.014	0.039	0.082	0.027	0.041
K_s (mg/L)	1.76	2.35	0.71	2.16	4.67	1.66	1.56
K_i (mg/L)	0.01	0.011	0.009	0.007	62.38	0.012	0.021
R^2	0.98	0.963	0.989	0.953	0.94	0.978	0.973

Appendix Table 11: Biomass growth kinetics of isolates strains in toluene supplemented MSM.

Pollutant: Toluene, Monod's model							
Strains/ parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
μ_m (d ⁻¹)	0.05	0.032	0.018	0.033	0.016	0.019	0.019
K_s (mg/L)	120.52	42.82	110.06	86.06	39.15	8.72	23.43
K_i (mg/L)	-	-	-	-	-	-	-
R^2	0.994	0.924	0.982	0.99	0.973	0.957	0.946

Appendix Table 12: Biomass growth kinetics of isolated strains in xylene supplemented MSM.

Pollutant: Xylene, Monod's model							
Strains/ parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
μ_m (d ⁻¹)	0.022	0.027	0.017	0.027	0.025	0.02	0.02
K_s (mg/L)	22.37	18.28	71.94	15.84	45.61	17.69	55.62
K_i (mg/L)	-	-	-	-	-	-	-
R^2	0.989	0.967	0.977	0.963	0.962	0.886	0.97

■ COD removal of hydrocarbons(s) (HCs)

Appendix Figure 7: Standard curve of COD determination in terms of glucose.



Appendix Table 13: Summary of growth study of isolates in different classes of HCs.

Hydrocarbon(s) (HCs)	MTC	Optimum inoculum (% v/v)	Removal efficiency		Kinetic parameters					
			At MTC	Maximum	μ_{max} (d ⁻¹)		K_s (mg/L)		K_i (mg/L)	
					Min.	Max.	Min.	Max.	Min.	Max.
<i>n</i> -hexadecane	GR2, GS1 (500 mg/L)	4%	87%, GR2	93%, GE1 (250 mg/L)	0.003 (GR3)	0.094 (GR2)	0.449 (GR3)	91.58 (GR2)	-	-
Anthracene	GE1 (50 mg/L)	4%	34%, GE1	59%, GE1 (10 mg/L)	0.037 (GR4)	0.09 (GE1)	1.91 (GR4)	14.33 (GE1)	3.11 (GR4)	22.44 (GR1)
Phenanthrene	GE1 (10 mg/L)	3%	17%, GE1	47%, GE1 (5 mg/L)	0.115 (GR1)	1.475 (GE1)	3.063 (GR4)	12.69 (GE1)	4.11 (GR1)	23.76 (GE1)
Benzene		4%	93%, GR2	96%, GR2 (1000 mg/L)	0.034 (GE1)	0.066 (GR2)	55.99 (GR4)	250.62 (GR1)	-	-
	All strains (1000 mg/L)	4%	94%, GR2	97%, GR2 (1000 mg/L)	0.016 (GE1)	0.05 (GR1)	8.72 (GE2)	120.52 (GR1)	-	-
Toluene										
Xylene		3%	94%, GR2	96%, GR2 (1000 mg/L)	0.017 (GR3)	0.027 (GR4)	15.836 (GR4)	71.94 (GR3)	-	-

➤ **Chapter 3: Statistical optimization for designing optimum consortia for remediation of waste sludge**

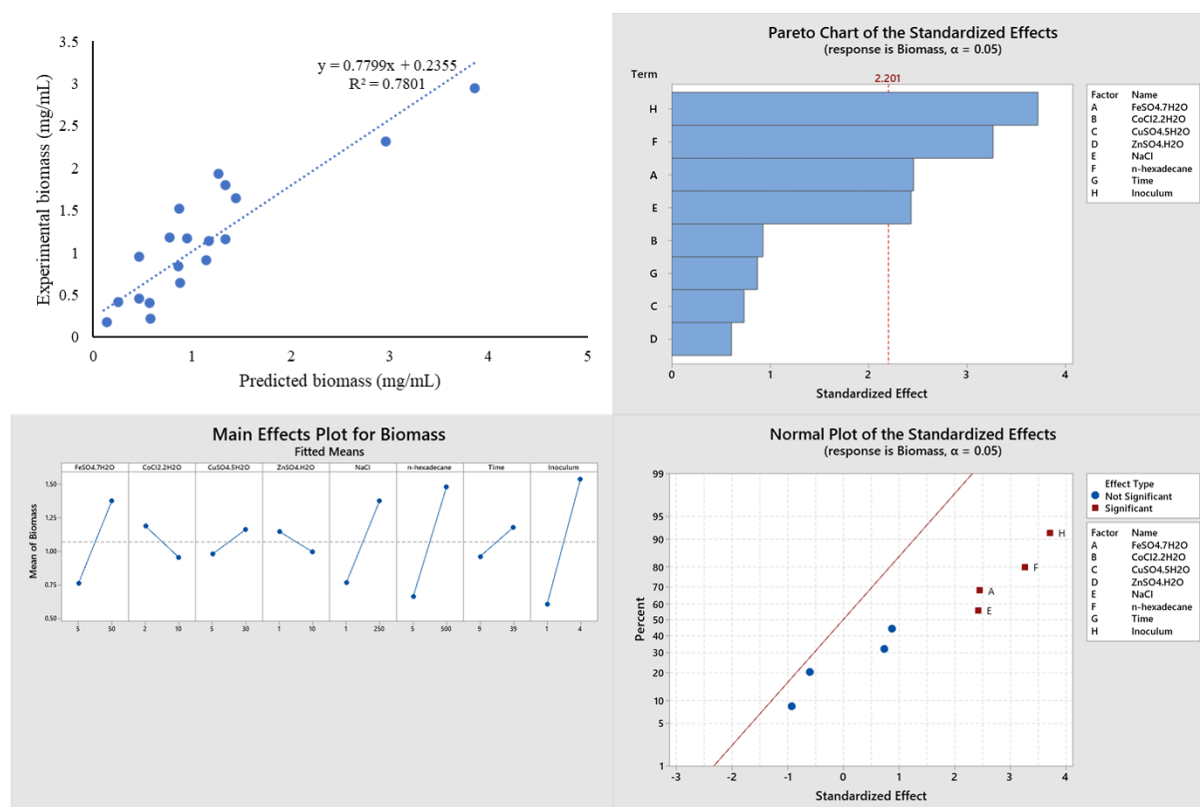
- Screening, optimization, statistical analysis and mutual interaction of significant factors for biomass production of selected strains in different class of hydrocarbons (HCs)

- *n*-hexadecane

- *Staphylococcus sp.* GR2

Appendix Table 14: PBD of *Staphylococcus sp.* GR2 for *n*-hexadecane supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	<i>n</i> - hexadecane (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	5	39	4	0.875
2	5	10	30	1	250	500	9	1	0.466
3	50	10	5	10	250	5	9	1	0.253
4	50	2	5	10	250	5	39	4	1.348
5	50	2	30	1	250	500	39	4	3.861
6	50	10	30	1	1	500	39	1	0.773
7	5	10	5	10	250	500	39	1	0.869
8	50	2	5	1	1	500	9	4	1.277
9	5	2	30	10	1	500	39	1	0.883
10	5	2	5	1	1	5	9	1	0.114
11	5	10	30	1	1	5	9	4	0.466
12	5	2	5	1	250	5	39	1	0.571
13	50	10	5	1	1	5	39	1	0.147
14	5	2	5	10	1	500	9	4	0.954
15	50	2	30	10	250	500	9	1	1.441
16	5	10	5	10	1	500	39	4	1.347
17	5	10	30	10	250	5	9	4	1.145
18	50	10	30	10	1	5	39	4	1.172
19	50	10	5	1	250	500	9	4	2.968
20	50	2	30	10	1	5	9	1	0.583



Appendix Figure 8: Precision, pareto chart, main effects plot and normal plot of PBD for *Staphylococcus sp.* GR2 in *n*-hexadecane supplemented MSM.

Appendix Table 15: ANOVA and model summary for PBD of *Staphylococcus sp.* GR2 in *n*-hexadecane MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	12.179	1.522	4.88	0.009
Linear	8	12.179	1.522	4.88	0.009
FeSO ₄ .7H ₂ O	1	1.885	1.885	6.04	0.032
CoCl ₂ .2H ₂ O	1	0.269	0.269	0.86	0.373
CuSO ₄ .5H ₂ O	1	0.169	0.169	0.54	0.477
ZnSO ₄ .H ₂ O	1	0.116	0.116	0.37	0.555
NaCl	1	1.848	1.848	5.92	0.033
<i>n</i> -hexadecane		3.329	3.329	10.67	0.008
Time		0.238	0.238	0.76	0.402
Inoculum		4.325	4.325	13.86	0.003
Error	11		3.433		0.312
Total	19		15.612		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.559	78.01%	62.02%	37.31%

$$\begin{aligned} \text{Biomass} = & -0.838 + 0.014 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.029 \text{CoCl}_2 \cdot 2\text{H}_2\text{O} \\ & + 0.007 \text{CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.017 \text{ZnSO}_4 \cdot \text{H}_2\text{O} + 0.002 \text{NaCl} + 0.002 n\text{-hexadecane} \\ & + 0.007 \text{Time} + 0.31 \text{Inoculum} \end{aligned}$$

**Appendix
Equation 1**

Appendix Table 16: CCD of *Staphylococcus sp.* GR2 in *n*-hexadecane MSM.

- Concentration of *n*-hexadecane = 500 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	125.5	2.5	4.761
2	65.34	125.5	2.5	1.194
3	27.5	125.5	2.5	4.814
4	50	1	1	0.663
5	5	250	1	0.78
6	50	1	4	1.33
7	27.5	125.5	2.5	4.791
8	27.5	125.5	2.5	4.814
9	27.5	334.883	2.5	4.282
10	5	1	1	0.372
11	50	250	1	0.994
12	27.5	125.5	5.023	2.764
13	27.5	125.5	2.5	4.831
14	-10.34	125.5	2.5	0.492
15	5	1	4	1.533
16	50	250	4	5.062
17	27.5	125.5	-0.023	0.041
18	5	250	4	1.56
19	27.5	125.5	2.5	4.761
20	27.5	-83.883	2.5	0.074

Appendix Table 17: ANOVA of CCD for *Staphylococcus sp.* GR2 in *n*-hexadecane MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	30.025	3.336	10.92	0
Linear	3	21.437	7.146	23.39	0
FeSO ₄ .7H ₂ O	1	1.807	1.807	5.92	0.035
NaCl	1	5.953	5.953	19.49	0.001
Inoculum	1	13.678	13.678	44.78	0
Square	3	5.414	1.805	5.91	0.014
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	2.608	2.608	8.54	0.015
NaCl*NaCl	1	2.193	2.193	7.18	0.023
Inoculum*Inoculum	1	1.672	1.672	5.47	0.041
2-Way Interaction	3	3.173	1.058	3.46	0.059
FeSO ₄ .7H ₂ O*NaCl	1	0.858	0.858	2.81	0.125
FeSO ₄ .7H ₂ O*Inoculum	1	1.805	1.805	5.91	0.035
NaCl*Inoculum	1	0.51	0.51	1.67	0.225
Error	10	3.055	0.306		
Lack-of-Fit	5	3.052	0.61	1137.34	0
Pure Error	5	0.003	0		
Total	19	33.08			

Appendix Table 18: Model summary of CCD for *Staphylococcus sp.* GR2 in *n*-hexadecane MSM.

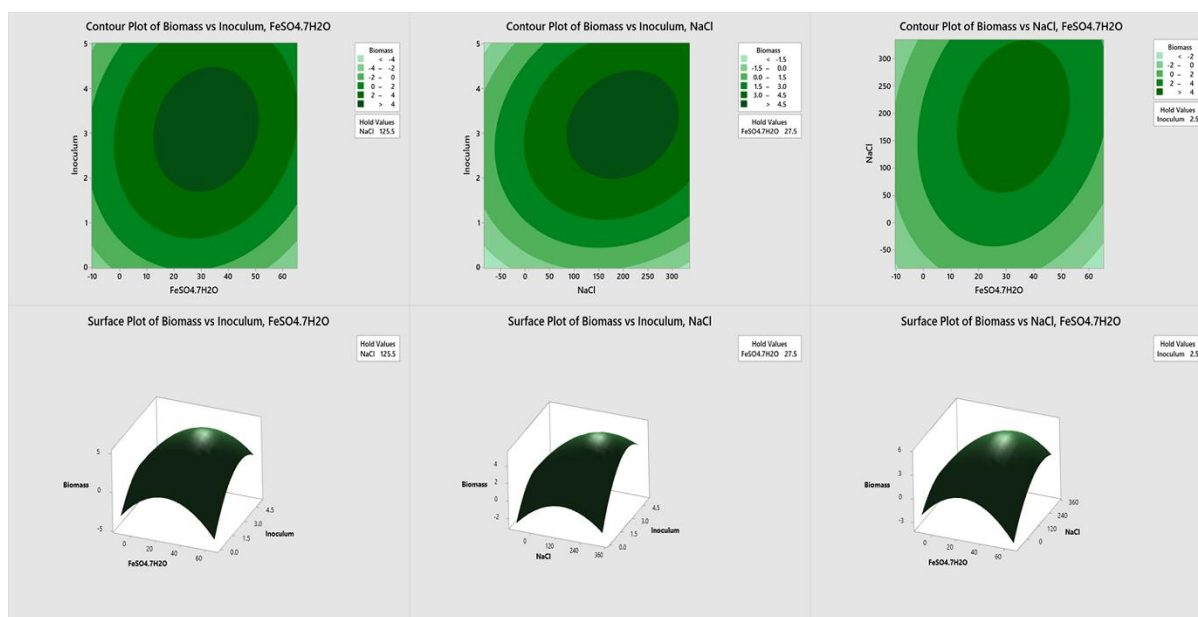
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	1.998	2.486	0.803	0.948	0.901	0.567

Appendix Table 19: Multiple response prediction of CCD for *Staphylococcus sp.* GR2 in *n*-hexadecane MSM.

Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	3.29	212.214	34.762	5.06	5.348

$$\begin{aligned} \text{Biomass} = & 0.083 + 0.005 \text{ Time} + 0.000 \text{ NaCl} - 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - \\ & 0.000 \text{ Time} * \text{Time} + 0.000 \text{ NaCl} * \text{NaCl} - 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + \\ & 0.000 \text{ Time} * \text{NaCl} + 0.000 \text{ Time} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \end{aligned}$$

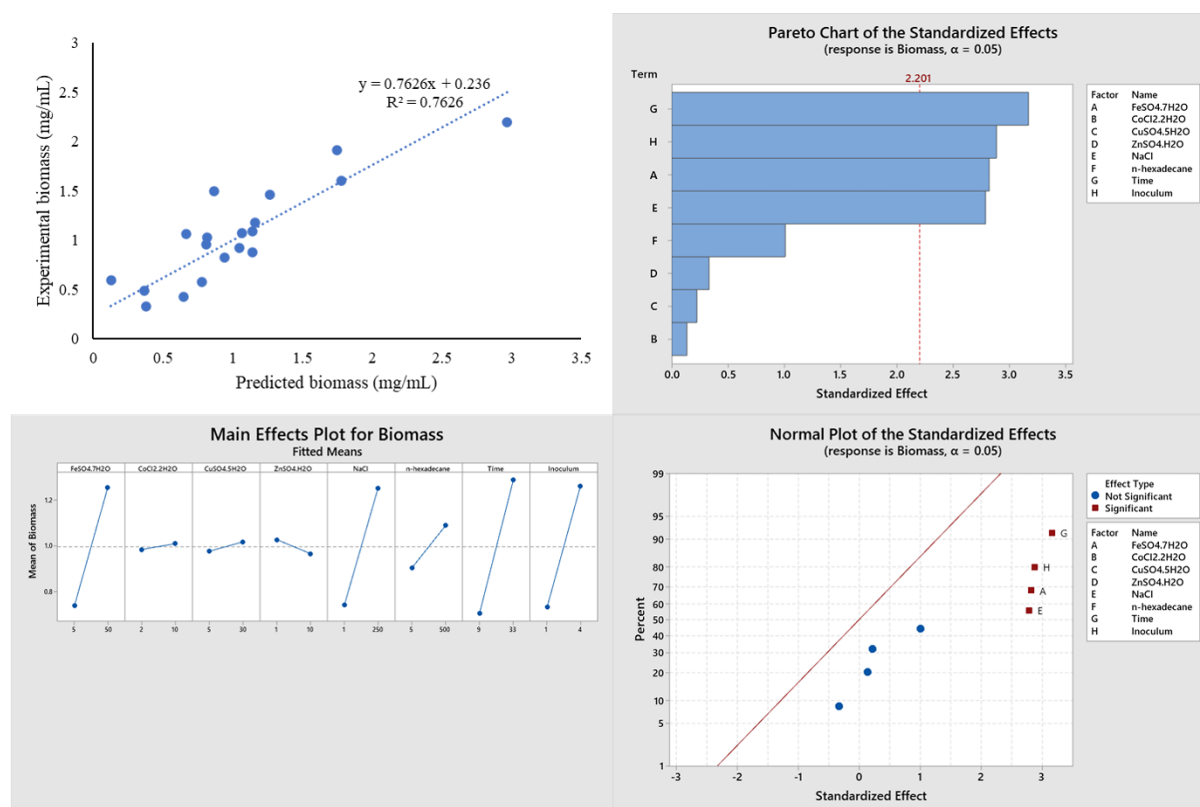
**Appendix
Equation 2**



Appendix Figure 9: Contour and surface plot of significant factors for *Staphylococcus sp.* GR2 in n-hexadecane MSM.

○ *Bacillus* sp. GE1Appendix Table 20: PBD of *Bacillus* sp. GE1 in *n*-hexadecane supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	<i>n</i> - hexadecane (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	5	39	4	0.871
2	5	10	30	1	250	500	9	1	0.132
3	50	10	5	10	250	5	9	1	0.945
4	50	2	5	10	250	5	39	4	1.752
5	50	2	30	1	250	500	39	4	2.973
6	50	10	30	1	1	500	39	1	1.164
7	5	10	5	10	250	500	39	1	1.072
8	50	2	5	1	1	500	9	4	0.672
9	5	2	30	10	1	500	39	1	0.78
10	5	2	5	1	1	5	9	1	0.181
11	5	10	30	1	1	5	9	4	0.653
12	5	2	5	1	250	5	39	1	1.055
13	50	10	5	1	1	5	39	1	0.812
14	5	2	5	10	1	500	9	4	0.377
15	50	2	30	10	250	500	9	1	0.823
16	5	10	5	10	1	500	39	4	1.149
17	5	10	30	10	250	5	9	4	1.141
18	50	10	30	10	1	5	39	4	1.272
19	50	10	5	1	250	500	9	4	1.785
20	50	2	30	10	1	5	9	1	0.381



Appendix Figure 10: Precision, pareto chart, main effects plot and normal plot for PBD for *Bacillus* sp. GE1 in *n*-hexadecane MSM.

Appendix Table 21: ANOVA and model for PBD for *Bacillus sp.* GE1 in *n*-hexadecane MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	5.93	0.7412	4.42	0.013
Linear	8	5.93	0.7412	4.42	0.013
FeSO ₄ .7H ₂ O	1	1.336	0.023	7.96	0.017
CoCl ₂ .2H ₂ O	1	0.003	0.002	0.02	0.894
CuSO ₄ .5H ₂ O	1	0.008	0.003	0.05	0.827
ZnSO ₄ .H ₂ O	1	0.019	0	0.11	0.745
NaCl	1	1.306	0.022	7.78	0.018
<i>n</i> -hexadecane		0.171	0.037	1.02	0.334
Time		1.688	0.054	10.06	0.009
Inoculum		1.399	0	8.34	0.015
Error			11	1.846	0.168
Total			19	7.776	

Model Summary			
S	R-sq	R-sq(adj)	R-sq(pred)
0.41	76.26%	59%	31.53%

$$\begin{aligned} \text{Biomass} = & -0.631 + 0.011 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.003 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} \\ & + 0.002 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.007 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.002 \text{ NaCl} + 0.000 \text{ } n\text{-hexadecane} \\ & + 0.024 \text{ Time} + 0.176 \text{ Inoculum} \end{aligned}$$

**Appendix
Equation 3**

Appendix Table 22: CCD of *Bacillus sp.* GE1 in *n*-hexadecane supplemented MSM.

- Concentration of *n*-hexadecane = 250 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Time (days)	Biomass (mg/mL)
1	27.5	125.5	0.818	0.011
2	65.34	125.5	21	0.554
3	27.5	125.5	9	0.134
4	50	1	41.182	3.463
5	5	250	21	2.994
6	50	1	21	3.081
7	27.5	125.5	21	3.06
8	27.5	125.5	33	1.05
9	27.5	334.883	21	1.053
10	5	1	21	0.093
11	50	250	21	3.071
12	27.5	125.5	33	0.244
13	27.5	125.5	33	3.422
14	-10.34	125.5	21	3.07
15	5	1	21	3.183
16	50	250	9	0.241
17	27.5	125.5	9	0.114
18	5	250	9	0.132
19	27.5	125.5	33	0.462
20	27.5	-83.883	21	2.991

Appendix Table 23: ANOVA for CCD of *Bacillus sp.* GE1 in *n*-hexadecane MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	35.719	3.969	15.17	0
Linear	3	17.038	5.679	21.71	0
FeSO ₄ .7H ₂ O	1	2.042	2.042	7.81	0.019
NaCl	1	3.483	3.483	13.31	0.004
Time	1	11.513	11.513	44	0
Square	3	16.169	5.39	20.6	0
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	10.547	10.547	40.31	0
NaCl*NaCl	1	4.524	4.524	17.29	0.002
Time*Time	1	3.998	3.998	15.28	0.003
2-Way Interaction	3	2.512	0.837	3.2	0.071
FeSO ₄ .7H ₂ O*NaCl	1	0.039	0.039	0.15	0.707
FeSO ₄ .7H ₂ O*Time	1	2.184	2.184	8.35	0.016
NaCl*Time	1	0.289	0.289	1.1	0.318
Error	10	2.616	0.262		
Lack-of-Fit	5	2.608	0.522	298.57	0
Pure Error	5	0.009	0.002		
Total	19	38.335			

Appendix Table 24: Model summary of CCD for *Bacillus sp.* GE1 in *n*-hexadecane MSM.

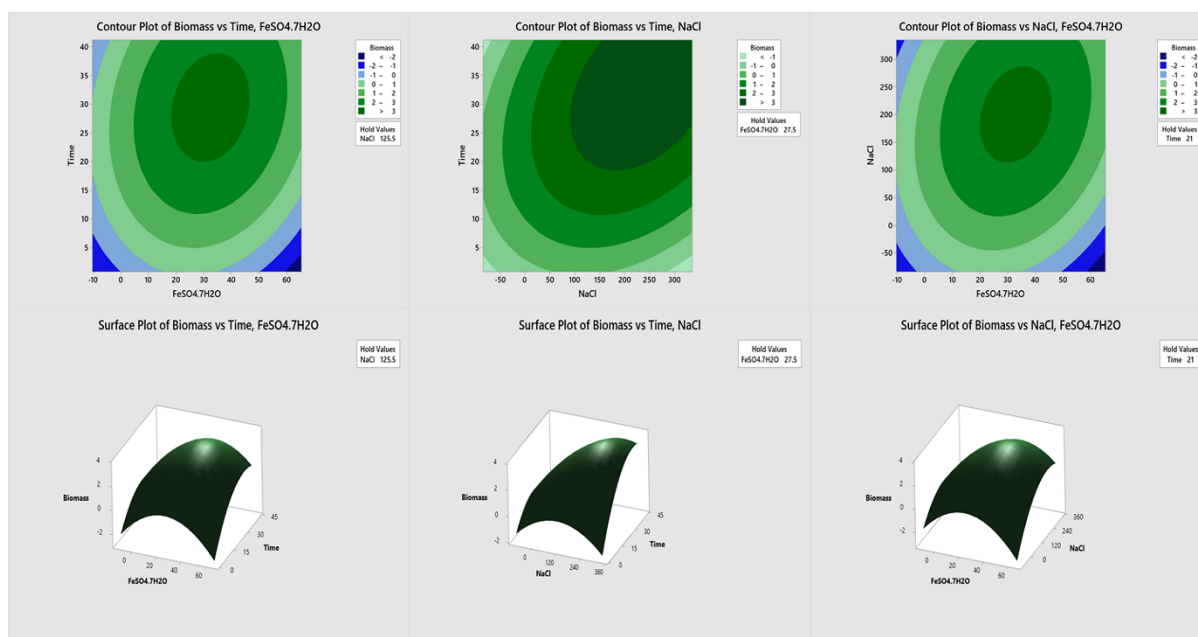
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	1.448	1.618	0.895	0.92	0.849	0.568

Appendix Table 25: Multiple response prediction for CCD of *Bacillus sp.* GE1 in *n*-hexadecane supplemented MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	35.474	267.204	38.585	3.46	3.948

$$\begin{aligned} \text{Biomass} = & -1.223 + 0.074 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.006 \text{NaCl} + 0.169 \text{Time} - \\ & 0.002 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} - 0.004 \text{Time} * \text{Time} \\ & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.001 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Time} + 0.000 \text{NaCl} * \text{Time} \end{aligned}$$

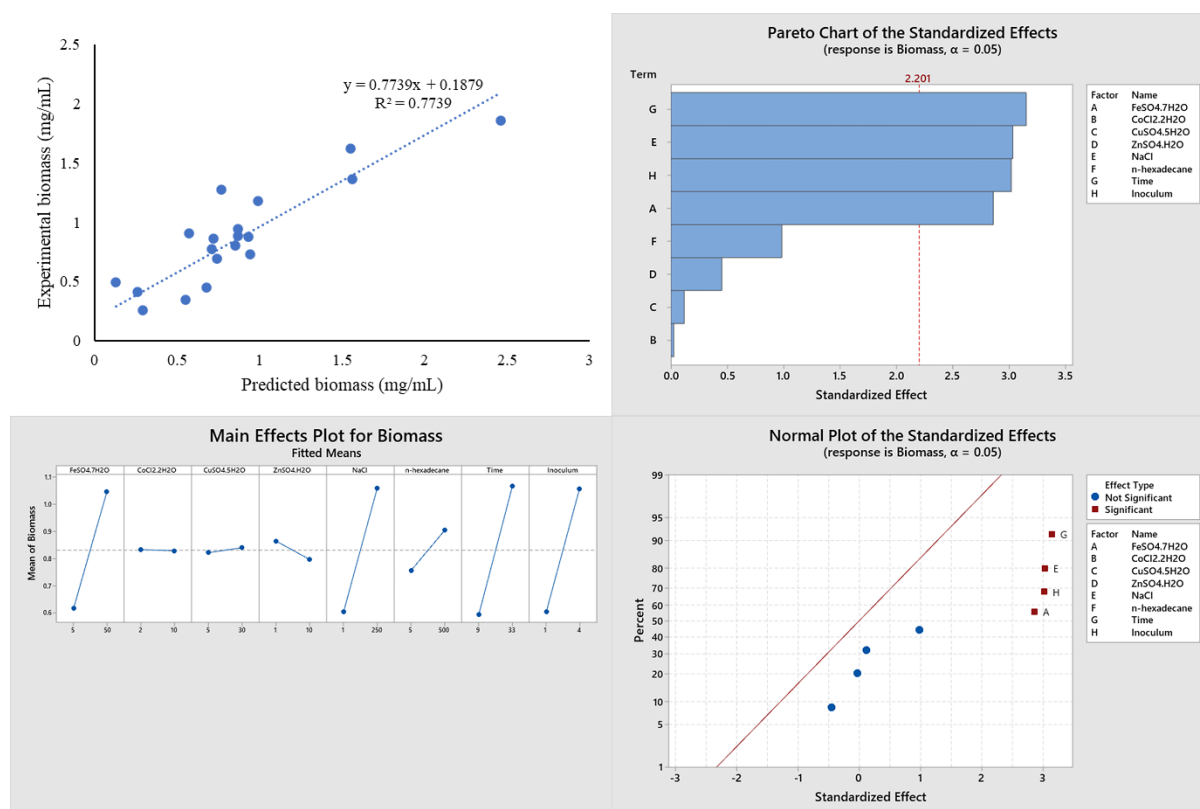
**Appendix
Equation 4**



Appendix Figure 11: Contour and surface plots of significant factors for *Bacillus sp.* GE1 in *n*-hexadecane MSM.

○ *Aquamicrobium* sp. GE2Appendix Table 26: PBD of *Aquamicrobium* sp. GE2 in *n*-hexadecane supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	<i>n</i> - hexadecane (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	5	39	4	0.773
2	5	10	30	1	250	500	9	1	0.132
3	50	10	5	10	250	5	9	1	0.746
4	50	2	5	10	250	5	39	4	1.553
5	50	2	30	1	250	500	39	4	2.468
6	50	10	30	1	1	500	39	1	0.871
7	5	10	5	10	250	500	39	1	0.872
8	50	2	5	1	1	500	9	4	0.575
9	5	2	30	10	1	500	39	1	0.683
10	5	2	5	1	1	5	9	1	0.182
11	5	10	30	1	1	5	9	4	0.556
12	5	2	5	1	250	5	39	1	0.851
13	50	10	5	1	1	5	39	1	0.712
14	5	2	5	10	1	500	9	4	0.261
15	50	2	30	10	250	500	9	1	0.721
16	5	10	5	10	1	500	39	4	0.931
17	5	10	30	10	250	5	9	4	0.943
18	50	10	30	10	1	5	39	4	0.991
19	50	10	5	1	250	500	9	4	1.561
20	50	2	30	10	1	5	9	1	0.294



Appendix Figure 12: Precision, pareto chart, main effects plot and normal plot for PBD of *Aquamicrobium sp.* GE2 in *n*-hexadecane supplemented MSM.

Appendix Table 27: ANOVA and model summary of PBD for *Aquamicrobium sp.* GE2 in *n*-hexadecane MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	4.252	0.532	4.71	0.01
Linear	8	4.252	0.532	4.71	0.01
FeSO ₄ .7H ₂ O	1	0.925	0.023	8.18	0.015
CoCl ₂ .2H ₂ O	1	0	0.002	0.00	0.979
CuSO ₄ .5H ₂ O	1	0.002	0.003	0.01	0.907
ZnSO ₄ .H ₂ O	1	0.023	0	0.20	0.66
NaCl	1	0.04	0.022	9.20	0.011
<i>n</i> -hexadecane		0.11	0.037	0.97	0.346
Time		1.123	0.054	9.94	0.009
Inoculum		1.031	0	9.12	0.012
Error			11	1.243	0.113
Total			19	5.495	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.336	77.39%	60.94%	35.24%

Biomass = $-0.498 + 0.01 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.001 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O}$
 $+ 0.001 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.008 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.002 \text{ NaCl} + 0.000 \text{ n-hexadecane}$
 $+ 0.02 \text{ Time} + 0.151 \text{ Inoculum}$

**Appendix
Equation 5**

Appendix Table 28: CCD of *Aquamicrobium* sp. GE2 in *n*-hexadecane MSM.

- Concentration of *n*-hexadecane = 250 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (days)	Biomass (mg/mL)
1	27.5	125.5	21	3.081
2	65.34	125.5	33	0.64
3	27.5	125.5	21	2.344
4	50	1	21	3.124
5	5	250	9	0.243
6	50	1	21	3.17
7	27.5	125.5	21	3.22
8	27.5	125.5	33	0.471
9	27.5	334.883	21	3.19
10	5	1	21	0.166
11	50	250	9	0.32
12	27.5	125.5	9	0.212
13	27.5	125.5	0.818	0.02
14	-10.34	125.5	41.182	3.712
15	5	1	9	0.144
16	50	250	33	1.266
17	27.5	125.5	21	0.225
18	5	250	21	3.161
19	27.5	125.5	33	3.467
20	27.5	-83.883	21	3.183

Appendix Table 29: ANOVA for CCD of *Aquamicrobium sp.* GE2 in *n*-hexadecane MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	38.094	4.233	15.4	0
Linear	3	20.811	6.937	25.25	0
FeSO ₄ .7H ₂ O	1	6.099	6.099	22.2	0.001
NaCl	1	9.713	9.713	35.35	0
Time	1	4.999	4.999	18.19	0.002
Square	3	14.151	14.151	17.17	0
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	7.851	7.851	28.57	0
NaCl*NaCl	1	5.01	5.01	18.23	0.002
Time*Time	1	3.987	3.987	14.51	0.003
2-Way Interaction	3	3.133	3.133	3.8	0.047
FeSO ₄ .7H ₂ O*NaCl	1	3.023	3.023	11.01	0.008
FeSO ₄ .7H ₂ O*Time	1	0.065	0.065	0.24	0.638
NaCl*Time	1	0.042	0.042	0.15	0.704
Error	10	2.748	2.748		
Lack-of-Fit	5	2.738	0.548	293.4	0
Pure Error	5	0.009	0.002		
Total	19	40.841			

Appendix Table 30: Model summary for CCD of *Aquamicrobium sp.* GE2 in *n*-hexadecane MSM.

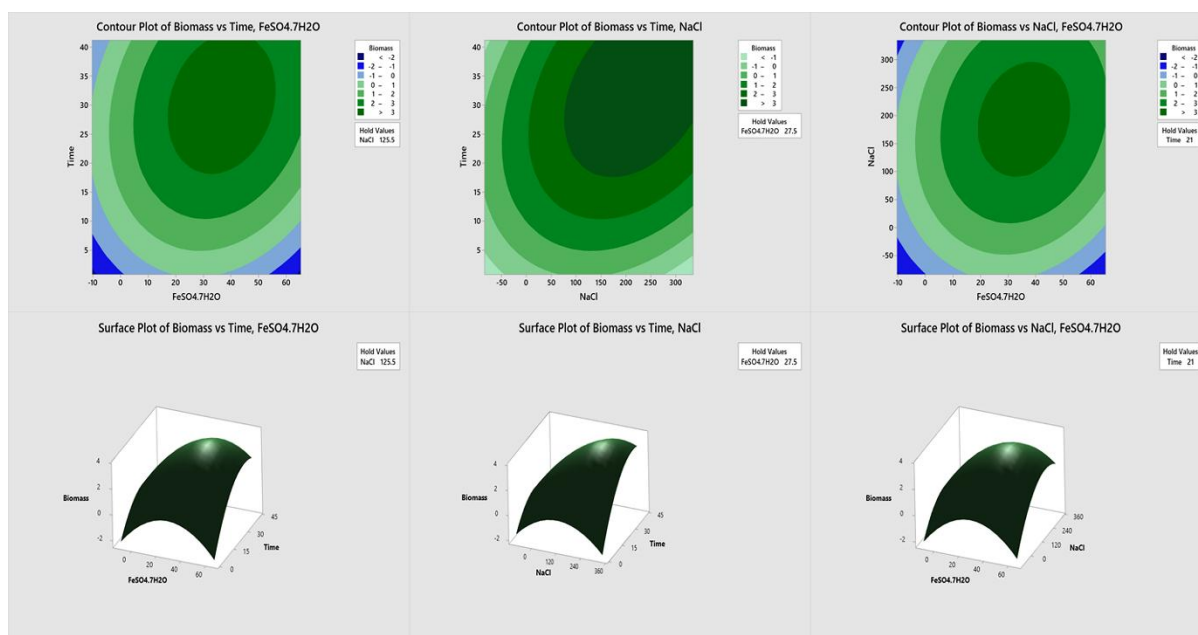
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	1.475	1.764	0.836	0.914	0.837	0.434

Appendix Table 31: Multiple response prediction of CCD for *Aquamicrobium sp.* GE2 in *n*-hexadecane MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	41.642	246.054	41.642	3.71	4.116

$$\begin{aligned} \text{Biomass} = & -1.520 + 0.076 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.007 \text{NaCl} + 0.179 \text{Time} \\ & - 0.002 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} - 0.004 \text{Time} * \text{Time} \\ & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.001 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Time} + 0.000 \text{NaCl} * \text{Time} \end{aligned}$$

Appendix Equation 6



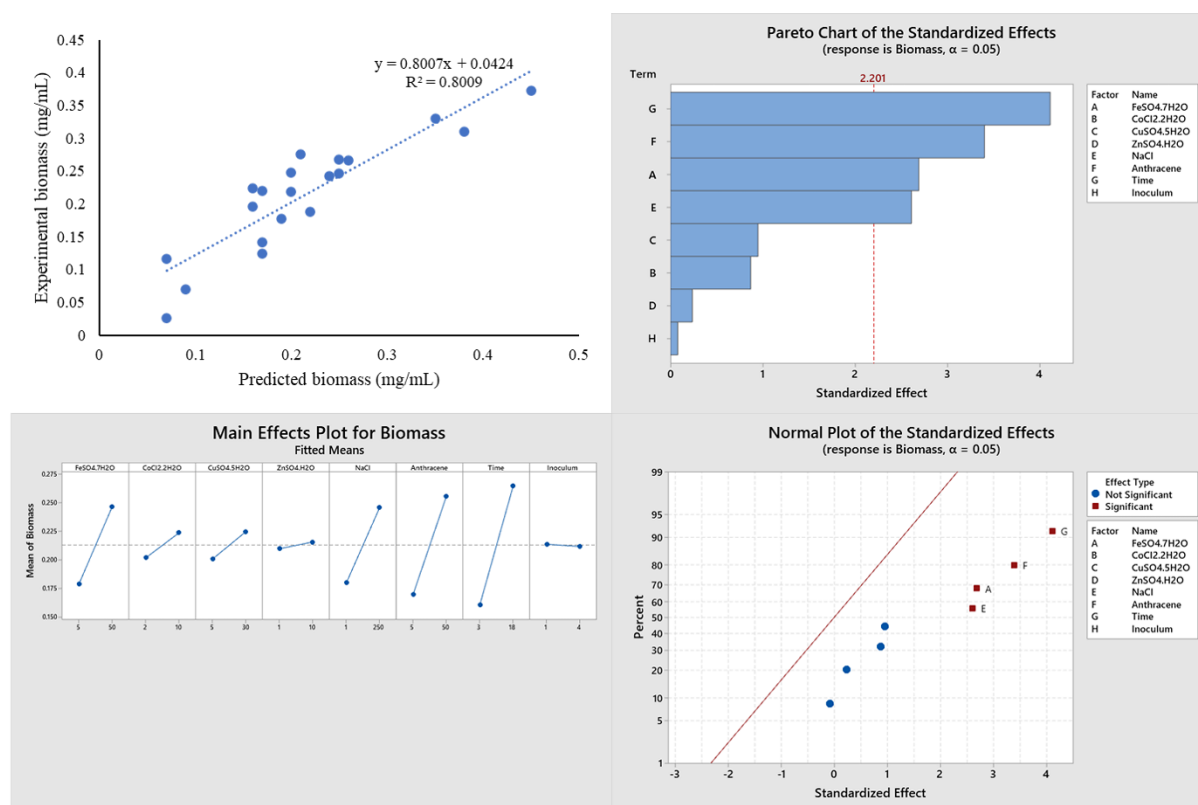
Appendix Figure 13: Contour and surface plots of significant factors for *Aquamicrobium sp. GE2* in *n*-hexadecane MSM.

- Anthracene

- *Staphylococcus sp.* GR2

Appendix Table 32: PBD of *Staphylococcus sp.* GR2 in anthracene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Anthracene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	10	5	10	1	50	18	4	0.245
2	50	10	30	10	1	5	18	4	0.27
3	5	10	5	10	250	50	18	1	0.383
4	5	10	30	1	1	5	3	4	0.092
5	50	10	5	1	250	50	3	4	0.261
6	50	2	30	10	1	5	3	1	0.178
7	5	2	5	1	1	5	3	1	0.075
8	5	2	5	1	250	5	18	1	0.169
9	50	10	5	10	250	5	3	1	0.223
10	50	10	5	1	1	5	18	1	0.175
11	50	2	5	10	250	5	18	4	0.251
12	5	2	30	10	1	50	18	1	0.257
13	50	10	30	1	1	50	18	1	0.353
14	5	2	5	10	1	50	3	4	0.072
15	5	10	30	1	250	50	3	1	0.164
16	5	10	30	10	250	5	3	4	0.171
17	50	2	5	1	1	50	3	4	0.196
18	50	2	30	1	250	50	18	4	0.452
19	5	2	30	1	250	5	18	4	0.212
20	50	2	30	10	250	50	3	1	0.211



Appendix Figure 14: Precision, pareto chart, main effects plot and normal plot for PBD of *Staphylococcus sp.* GR2 in anthracene supplemented MSM.

Appendix Table 33: ANOVA and model summary for PBD of *Staphylococcus sp.* GR2 in anthracene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.1415	0.018	5.53	0.006
Linear	8	0.1415	0.018	5.53	0.006
FeSO ₄ ·7H ₂ O	1	0.023	0.023	7.23	0.021
CoCl ₂ ·2H ₂ O	1	0.002	0.002	0.76	0.403
CuSO ₄ ·5H ₂ O	1	0.003	0.003	0.9	0.363
ZnSO ₄ ·H ₂ O	1	0	0	0.06	0.817
NaCl	1	0.022	0.022	6.81	0.024
Anthracene		0.037	0.037	11.57	0.006
Time		0.054	0.054	16.92	0.002
Inoculum		0	0	0.01	0.938
Error			11	0.035	0.003
Total			19	0.177	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.057	80.09%	65.61%	44.19%

Biomass = $-0.023 + 0.002 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.003 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O}$
 $+ 0.001 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O} + 0.001 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.002 \text{ Anthracene}$
 $+ 0.007 \text{ Time} - 0.001 \text{ Inoculum}$

**Appendix
Equation 7**

Appendix Table 34: CCD of *Staphylococcus sp.* GR2 in anthracene supplemented MSM.

- Concentration of anthracene = 50 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (days)	Biomass (mg/mL)
1	27.5	125.5	18	0.433
2	65.34	125.5	10.5	0.217
3	27.5	125.5	23.113	0.312
4	50	1	3	0.079
5	5	250	10.5	0.273
6	50	1	3	0.116
7	27.5	125.5	10.5	0.211
8	27.5	125.5	18	0.245
9	27.5	334.883	3	0.176
10	5	1	10.5	0.153
11	50	250	3	0.117
12	27.5	125.5	10.5	0.211
13	27.5	125.5	18	0.15
14	-10.34	125.5	10.5	0.133
15	5	1	-2.113	0.079
16	50	250	18	0.217
17	27.5	125.5	10.5	0.214
18	5	250	10.5	0.201
19	27.5	125.5	10.5	0.225
20	27.5	-83.883	10.5	0.191

Appendix Table 35: ANOVA for CCD of *Staphylococcus sp.* GR2 in anthracene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.128	0.014	30.87	0
Linear	3	0.115	0.038	83.16	0
Time	1	0.069	0.069	150.83	0
NaCl	1	0.015	0.015	32.35	0
FeSO ₄ .7H ₂ O	1	0.031	0.031	66.29	0
Square	3	0.002	0	1.38	0.304
Time*Time	1	0.001	0	1.31	0.279
NaCl*NaCl	1	0.001	0.001	3.14	0.107
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.27	0.615
2-Way Interaction	3	0.011	0.004	8.07	0.005
Time*NaCl	1	0.003	0.003	6.11	0.033
Time*FeSO ₄ .7H ₂ O	1	0.006	0.006	11.98	0.006
NaCl*FeSO ₄ .7H ₂ O	1	0.003	0.003	6.11	0.033
Error	10	0.005	0		
Lack-of-Fit	5	0.004	0.001	7.63	0.022
Pure Error	5	0.001	0		
Total	19	0.132			

Appendix Table 36: Model summary for CCD of *Staphylococcus sp.* GR2 in anthracene MSM.

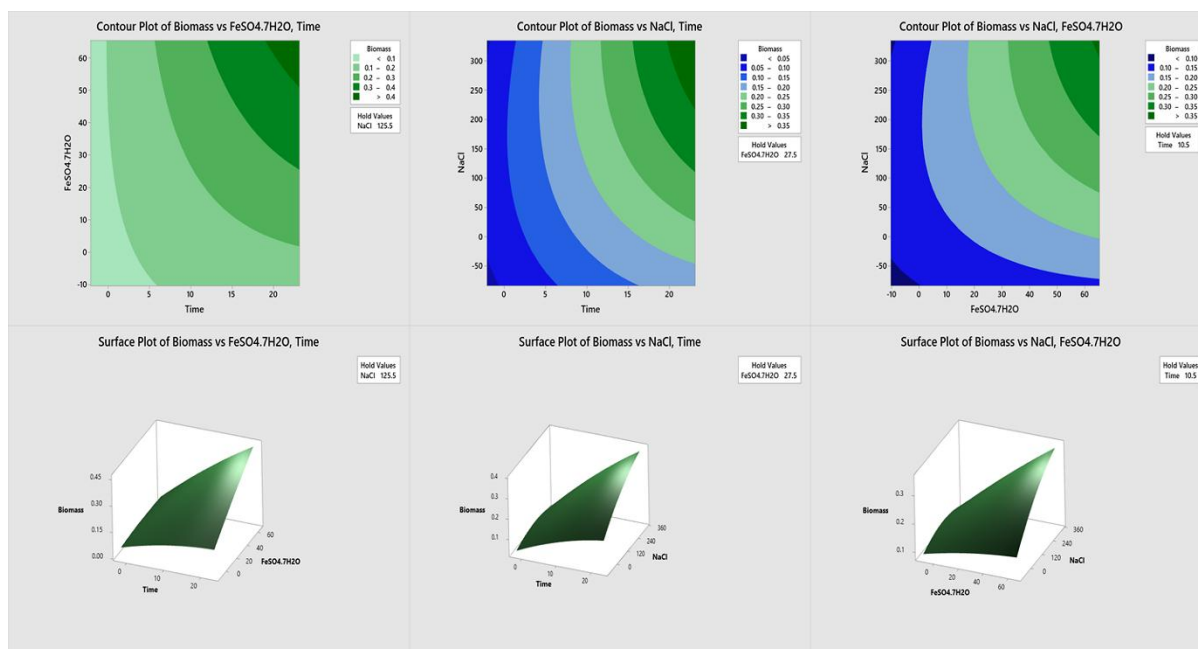
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.084	0.194	0.432	0.965	0.934	0.743

Appendix Table 37: Multiple response prediction for CCD of *Staphylococcus sp.* GR2 in anthracene MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	23.114	334.883	65.34	0.43	0.587

$$\begin{aligned}
 \text{Biomass} = & -1.827 + 0.102 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.01 \text{NaCl} + 2.277 \text{Inoculum} \\
 & - 0.002 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} - \\
 & 0.447 \text{Inoculum} * \text{Inoculum} + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} \\
 & + 0.01 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Inoculum} + 0.002 \text{NaCl} * \text{Inoculum}
 \end{aligned}$$

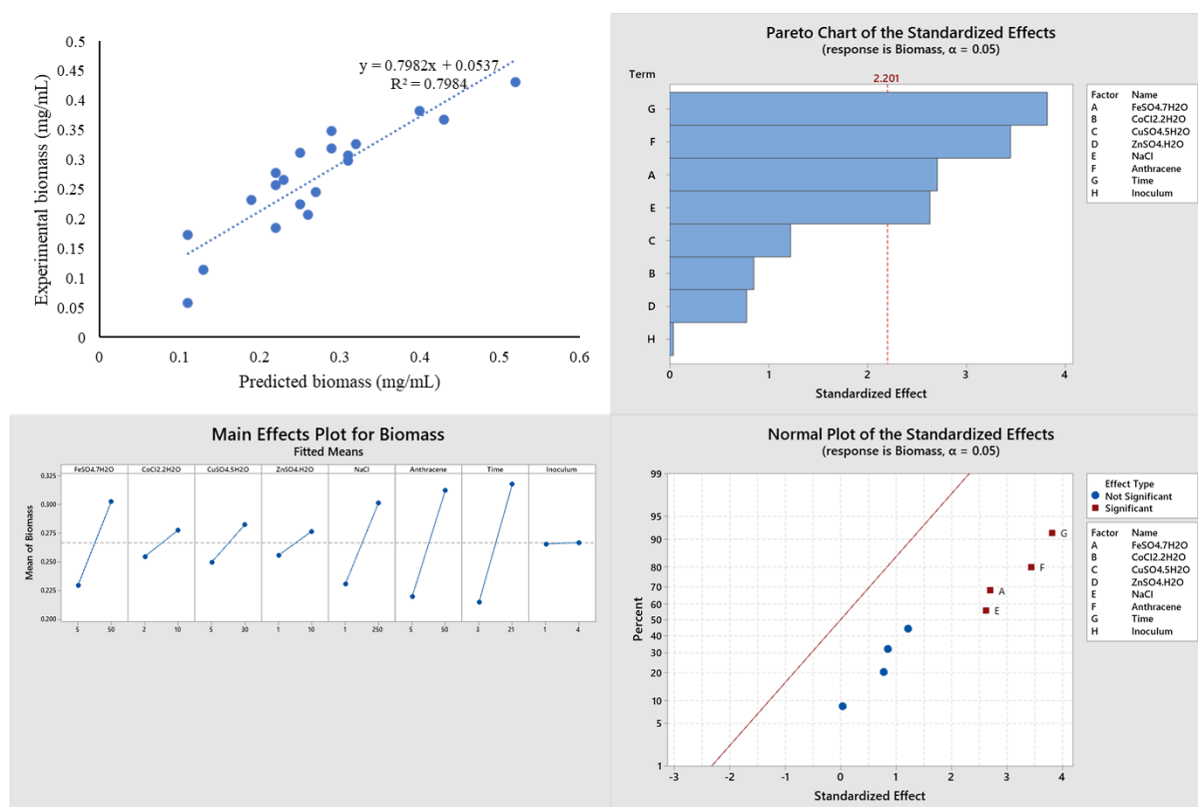
**Appendix
Equation 8**



Appendix Figure 15: Contour and surface plots of significant factors for *Staphylococcus sp.* GR2 in anthracene MSM.

○ *Aquamicrobium* sp. GE2Appendix Table 38: PBD of *Aquamicrobium* sp. GE2 in anthracene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Anthracene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	10	5	10	1	50	24	4	0.311
2	50	10	30	10	1	5	24	4	0.256
3	5	10	5	10	250	50	24	1	0.433
4	5	10	30	1	1	5	3	4	0.138
5	50	10	5	1	250	50	3	4	0.294
6	50	2	30	10	1	5	3	1	0.229
7	5	2	5	1	1	5	3	1	0.115
8	5	2	5	1	250	5	24	1	0.191
9	50	10	5	10	250	5	3	1	0.279
10	50	10	5	1	1	5	24	1	0.224
11	50	2	5	10	250	5	24	4	0.322
12	5	2	30	10	1	50	24	1	0.316
13	50	10	30	1	1	50	24	1	0.412
14	5	2	5	10	1	50	3	4	0.117
15	5	10	30	1	250	50	3	1	0.226
16	5	10	30	10	250	5	3	4	0.261
17	50	2	5	1	1	50	3	4	0.251
18	50	2	30	1	250	50	24	4	0.526
19	5	2	30	1	250	5	24	4	0.234
20	50	2	30	10	250	50	3	1	0.299



Appendix Figure 16: Precision, pareto chart, main effects plot and normal plot for PBD of *Aquamicrobium* sp. GE2 in anthracene supplemented MSM.

Appendix Table 39: ANOVA and model summary for PBD of *Aquamicrobium* sp. GE2 in anthracene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.158	0.02	5.44	0.006
Linear	8	0.158	0.02	5.44	0.006
FeSO ₄ .7H ₂ O	1	0.027	0.027	7.32	0.02
CoCl ₂ .2H ₂ O	1	0.003	0.003	0.73	0.412
CuSO ₄ .5H ₂ O	1	0.005	0.005	1.5	0.247
ZnSO ₄ .H ₂ O	1	0.002	0.002	0.61	0.453
NaCl	1	0.025	0.025	6.93	0.023
Anthracene		0.043	0.043	11.89	0.005
Time		0.053	0.053	14.58	0.003
Inoculum		0	0	0	0.971
Error			11	0.04	0.004
Total			19	0.198	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.06	79.84%	65.15%	43.34%

Biomass = 0.007 + 0.002 FeSO₄·7H₂O + 0.003 CoCl₂·2H₂O
+ 0.001 CuSO₄·5H₂O + 0.002 ZnSO₄·H₂O + 0.000 NaCl + 0.002 Anthracene
+ 0.00572 Time + 0.000 Inoculum

Appendix
Equation 9Appendix Table 40: CCD of *Aquamicrobium* sp. GE2 in anthracene supplemented MSM.

- Concentration of anthracene = 50 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Time (days)	Biomass (mg/mL)
1	27.5	125.5	12	0.171
2	65.34	125.5	12	0.216
3	27.5	125.5	21	0.113
4	50	1	12	0.207
5	5	250	-3.136	0.071
6	50	1	3	0.142
7	27.5	125.5	3	0.161
8	27.5	125.5	3	0.076
9	27.5	334.883	12	0.205
10	5	1	27.136	0.289
11	50	250	12	0.181
12	27.5	125.5	12	0.214
13	27.5	125.5	21	0.452
14	-10.34	125.5	12	0.208
15	5	1	3	0.158
16	50	250	21	0.211
17	27.5	125.5	12	0.276
18	5	250	12	0.201
19	27.5	125.5	21	0.269
20	27.5	-83.883	12	0.294

Appendix Table 41: ANOVA for CCD of *Aquamicrobium sp.* GE2 in anthracene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.129	0.014	19.29	0
Linear	3	0.109	0.036	48.88	0
Time	1	0.055	0.055	73.31	0
NaCl	1	0.021	0.021	28.5	0
FeSO ₄ .7H ₂ O	1	0.033	0.033	44.83	0
Square	3	0.004	0.004	1.77	0.217
Time*Time	1	0.003	0.003	3.55	0.089
NaCl*NaCl	1	0	0	0.11	0.748
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	1.14	0.31
2-Way Interaction	3	0.016	0.016	7.23	0.007
Time*NaCl	1	0.006	0.006	7.41	0.022
Time*FeSO ₄ .7H ₂ O	1	0.011	0.011	14.13	0.004
NaCl*FeSO ₄ .7H ₂ O	1	0	0	0.15	0.706
Error	10	0.007	0.007		
Lack-of-Fit	5	0.007	0.001	54.82	0
Pure Error	5	0	0		
Total	19	0.137			

Appendix Table 42: Model summary for CCD of *Aquamicrobium sp.* GE2 in anthracene MSM.

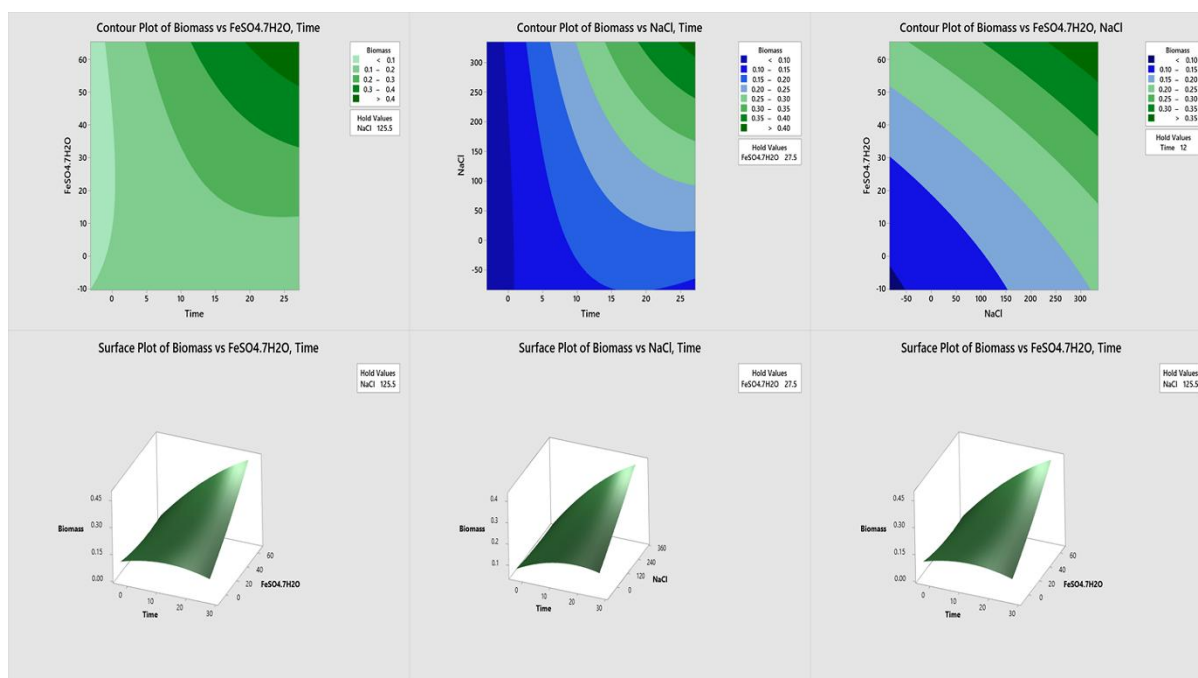
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.085	0.202	0.421	0.946	0.897	0.56

Appendix Table 43: Multiple response prediction for CCD of *Aquamicrobium sp.* GE2 in anthracene MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	27.136	334.883	65.34	0.45	0.637

$$\begin{aligned} \text{Biomass} = & 0.108 + 0.003 \text{ Time} - 0.000 \text{ NaCl} - 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - \\ & 0.000 \text{ Time} * \text{Time} + 0.000 \text{ NaCl} * \text{NaCl} + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \\ & + 0.000 \text{ Time} * \text{NaCl} + 0.000 \text{ Time} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \end{aligned}$$

**Appendix
Equation 10**

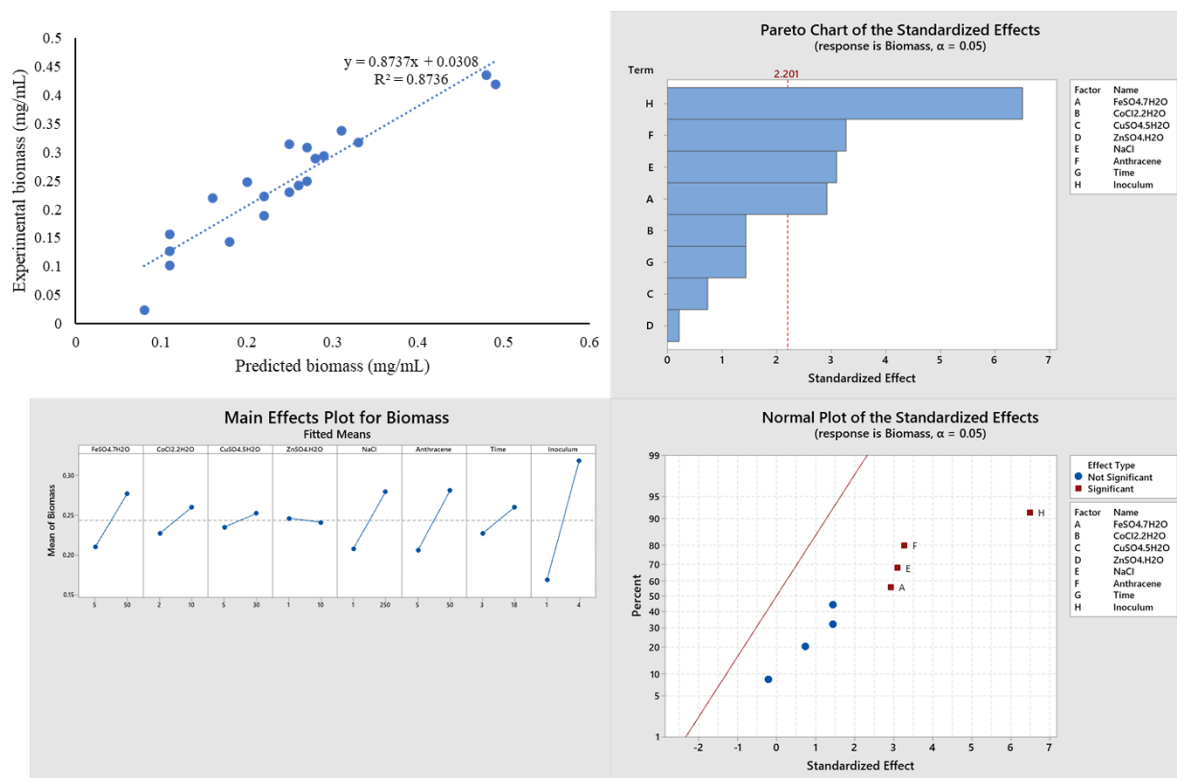


Appendix Figure 17: Contour and surface plot of significant factors for *Aquamicrobium* sp. in anthracene MSM.

○ *Bacillus sp.* GS1

Appendix Table 44: PBD of *Bacillus sp.* GS1 in anthracene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Anthracene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	10	5	10	1	50	24	4	0.273
2	50	10	30	10	1	5	24	4	0.333
3	5	10	5	10	250	50	24	1	0.256
4	5	10	30	1	1	5	3	4	0.223
5	50	10	5	1	250	50	3	4	0.498
6	50	2	30	10	1	5	3	1	0.115
7	5	2	5	1	1	5	3	1	0.086
8	5	2	5	1	250	5	24	1	0.119
9	50	10	5	10	250	5	3	1	0.221
10	50	10	5	1	1	5	24	1	0.116
11	50	2	5	10	250	5	24	4	0.314
12	5	2	30	10	1	50	24	1	0.188
13	50	10	30	1	1	50	24	1	0.273
14	5	2	5	10	1	50	3	4	0.262
15	5	10	30	1	250	50	3	1	0.165
16	5	10	30	10	250	5	3	4	0.282
17	50	2	5	1	1	50	3	4	0.251
18	50	2	30	1	250	50	24	4	0.488
19	5	2	30	1	250	5	24	4	0.299
20	50	2	30	10	250	50	3	1	0.225



Appendix Figure 18: Precision, pareto chart, main effects plot and normal plot for PBD of *Bacillus sp.* GS1 in anthracene supplemented MSM.

Appendix Table 45: ANOVA and model summary for PBD of *Bacillus sp.* GS1 in anthracene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.199	0.025	9.51	0.001
Linear	8	0.199	0.025	9.51	0.001
FeSO ₄ .7H ₂ O	1	0.022	0.022	8.57	0.014
CoCl ₂ .2H ₂ O	1	0.005	0.005	2.08	0.177
CuSO ₄ .5H ₂ O	1	0.001	0.001	0.55	0.473
ZnSO ₄ .H ₂ O	1	0	0	0.05	0.831
NaCl	1	0.025	0.025	9.62	0.01
Anthracene		0.028	0.028	10.74	0.007
Time		0.005	0.005	2.08	0.177
Inoculum		0.111	0.111	42.38	0
Error	11		0.039		0.004
Total	19		0.157		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.051	87.36%	78.18%	58.23%

Biomass = $-0.06 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.004 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O}$
 $+ 0.001 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.001 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.002 \text{ Anthracene}$
 $+ 0.002 \text{ Time} + 0.05 \text{ Inoculum}$

Appendix
Equation 11Appendix Table 46: CCD of *Bacillus* sp. GS1 in anthracene supplemented MSM.

- Concentration of anthracene = 50 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (days)	Biomass (mg/mL)
1	27.5	125.5	12	0.383
2	65.34	125.5	12	0.211
3	27.5	125.5	21	0.287
4	50	1	12	0.074
5	5	250	-3.136	0.241
6	50	1	3	0.118
7	27.5	125.5	3	0.205
8	27.5	125.5	3	0.212
9	27.5	334.883	12	0.179
10	5	1	27.136	0.154
11	50	250	12	0.111
12	27.5	125.5	12	0.187
13	27.5	125.5	21	0.155
14	-10.34	125.5	12	0.131
15	5	1	3	0.078
16	50	250	21	0.204
17	27.5	125.5	12	0.19
18	5	250	12	0.193
19	27.5	125.5	21	0.182
20	27.5	-83.883	12	0.164

Appendix Table 47: ANOVA for CCD of *Bacillus sp.* GS1 in anthracene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.091	0.01	23.94	0
Linear	3	0.084	0.028	66.31	0
Inoculum	1	0.051	0.051	120.39	0
NaCl	1	0.013	0.013	30.73	0
FeSO ₄ .7H ₂ O	1	0.02	0.02	47.8	0
Square	3	0	0	0.22	0.883
Inoculum*Inoculum	1	0	0	0.53	0.481
NaCl*NaCl	1	0	0	0.16	0.694
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.01	0.939
2-Way Interaction	3	0.007	0.002	5.29	0.019
Inoculum*NaCl	1	0.002	0.002	4.26	0.066
Inoculum*FeSO ₄ .7H ₂ O	1	0.002	0.002	5.8	0.037
NaCl*FeSO ₄ .7H ₂ O	1	0.002	0.002	5.8	0.037
Error	10	0.004	0		
Lack-of-Fit	5	0.003	0.001	3.52	0.097
Pure Error	5	0.001	0		
Total	19	0.095			

Appendix Table 48: Model summary for CCD of *Bacillus sp.* GS1 in anthracene MSM.

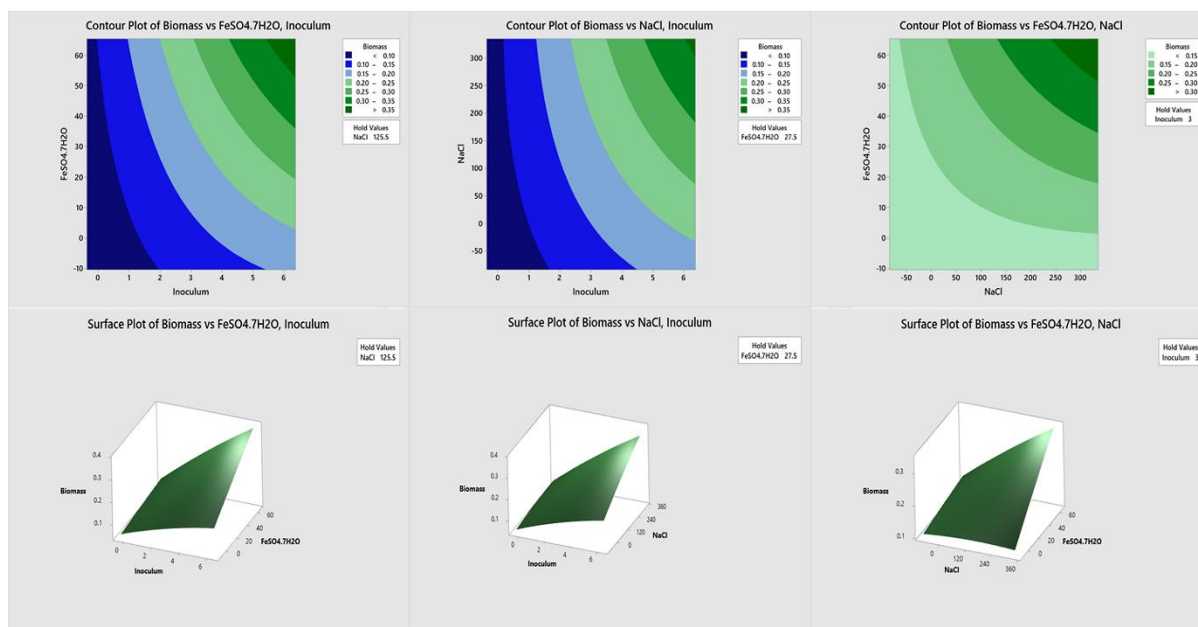
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.071	0.179	0.395	0.956	0.916	0.703

Appendix Table 49: Multiple response prediction for CCD of *Bacillus sp.* GS1 in anthracene MSM.

Multiple Response Prediction					
Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	6.366	334.883	65.34	0.45	0.525

$$\begin{aligned} \text{Biomass} = & 0.079 + 0.018 \text{ Inoculum} - 0.000 \text{ NaCl} - 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - \\ & 0.001 \text{ Inoculum} * \text{Inoculum} - 0.000 \text{ NaCl} * \text{NaCl} - \\ & 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ Inoculum} * \text{NaCl} \\ & + 0.000 \text{ Inoculum} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \end{aligned}$$

**Appendix
Equation 12**



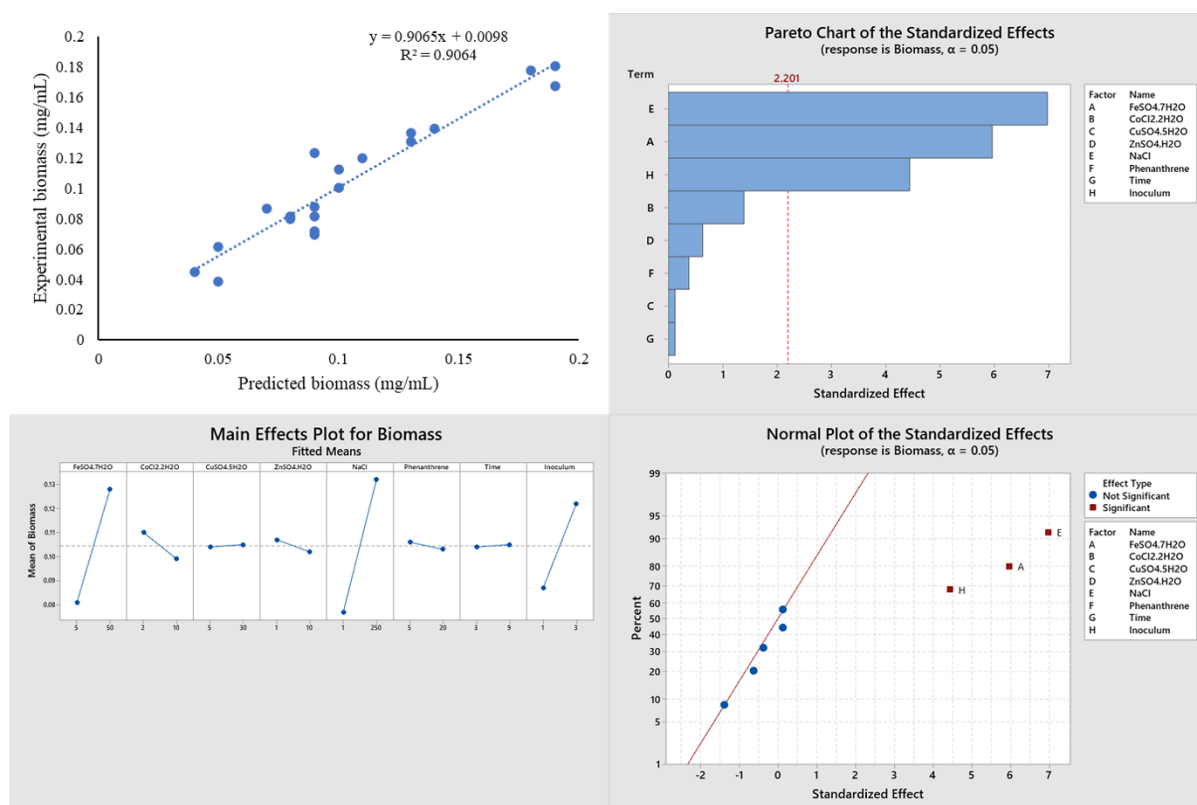
Appendix Figure 19: Contour and surface plots of significant factors for *Bacillus sp. GS1* in anthracene MSM.

• Phenanthrene

○ *Bacillus sp.* GE1

Appendix Table 50: PBD of *Bacillus sp.* GE1 in phenanthrene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Phenanthrene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	50	2	30	10	1	20	3	3	0.103
2	50	10	5	10	250	5	9	1	0.091
3	5	10	30	1	250	5	9	3	0.116
4	5	2	30	10	1	20	9	3	0.142
5	50	2	5	10	250	20	3	1	0.138
6	50	10	5	1	250	5	3	3	0.182
7	50	10	30	1	1	5	9	1	0.091
8	50	10	30	10	1	20	9	1	0.116
9	5	10	30	10	250	5	9	3	0.133
10	50	2	30	10	250	5	3	1	0.071
11	5	10	5	10	250	5	3	1	0.091
12	50	2	30	1	250	5	3	3	0.196
13	5	10	5	10	1	5	9	3	0.193
14	5	2	30	1	250	20	9	1	0.051
15	5	2	5	10	1	20	3	3	0.098
16	5	2	5	1	250	20	9	1	0.042
17	50	2	5	1	1	5	3	1	0.055
18	50	10	5	1	1	20	9	3	0.083
19	5	10	30	1	1	20	3	3	0.097
20	5	2	5	1	1	20	3	1	0.081



Appendix Figure 20: Precision, pareto chart, main effects plot and normal plot for PBD of *Bacillus* sp. GE1 in phenanthrene supplemented MSM.

Appendix Table 51: ANOVA and model summary for PBD of *Bacillus* sp. GE1 in phenanthrene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.033	0.004	13.32	0
Linear	8	0.033	0.004	13.32	0
FeSO ₄ .7H ₂ O	1	0.011	0.011	35.58	0
CoCl ₂ .2H ₂ O	1	0.001	0.001	1.95	0.19
CuSO ₄ .5H ₂ O	1	0	0	0.02	0.901
ZnSO ₄ .H ₂ O	1	0	0	0.4	0.539
NaCl	1	0.015	0.015	48.72	0
Phenanthrene		0	0	0.14	0.711
Time		0	0	0.02	0.901
Inoculum		0.006	0.006	19.73	0.001
Error			11	0.003	0
Total			19	0.036	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.018	90.64%	83.84%	69.07%

Biomass = 0.025 + 0.001 FeSO₄.7H₂O – 0.001 CoCl₂.2H₂O + 0.000
 CuSO₄.5H₂O – 0.001 ZnSO₄.H₂O + 0.000 NaCl + 0.000 Phenanthrene + 0.000
 Time + 0.018 Inoculum

Appendix
Equation 13Appendix Table 52: CCD of *Bacillus* sp. GE1 in phenanthrene supplemented MSM.

- Concentration of phenanthrene = 20 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ .7H ₂ O (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	125.5	3	0.151
2	65.34	125.5	2	0.133
3	27.5	125.5	2	0.121
4	50	1	1	0.144
5	5	250	1	0.082
6	50	1	2	0.051
7	27.5	125.5	3	0.142
8	27.5	125.5	1	0.071
9	27.5	334.883	2	0.119
10	5	1	2	0.146
11	50	250	3	0.212
12	27.5	125.5	1	0.054
13	27.5	125.5	2	0.12
14	–10.34	125.5	0.318	0.091
15	5	1	2	0.114
16	50	250	2	0.088
17	27.5	125.5	2	0.138
18	5	250	3.682	0.142
19	27.5	125.5	3	0.083
20	27.5	–83.883	2	0.123

Appendix Table 53: ANOVA for CCD of *Bacillus sp.* GE1 in phenanthrene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.026	0.003	14.02	0
Linear	3	0.025	0.008	40.31	0
FeSO ₄ .7H ₂ O	1	0.01	0.01	47.43	0
NaCl	1	0.008	0.008	36.75	0
Inoculum	1	0.008	0.008	36.75	0
Square	3	0.001	0	0.8	0.524
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0.001	0.001	2.39	0.153
NaCl*NaCl	1	0	0	0.02	0.881
Inoculum*Inoculum	1	0	0	0.02	0.881
2-Way Interaction	3	0	0	0.96	0.451
FeSO ₄ .7H ₂ O*NaCl	1	0	0	0.96	0.351
FeSO ₄ .7H ₂ O*Inoculum	1	0	0	0.96	0.351
NaCl*Inoculum	1	0	0	0.96	0.351
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	9.46	0.014
Pure Error	5	0	0		
Total	19	0.029			

Appendix Table 54: Model summary for CCD of *Bacillus sp.* GE1 in phenanthrene MSM.

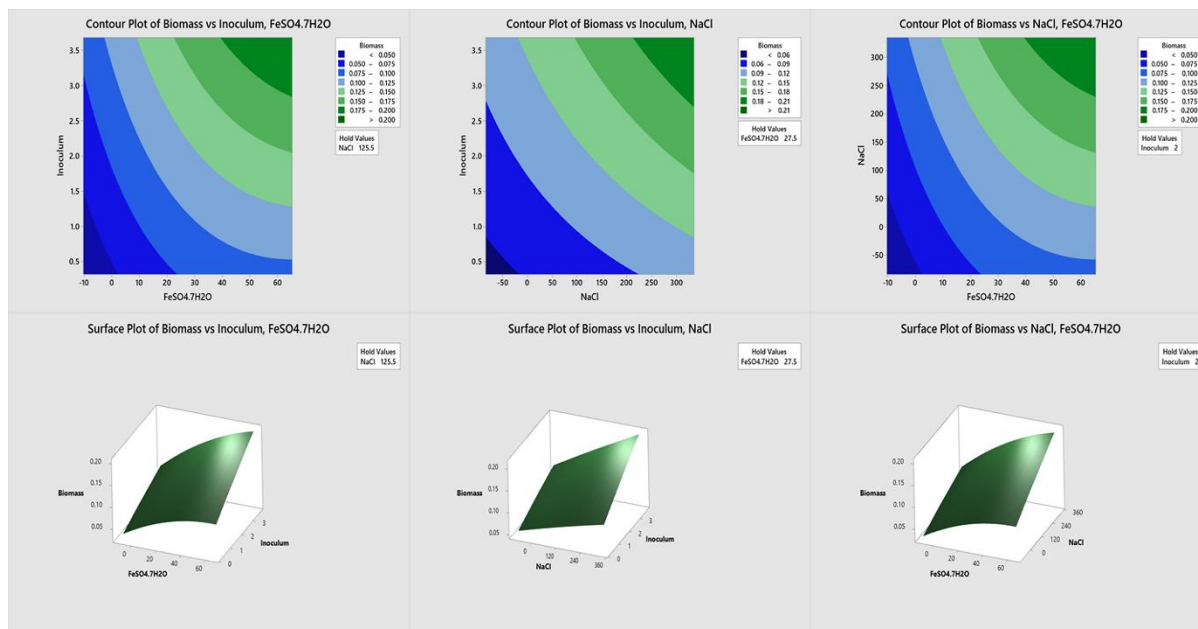
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.038	0.115	0.337	0.927	0.861	0.575

Appendix Table 55: Multiple response prediction for CCD of *Bacillus sp.* GE1 in phenanthrene MSM.

Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	3.682	334.883	65.34	0.21	0.267

$$\begin{aligned} \text{Biomass} = & 0.032 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.015 \text{ Inoculum} \\ & - 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{ NaCl} * \text{NaCl} - \\ & 0.001 \text{ Inoculum} * \text{Inoculum} + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} \\ & + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Inoculum} + 0.000 \text{ NaCl} * \text{Inoculum} \end{aligned}$$

**Appendix
Equation 14**



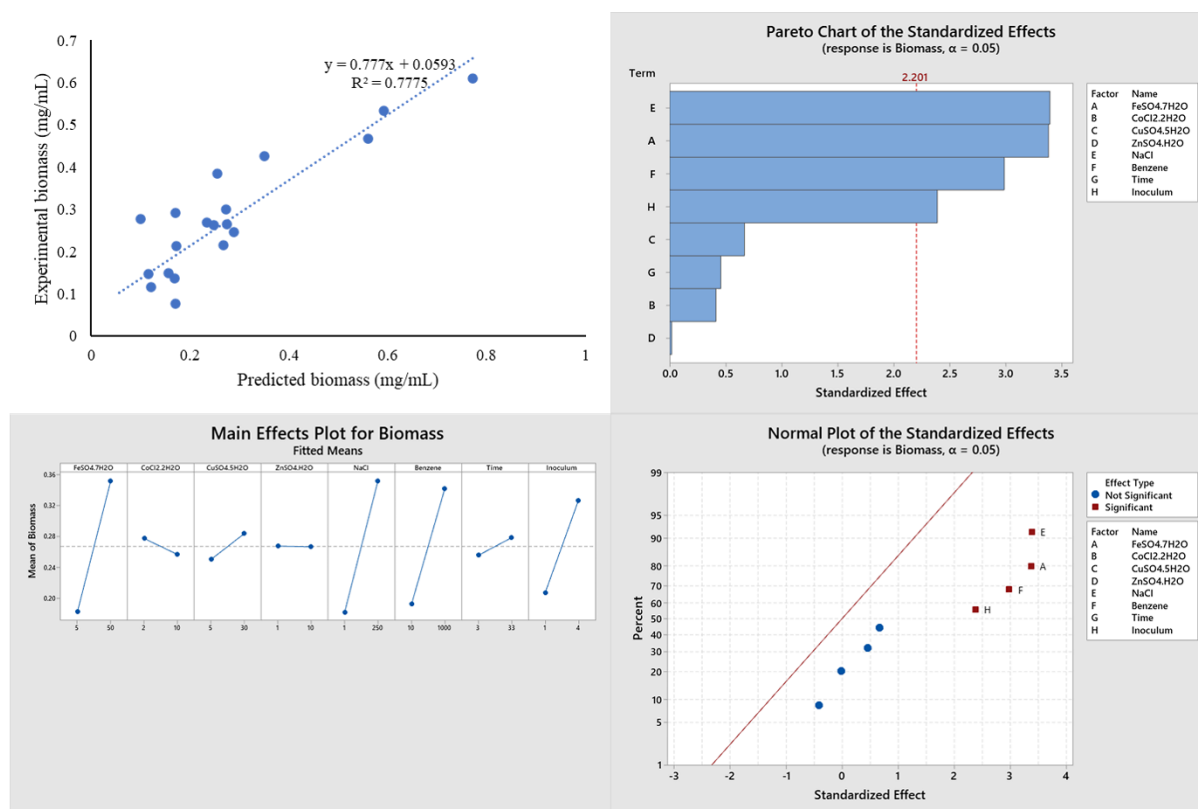
Appendix Figure 21: Contour and surface plots of significant factors for *Bacillus sp. GE1* in phenanthrene MSM.

- Benzene

- *Aquamicrobium* sp. GR4

Appendix Table 56: PBD of *Aquamicrobium* sp. GR4 in benzene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Benzene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	10	33	4	0.17
2	5	10	30	1	250	1000	3	1	0.1
3	50	10	5	10	250	10	3	1	0.248
4	50	2	5	10	250	10	33	4	0.35
5	50	2	30	1	250	1000	33	4	0.772
6	50	10	30	1	1	1000	33	1	0.272
7	5	10	5	10	250	1000	33	1	0.274
8	50	2	5	1	1	1000	3	4	0.254
9	5	2	30	10	1	1000	33	1	0.156
10	5	2	5	1	1	10	3	1	0.056
11	5	10	30	1	1	10	3	4	0.17
12	5	2	5	1	250	10	33	1	0.168
13	50	10	5	1	1	10	33	1	0.121
14	5	2	5	10	1	1000	3	4	0.172
15	50	2	30	10	250	1000	3	1	0.56
16	5	10	5	10	1	1000	33	4	0.268
17	5	10	30	10	250	10	3	4	0.288
18	50	10	30	10	1	10	33	4	0.234
19	50	10	5	1	250	1000	3	4	0.592
20	50	2	30	10	1	10	3	1	0.116



Appendix Figure 22: Precision, pareto chart, main effects plot and normal plot for PBD of *Aquamicrobium sp.* GR4 in benzene supplemented MSM

Appendix Table 57: ANOVA and model summary for PBD of *Aquamicrobium sp.* GR4 in benzene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.484	0.06	4.8	0.01
Linear	8	0.484	0.06	4.8	0.01
FeSO ₄ .7H ₂ O	1	0.144	0.144	11.44	0.006
CoCl ₂ .2H ₂ O	1	0.002	0.002	0.17	0.688
CuSO ₄ .5H ₂ O	1	0.006	0.006	0.45	0.518
ZnSO ₄ .H ₂ O	1	0	0	0	0.986
NaCl	1	0.145	0.145	11.53	0.006
Benzene		0.112	0.112	8.93	0.012
Time		0.003	0.003	0.21	0.657
Inoculum		0.072	0.072	5.71	0.036
Error	11		0.138		0.013
Total	19		0.062		

Model Summary			
S	R-sq	R-sq(adj)	R-sq(pred)
0.112	77.75%	61.57%	46.44%

$$\begin{aligned} \text{Biomass} = & -0.12 + 0.004 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.003 \text{CoCl}_2 \cdot 2\text{H}_2\text{O} \\ & + 0.001 \text{CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.000 \text{ZnSO}_4 \cdot \text{H}_2\text{O} + 0.001 \text{NaCl} + 0.000 \text{Benzene} \\ & + 0.001 \text{Time} + 0.04 \text{Inoculum} \end{aligned}$$

**Appendix
Equation 15**

Appendix Table 58: CCD for *Aquamicrobium sp.* GR4 in benzene supplemented MSM.

- Concentration of benzene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	125.5	2	0.167
2	50	1	1	0.102
3	50	250	3	0.289
4	27.5	125.5	2	0.172
5	5	250	1	0.11
6	50	1	3	0.182
7	27.5	125.5	0.318	0.123
8	27.5	125.5	2	0.173
9	27.5	125.5	2	0.168
10	27.5	334.883	2	0.193
11	65.34	125.5	2	0.195
12	5	250	3	0.193
13	5	1	3	0.114
14	27.5	-83.883	2	0.113
15	27.5	125.5	2	0.172
16	27.5	125.5	3.682	0.196
17	5	1	1	0.095
18	-10.34	125.5	2	0.121
19	27.5	125.5	2	0.176
20	50	250	1	0.194

Appendix Table 59: ANOVA for CCD of *Aquamicrobium sp.* GR4 in benzene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.039	0.004	23.82	0
Linear	3	0.036	0.012	64.99	0
FeSO ₄ .7H ₂ O	1	0.011	0.011	57.69	0
NaCl	1	0.013	0.013	73.25	0
Inoculum	1	0.012	0.012	64.04	0
Square	3	0.001	0	1.3	0.328
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	1.19	0.301
NaCl*NaCl	1	0	0	2.52	0.143
Inoculum*Inoculum	1	0	0	0.89	0.368
2-Way Interaction	3	0.003	0.001	5.15	0.021
FeSO ₄ .7H ₂ O*NaCl	1	0.001	0.001	7.54	0.021
FeSO ₄ .7H ₂ O*Inoculum	1	0.001	0.001	3.65	0.085
NaCl*Inoculum	1	0.001	0	4.27	0.066
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	32.03	0.001
Pure Error	5	0	0		
Total	19	0.041			

Appendix Table 60: Model summary for CCD of *Aquamicrobium sp.* GR4 in benzene MSM.

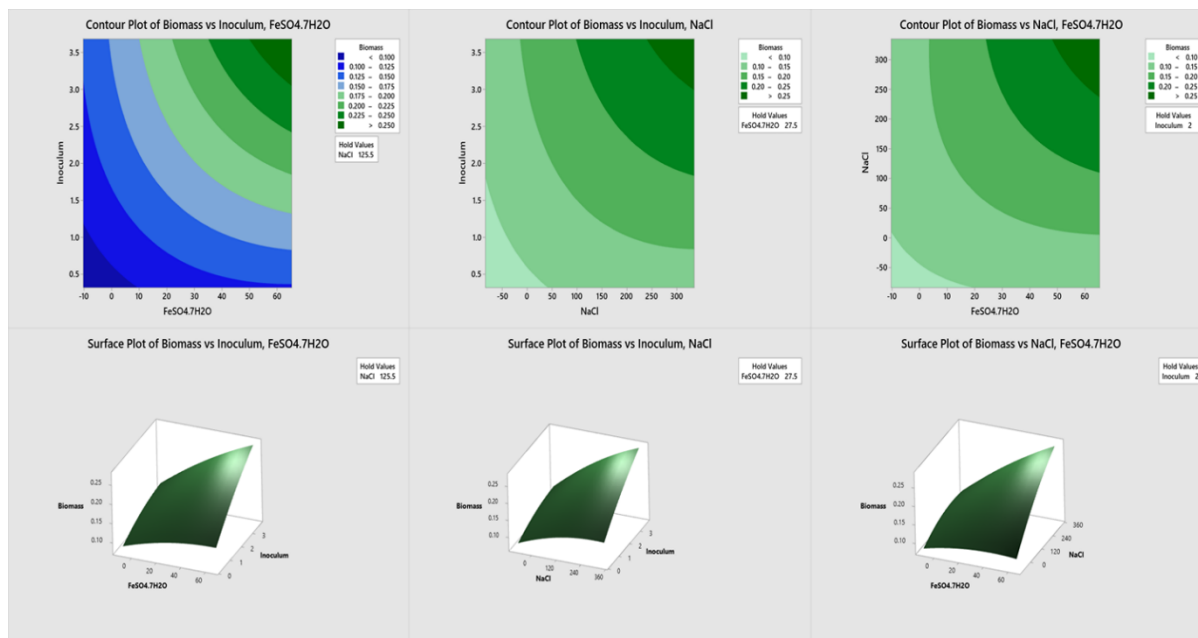
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	0.047	0.162	0.286	0.955	0.915	0.658

Appendix Table 61: Multiple response prediction for CCD of *Aquamicrobium sp.* GR4 in benzene MSM.

Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	3.682	334.88	65.34	0.269	0.324

$$\begin{aligned}
 \text{Biomass} = & 0.081 + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{NaCl} + 0.022 \text{Inoculum} \\
 & - 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} - \\
 & 0.003 \text{Inoculum} * \text{Inoculum} + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} \\
 & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Inoculum} + 0.000 \text{NaCl} * \text{Inoculum}
 \end{aligned}$$

**Appendix
Equation 16**

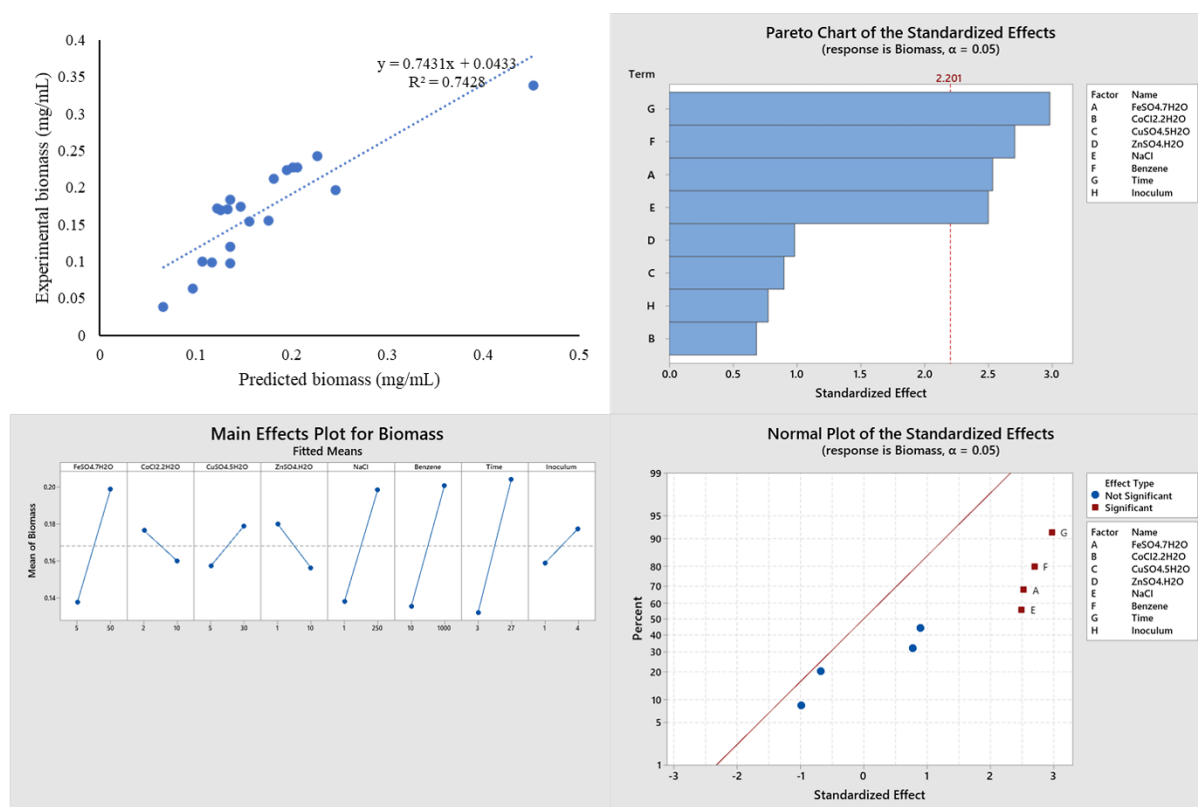


Appendix Figure 23: Contour and surface plots of significant factors for *Aquamicrobium sp. GR4* in benzene MSM.

○ *Bacillus* sp. GE1

Appendix Table 62: PBD of *Bacillus* sp. GE1 in benzene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Benzene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	5	2	30	1	250	10	27	4	0.181
2	5	10	30	1	250	1000	3	1	0.126
3	50	10	5	10	250	10	3	1	0.136
4	50	2	5	10	250	10	27	4	0.201
5	50	2	30	1	250	1000	27	4	0.452
6	50	10	30	1	1	1000	27	1	0.227
7	5	10	5	10	250	1000	27	1	0.246
8	50	2	5	1	1	1000	3	4	0.136
9	5	2	30	10	1	1000	27	1	0.147
10	5	2	5	1	1	10	3	1	0.066
11	5	10	30	1	1	10	3	4	0.097
12	5	2	5	1	250	10	27	1	0.133
13	50	10	5	1	1	10	27	1	0.176
14	5	2	5	10	1	1000	3	4	0.117
15	50	2	30	10	250	1000	3	1	0.195
16	5	10	5	10	1	1000	27	4	0.156
17	5	10	30	10	250	10	3	4	0.107
18	50	10	30	10	1	10	27	4	0.122
19	50	10	5	1	250	1000	3	4	0.206
20	50	2	30	10	1	10	3	1	0.136



Appendix Figure 24: Precision, pareto chart, main effects plot and normal plot for PBD of *Bacillus sp.* GE1 in benzene supplemented MSM.

Appendix Table 63: ANOVA and model summary for *Bacillus sp.* GE1 in benzene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.092	0.012	3.97	0.019
Linear	8	0.092	0.012	3.97	0.019
FeSO ₄ .7H ₂ O	1	0.019	0.019	6.43	0.028
CoCl ₂ .2H ₂ O	1	0.001	0.001	0.47	0.508
CuSO ₄ .5H ₂ O	1	0.002	0.002	0.81	0.387
ZnSO ₄ .H ₂ O	1	0.003	0.003	0.97	0.347
NaCl	1	0.018	0.018	6.26	0.029
Benzene		0.021	0.021	7.34	0.02
Time		0.026	0.026	8.9	0.012
Inoculum		0.002	0.002	0.6	0.454
Error	11		0.032		0.003
Total	19		0.124		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.054	74.28%	55.57%	34.97%

Biomass = $0.018 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.002 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O}$
 $+ 0.001 \text{ CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.003 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.000 \text{ Benzene}$
 $+ 0.003 \text{ Time} + 0.006 \text{ Inoculum}$

Appendix
Equation 17Appendix Table 64: CCD of *Bacillus* sp. GE1 in benzene supplemented MSM.

- Concentration of benzene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (Days)	Biomass (mg/mL)
1	27.5	125.5	15	0.188
2	27.5	125.5	15	0.185
3	50	250	3	0.195
4	50	1	27	0.199
5	27.5	125.5	15	0.183
6	27.5	125.5	35.18	0.192
7	27.5	125.5	15	0.185
8	27.5	125.5	15	0.182
9	5	250	3	0.101
10	27.5	125.5	-5.18	0.143
11	50	1	3	0.129
12	5	1	3	0.099
13	5	250	27	0.203
14	50	250	27	0.299
15	27.5	334.883	15	0.192
16	27.5	-83.883	15	0.133
17	-10.34	125.5	15	0.129
18	27.5	125.5	15	0.186
19	5	1	27	0.109
20	65.34	125.5	15	0.199

Appendix Table 65: ANOVA for CCD of *Bacillus sp.* GE1 in benzene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.037	0.004	10.97	0
Linear	3	0.033	0.011	28.87	0
FeSO ₄ .7H ₂ O	1	0.013	0.013	35.27	0
NaCl	1	0.01	0.01	25.16	0.001
Time	1	0.01	0.01	26.17	0
Square	3	0.002	0.002	1.33	0.318
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0.001	0.001	1.69	0.223
NaCl*NaCl	1	0.001	0.001	1.97	0.191
Time*Time	1	0	0	1.12	0.315
2-Way Interaction	3	0.003	0.003	2.7	0.102
FeSO ₄ .7H ₂ O*NaCl	1	0.001	0.001	1.61	0.233
FeSO ₄ .7H ₂ O*Time	1	0	0	1.27	0.287
NaCl*Time	1	0.002	0.002	5.23	0.045
Error	10	0.004	0		
Lack-of-Fit	5	0.004	0	165.33	0
Pure Error	5	0	0		
Total	19	0.041			

Appendix Table 66: Model summary for CCD of *Bacillus sp.* GE1 in benzene MSM.

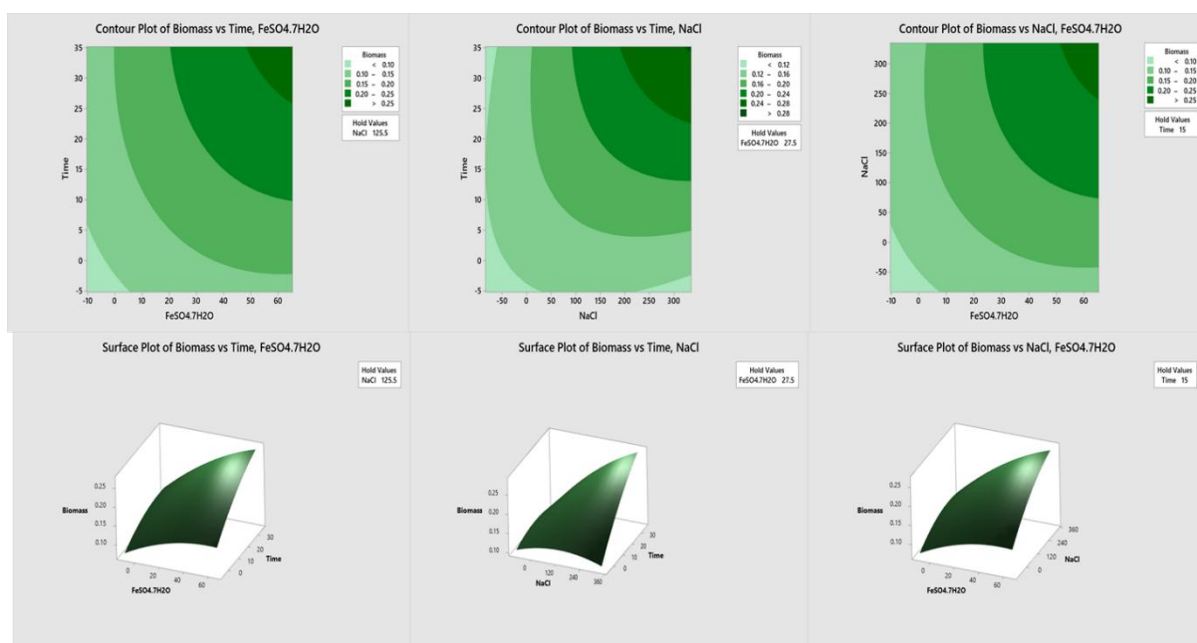
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.047	0.172	0.272	0.912	0.832	0.507

Appendix Table 67: Multiple response prediction for CCD of *Bacillus sp.* GE1 in benzene MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	35.182	334.88	65.34	0.289	0.364

$$\begin{aligned} \text{Biomass} = & 0.1 + 0.001 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{NaCl} + 0.001 \text{Time} \\ & - 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} - 0.000 \text{Time} * \text{Time} \\ & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Time} + 0.000 \text{NaCl} * \text{Time} \end{aligned}$$

**Appendix
Equation 18**



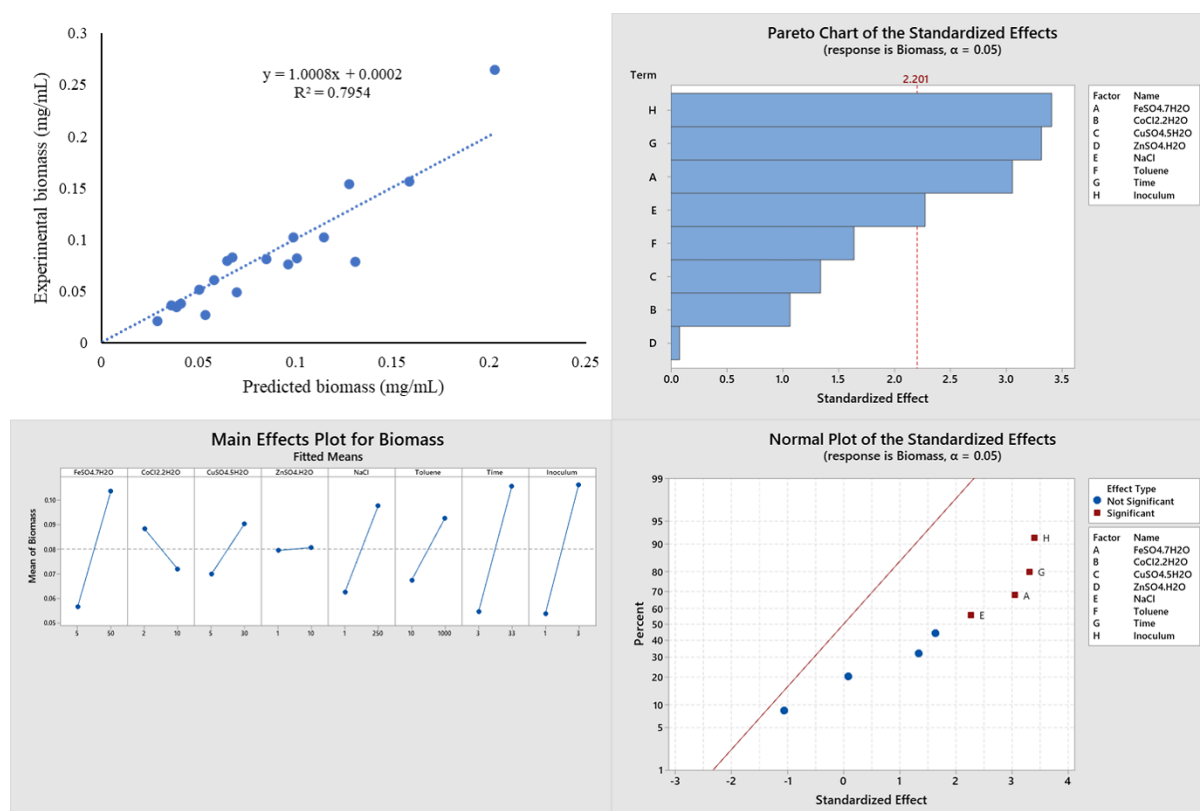
Appendix Figure 25: Contour and surface plots of significant factors for *Bacillus sp. GE1* in benzene MSM.

• Toluene

○ *Aquamicrobium* sp. GR1

Appendix Table 68: PBD of *Aquamicrobium* sp. GR1 in toluene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Toluene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	50	2	30	10	1	1000	3	3	0.051
2	50	10	5	10	250	10	33	1	0.027
3	5	10	30	1	250	10	33	3	0.156
4	5	2	30	10	1	1000	33	3	0.081
5	50	2	5	10	250	1000	3	1	0.082
6	50	10	5	1	250	10	3	3	0.021
7	50	10	30	1	1	10	33	1	0.061
8	50	10	30	10	1	1000	33	1	0.083
9	5	10	30	10	250	10	33	3	0.154
10	50	2	30	10	250	10	3	1	0.034
11	5	10	5	10	250	10	3	1	0.027
12	50	2	30	1	250	10	3	3	0.079
13	5	10	5	10	1	10	33	3	0.078
14	5	2	30	1	250	1000	33	1	0.102
15	5	2	5	10	1	1000	3	3	0.102
16	5	2	5	1	250	1000	33	1	0.049
17	50	2	5	1	1	10	3	1	0.038
18	50	10	5	1	1	1000	33	3	0.265
19	5	10	30	1	1	1000	3	3	0.076
20	5	2	5	1	1	1000	3	1	0.036



Appendix Figure 26: Precision, pareto chart, main effects plot and normal plot for PBD of *Aquamicrobium sp.* GR1 in toluene supplemented MSM.

Appendix Table 69: ANOVA and model summary for PBD of *Aquamicrobium sp.* GR1 in toluene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.051	0.006	5.34	0.005
Linear	8	0.051	0.006	5.34	0.005
FeSO ₄ ·7H ₂ O	1	0.011	0.011	9.34	0.006
CoCl ₂ ·2H ₂ O	1	0.001	0.001	1.14	0.309
CuSO ₄ ·5H ₂ O	1	0.002	0.002	1.79	0.207
ZnSO ₄ ·H ₂ O	1	0	0	0.01	0.939
NaCl	1	0.006	0.006	5.18	0.034
Toluene		0.003	0.003	2.69	0.13
Time		0.013	0.013	11	0.006
Inoculum		0.014	0.014	11.61	0.005
Error			11	0.013	0.001
Total			19	0.064	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.034	79.54%	64.66%	52.36%

Biomass = $-0.065 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.002 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} + 0.001$
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O} + 0.000 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.000 \text{ Toluene} + 0.002$
 $\text{Time} + 0.026 \text{ Inoculum}$

Appendix
Equation 19Appendix Table 70: CCD of *Aquamicrobium* sp. GR1 in toluene supplemented MSM.

- Concentration of toluene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (Days)	Biomass (mg/mL)
1	27.5	-83.883	18	0.104
2	5	250	3	0.099
3	50	250	33	0.265
4	65.34	125.5	18	0.171
5	50	1	33	0.178
6	50	250	3	0.169
7	-10.34	125.5	18	0.105
8	5	250	33	0.177
9	27.5	125.5	18	0.152
10	27.5	125.5	18	0.149
11	27.5	125.5	18	0.153
12	27.5	125.5	18	0.148
13	27.5	125.5	18	0.15
14	5	1	33	0.102
15	50	1	3	0.098
16	27.5	125.5	18	0.155
17	27.5	125.5	43.227	0.174
18	27.5	334.883	18	0.169
19	27.5	125.5	-7.227	0.099
20	5	1	3	0.089

Appendix Table 71: ANOVA for CCD of *Aquamicrobium sp.* GR1 in toluene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.032	0.004	18.71	0
Linear	3	0.03	0.01	51.5	0
FeSO ₄ .7H ₂ O	1	0.009	0.01	47.92	0
NaCl	1	0.009	0.01	47.47	0
Time	1	0.011	0.011	59.11	0
Square	3	0	0	0.46	0.716
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.41	0.536
NaCl*NaCl	1	0	0	0.62	0.45
Time*Time	1	0	0	0.62	0.45
2-Way Interaction	3	0.002	0.001	4.16	0.037
FeSO ₄ .7H ₂ O*NaCl	1	0.001	0.001	3.48	0.092
FeSO ₄ .7H ₂ O*Time	1	0.001	0.001	4.72	0.055
NaCl*Time	1	0.001	0.001	4.28	0.065
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	53.97	0
Pure Error	5	0	0		
Total	19	0.034			

Appendix Table 72: Model summary for CCD of *Aquamicrobium sp.* GR1 in toluene MSM.

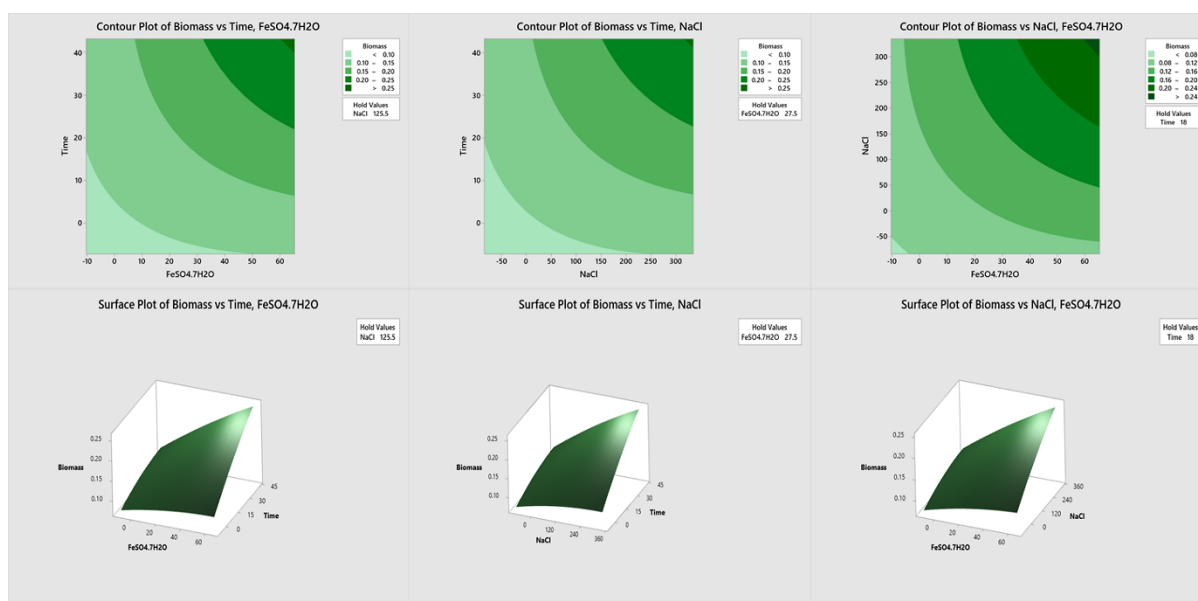
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.042	0.145	0.292	0.944	0.894	0.567

Appendix Table 73: Multiple response prediction for CCD of *Aquamicrobium sp.* GR1 in toluene MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	43.227	334.883	65.34	0.265	0.348

$$\begin{aligned} \text{Biomass} = & 0.087 + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{NaCl} + 0.001 \text{Time} \\ & - 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} - 0.000 \text{Time} * \text{Time} \\ & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Time} + 0.000 \text{NaCl} * \text{Time} \end{aligned}$$

**Appendix
Equation 20**

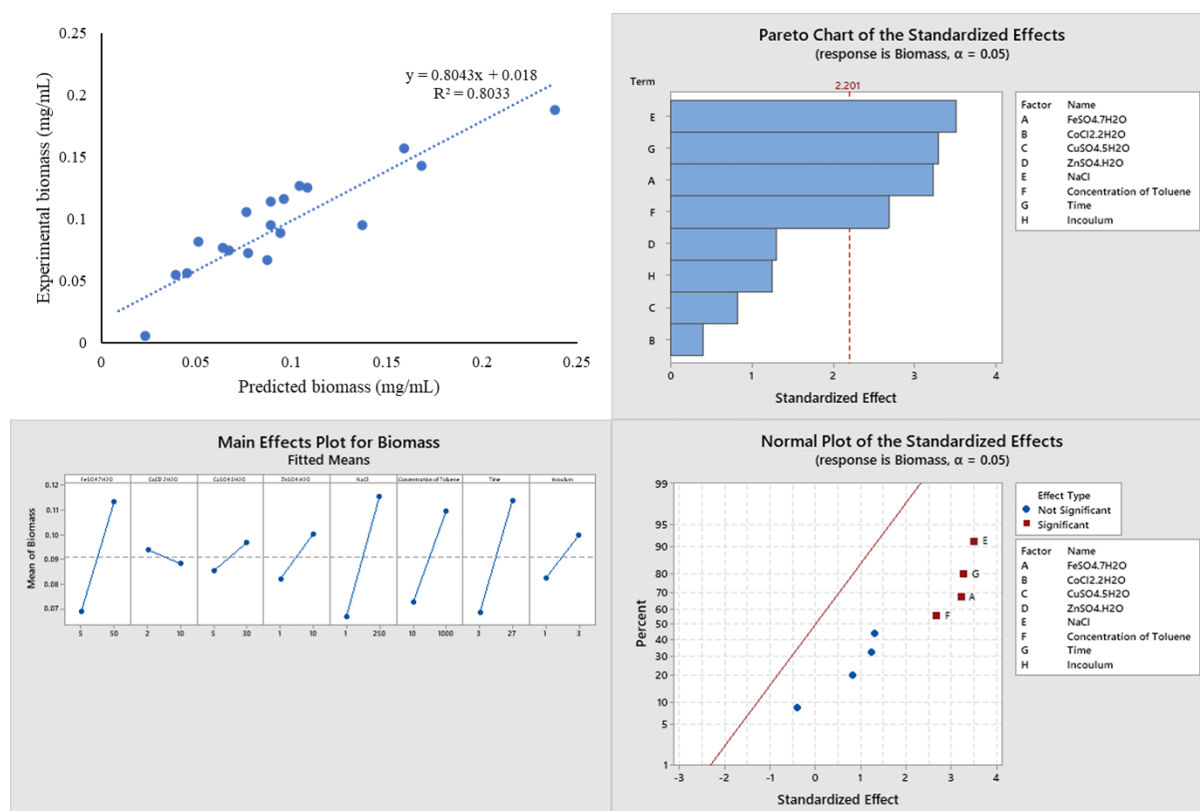


Appendix Figure 27: Contour and surface plots of significant factors for *Aquamicrobium sp. GR1* in toluene MSM.

○ *Staphylococcus sp.* GR2

Appendix Table 74: PBD of *Staphylococcus sp.* GR2 in toluene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Toluene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	50	2	30	10	1	1000	3	3	0.067
2	50	10	5	10	250	10	27	1	0.045
3	5	10	30	1	250	10	27	3	0.159
4	5	2	30	10	1	1000	27	3	0.168
5	50	2	5	10	250	1000	3	1	0.064
6	50	10	5	1	250	10	3	3	0.089
7	50	10	30	1	1	10	27	1	0.089
8	50	10	30	10	1	1000	27	1	0.087
9	5	10	30	10	250	10	27	3	0.137
10	50	2	30	10	250	10	3	1	0.039
11	5	10	5	10	250	10	3	1	0.051
12	50	2	30	1	250	10	3	3	0.238
13	5	10	5	10	1	10	27	3	0.096
14	5	2	30	1	250	1000	27	1	0.104
15	5	2	5	10	1	1000	3	3	0.077
16	5	2	5	1	250	1000	27	1	0.076
17	50	2	5	1	1	10	3	1	0.023
18	50	10	5	1	1	1000	27	3	0.009
19	5	10	30	1	1	1000	3	3	0.094
20	5	2	5	1	1	1000	3	1	0.108



Appendix Figure 28: Precision, pareto chart, main effects plot and normal plot for PBD of *Staphylococcus sp. GR2* in toluene supplemented MSM.

Appendix Table 75: ANOVA and model summary for PBD of *Staphylococcus sp. GR2* in toluene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.044	0.005	5.62	0.005
Linear	8	0.044	0.005	5.62	0.005
FeSO ₄ ·7H ₂ O	1	0.01	0.01	10.43	0.006
CoCl ₂ ·2H ₂ O	1	0	0	0.16	0.695
CuSO ₄ ·5H ₂ O	1	0	0	0.69	0.423
ZnSO ₄ ·H ₂ O	1	0.002	0.002	1.71	0.218
NaCl	1	0.012	0.012	12.37	0.005
Toluene		0.007	0.007	7.21	0.021
Time		0.01	0.01	10.81	0.006
Inoculum		0.002	0.002	1.56	0.238
Error	11		0.011		0.001
Total	19		0.054		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.031	80.33%	66.03%	44.99%

Biomass = $-0.041 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.001 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} + 0.000$
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O} + 0.002 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.000 \text{ Toluene} + 0.002$
 $\text{Time} + 0.009 \text{ Inoculum}$

Appendix
Equation 21Appendix Table 76: CCD of *Staphylococcus sp.* GR2 in toluene supplemented MSM.

- Concentration of toluene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (Days)	Biomass (mg/mL)
1	27.5	125.5	15	0.167
2	-10.34	125.5	15	0.122
3	27.5	334.883	15	0.194
4	5	250	3	0.134
5	5	250	27	0.205
6	27.5	125.5	15	0.166
7	27.5	-83.883	15	0.119
8	5	1	3	0.104
9	27.5	125.5	15	0.166
10	27.5	125.5	15	0.167
11	27.5	125.5	15	0.167
12	65.34	125.5	15	0.187
13	50	250	27	0.252
14	50	1	27	0.208
15	27.5	125.5	35.182	0.188
16	5	1	27	0.135
17	27.5	125.5	15	0.167
18	27.5	125.5	-5.182	0.123
19	50	1	3	0.135
20	50	250	3	0.207

Appendix Table 77: ANOVA for CCD of *Staphylococcus sp.* GR2 in toluene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.025	0.003	12.95	0
Linear	3	0.025	0.008	38.73	0
FeSO ₄ .7H ₂ O	1	0.008	0.008	38.35	0
NaCl	1	0.009	0.009	40.4	0
Time	1	0.008	0.008	37.43	0
Square	3	0	0	0.01	0.999
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.02	0.885
NaCl*NaCl	1	0	0	0	0.972
Time*Time	1	0	0	0	0.956
2-Way Interaction	3	0	0	0.13	0.941
FeSO ₄ .7H ₂ O*NaCl	1	0	0	0.15	0.706
FeSO ₄ .7H ₂ O*Time	1	0	0	0.15	0.706
NaCl*Time	1	0	0	0.08	0.777
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	1590.12	0
Pure Error	5	0	0		
Total	19	0.027			

Appendix Table 78: Model summary for CCD of *Staphylococcus sp.* GR2 in toluene MSM.

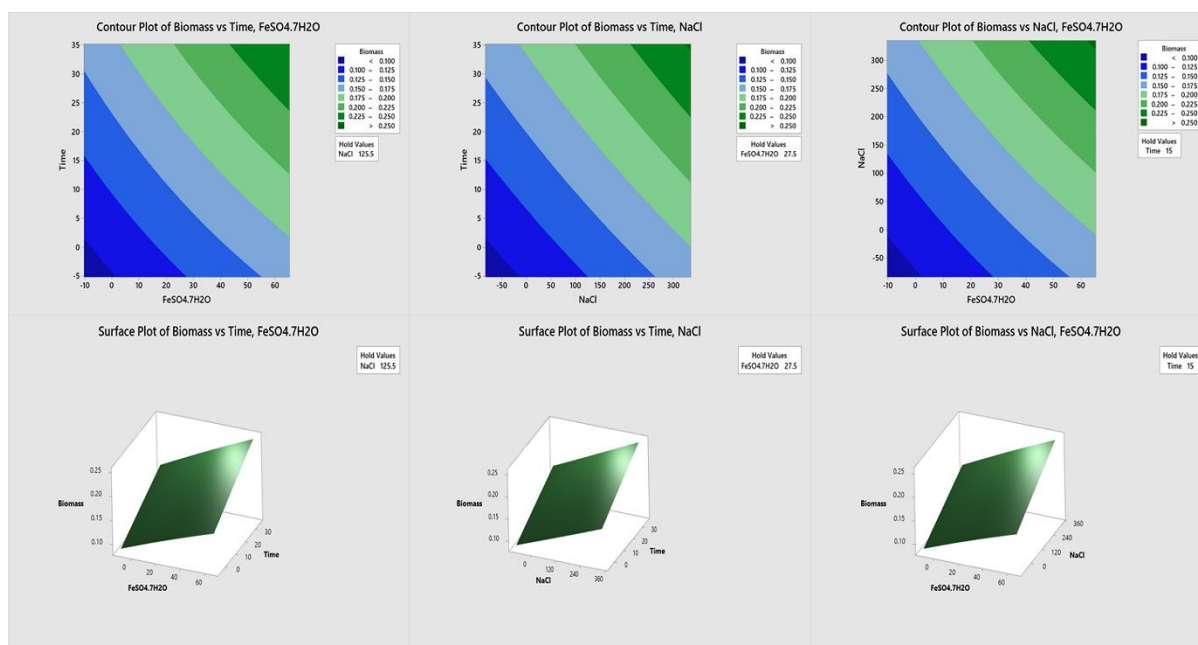
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.038	0.166	0.227	0.921	0.85	0.468

Appendix Table 79: Multiple response prediction for CCD of *Staphylococcus sp.* GR2 in toluene MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	35.182	334.883	65.34	0.252	0.304

$$\begin{aligned} \text{Biomass} = & 0.087 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.002 \text{ Time} \\ & - 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} * \text{NaCl} - 0.000 \text{ Time} * \text{Time} \\ & + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Time} + 0.000 \text{ NaCl} * \text{Time} \end{aligned}$$

Appendix Equation 22

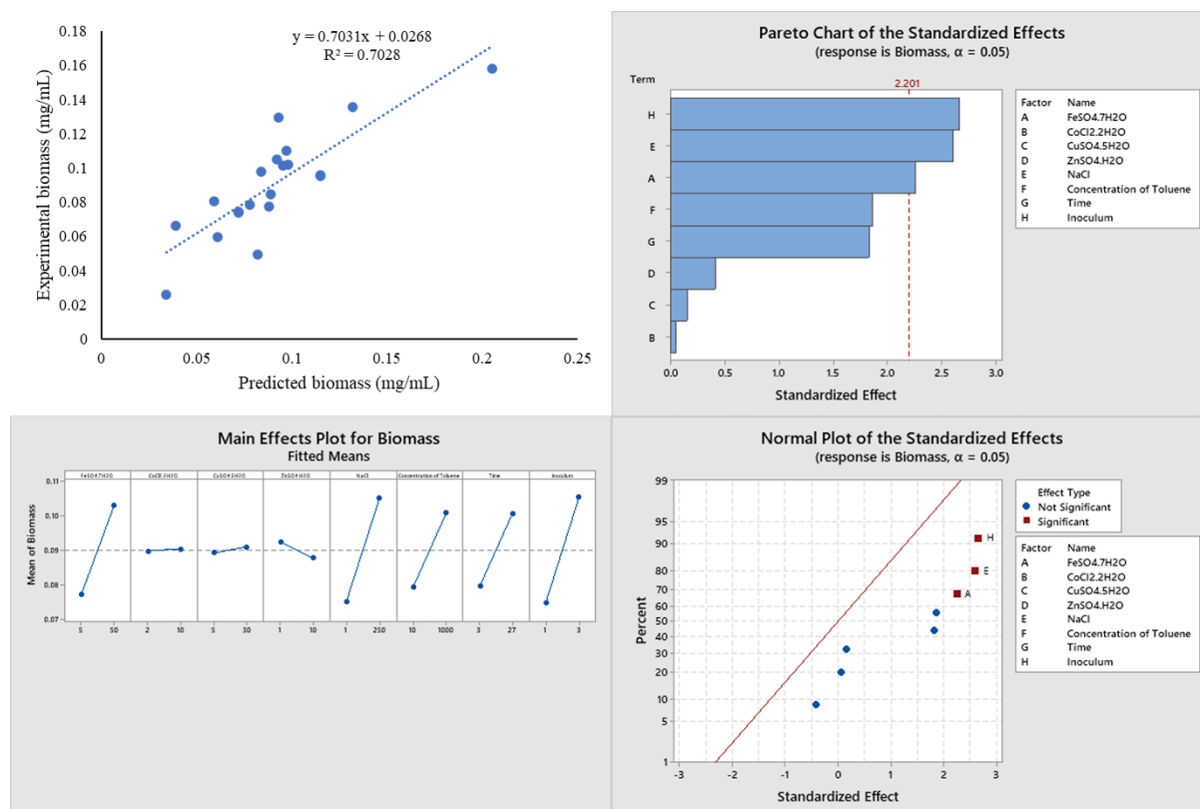


Appendix Figure 29: Contour and surface plots of significant factors for *Staphylococcus sp. GR2* in toluene MSM.

○ *Aquamicrobium* sp. GR4

Appendix Table 80: PBD of *Aquamicrobium* sp. GR4 in toluene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Toluene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	50	2	30	10	1	1000	3	3	0.082
2	50	10	5	10	250	10	27	1	0.093
3	5	10	30	1	250	10	27	3	0.072
4	5	2	30	10	1	1000	27	3	0.072
5	50	2	5	10	250	1000	3	1	0.095
6	50	10	5	1	250	10	3	3	0.098
7	50	10	30	1	1	10	27	1	0.089
8	50	10	30	10	1	1000	27	1	0.061
9	5	10	30	10	250	10	27	3	0.039
10	50	2	30	10	250	10	3	1	0.078
11	5	10	5	10	250	10	3	1	0.088
12	50	2	30	1	250	10	3	3	0.205
13	5	10	5	10	1	10	27	3	0.034
14	5	2	30	1	250	1000	27	1	0.115
15	5	2	5	10	1	1000	3	3	0.132
16	5	2	5	1	250	1000	27	1	0.059
17	50	2	5	1	1	10	3	1	0.084
18	50	10	5	1	1	1000	27	3	0.092
19	5	10	30	1	1	1000	3	3	0.115
20	5	2	5	1	1	1000	3	1	0.097



Appendix Figure 30: Precision, pareto chart, main effects plot and normal plot for PBD of *Aquamicrobium sp.* GR4 in toluene supplemented MSM.

Appendix Table 81: ANOVA and model summary for PBD of *Aquamicrobium sp.* GR4 in toluene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.017	0.002	3.25	0.037
Linear	8	0.017	0.002	3.25	0.037
FeSO ₄ ·7H ₂ O	1	0.0034	0.0034	5.12	0.045
CoCl ₂ ·2H ₂ O	1	0	0	0	0.96
CuSO ₄ ·5H ₂ O	1	0	0	0.02	0.879
ZnSO ₄ ·H ₂ O	1	0	0	0.17	0.687
NaCl	1	0.005	0.005	6.8	0.024
Toluene		0.002	0.002	3.48	0.089
Time		0.002	0.002	3.35	0.094
Inoculum		0.005	0.005	7.07	0.022
Error			11	0.007	0.007
Total			19	0.025	

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.026	70.28%	58.67%	41.77%

Biomass = 0.005 + 0.001 FeSO₄·7H₂O + 0.000 CoCl₂·2H₂O + 0.000
 CuSO₄·5H₂O – 0.001 ZnSO₄·H₂O + 0.000 NaCl + 0.000 Toluene + 0.001
 Time + 0.015 Inoculum

Appendix
Equation 23Appendix Table 82: CCD of *Aquamicrobium* sp. GR4 in toluene supplemented MSM.

- Concentration of toluene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	FeSO ₄ ·7H ₂ O (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	334.883	2	0.187
2	65.34	125.5	2	0.192
3	27.5	125.5	2	0.15
4	27.5	–83.883	2	0.118
5	27.5	125.5	2	0.149
6	5	1	3	0.129
7	27.5	125.5	2	0.151
8	5	250	3	0.199
9	–10.34	125.5	2	0.115
10	50	1	1	0.125
11	50	250	3	0.24
12	27.5	125.5	2	0.148
13	50	1	3	0.201
14	5	250	1	0.129
15	50	250	1	0.197
16	27.5	125.5	3.682	0.191
17	5	1	1	0.09
18	27.5	125.5	2	0.151
19	27.5	125.5	2	0.147
20	27.5	125.5	0.318	0.117

Appendix Table 83: ANOVA for CCD of *Aquamicrobium sp.* GR4 in toluene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.025	0.003	17.54	0
Linear	3	0.025	0.008	51.79	0
FeSO ₄ .7H ₂ O	1	0.008	0.008	51.5	0
NaCl	1	0.008	0.008	49.14	0
Inoculum	1	0.009	0.009	54.72	0
Square	3	0	0	0.8	0.523
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0.88	0.371
NaCl*NaCl	1	0	0	0.78	0.398
Inoculum*Inoculum	1	0	0	1.2	0.299
2-Way Interaction	3	0	0	0.03	0.993
FeSO ₄ .7H ₂ O*NaCl	1	0	0	0	0.957
FeSO ₄ .7H ₂ O*Inoculum	1	0	0	0.08	0.786
NaCl*Inoculum	1	0	0	0	0.957
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	118.96	0
Pure Error	5	0	0		
Total	19	0.027			

Appendix Table 84: Model summary for CCD of *Aquamicrobium sp.* GR4 in toluene MSM.

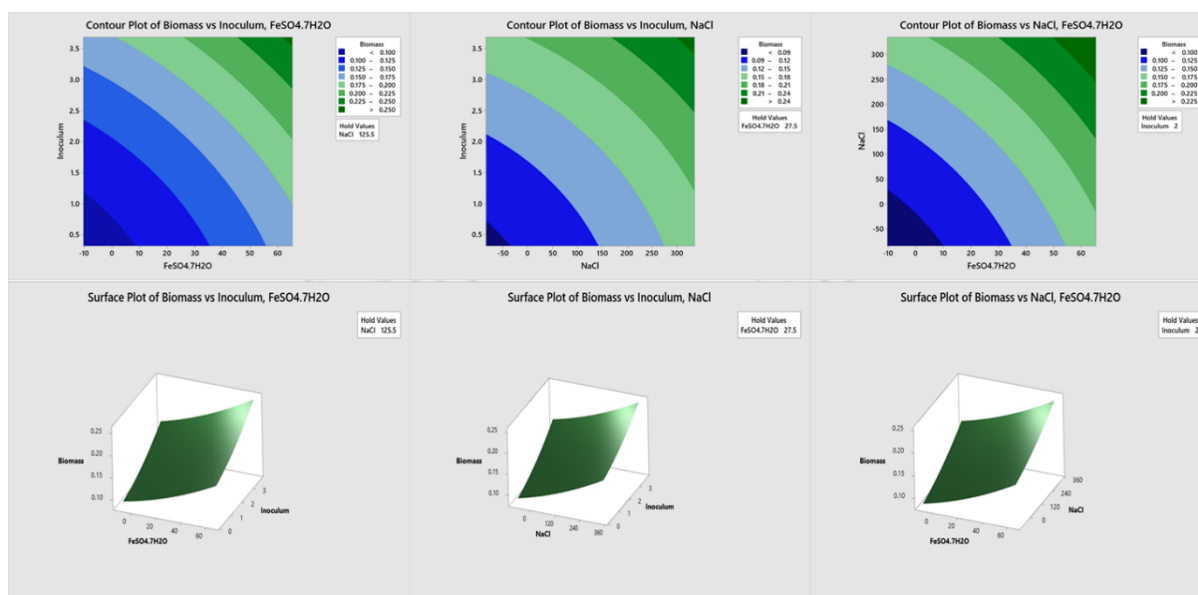
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.038	0.156	0.242	0.94	0.887	0.521

Appendix Table 85: Multiple response prediction for CCD of *Aquamicrobium sp.* GR4 in toluene MSM.

Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	3.682	334.883	65.34	0.24	0.304

$$\begin{aligned}
\text{Biomass} = & 0.069 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.009 \text{ Inoculum} \\
& + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} * \text{NaCl} \\
& + 0.004 \text{ Inoculum} * \text{Inoculum} + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} \\
& + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Inoculum} - 0.000 \text{ NaCl} * \text{Inoculum}
\end{aligned}$$

**Appendix
Equation 24**



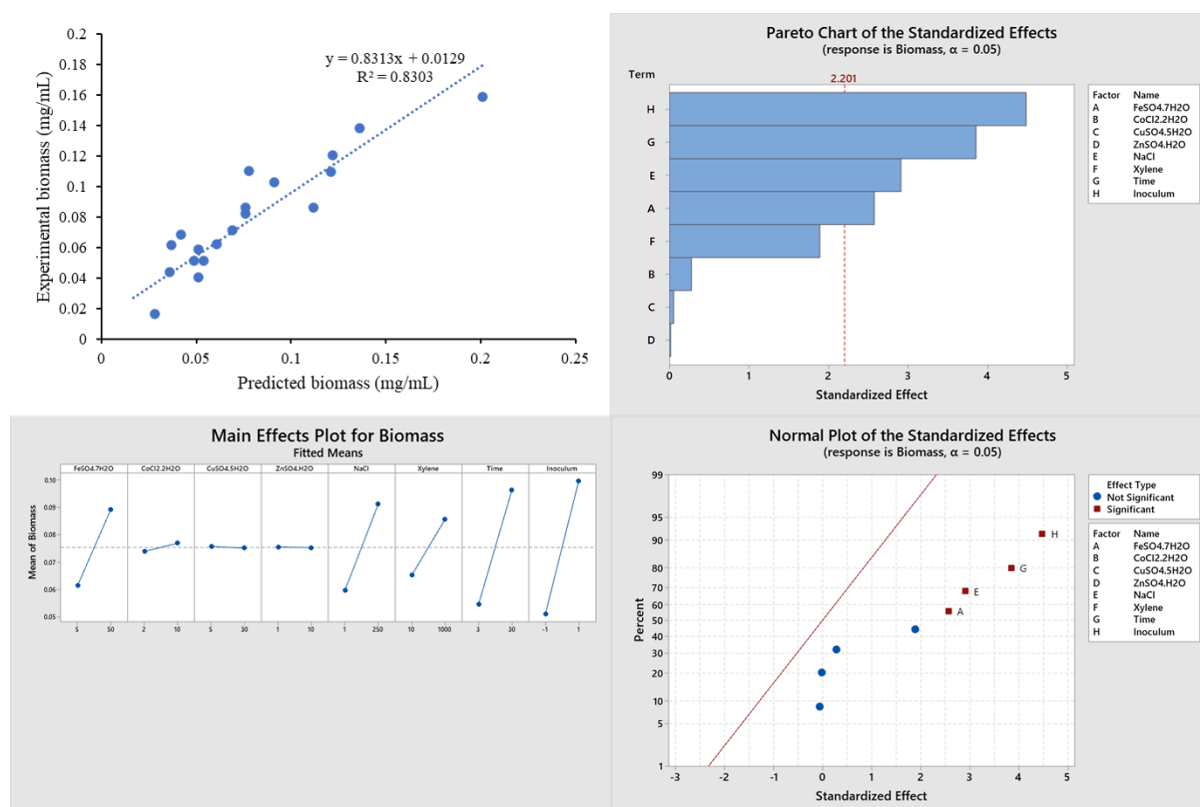
Appendix Figure 31: Contour and surface plot of significant factors for *Aquamicrobium sp. GR4* in toluene MSM.

- Xylene

- *Staphylococcus sp.* GR2

Appendix Table 86: PBD of *Staphylococcus sp.* GR2 in xylene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Xylene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	50	2	30	10	1	1000	3	2	0.051
2	50	10	5	10	250	10	30	1	0.037
3	5	10	30	1	250	10	30	2	0.136
4	5	2	30	10	1	1000	30	2	0.091
5	50	2	5	10	250	1000	3	1	0.042
6	50	10	5	1	250	10	3	2	0.051
7	50	10	30	1	1	10	30	1	0.061
8	50	10	30	10	1	1000	30	1	0.112
9	5	10	30	10	250	10	30	2	0.121
10	50	2	30	10	250	10	3	1	0.054
11	5	10	5	10	250	10	3	1	0.017
12	50	2	30	1	250	10	3	2	0.069
13	5	10	5	10	1	10	30	2	0.078
14	5	2	30	1	250	1000	30	1	0.076
15	5	2	5	10	1	1000	3	2	0.122
16	5	2	5	1	250	1000	30	1	0.049
17	50	2	5	1	1	10	3	1	0.028
18	50	10	5	1	1	1000	30	2	0.201
19	5	10	30	1	1	1000	3	2	0.076
20	5	2	5	1	1	1000	3	1	0.036



Appendix Figure 32: Precision, pareto chart, main effects plot and normal plot for PBD of *Staphylococcus sp.* GR2 in xylene supplemented MSM.

Appendix Table 87: ANOVA and model summary for PBD of *Staphylococcus sp.* GR2 in xylene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.031	0.004	6.73	0.003
Linear	8	0.031	0.004	6.73	0.003
FeSO ₄ .7H ₂ O	1	0.004	0.01	6.65	0.026
CoCl ₂ .2H ₂ O	1	0	0	0.08	0.786
CuSO ₄ .5H ₂ O	1	0	0.001	0	0.957
ZnSO ₄ .H ₂ O	1	0	0.002	0	0.986
NaCl	1	0.005	0.012	8.48	0.014
Xylene		0.002	0.007	3.58	0.085
Time		0.009	0.01	14.88	0.003
Inoculum		0.012	0.002	20.15	0.001
Error	11		0.006		0.001
Total	19		0.038		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.024	83.03%	70.69%	53.9%

Biomass = $-0.068 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} - 0.000$
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.000 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.000 \text{ Xylene} + 0.002 \text{ Time}$
 $+ 0.005 \text{ Inoculum}$

**Appendix
Equation 25**

Appendix Table 88: CCD of *Staphylococcus sp.* GR2 in xylene supplemented MSM.

- Concentration of xylene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Time (Days)	Biomass (mg/mL)
1	27.5	125.5	16.5	0.171
2	65.34	125.5	30	0.266
3	27.5	125.5	16.5	0.135
4	50	1	16.5	0.085
5	5	250	3	0.089
6	50	1	16.5	0.169
7	27.5	125.5	3	0.18
8	27.5	125.5	30	0.087
9	27.5	334.883	3	0.089
10	5	1	-6.2	0.088
11	50	250	16.5	0.136
12	27.5	125.5	3	0.088
13	27.5	125.5	39.2	0.176
14	-10.34	125.5	16.5	0.085
15	5	1	16.5	0.136
16	50	250	16.5	0.137
17	27.5	125.5	16.5	0.137
18	5	250	16.5	0.135
19	27.5	125.5	30	0.171
20	27.5	-83.883	30	0.173

Appendix Table 89: ANOVA for CCD of *Staphylococcus sp.* GR2 in xylene MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	9	0.04	0.004	21.55	0
Linear	3	0.037	0.012	59.53	0
FeSO ₄ .7H ₂ O	1	0.013	0.013	62.06	0
NaCl	1	0.012	0.012	59.89	0
Time	1	0.012	0.012	56.65	0
Square	3	0	0	0.06	0.978
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0	0	0	0.961
NaCl*NaCl	1	0	0	0	0.966
Time*Time	1	0	0	0.18	0.68
2-Way Interaction	3	0.003	0.001	5.05	0.022
FeSO ₄ .7H ₂ O*NaCl	1	0.001	0.001	5.95	0.035
FeSO ₄ .7H ₂ O*Time	1	0.001	0.001	4.81	0.053
NaCl*Time	1	0.001	0.001	4.39	0.063
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	513.47	0
Pure Error	5	0	0		
Total	19	0.041969			

Appendix Table 90: Model summary for CCD of *Staphylococcus sp.* GR2 in xylene MSM.

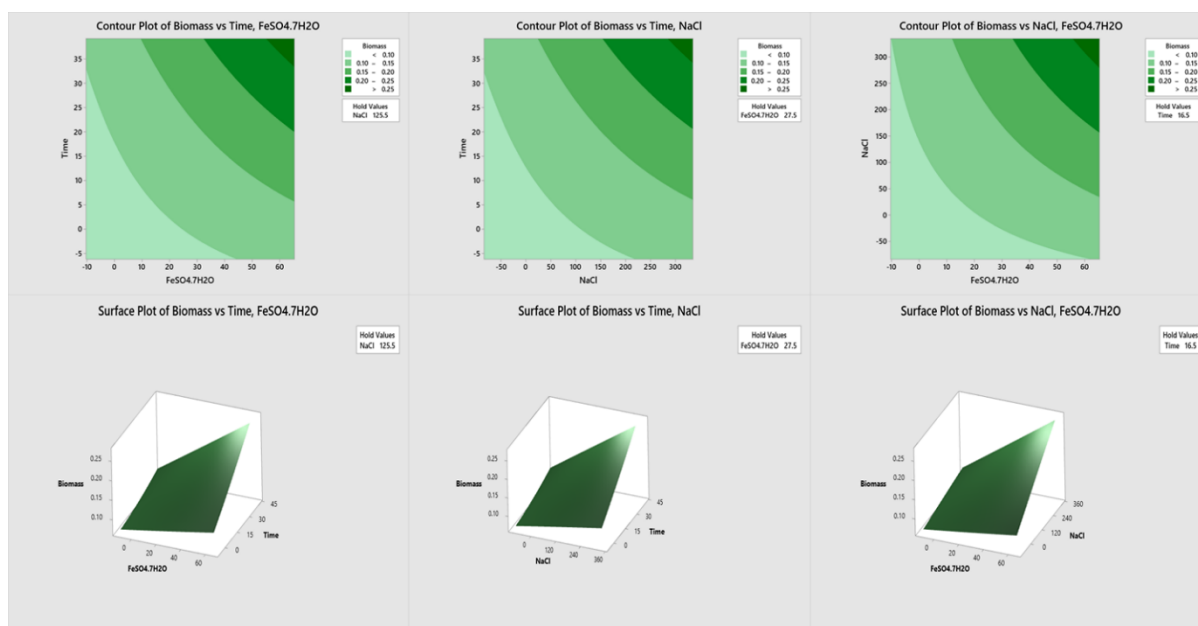
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	Adjusted R ²	Predicted R ²
Predicted response	0.047	0.137	0.344	0.907	0.598

Appendix Table 91: Multiple response prediction for CCD of *Staphylococcus sp.* GR2 in xylene MSM.

Variable	Time	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	39.204	334.883	65.34	0.266	0.288

$$\begin{aligned} \text{Biomass} = & 0.08 + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{NaCl} + 0.000 \text{Time} \\ & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{NaCl} * \text{NaCl} + 0.000 \text{Time} * \text{Time} \\ & + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} + 0.000 \text{FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Time} + 0.000 \text{NaCl} * \text{Time} \end{aligned}$$

Appendix Equation 26

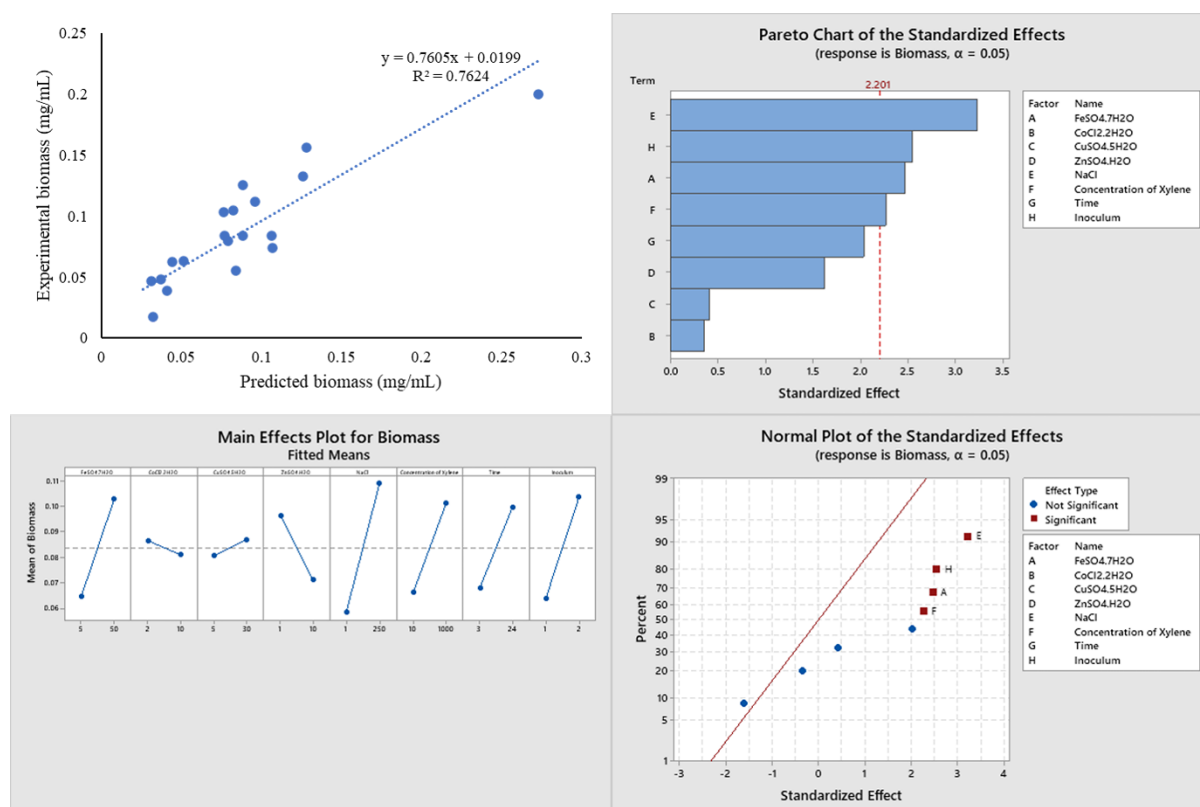


Appendix Figure 33: Contour and surface plots of significant factors for *Staphylococcus sp.* GR2 in xylene MSM.

○ *Aquamicrobium* sp. GR4

Appendix Table 92: PBD of *Aquamicrobium* sp. GR4 in xylene supplemented MSM.

Run	FeSO ₄ ·7H ₂ O (mg/L)	CoCl ₂ ·2H ₂ O (mg/L)	CuSO ₄ ·5H ₂ O (mg/L)	ZnSO ₄ ·H ₂ O (mg/L)	NaCl (mg/L)	Xylene (mg/L)	Time (Days)	Inoculum (%)	Biomass (mg/mL)
1	50	2	30	10	1	1000	3	2	0.037
2	50	10	5	10	250	10	24	1	0.084
3	5	10	30	1	250	10	24	2	0.026
4	5	2	30	10	1	1000	24	2	0.107
5	50	2	5	10	250	1000	3	1	0.077
6	50	10	5	1	250	10	3	2	0.088
7	50	10	30	1	1	10	24	1	0.126
8	50	10	30	10	1	1000	24	1	0.076
9	5	10	30	10	250	10	24	2	0.082
10	50	2	30	10	250	10	3	1	0.128
11	5	10	5	10	250	10	3	1	0.032
12	50	2	30	1	250	10	3	2	0.051
13	5	10	5	10	1	10	24	2	0.106
14	5	2	30	1	250	1000	24	1	0.088
15	5	2	5	10	1	1000	3	2	0.044
16	5	2	5	1	250	1000	24	1	0.273
17	50	2	5	1	1	10	3	1	0.041
18	50	10	5	1	1	1000	24	2	0.096
19	5	10	30	1	1	1000	3	2	0.079
20	5	2	5	1	1	1000	3	1	0.031



Appendix Figure 34: Precision, pareto chart, main effects plot and normal plot for PBD of *Aquamicrobium sp.* GR4 in xylene supplemented MSM.

Appendix Table 93: ANOVA and model summary for PBD of *Aquamicrobium sp.* GR4 in xylene supplemented MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	8	0.043	0.005	4.41	0.013
Linear	8	0.043	0.005	4.41	0.013
FeSO ₄ .7H ₂ O	1	0.007	0.007	6.12	0.031
CoCl ₂ .2H ₂ O	1	0	0	0.13	0.726
CuSO ₄ .5H ₂ O	1	0	0	0.17	0.69
ZnSO ₄ .H ₂ O	1	0.003	0.003	2.65	0.132
NaCl	1	0.013	0.013	10.43	0.008
Xylene		0.006	0.006	5.15	0.044
Time		0.005	0.005	4.15	0.066
Inoculum		0.008	0.008	6.51	0.027
Error	11		0.013		0.001
Total	19		0.056		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.035	76.24%	58.96%	41.46%

Biomass = $-0.048 + 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.001 \text{ CoCl}_2 \cdot 2\text{H}_2\text{O} + 0.000$
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O} - 0.003 \text{ ZnSO}_4 \cdot \text{H}_2\text{O} + 0.000 \text{ NaCl} + 0.000 \text{ Toluene} + 0.002$
Time + 0.04 Inoculum

**Appendix
Equation 27**

Appendix Table 94: CCD of *Aquamicrobium* sp. GR4 in xylene supplemented MSM.

- Concentration of xylene = 1000 mg/L
- Design: CCD ($\alpha = 1.633$)

Run	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/L)	NaCl (mg/L)	Inoculum (%)	Biomass (mg/mL)
1	27.5	125.5	2	0.137
2	65.34	125.5	2	0.138
3	27.5	125.5	2	0.136
4	50	1	2	0.192
5	5	250	2	0.197
6	50	1	2	0.137
7	27.5	125.5	1	0.079
8	27.5	125.5	1	0.092
9	27.5	334.883	0.318	0.112
10	5	1	3	0.084
11	50	250	3	0.179
12	27.5	125.5	1	0.183
13	27.5	125.5	1	0.091
14	-10.34	125.5	3	0.273
15	5	1	2	0.137
16	50	250	2	0.122
17	27.5	125.5	3	0.181
18	5	250	3.682	0.177
19	27.5	125.5	2	0.138
20	27.5	-83.883	2	0.089

Appendix Table 95: ANOVA for CCD of *Aquamicrobium sp.* GR4 in xylene MSM.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	9	0.043	0.005	19.06	0
Linear	3	0.04	0.013	52.99	0
FeSO ₄ .7H ₂ O	1	0.012	0.012	49.47	0
NaCl	1	0.0166	0.017	66.66	0
Inoculum	1	0.01	0.011	42.84	0
Square	3	0.001	0	0.79	0.526
FeSO ₄ .7H ₂ O*FeSO ₄ .7H ₂ O	1	0.001	0.001	2.27	0.163
NaCl*NaCl	1	0	0	0.1	0.76
Inoculum*Inoculum	1	0	0	0.2	0.668
2-Way Interaction	3	0.003	0.001	3.39	0.062
FeSO ₄ .7H ₂ O*NaCl	1	0.001	0.001	2.91	0.119
FeSO ₄ .7H ₂ O*Inoculum	1	0.001	0.001	3.38	0.096
NaCl*Inoculum	1	0.001	0	3.9	0.077
Error	10	0.002	0		
Lack-of-Fit	5	0.002	0	876.07	0
Pure Error	5	0	0		
Total	19	0.045			

Appendix Table 96: Model summary for CCD of *Aquamicrobium sp.* GR4 in xylene MSM.

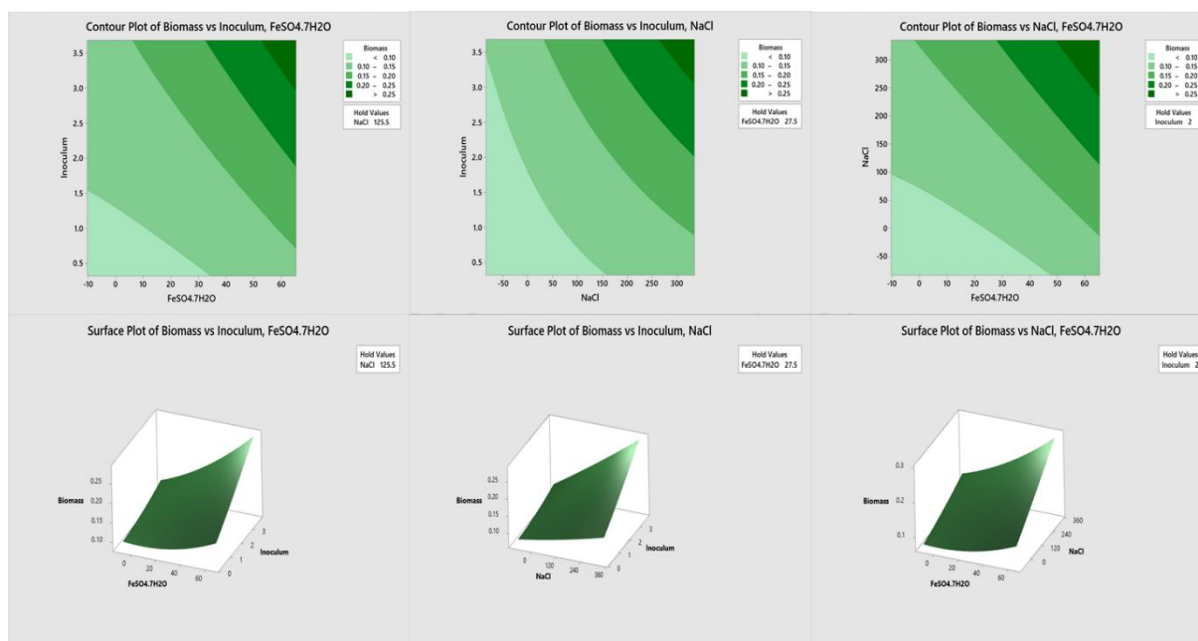
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R ²	Adjusted R ²	Predicted R ²
Predicted response	0.049	0.144	0.339	0.945	0.895	0.553

Appendix Table 97: Multiple response prediction for CCD of *Aquamicrobium sp.* GR4 in xylene MSM.

Variable	Inoculum	NaCl	FeSO ₄ .7H ₂ O	Biomass fit	
				Target	Predict
Optimized setting	3.682	334.88	65.34	0.273	0.305

$$\begin{aligned}
\text{Biomass} = & 0.087 - 0.001 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} - 0.000 \text{ NaCl} - 0.003 \text{ Inoculum} \\
& + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{FeSO}_4 \cdot 7\text{H}_2\text{O} + 0.000 \text{ NaCl} * \text{NaCl} \\
& + 0.00 \text{ Inoculum} * \text{Inoculum} + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{NaCl} \\
& + 0.000 \text{ FeSO}_4 \cdot 7\text{H}_2\text{O} * \text{Inoculum} + 0.000 \text{ NaCl} * \text{Inoculum}
\end{aligned}$$

**Appendix
Equation 28**



Appendix Figure 35: Contour and surface plots of significant factors for *Aquamicrobium sp. GR4* in xylene MSM.

▪ Screening, optimization and statistical analysis of significant strains for biomass production for optimum consortia design

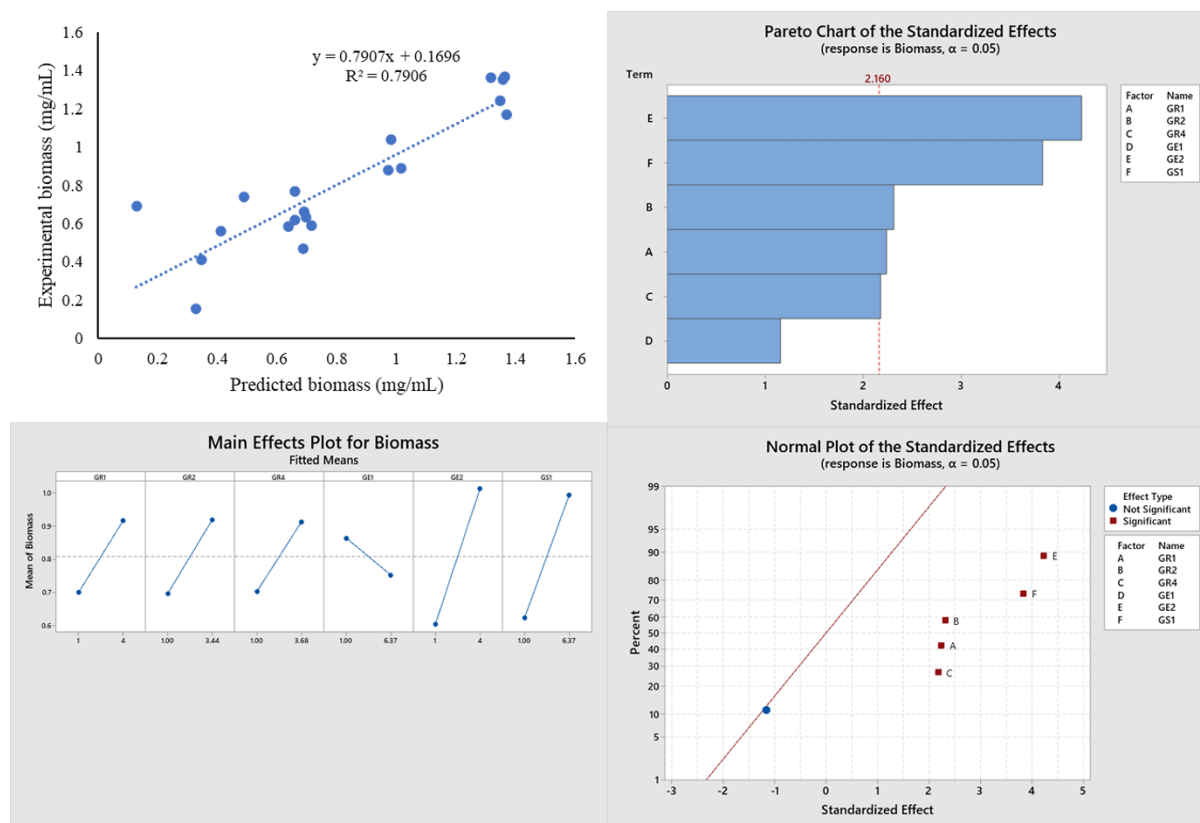
• Set 2: 1st order Placket-Burman Design screening

Appendix Table 98: PBD of Set 2 of hydrocarbon (HC) mixture supplemented in modified MSM.

10 mg/L *n*-hexadecane, 5 mg/L Anthracene, 5 mg/L Phenanthrene, 10 mg/L Benzene, 10 mg/L Toluene, 10 mg/L Xylene

Design: PBD

Run	GR1	GR2	GR4	GE1	GE2	GS1	Biomass (mg/mL)
1	1	1	3.68	1	4	1	0.658
2	4	1	3.68	1	4	6.37	1.357
3	1	1	1	1	1	1	0.327
4	4	3.44	1	1	1	1	0.715
5	1	1	1	1	4	1	0.412
6	4	1	1	1	1	6.37	0.489
7	4	3.44	3.68	6.37	1	1	0.128
8	1	3.44	3.68	1	1	1	0.637
9	1	3.44	3.68	6.37	4	1	0.973
10	4	1	3.68	6.37	1	1	0.688
11	4	3.44	1	6.37	4	1	1.017
12	1	3.44	1	6.37	4	6.37	0.983
13	1	3.44	3.68	1	4	6.37	1.318
14	4	3.44	3.68	1	1	6.37	1.368
15	1	1	1	6.37	1	6.37	0.347
16	1	1	3.68	6.37	1	6.37	0.658
17	4	1	1	6.37	4	1	0.69
18	1	3.44	1	6.37	1	6.37	0.697
19	4	3.44	1	1	4	6.37	1.362
20	4	1	3.68	6.37	4	6.37	1.349



Appendix Figure 36: Precision, pareto chart, main effects plot and normal plot for PBD of Set 2 mixture of HCs in modified MSM.

Appendix Table 99: ANOVA and model summary for PBD of Set 2 mixture of HCs in modified MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	6	2.265	0.377	8.18	0.001
Linear	6	2.265	0.377	8.18	0.001
GR1	1	0.232	0.232	5.02	0.043
GR2	1	0.247	0.247	5.35	0.038
GR4	1	0.219	0.219	4.76	0.048
GE1	1	0.069	0.069	1.34	0.267
GE2	1	0.826	0.826	17.9	0.001
GS1	1	0.678	0.678	14.7	0.002
Error	13		0.6		0.046
Total	19		2.865		

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.031	79.06%	69.39%	50.43%

$$\text{Biomass} = -0.271 + 0.072 \text{ GR1} + 0.091 \text{ GR2} + 0.078 \text{ GR4} - 0.021 \text{ GE1} + 0.136 \text{ GE2} + 0.069 \text{ GS1}$$

**Appendix
Equation 29**

• **Set 2: 2nd order Central Composite Design optimization**

○ **Set 2(i)**

Appendix Table 100: CCD of Set 2(i) mixture of HCs in modified MSM.

- 10 mg/L *n*-hexadecane, 5 mg/L Anthracene, 5 mg/L Phenanthrene, 10 mg/L Benzene, 10 mg/L Toluene, 10 mg/L Xylene
- Design: CCD ($\alpha = 1.633$)

Run	GR1	GR2	GS1	Biomass (mg/mL)
1	1	3.44	1	0.218
2	1	3.44	6.37	0.56
3	4	3.44	1	0.437
4	2.5	2.22	3.685	0.247
5	2.5	2.22	3.685	0.251
6	1	1	6.37	0.431
7	4	3.44	6.37	0.742
8	4	1	1	0.188
9	1	1	1	0.183
10	4	1	6.37	0.587
11	2.5	2.22	3.685	0.253
12	2.5	2.22	3.685	0.231
13	2.5	4.212	3.685	0.331
14	2.5	2.22	-0.7	0.172
15	2.5	2.22	8.07	0.522
16	4.95	2.22	3.685	0.52
17	2.5	2.22	3.685	0.253
18	2.5	2.22	3.685	0.253
19	0.051	2.22	3.685	0.194
20	2.5	0.228	3.685	0.202

Appendix Table 101: ANOVA for CCD of Set 2(i) mixture of HCs in modified MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	10	0.509	0.051	16.73	0
Blocks	1	0.014	0.014	4.74	0.058
Linear	3	0.396	0.132	43.41	0
GR1	1	0.09	0.09	29.52	0
GR2	1	0.0455	0.045	14.94	0.004
GS1	1	0.261	0.261	85.77	0
Square	3	0.09	0.03	9.83	0.003
GR1*GR1	1	0.048	0.048	15.92	0.003
GR2*GR2	1	0.009	0.009	3.08	0.113
GS1*GS1	1	0.043	0.043	14.01	0.005
2-Way Interaction	3	0.009	0.003	0.97	0.45
GR1*GR2	1	0.007	0.007	2.37	0.158
GR1*GS1	1	0.002	0.002	0.53	0.484
GR2*GS1	1	0	0	0	1
Error	9	0.027	0.003		
Lack-of-Fit	5	0.027	0.005	72.48	0.001
Pure Error	4	0	0		
Total	19	0.537			

Appendix Table 102: Model summary for CCD of Set 2(i) mixture of HCs in modified MSM.

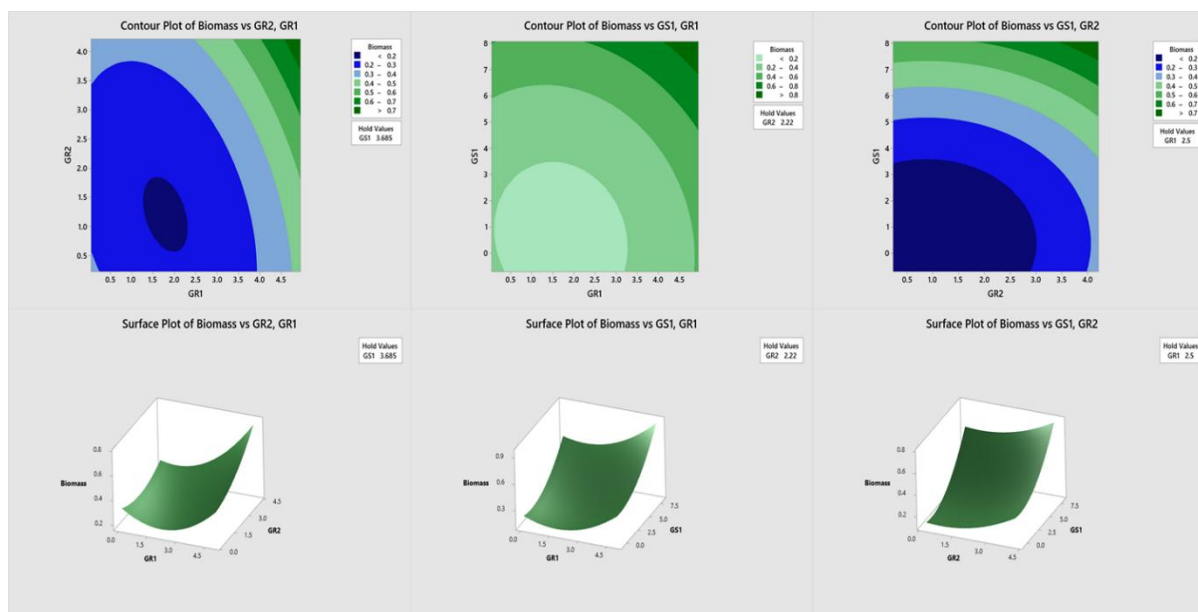
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	0.168	0.339	0.496	0.949	0.892	0.546

Appendix Table 103: Multiple response prediction for CCD of Set 2(i) mixture of HCs.

Variable	GR1	GR2	GS1	Biomass fit	
				Target	Predict
Optimized setting	4.237	3.891	6.113	0.779	0.857

$$\begin{aligned} \text{Biomass} = & 0.289 - 0.129 \text{ GR1} - 0.073 \text{ GR2} - 0.015 \text{ GS1} + 0.027 \text{ GR1*GR1} \\ & + 0.018 \text{ GR2*GR2} + 0.008 \text{ GS1*GS1} + 0.016 \text{ GR1*GR2} + 0.004 \text{ GR1*GS1} \\ & + 0.000 \text{ GR2*GS1} \end{aligned}$$

**Appendix
Equation 30**



Appendix Figure 37: Contour and surface plot of significant factors for Set 2(i) mixture of HCs in modified MSM.



o Set 2(ii)

Appendix Table 104: CCD of Set 2 (ii) mixture of HCs in modified MSM.

- 10 mg/L *n*-hexadecane, 5 mg/L Anthracene, 5 mg/L Phenanthrene, 10 mg/L Benzene, 10 mg/L Toluene, 10 mg/L Xylene
- Design: CCD ($\alpha = 1.633$)

Run	GR2	GR4	GE1	Biomass (mg/mL)
1	0.228	2.34	3.685	0.187
2	2.22	0.152	3.685	0.197
3	4.212	2.34	3.685	0.497
4	2.22	2.34	3.685	0.213
5	2.22	2.34	3.685	0.211
6	2.22	4.528	3.685	0.302
7	2.22	2.34	-0.7	0.163
8	2.22	2.34	8.07	0.502
9	2.22	2.34	3.685	0.208
10	2.22	2.34	3.685	0.211
11	1	3.68	6.37	0.544
12	3.44	1	1	0.165
13	1	1	1	0.152
14	3.44	3.68	6.37	0.789
15	1	1	6.37	0.394
16	2.22	2.34	3.685	0.214
17	2.22	2.34	3.685	0.218
18	1	3.68	1	0.211
19	3.44	1	6.37	0.496
20	3.44	3.68	1	0.378

Appendix Table 105: ANOVA for CCD of Set 2 (ii) mixture of HCs in modified MSM.

Source	DF	Adj SS	Adj MS	F-value	p-value
Model	10	0.535	0.054	16.53	0
Blocks	1	0.011	0.011	3.37	0.100
Linear	3	0.401	0.134	41.31	0
GR2	1	0.08	0.08	24.72	0.001
GR4	1	0.059	0.059	18.19	0.002
GE1	1	0.262	0.262	81.01	0
Square	3	0.105	0.035	10.8	0.002
GR2*GR2	1	0.056	0.056	17.22	0.002
GR4*GR4	1	0.012	0.012	3.74	0.085
GE1*GE1	1	0.05	0.05	15.38	0.003
2-Way Interaction	3	0.018	0.006	1.87	0.205
GR2*GR4	1	0.011	0.011	3.4	0.098
GR2*GE1	1	0.003	0.003	1.08	0.327
GR4*GE1	1	0.004	0.004	1.13	0.316
Error	9	0.03	0.003		
Lack-of-Fit	5	0.029	0.006	410.19	0
Pure Error	4	0	0		
Total	19	0.565			

Appendix Table 106: Model summary for CCD of Set 2 (ii) mixture of HCs in modified MSM.

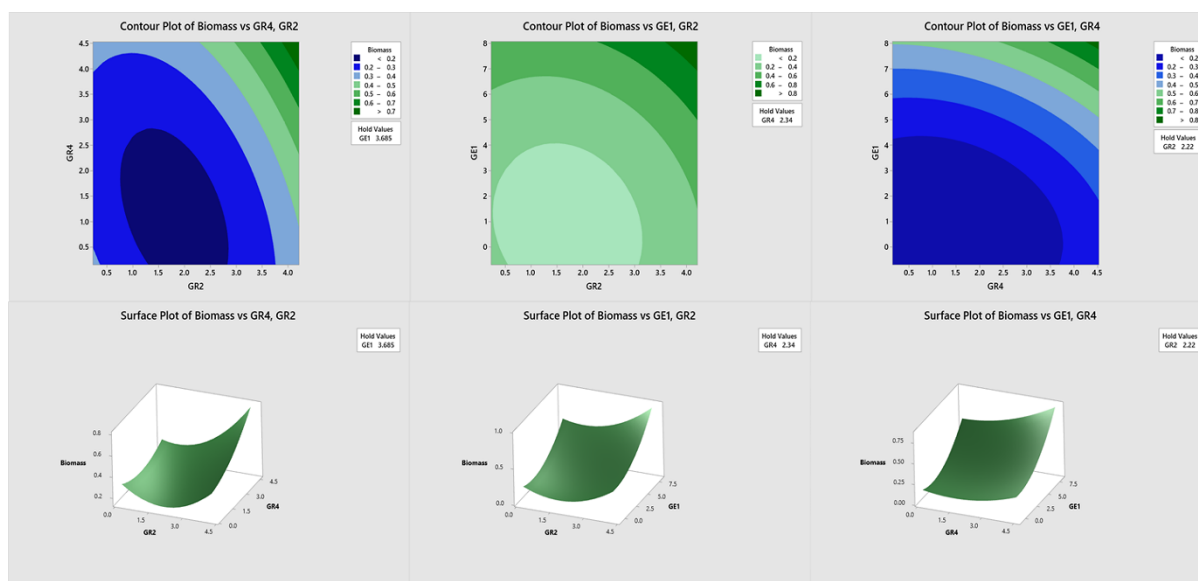
Statistical parameters	Standard Deviation (SD)	Mean	Coefficient of variance (C.V)	R^2	Adjusted R^2	Predicted R^2
Predicted response	0.172	0.313	0.552	0.948	0.891	0.511

Appendix Table 107: Multiple response prediction for CCD of Set 2(ii) mixture of HCs in modified MSM.

Variable	GR2	GR4	GE1	Biomass fit	
				Target	Predict
Optimized setting	3.506	3.999	6.307	0.777	0.789

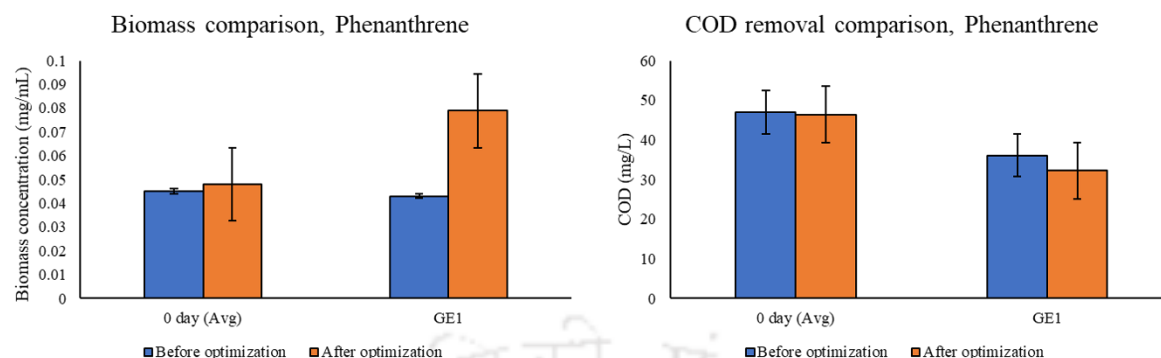
$$\begin{aligned} \text{Biomass} = & 0.398 - 0.207 \text{ GR2} - 0.102 \text{ GR4} - 0.039 \text{ GE1} + 0.044 \text{ GR2*GR2} \\ & + 0.017 \text{ GR4*GR4} + 0.009 \text{ GE1*GE1} + 0.023 \text{ GR2*GR4} + 0.006 \text{ GR2*GE1} \\ & + 0.006 \text{ GR4*GE1} \end{aligned}$$

**Appendix
Equation 31**

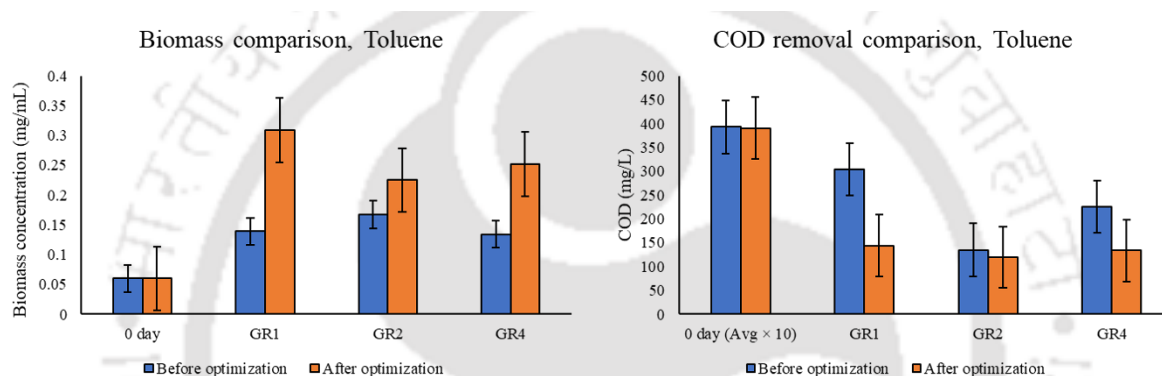


Appendix Figure 38: Contour and surface plots for significant factors of Set 2(ii) mixture of HCs in modified MSM.

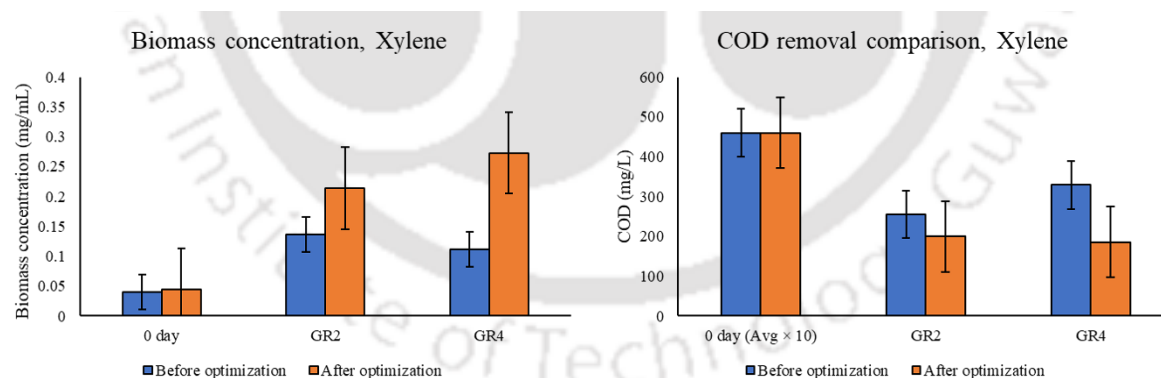
■ **Biomass and COD removal comparison for assessing enhanced growth in optimized media**



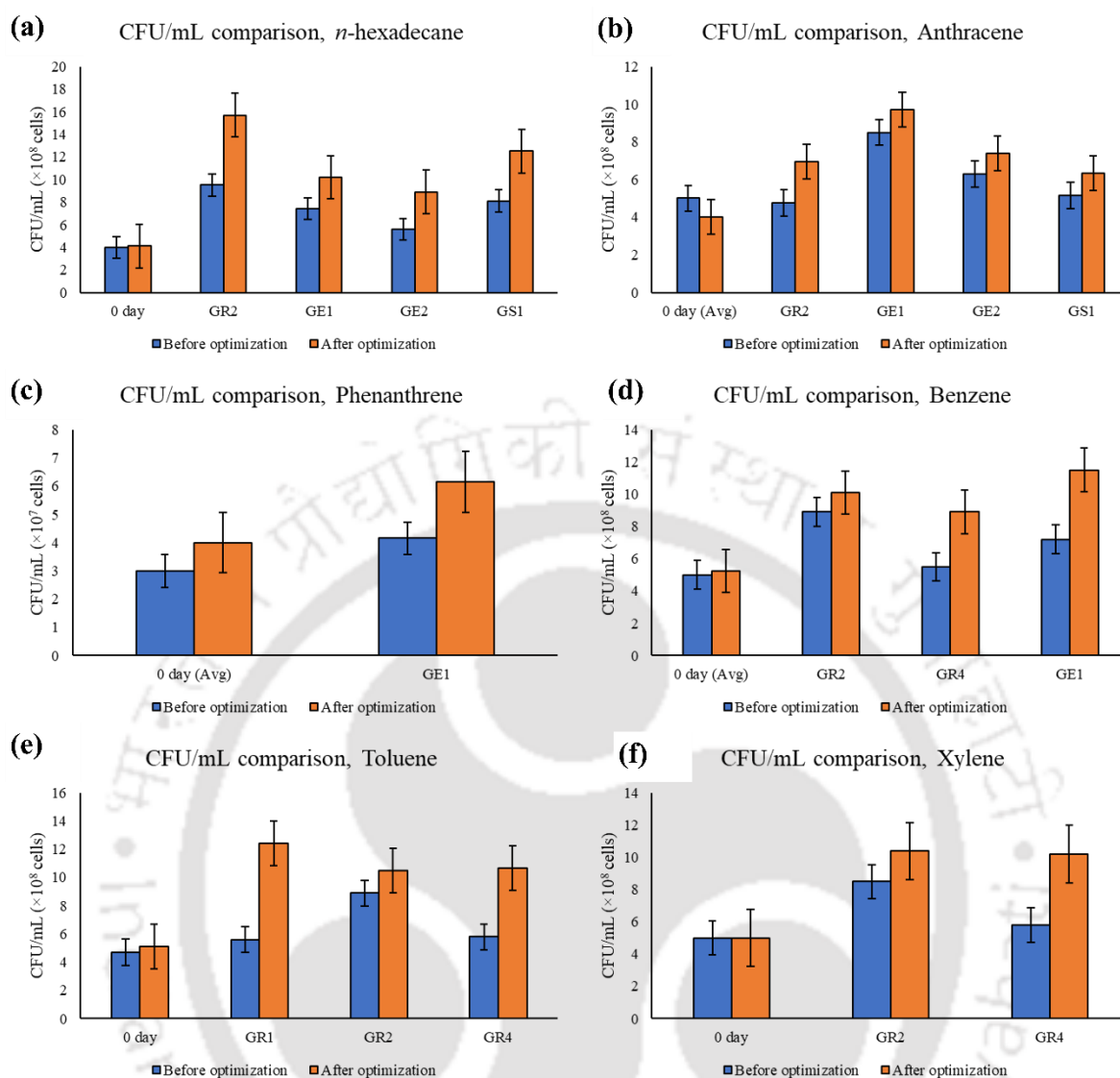
Appendix Figure 39: Comparison of biomass concentration and COD removal of selected strain(s) in phenanthrene MSM.



Appendix Figure 40: Comparison of biomass concentration and COD removal of selected strain(s) in toluene MSM.



Appendix Figure 41: Comparison of biomass concentration and COD removal of selected strain(s) in xylene MSM.



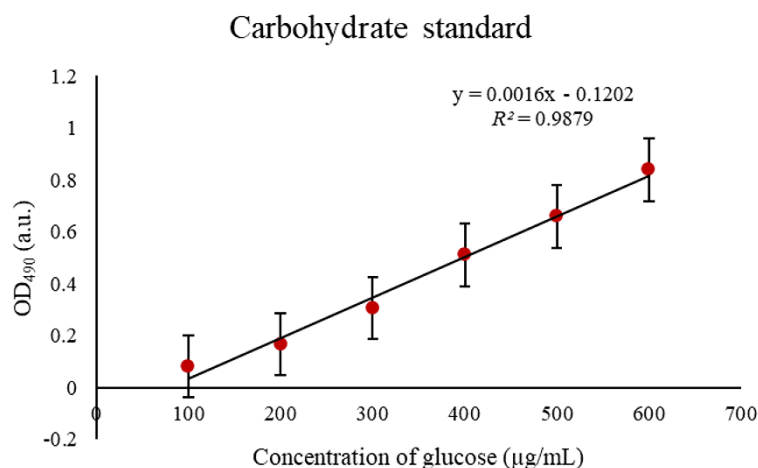
Appendix Figure 42: Comparison of CFU/mL to check biomass viability of selected strain(s) in (a) *n*-hexadecane; (b) anthracene; (c) phenanthrene; (d) benzene; (e) toluene; and (f) xylene supplemented MSM.

Appendix Table 108: Summary of media optimization carried out for the selected strains in different classes of HCs.

Hydrocarbon(s) (HCs)	1 st and 2 nd order optimization using PBD and CCD		Max. biomass (mg/mL)	
	Significant factors PBD	Optimized factors CCD	Before	After
<i>n</i> -hexadecane	FeSO ₄ .7H ₂ O, NaCl, Inoculum, <i>n</i> -hexadecane (GR2, GS1), Time (GE1, GE2)	FeSO ₄ .7H ₂ O: 41.64 mg/L, NaCl: 246.05 mg/L, Time: 41.64 days, Inoculum: 3.44%	2.96 (<i>Staphylococcus</i> sp. GR2)	5.06 (<i>Staphylococcus</i> sp. GR2)
Anthracene	FeSO ₄ .7H ₂ O, NaCl, Anthracene, Inoculum (GE1, GS1), Time (GR2, GE2)	FeSO ₄ .7H ₂ O: 65.34 mg/L, NaCl: 334.88 mg/L, Time: 27.14 days, Inoculum: 6.37%	0.31 (<i>Bacillus</i> sp. GE1)	0.45 (<i>Bacillus</i> sp. GE1)
Benzene	FeSO ₄ .7H ₂ O, NaCl, Benzene, Time (GR2, GE1), Inoculum (GR4)	FeSO ₄ .7H ₂ O: 65.34 mg/L, NaCl: 334.88 mg/L, Time: 47.25 days, Inoculum: 3.68%	0.137 (<i>Bacillus</i> sp. GE1)	0.289 (<i>Bacillus</i> sp. GE1)
Toluene	FeSO ₄ .7H ₂ O, NaCl, Toluene, Time (GR1, GR2), Inoculum (GR4)	FeSO ₄ .7H ₂ O: 65.34 mg/L, NaCl: 334.88 mg/L, Time: 43.23 days, Inoculum: 3.68%	0.137 (<i>Aquamicrobium</i> sp. GR1)	0.265 (<i>Aquamicrobium</i> sp. GR1)
Xylene	FeSO ₄ .7H ₂ O, NaCl, Inoculum, Time (GR2), Xylene (GR4)	FeSO ₄ .7H ₂ O: 65.34 mg/L, NaCl: 334.88 mg/L, Time: 39.2 days, Inoculum: 3.68%	0.136 (<i>Aquamicrobium</i> sp GR4)	0.273 (<i>Aquamicrobium</i> sp GR4)
Phenanthrene	FeSO ₄ .7H ₂ O, NaCl, Inoculum (GE1)	FeSO ₄ .7H ₂ O: 65.34 mg/L, NaCl: 334.88 mg/L, Inoculum: 3.68%	0.12 (<i>Bacillus</i> sp. GE1)	0.21 (<i>Bacillus</i> sp. GE1)

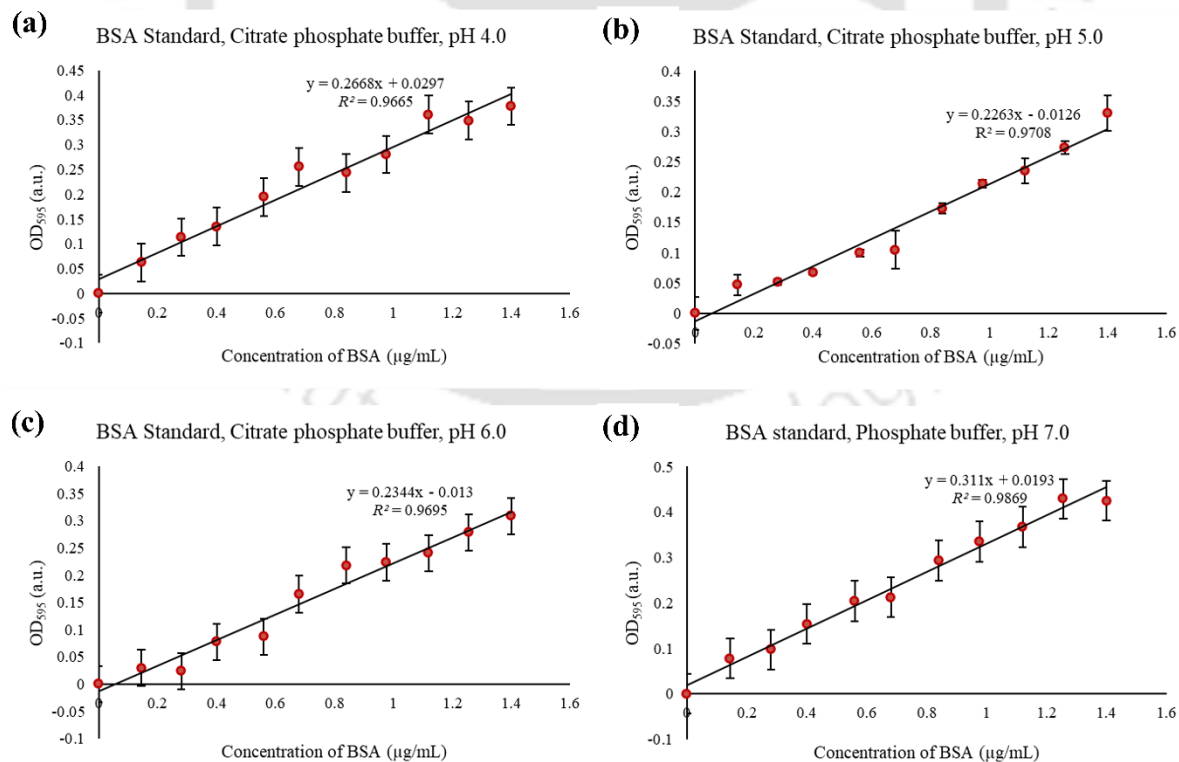
➤ Chapter 4: Analysis of biomass characteristics and enzyme activities for insight into bioremediation of waste sludge

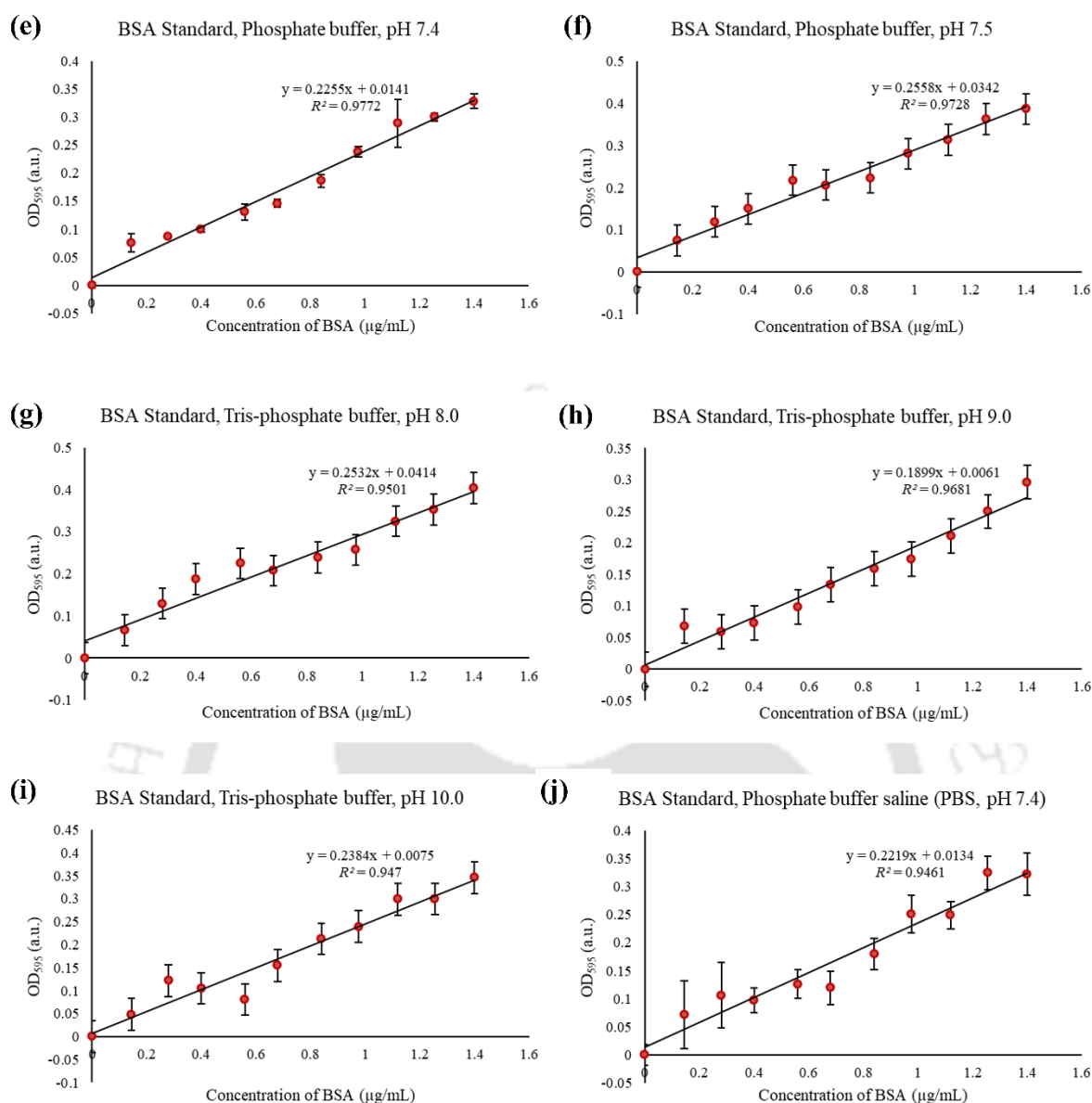
▪ Estimation of total carbohydrate content in microbial biomass



Appendix Figure 43: Standard curve for total carbohydrate estimation.

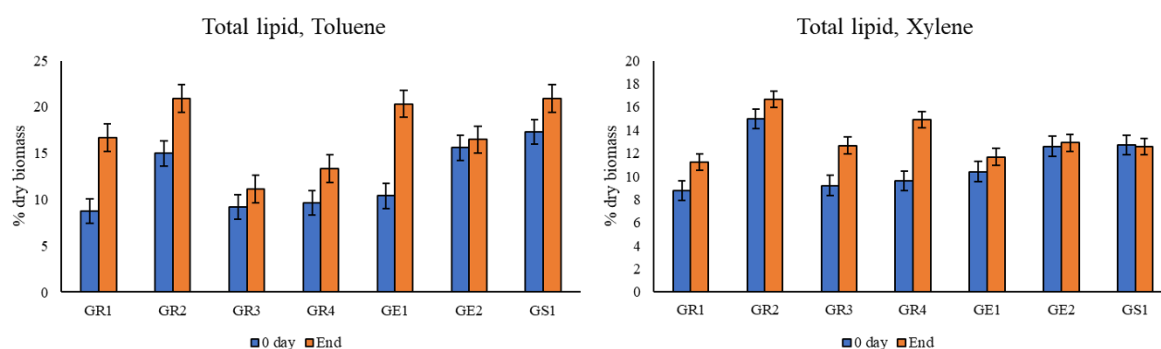
▪ Estimation of total protein content in microbial biomass



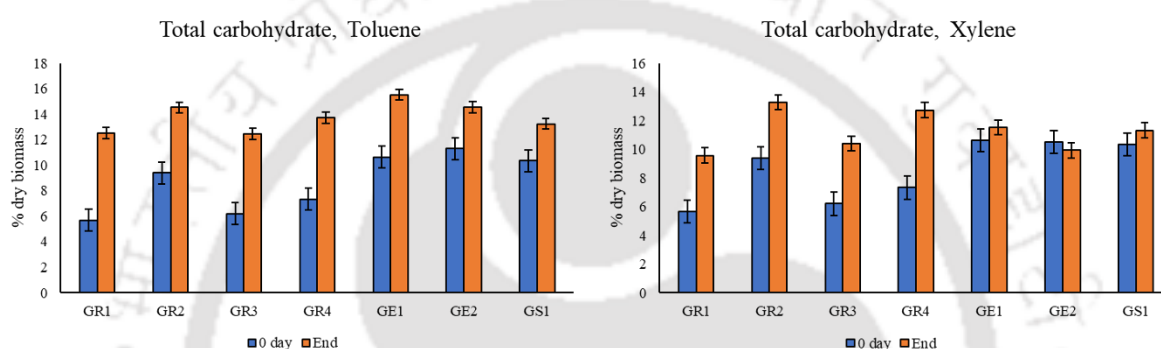


Appendix Figure 44: BSA standard in respective buffers at pH (a) 4.0; (b) 5.0; (c) 6.0; (d) 7.0; (e) 7.4; (f) 7.5; (g) 8.0; (h) 9.0; (i) 10.0; (j) 1X PBS, pH=7.4.

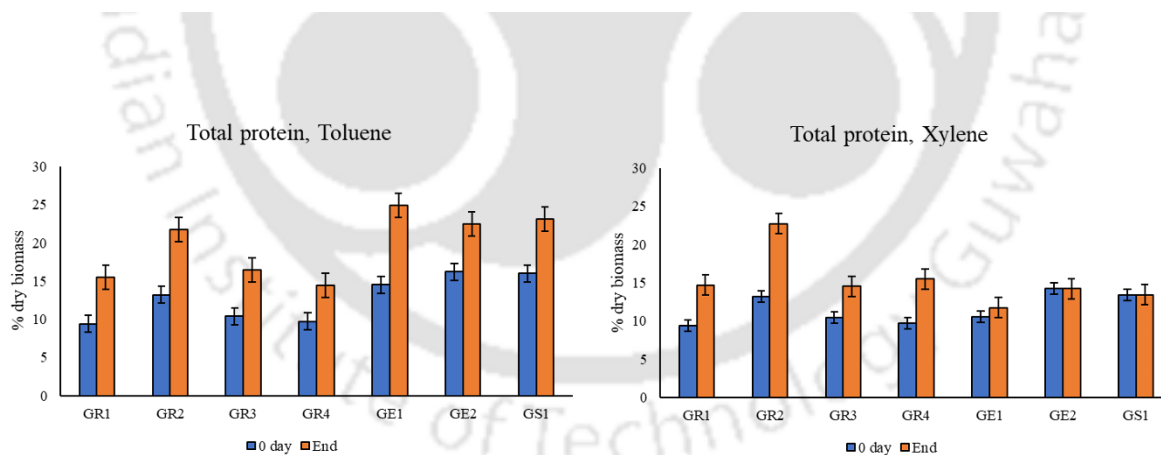
■ Estimation of lipid, carbohydrate and protein content



Appendix Figure 45: Total lipid from biomass of isolates after incubation in toluene and xylene MSM.

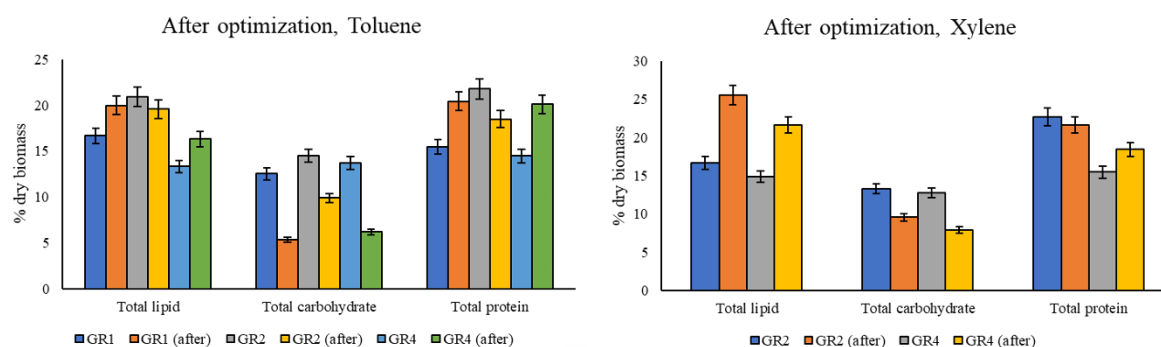


Appendix Figure 46: Total carbohydrate from biomass of isolates after incubation in toluene and xylene MSM.



Appendix Figure 47: Total protein from biomass of isolates after incubation in toluene and xylene MSM.

■ Estimation of lipid, carbohydrate and protein content after media optimization



Appendix Figure 48: Comparison of total lipid, carbohydrate and protein from biomass of isolates before and after statistical media optimization in toluene and xylene MSM.

■ Summary of biomass characteristics and enzyme assay

Appendix Table 109: Summary of lipid, carbohydrate and protein contents from biomass of isolates.

Pollutants	Maximum concentration before optimization (% dry biomass)			Maximum concentration before optimization (% dry biomass)		
	Lipid	Carbohydrate	Protein	Lipid	Carbohydrate	Protein
<i>n</i>-hexadecane	19.44 (GR2)	16.67 (GE2)	21.37 (GS1)	21.11 (GR2)	35.66 (GS1)	17.54 (GR2)
Anthracene	22.94 (GS1)	12.22 (GE1)	20.19 (GE1)	23.58 (GS1)	8.58 (GS1)	11.79 (GS1)
Phenanthrene	23.28 (GE1)	17.48 (GE1)	18.29 (GS1)	17.72 (GE1)	12.65 (GE1)	26.58 (GE1)
Benzene	28.99 (GR2)	18.55 (GE2)	31.11 (GS1)	25.5 (GR2)	10.72 (GR2)	23.32 (GR2)
Toluene	20.91 (GR2 & GS1)	15.52 (GE1)	24.94 (GE1)	20 (GR1)	9.9 (GR2)	20.42 (GR1)
Xylene	16.67 (GR2)	13.27 (GR2)	22.71 (GR2)	25.51 (GR2)	9.54 (GR2)	21.62 (GR2)

Appendix Table 110: Lipid, carbohydrate and protein concentrations from biomass before statistical optimization.

Pollutants	Parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
<i>n</i>-hexadecane	Lipid (mg/mL)	0.012	0.051	0.01	0.006	0.043	0.036	0.04
	Carbohydrate (mg/mL)	0.006	0.031	0.005	0.002	0.033	0.032	0.034
	Protein (mg/mL)	0.012	0.049	0.009	0.007	0.045	0.038	0.052
Anthracene	Lipid (mg/mL)	0.007	0.014	0.005	0.006	0.059	0.023	0.025
	Carbohydrate (mg/mL)	0.006	0.01	0.005	0.002	0.038	0.017	0.012
	Protein (mg/mL)	0.009	0.014	0.006	0.007	0.055	0.029	0.023
Phenanthrene	Lipid (mg/mL)	0.003	0.007	0.002	0.002	0.01	0.007	0.007
	Carbohydrate (mg/mL)	0.003	0.005	0.002	0.002	0.008	0.005	0.005
	Protein (mg/mL)	0.004	0.006	0.003	0.002	0.008	0.006	0.006
Benzene	Lipid (mg/mL)	0.034	0.045	0.022	0.027	0.045	0.031	0.035
	Carbohydrate (mg/mL)	0.022	0.028	0.021	0.019	0.028	0.022	0.023
	Protein (mg/mL)	0.037	0.048	0.03	0.028	0.046	0.033	0.042
Toluene	Lipid (mg/mL)	0.025	0.035	0.015	0.018	0.031	0.02	0.028
	Carbohydrate (mg/mL)	0.018	0.024	0.017	0.019	0.024	0.017	0.018
	Protein (mg/mL)	0.023	0.036	0.023	0.02	0.038	0.027	0.031
Xylene	Lipid (mg/mL)	0.016	0.024	0.013	0.015	0.018	0.015	0.017
	Carbohydrate (mg/mL)	0.013	0.019	0.011	0.013	0.018	0.012	0.015
	Protein (mg/mL)	0.02	0.032	0.015	0.016	0.018	0.017	0.018

Appendix Table 111: Lipid, carbohydrate and protein concentration from biomass after statistical optimization.

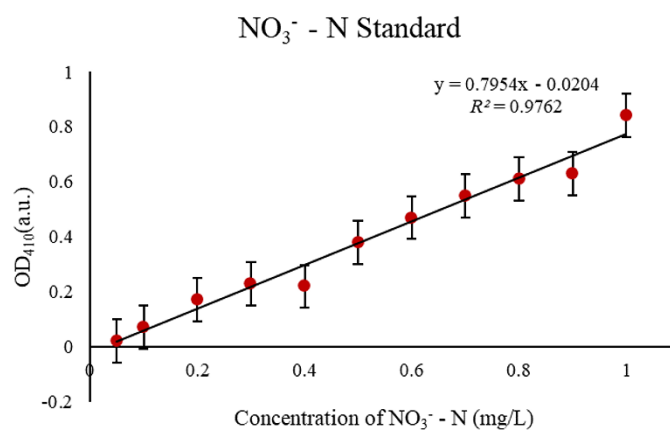
Pollutants	Parameters	GR1	GR2	GR3	GR4	GE1	GE2	GS1
n-hexadecane	Lipid (mg/mL)	-	0.108	-	-	0.07	0.049	0.094
	Carbohydrate (mg/mL)	-	0.028	-	-	0.026	0.015	0.027
	Protein (mg/mL)	-	0.09	-	-	0.081	0.042	0.077
Anthracene	Lipid (mg/mL)	-	0.018	-	-	0.09	0.039	0.058
	Carbohydrate (mg/mL)	-	0.011	-	-	0.047	0.02	0.021
	Protein (mg/mL)	-	0.019	-	-	0.085	0.035	0.029
Phenanthrene	Lipid (mg/mL)	-	-	-	-	0.014	-	-
	Carbohydrate (mg/mL)	-	-	-	-	0.01	-	-
	Protein (mg/mL)	-	-	-	-	0.021	-	-
Benzene	Lipid (mg/mL)	-	0.057	-	0.044	0.062	-	-
	Carbohydrate (mg/mL)	-	0.024	-	0.022	0.029	-	-
	Protein (mg/mL)	-	0.052	-	0.058	0.059	-	-
Toluene	Lipid (mg/mL)	0.062	0.044	-	0.041	-	-	-
	Carbohydrate (mg/mL)	0.016	0.022	-	0.016	-	-	-
	Protein (mg/mL)	0.063	0.042	-	0.051	-	-	-
Xylene	Lipid (mg/mL)	-	0.055	-	0.059	-	-	-
	Carbohydrate (mg/mL)	-	0.02	-	0.022	-	-	-
	Protein (mg/mL)	-	0.046	-	0.05	-	-	-

Appendix Table 112: Summary of enzyme assays of crude protein obtain from the biomass of isolates.

Enzyme assay	Optimum conditions			Maximum enzyme activity (U mg ⁻¹)	Kinetic parameters	
	Temperature (°C)	pH	Stability (minutes)		Max V_{max} (U mg ⁻¹)	Max. K_m
<i>alkB</i>	37.5	7.4	120	104.35 (GR2)	316.16 (GS1)	0.117 M <i>n</i> -hexadecane (GR4)
<i>ndo</i>	32.5	7.5	120	6.93 (GE1)	11.52 (GE1)	5.63 mM naphthalene (GE1)
<i>catA</i>	30	7	180	15.7 (GR4)	38.3 (GE1)	299.58 µM catechol (GE1)
<i>catE</i>	30	7.5	120-180	28.33 (GE1)	59.3 (GE1 & GS1)	424.81 µM catechol (GS1)

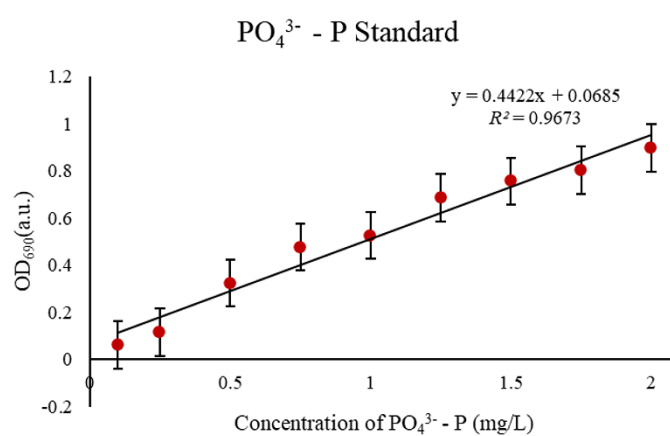
➤ Chapter 5: Application of designed consortia for bioremediation of waste sludge

▪ Estimation of nitrate (NO_3^- -N)



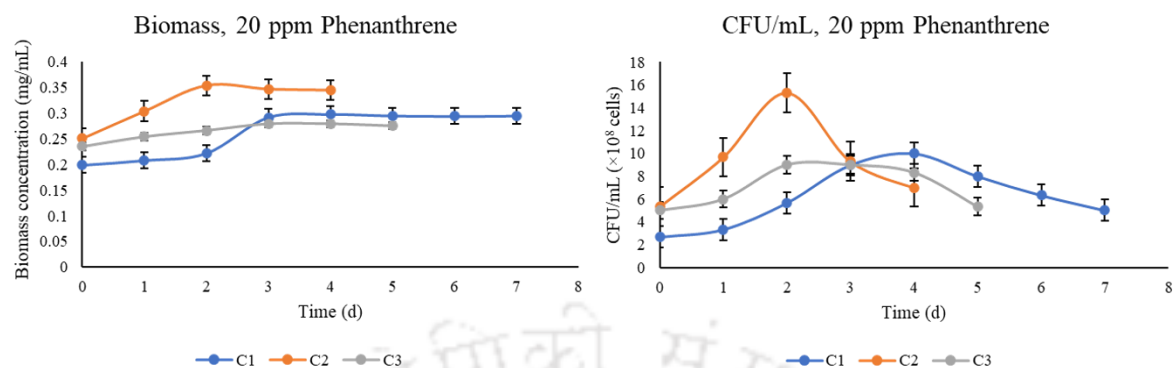
Appendix Figure 49: Standard curve for NO_3^- -N estimation.

▪ Estimation of phosphate (PO_4^{3-} -P)

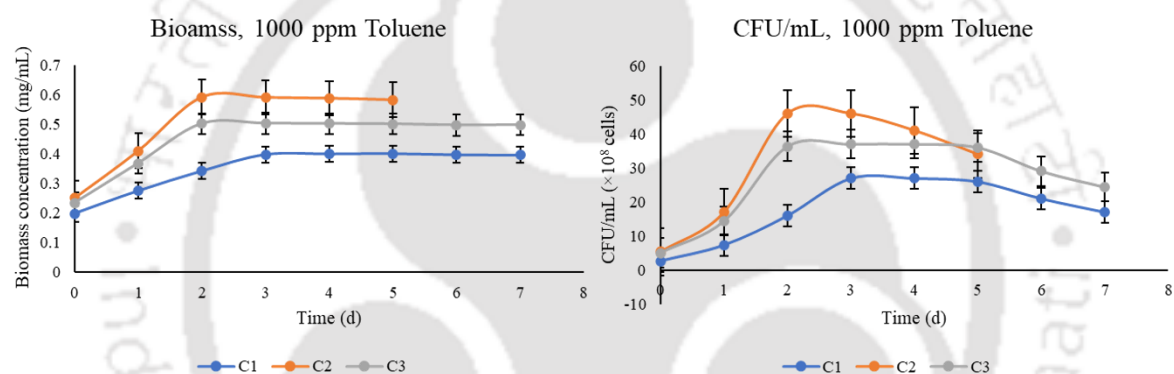


Appendix Figure 50: Standard curve for PO_4^{3-} -P estimation.

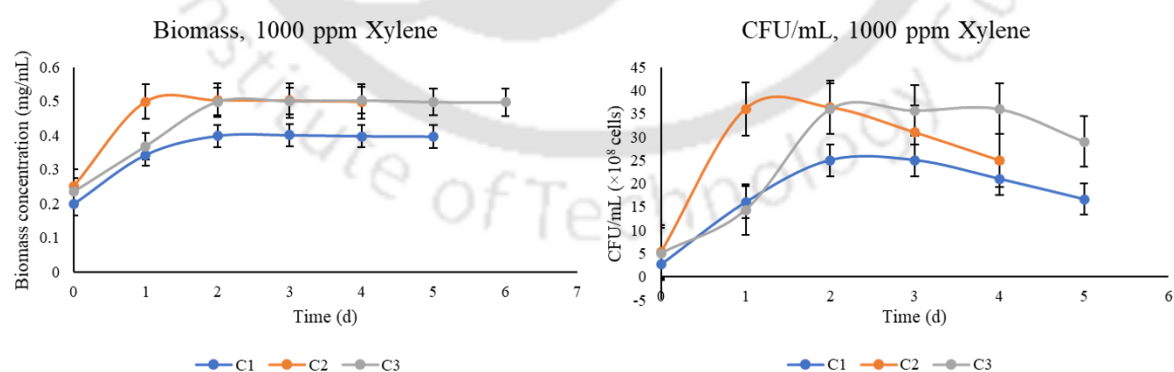
■ Growth profile of consortia in different class of hydrocarbons (HCs)



Appendix Figure 51: Biomass and CFU/mL of consortia in phenanthrene mMSM.

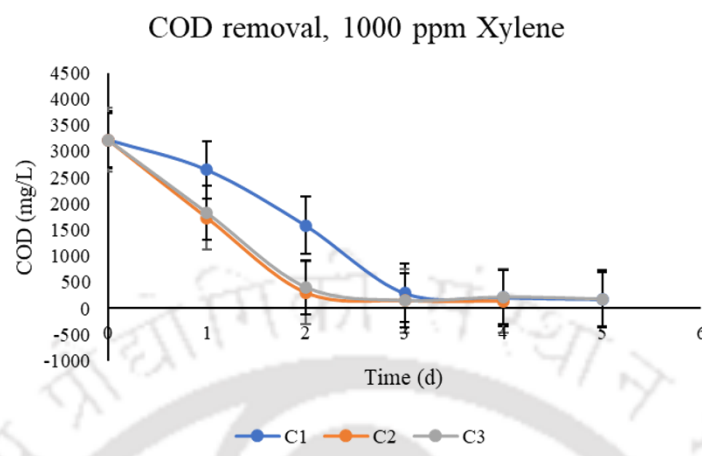


Appendix Figure 52: Biomass and CFU/mL of consortia in toluene mMSM.



Appendix Figure 53: Biomass and CFU/mL of consortia in xylene mMSM.

▪ COD removal of hydrocarbon(s) (HCs)



Appendix Figure 54: COD removal by consortia in xylene mMSM.

▪ Biomass growth and COD utilization kinetics of consortia in different class of hydrocarbons (HCs)

Appendix Table 113: Biomass growth and HC utilization of consortia in **phenanthrene** mMSM.

Biomass growth: Exponential growth ($R^2 = 0.88$) for C1, Logarithmic growth ($R^2 = 0.77, 0.96$), Andrew's kinetic model for C1, C2 and C3
Hydrocarbon (HC) utilization: Logarithmic COD removal ($R^2 = 0.86, 0.93$) for C1 and C2, Exponential COD removal for C3 ($R^2 = 0.96$), Haldane's kinetic model for C1, C2 and C3

Consortia	Biomass growth						Hydrocarbon (HC) utilization rate						Yield (Y_B) (g/g)
	k (d ⁻¹)	t_d (d)	μ_{max} (d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	k (d ⁻¹)	$t_{1/2}$ (d)	q_{max} (g/g d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	
C1	0.135	5.13	1844.93	544.68	0.66	0.78	8.88	720.37	0.778	1.49	1.76	0.86	0.8
C2	0.043	5.84	1236.43	68.9	63.2	0.8	15.85	151.5	2.47	56.56	0.99	0.78	0.6
C3	0.019	12.37	1152.39	153.74	2.01	0.87	0.043	16.12	6.63	76.02	0.343	0.84	0.2

Appendix Table 114: Biomass growth and HC utilization of consortia in **toluene** mMSM.

Biomass growth: Logarithmic growth ($R^2 = 0.87-0.97$) for C1, C2 and C3, Andrew's kinetic model for C1, C2 and C3
Hydrocarbon (HC) utilization: Logarithmic COD removal ($R^2 = 0.9$) for C1, Exponential COD removal ($R^2 = 0.96$) for C2 and C3, Haldane's kinetic model for C1 and C2, Monod's kinetic model for C3

Consortia	Biomass growth						Hydrocarbon (HC) utilization rate						Yield (Y_B) (g/g)
	k (d ⁻¹)	t_d (d)	μ_{max} (d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	k (d ⁻¹)	$t_{1/2}$ (d)	q_{max} (g/g d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	
C1	0.097	7.77	5.41	7.56	141.92	0.97	0.265	1.93	94.7	7.34	47.94	0.83	0.061
C2	0.175	4.18	101.17	74	8.77	0.93	1.28	0.54	147.4	11.27	54.98	0.92	0.105
C3	0.131	5.99	24.46	20.86	36.18	0.92	1.26	0.55	198.5	13.92	44.84	0.85	0.083

Appendix Table 115: Biomass growth and HC utilization by consortia in **xylene** mMSM.

Biomass growth: Logarithmic growth ($R^2 = 0.86-0.87$) for C1, C2 and C3, Andrew's kinetic model for C1, C2 and C3
Hydrocarbon (HC) utilization: Linear COD removal ($R^2 = 0.997$) for C1, Exponential COD removal ($R^2 = 0.96, 0.97$) for C2 and C3, Haldane's kinetic model for C1, C2 and C3

Consortia	Biomass growth						Hydrocarbon (HC) utilization rate						Yield (Y_B) (g/g)
	k (d ⁻¹)	t_d (d)	μ_{max} (d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	k (d ⁻¹)	$t_{1/2}$ (d)	q_{max} (g/g d ⁻¹)	K_s (g/L)	K_i (mg/L)	R^2	
C1	0.036	19.25	0.183	1072.82	42.76	0.95	2.775	1.37	1113.68	79.41	2732.75	0.99	0.069
C2	0.05	13.86	0.327	804.28	3302.29	0.995	1.23	0.56	2019.58	282.28	2001.2	0.995	0.082
C3	0.127	6.36	2.16	8714.75	151.82	0.99	0.997	0.7	38.5	14.35	84.08	0.999	0.089

▪ Biomass characteristics of consortia in different hydrocarbons (HCs)

Appendix Table 116: Lipid, carbohydrate and protein concentrations from biomass of consortia in different HC supplemented mMSM.

Pollutants	Parameters	C1	C2	C3
<i>n</i>-hexadecane	Lipid (mg/mL)	0.086	0.149	0.164
	Carbohydrate (mg/mL)	0.075	0.134	0.14
	Protein (mg/mL)	0.087	0.157	0.176
Anthracene	Lipid (mg/mL)	0.064	0.097	0.092
	Carbohydrate (mg/mL)	0.057	0.083	0.08
	Protein (mg/mL)	0.064	0.097	0.097
Phenanthrene	Lipid (mg/mL)	0.051	0.078	0.054
	Carbohydrate (mg/mL)	0.054	0.075	0.053
	Protein (mg/mL)	0.064	0.078	0.059
Benzene	Lipid (mg/mL)	0.109	0.193	0.132
	Carbohydrate (mg/mL)	0.077	0.129	0.108
	Protein (mg/mL)	0.13	0.223	0.157
Toluene	Lipid (mg/mL)	0.081	0.137	0.097
	Carbohydrate (mg/mL)	0.09	0.163	0.122
	Protein (mg/mL)	0.1	0.162	0.123
Xylene	Lipid (mg/mL)	0.091	0.152	0.137
	Carbohydrate (mg/mL)	0.077	0.107	0.112
	Protein (mg/mL)	0.103	0.137	0.127

▪ **Biomass characteristics and enzyme activities after N and P addition**

Appendix Table 117: Biomass characteristics and enzyme activities after N and P addition.

Consortia	Biomass characteristics (% dry biomass)			Enzyme activities (U mg ⁻¹)			
	Lipid	Carbohydrate	Protein	<i>alkB</i>	<i>ndo</i>	<i>catA</i>	<i>catE</i>
C1	29.36	12.31	22.35	37.55	1.08	1.67	2.46
C2	32.19	13.39	25.63	48.6	1.64	2.06	1.06
C3	30.2	21.55	14.42	42.79	1.33	1.82	0.93
C1+N+P	39.44	19.33	28.33	62.37	1.88	4.26	11.63
C2+N+P	45.32	17.36	33.17	92.5	2.86	7.99	5.22
C3+N+P	33.12	32.44	20.37	77.38	2.42	6.35	6.96

Appendix Table 118: Lipid, carbohydrate and protein concentrations from biomass after N and P addition.

C:N:P	Parameters	C1	C2	C3
Without N and P	Lipid (mg/mL)	0.236	0.366	0.244
	Carbohydrate (mg/mL)	0.1	0.152	0.1
	Protein (mg/mL)	0.18	0.291	0.175
N and P (100:5:1)	Lipid (mg/mL)	0.309	0.927	0.412
	Carbohydrate (mg/mL)	0.142	0.395	0.179
	Protein (mg/mL)	0.235	0.681	0.321
N and P (100:10:1)	Lipid (mg/mL)	0.387	0.609	0.74
	Carbohydrate (mg/mL)	0.186	0.231	0.383
	Protein (mg/mL)	0.298	0.42	0.608

■ Biomass growth and COD removal after N and P addition

Appendix Table 119: Summary of removal and growth profile of consortia after N and P addition as C:N:P = 100:5:1.

Consortia	COD removal				Biomass growth				Yield (Y_B) (mg/g)
	q (g/g.d)	k (d ⁻¹)	$t_{1/2}$ (d)	% Removal	μ (d ⁻¹)	k (d ⁻¹)	t_d (d)	Fractional increase	
C1	0.618	0.103	6.73	78.4	0.064	0.137	5.06	3.39	0.045
C1+N+P	0.853	0.401	1.72	87.36	0.072	0.229	3.03	6.67	0.079
C2	0.429	0.149	4.65	86.27	0.067	0.446	1.47	3.95	0.042
C2+N+P	0.879	0.21	3.33	92.44	0.076	1.29	1.14	9.78	0.088
C3	0.718	0.103	8.43	82.65	0.064	0.097	4.06	2.9	0.045
C3+N+P	0.92	0.437	4.48	84.28	0.088	0.258	2.12	4.45	0.089

Appendix Table 120: Residual COD, N, P; biomass and bacterial count after N and P addition.

C:N:P	Consortia	COD (mg/L)	NO ₃ ⁻ (mg/L)	PO ₄ ³⁻ (mg/L)	Biomass (mg/mL)	CFU/mL ($\times 10^8$ cells)
Without N and P	C1	2442.74	-	139.02	0.816	52.33
	C2	1557.25	-	123.14	1.151	77.67
	C3	1962.11	-		0.811	66
100:5:1	C1	1429.46	2117.18	322.67	1.467	74
	C2	854.96	1200.02	228.96	2.247	107
	C3	1777.77	1910.44	300.59	1.241	93
100:10:1	C1	618.6	3053.17	206.82	1.889	88
	C2	972.57	3546.8	277.26	1.729	109
	C3	815.38	3317.79	249.09	1.679	95

Appendix Table 121: Summary of removal and growth profile of consortia after N and P addition as C:N:P=100:10:1.

Consortia	COD removal				Biomass growth				Yield (Y_B) (mg/g)
	q (g/g.d)	k (d ⁻¹)	$t_{1/2}$ (d)	% Removal	μ (d ⁻¹)	k (d ⁻¹)	t_d (d)	Fractional increase	
C1	0.618	0.103	6.73	78.4	0.064	0.137	5.06	3.39	0.045
C1+N+P	0.889	0.463	1.32	94.53	0.089	0.269	2.75	12.39	0.084
C2	0.429	0.149	4.65	86.23	0.067	0.446	1.47	3.95	0.042
C2+N+P	0.764	0.197	3.42	91.4	0.071	1.05	1.39	9.82	0.071
C3	0.718	0.103	8.43	82.65	0.064	0.097	4.06	2.9	0.045
C3+N+P	0.96	0.437	4.02	92.79	0.096	0.295	1.91	10.56	0.095

Appendix Table 122: Lipid, carbohydrate and protein concentrations from biomass after experimentation in sludge sample.

Consortia	Parameters	Trial study	Undiluted sludge
C1 (100:10:1)	Lipid (mg/mL)	0.439	0.707
	Carbohydrate (mg/mL)	0.283	0.522
	Protein (mg/mL)	0.4	0.444
C2 (100:5:1)	Lipid (mg/mL)	0.745	0.903
	Carbohydrate (mg/mL)	0.501	0.708
	Protein (mg/mL)	0.717	0.605
C3 (100:10:1)	Lipid (mg/mL)	0.57	0.364
	Carbohydrate (mg/mL)	0.378	0.577
	Protein (mg/mL)	0.539	0.407

- Trial study of optimum C:N:P on diluted sludge sample to investigate the treatment efficiency of the designed consortia

Appendix Table 123: Summary of COD, N, P utilization and biomass growth of consortia after trial experiment in diluted sludge sample.

TRIAL: Input COD: ~11500 mg/L, C1: 100:10:1; C2: 100:5:1; and C3: 100:10:1												
COD removal, N and P utilization and biomass growth summary												
Consortia	COD removal				N and P utilization			Biomass growth				
	q (mg/g.d)	k (d ⁻¹)	$t_{1/2}$ (d)	R^2	% removal	% N	% P	μ (d ⁻¹)	k (d ⁻¹)	t_d (d)	R^2	Fractional increase
C1+N+P	201.27	0.556	2.25	0.99	67.14	72.4	64.8	0.033	0.89	1.83	0.95	5.25
C2+N+P	258.7	0.676	1.55	0.99	74.12	75.62	72.4	0.035	1.27	1.36	0.95	7.42
C3+N+P	204.22	0.604	2.05	0.97	70.12	70.24	66.7	0.034	1.02	1.52	0.97	6.07

Appendix Table 124: Summary of biomass characteristics and enzyme activities of consortia after trial experiment in diluted sludge sample.

TRIAL: Input COD: ~11500 mg/L, C1: 100:10:1; C2: 100:5:1; and C3: 100:10:1								
Biomass characteristics and enzyme assay								
Consortia	Yield (Y_B) (g/gCOD)	Characteristics (% dry biomass)			Enzyme activity (U mg⁻¹)			
		Carbohydrate content	Protein content	Lipid content	<i>alkB</i>	<i>ndo</i>	<i>catA</i>	<i>catE</i>
C1+N+P	0.112	31.08	20.39	28.17	52.33	3.94	7.44	5.77
C2+N+P	0.137	36.27	23.56	33.82	85.27	5.22	8.23	11.57
C3+N+P	0.125	33.41	22.67	34.23	65.2	4.34	7.99	12.11



Research Outputs



Research Outputs

➤ Journal articles

1. *n*-hexadecane degrading microbial strains isolated from petroleum contaminated sites have potential for bioremediation of refinery waste (Manuscript under preparation)
2. Optimization of mineral nutrients and growth parameters of *n*-hexadecane degrading microbial strains for enhanced biomass production for bioremediation of petroleum refinery waste (Manuscript under preparation).

➤ Conferences/Seminars

▪ Thesis work related presentations

1. **Prithwi Chayan Chatterjee**, Debasree Kundu and Sanjukta Patra, *n*-hexadecane degrading strains isolated from a petroleum refinery waste contaminated site for potential application in refinery wastewater bioremediation, 59th Annual conference of “Association of Microbiologists of India (AMI-2018), School of Life Sciences, University of Hyderabad in association with Department of Microbiology, Osmania University, December 9, 2018-December 12, 2018 (Poster presentation).
2. **Prithwi Chayan Chatterjee**, Debasree Kundu and Sanjukta Patra, The scope of *n*-hexadecane degrading microbial strains isolated from petroleum waste contaminated site for bioremediation of refinery waste, Research Conclave 2019-An amalgamation of Academia, Industry and Start-ups, IIT Guwahati, March 14, 2019-March 17, 2019 (Poster presentation), **Awarded best poster presentation.**
3. **Prithwi Chayan Chatterjee** and Sanjukta Patra, Optimization of mineral nutrients and growth parameters of *n*-hexadecane degrading microbial strains for enhanced biomass production for bioremediation of petroleum refinery wastewater, WATER2020, Organized by Centre for the Environment, IIT Guwahati, January 23-25, 2020 (Poster Presentation).
4. **Prithwi Chayan Chatterjee**, Sanjukta Patra and Mohammad Jawed, Investigation of optimum nutrient factors and growth parameters for enhanced biomass production for bioremediation of petroleum refinery wastewater, International conference of Biotechnology for Sustainable Agriculture, Environment and Health (BSAEH-2021), organized by The Biotech Research Society, India and Malviya National Institute of Technology Jaipur, April 4, 2021-April 8, 2021 (Oral and poster presentation).
5. **Prithwi Chayan Chatterjee**, Sanjukta Patra and Mohammad Jawed, Multivariate optimization of nutrient and growth factors of anthracene degrading microbial strains to maximize biomass production for bioremediation of petroleum refinery wastewater, Research and Industrial Conclave 2022-An amalgamation of Academia, Industry and Start-ups, IIT Guwahati, January 20, 2022-January 23, 2022 (Oral presentation) **Awarded 2nd position in oral presentation.**

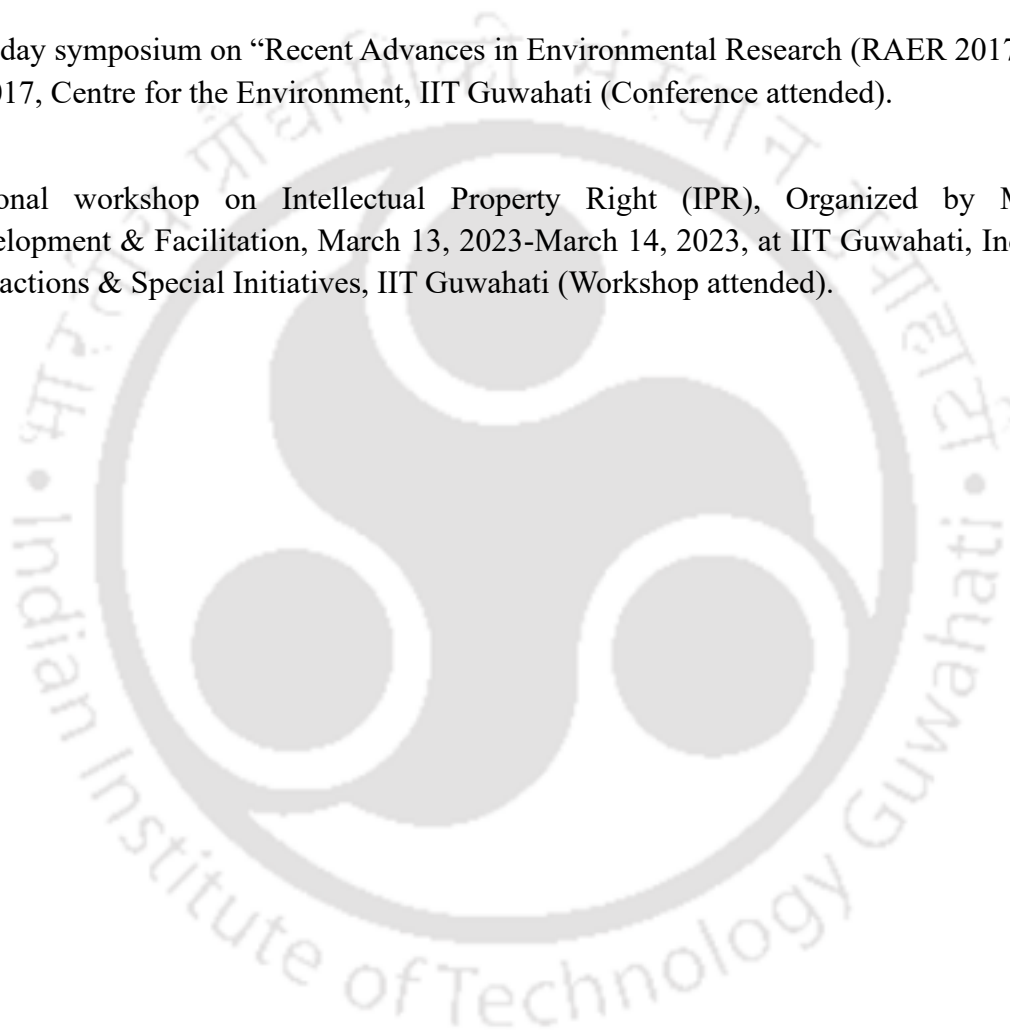
6. **Prithwi Chayan Chatterjee**, Sanjukta Patra and Mohammad Jawed, Enhanced biomass production of anthracene degrading microbial strains promote sustainability through accelerated bioremediation of petroleum refinery waste, North-East Research Conclave (NERC) 2022, May 20, 2022-May 22, 2022 (Poster presentation).
7. **Prithwi Chayan Chatterjee**, Sanjukta Patra and Mohammad Jawed, Microbial uptake of BTX promotes in situ bioremediation in a petroleum refinery waste contaminated site, International conference on Green Energy & Sustainable Energy Technology (GESET-2022), KIIT, Bhubaneswar and Save the Environment (STE), Kolkata, September 15, 2022-September 16, 2022 (Oral presentation).
8. **Prithwi Chayan Chatterjee**, Sanjukta Patra and Mohammad Jawed, BTX degrading microbial strains isolated from petroleum waste contaminated site have potential for bioremediation of refinery waste, 62nd Annual conference of “Association of Microbiologists of India (AMI-2022), University of Mysore, September 21, 2022-September 23, 2022 (Oral presentation).

■ **Other non-thesis work presentations**

1. **Prithwi Chayan Chatterjee**, Debasree Kundu and Sanjukta Patra, Multivariate optimization of process parameters for biomass and lipid production by *Chlorella pyrenoidosa* NCIM 2738, December 9, 2017-December 11, 2017, 5th Annual conference on “Recent Trends in Bioprocessing for Healthcare, Energy and Environment, Bioprocessing India (BPI-2017), Department of Biosciences and Bioengineering, IIT Guwahati (Poster presentation).
2. **Prithwi Chayan Chatterjee**, Debasree Kundu and Sanjukta Patra, Investigation of combined effect of various process parameters on biomass and lipid productivity of *Chlorella pyrenoidosa* NCIM 2738 using response surface methodology, March 8, 2018-March 11, 2018, Research Conclave 2018-An amalgamation of Academia, Industry and Start-ups, IIT Guwahati (Poster presentation) **Awarded 2nd position in poster presentation.**
3. **Prithwi Chayan Chatterjee**, Debasree Kundu and Sanjukta Patra, Optimizing the effect of various process parameters for enhanced biomass and lipid production in *Chlorella pyrenoidosa* NCIM 2738 using response surface methodology, June 2, 2018, Workshop on Advances in Environmental Protection and Sustainability (AEPS-2018), Centre for the Environment, IIT Guwahati (Poster presentation).

▪ Other seminars/workshops attended

1. Workshop on Intellectual Property Right, November 30, 2016-December 1, 2016, at IIT Guwahati, organized by IPR Cell, Research & Development, IIT Guwahati (Workshop attended).
2. Indo-Japan Bilateral Symposium (IJBS-2017) on Future Perspectives on Bioresource Utilization in North-East India, February 1, 2018-February 4, 2018, Jointly organized by IIT Guwahati, India and GIFU University, Japan (Conference attended).
3. One day symposium on “Recent Advances in Environmental Research (RAER 2017), June 1, 2017, Centre for the Environment, IIT Guwahati (Conference attended).
4. National workshop on Intellectual Property Right (IPR), Organized by MSME-Development & Facilitation, March 13, 2023-March 14, 2023, at IIT Guwahati, Industrial Interactions & Special Initiatives, IIT Guwahati (Workshop attended).





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