Sustainable biofuel production through effective wastewater treatment using indigenous algae-bacteria consortia

A Thesis

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DOCTOR OF PHILOSOPHY

by

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STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the supervision of Prof. Debasish Das.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: March 2020 **Bidhu Bhusan Makut**

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Sustainable biofuel production through effective wastewater treatment using indigenous algae-bacteria consortia**" by Mr. Bidhu Bhusan Makut for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Abstract

Microalgal biomass-based biofuel has gained importance as one of the most promising renewable energy sources, in recent years. Although microalgal biofuel technology is gaining ground, bottlenecks towards its commercialization and economic feasibility includes, the high production cost of microalgal biomass, lower biomass productivity and intensive downstream processing cost. Thus, sustainability and economic feasibility of microalgal biofuel production could be achieved by examining the following (i) co-cultivating microalgae and bacteria using wastewater as a cheap source of nutrients, and (ii) the thermochemical conversion of microbial biomass feedstock into bio-crude oil via hydrothermal liquefaction.

In the present study, indigenous microalgal and bacterial strains were isolated and screened for their ability to grow in artificial wastewater (AWW). Strains with significant growth potential were further characterized in the AWW to evaluate their performance efficiency based on the biomass titer and nutrient removal efficiency. In order to achieve higher biomass titer and wastewater treatment efficiency, eight different combinations of primary consortium were constructed from the pool of selected microalgae and bacteria strains. In the next step, four different secondary microalgae-bacteria consortium were constructed from the best performed primary consortium with the following objective: (a) improve the total biomass titer and (b) improve organic carbon and nutrient treatment efficiency. Finally, one tertiary combination of microalgae-bacteria consortium was considered from the best

performed secondary combinations, which were comprised of two microalgal strain and two bacterial strains. Characterization of the tertiary consortium was performed in AWW in order to assess its biomass productivity and wastewater treatment efficiency. Further improvement in total biomass titer of the tertiary consortium was achieved via statistical optimization of physico-chemical parameters of the medium as well as biological parameters. In the next step, with the aim of establishing the feasibility of application at industrial scale, performance of this tertiary consortium in terms of biomass titer and wastewater treatment efficiency was evaluated by employing the following wastewaters: paper industry wastewater (PWW), textile industry wastewater (TWW), leather industry wastewater (LWW) and municipal wastewater (MWW). Based on the microbial biomass titer and nutrient removal efficiency obtained for the different wastewaters, PWW was selected for subsequent experimentation. Further, with the objective of maximizing the microbial biomass titer, a process engineering strategy was implemented in an automated photobioreactor involving intermittent feeding of the limiting nutrients under fed-batch mode of cultivation of the consortium on PWW. The wet microbial biomass thus obtained from fed-batch cultivation was further subjected to HTL for direct conversion into bio-crude oil. Further improvement in bio-crude oil yield was carried out through optimizing the HTL process parameters. In the next step, for the process to be realized at a commercial scale, the performance of the consortium was evaluated in pilot scale outdoor condition where microbes were subjected to fluctuating environmental conditions such as illumination and temperature.

The key findings: Six microalgal (A1, A2, A3, A4, A5, and A6) and three bacterial (B1, B2, and B3) strains were isolated from an oil refinery wastewater. In the initial step of screening procedure in AWW, four microalgal (A1, A2, A4, and A6) and two

bacterial (B1 and B3) strains were found to grow substantially. Based on their growth potential in AWW containing a wide range of heavy metals, four microalgal (A1, A2, A4, and A6) and two bacterial (B1 and B3) strains were selected for further characterization with respect to their wastewater treatment efficiency and subsequent microalgae-bacteria consortium formation. While microalgal strains contributed predominant towards removal of nitrate (45−62% efficiency) and phosphate (100% efficiency), removal of COD was mainly supported by the bacterial strains with an efficiency of 31−33%. Moreover, AWW was supplemented with five different heavy metal ions (Cu, Cr, Cd, Ni, and Pb). From the profile of heavy metal ion removal efficiency by the selected individual strains, it was observed that while the microalgal strains could remove a majority of the heavy metal ions with higher efficacy, the removal efficiency of many metal ions were lower in case of using bacteria. Therefore, co-cultivation of microalgae and bacteria could be an effective strategy for biomass feedstock generation coupled with wastewater treatment. To that end, eight combinations of primary consortia, four combinations of secondary consortia and one tertiary consortium was considered. In all primary combinations of microalgae-bacteria consortia (A1B1, A1B3, A2B1, A2B3, A4B1, A4B3, A6B1, and A6B3), an increment in biomass titer in the range of 4−21% was observed when compared with the corresponding individual microalgal isolate. While improvement in nitrate removal efficiency was recorded to be in the range of 3−20%, improvement in COD removal efficiency was significantly higher within the range of 280− 380%. The highest biomass titer was 1.12 g L^{-1} in case of the primary consortium A2B1 followed by A4B3 (1.09 g L^{-1}). The nitrate and COD removal efficiency for these two best performing primary consortia A2B1 and A4B3 were found 72.61% & 71.08% and 57.02% & 56.69% respectively. Further, four different combinations of secondary

microalgae-bacteria consortia (A2A4B1, A2A4B3, B1B3A2, and B1B3A4) were formed using the microalgal and bacterial strains present in A2B1 and A4B3,, two best performing primary consortia. Out of four secondary consortia, A2A4B1 and A2A4B3 produced by combining two microalgae and one bacteria system showed enhanced biomass titer and nitrate removal efficiency of 1.21 g L^{-1} & 1.18 g L^{-1} and 74.11% & 72.44% respectively. However, the other two secondary consortia B1B3A2 and B1B3A4 comprising of two bacteria and one microalgal system exhibited higher COD removal efficiency of 59.67% & 58.66% respectively as compared to A2A4B1 and A2A4B3. When employing biomass titer as the key selection criteria, the secondary consortium A2A4B1 was considered to be the best amongst all four combinations followed by A2A4B3. In further step, the tertiary consortium A2A4B1B3 was formed using microalgal and bacterial strains present in the two best performing secondary consortia, A2A4B1 and A2A4B3. In comparison to best primary consortium and best secondary consortium under un-optimized growth conditions, the combined microalgae-bacteria tertiary consortium showed an improved total biomass titer of 1.27 g L^{-1} with nitrate and COD removal efficiencies of 79.78% and 62.43%, respectively. Moreover, the heavy metal ion removal efficiencies of tertiary consortium (Cu= 78.1%, Cr= 76.34%, Cd= 55.19%, Ni= 69.78%, and Pb= 65.12%) was comparatively higher than the best primary consortium (Cu= 72.56%, Cr= 69.18%, Cd= 48.49%, Ni= 61.29%, and Pb= 54.82%) and best secondary consortium (Cu= 60.39%, Cr= 63.41%, Cd= 49.91%, Ni= 55.9%, and Pb= 56.7%). While this tertiary consortium was characterized under optimized growth condition, a significant improvement in the total biomass titer of 1.73 g L^{-1} was observed. The microalgal and bacterial partners present in the tertiary consortium were identified as *Chlorella sorokiniana* strain DBWC2, *Chlorella* sp. strain

DBWC7, *Klebsiella pneumoniae* strain IITG-ORWB1, and *Acinetobacter calcoaceticus* strain IITG-ORWB3 respectively. The biomass titer and wastewater treatment efficiency of this tertiary consortium was further evaluated using four different wastewater samples. The total biomass titer, total nitrogen and COD removal efficiency was found 3.17 g L^{-1}, 99.95% and 95.16% respectively when this consortium was grown using PWW in a photobioreactor under batch mode conditions. The biomass titer was enhanced to 4.1 g L^{-1} through intermittent feeding with nitrogen and phosphate. The yield of bio-crude oil obtained from HTL of microbial biomass generated by growing the tertiary consortium in PWW was 15% (w/w) under un-optimized condition. Statistical optimization resulted in highest bio-crude oil yield of 21.73 (%, w/w) under optimal temperature, biomass loading and reaction time of 300ºC, 16.14 (%, w/v) and 65min, respectively. With an energy recovery of 42.95% and heating value of 33.11 MJ kg^{-1} of bio-crude oil, reflects 81.7% and 73.4% heating value of biodiesel and diesel, respectively. While, the high percentage of hydrocarbon content in the bio-crude oil indicates good oil quality, the presence of significant esters fraction indicates similarities with the chemical characteristics of biodiesel. The lower H/C ratio and higher O/C ratio in comparison to diesel indicates a requirement to up-grade the bio-crude oil before it can be used at commercial scale. Open pond cultivation of the tertiary consortium under fluctuating environmental conditions such as illumination and temperature, resulted in a maximum biomass titer of 3.96 g L^{-1} with 90% COD removal efficiency within 19 days of cultivation followed by an insignificant change in the titer.

The overall study illustrates that the co-cultivation of a microalgae-bacteria consortium fed with wastewater is an integrative approach to solve both energy and environmental issues. The present study is focused on developing a sustainable

process for the production of microbial (microalgae and bacteria) biomass which can be employed to produce bio-crude oil. The sustainability aspect of the process is employing a wastewater and naturally occurring microorganisms to produce fuel. The following combinatorial approach was used in this study (i) develop a strategy to improve biomass titer through co-cultivation of algae-bacteria consortium with respect to pure culture; (ii) use an inexpensive nutrient source such as a wastewater to reduce cultivation cost; (iii) produce a microbial feedstock which can be processed into a fuel coupled with efficient wastewater treatment; and, (iv) convert the microbial biomass feedstock into bio-crude oil using HTL.

CONTENTS

List of Figures

List of Tables

Research is formalized curiosity. It is poking and prying with a purpose.

Zora Neale Hurston

Chapter 1

Introduction

1.1 Background and motivation

Unfettered exploitation of the fossil fuels integrated with unimpeded population growth and brisk industrialization are the root causes of the escalating world energy crisis and the intensification of global warming. Global disquietude for energy security and environmental sustainability has propelled the scientific community to contemplate conceivable energy resources as prospective substitutes. Presently, several countries across the globe are capitalizing on biomass-, wastes-, solar-, wind-, hydro- and geothermal energy sources, with net-zero carbon emissions, as futuristic alternatives to fossil based fuels. Microalgal biofuel has been gaining impetus owing to their several advantages such as high photosynthetic efficiency coupled with the sequestration of carbon dioxide, high growth rate, capability to accumulate lipids/carbohydrates and tolerance towards different cultivation conditions. Besides the fuel counterpart, a wide range of value-added bio-products such as staple foods (e.g., carbohydrates, phycobiliproteins, phycocyanin, β-carotene, sulphated polysaccharides etc.), vitamins (e.g., A, B1, B2, B6, B12, C, E, nictitate, biotin, folic acid and pantothenic acid), and polyunsaturated fatty acids (γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid) can be obtained from these organisms. Though the concept of biofuel production using microalgal feedstock is gaining ground, yet certain challenges pose a major hindrance towards commercialization of this scientific know-how. The high production cost of

microalgal biomass using fresh water coupled with energy and cost intensive downstream processing are the dominant issues facing the economic feasibility of this technology.

Microalgae cultivation in a wastewater is an integrative approach which includes developing a cost effective and eco-friendly approach to sustainably produce a biofuel and bio-based chemicals. Reducing costs by not utilizing large volume of fresh water as well as avoid adding nutrients and trace elements is a path towards developing a sustainable process (Pittman et al., 2011). In addition, overcoming challenges such as developing robust microalgae strains which can tolerate a wide range of pollutants typically present in wastewater, increase the biomass titer, and reduce downstream processing cost will further lead to an improved process.

In recent years, exploitation of microalgae-bacteria consortium have been demonstrated to be an attractive approach which can offer enhanced biomass titer with improved wastewater treatment efficiency when compared to microalgae (Choi et al., 2017; Makut et al., 2019; Mujtaba et al., 2016). Such a microalgae-bacteria consortia can be used as robust biological systems with the ability to function under fluctuating growth environments and nutrient loads because of their diverse metabolic activities and adaptation to different environmental conditions. The advantageous association of photosynthetic microalgae and heterotrophic bacteria have been attributed to various factors such as for example, (i) enhanced microalgal growth by growth promoting substances secreted by bacteria (Dao et al., 2018; Gonzalez et al., 2000); (ii) bacteria mediated reduction in dissolved oxygen level of the medium resulting in alleviation of oxygen inhibition to microalgae (Mouget et al., 1995); (iii) exchange of O_2/CO_2, micro- or macro nutrients and signalling molecules etc. (De-Bashan et al., 2008; Du et al., 2013; Fuentes et al., 2016; Praveen et al., 2015) and (iv)

secretion of dissolved organic matter by microalgae which serves as a source of carbon, nitrogen and energy for the co-existing bacteria (Dao et al., 2018). Therefore, the microalgae-bacteria consortium utilizing wastewater can offer a sustainable technology enabling a step forward towards commercial application. High cost and energy intensive downstream processing (harvesting and drying) of the microbial biomass obtained from large scale cultivation remains another major obstacle towards commercialization of microalgae-based biofuel. Production of bio-crude oil via hydrothermal liquefaction (HTL) of microalgae biomass has been gaining the attentions of the researchers because it offers multiple advantages over conventional process such as biochemical and thermochemical conversion methods. These advantages include (i) ability to convert wet algal biomass into bio-crude oil and hence, bypassing the need for drying (Guo et al., 2015); (ii) high energy efficiency and low tar yield (Gollakota et al., 2018); (iii) use of a catalyst is not essential; (iv) higher conversion rates (Vardon et al., 2012); (v) convert non-lipid fraction of the biomass into bio-crude; and and (vi) nutrient rich aqueous phase collected after HTL can be recycled for microalgae growth. Before developing the bio-crude refining and upgrading processes, understanding the physical and chemical properties is essential before the bio-crude can be considered further for subsequent processing into transportation fuels.

1.2 Approach

Two different approaches will be examined for the sustainable production of biofuel by employing the co-cultivation of microalgae and bacteria consortium fed with a wastewater. The first approach will examine increasing the microbial biomass titer while enhancement of bio-crude oil yield will be assessed in the second approach. The first approach involves (1) isolation and characterization of indigenous

microalgal and bacterial strains from wastewater resources, (2) improving the biomass titer through co-cultivation of microalgae-bacteria consortium in a wastewater, and (3) optimization of the biological as well as the physico-chemical parameters. The second approach includes optimizing the HTL process parameters for maximum bio-crude oil yield.

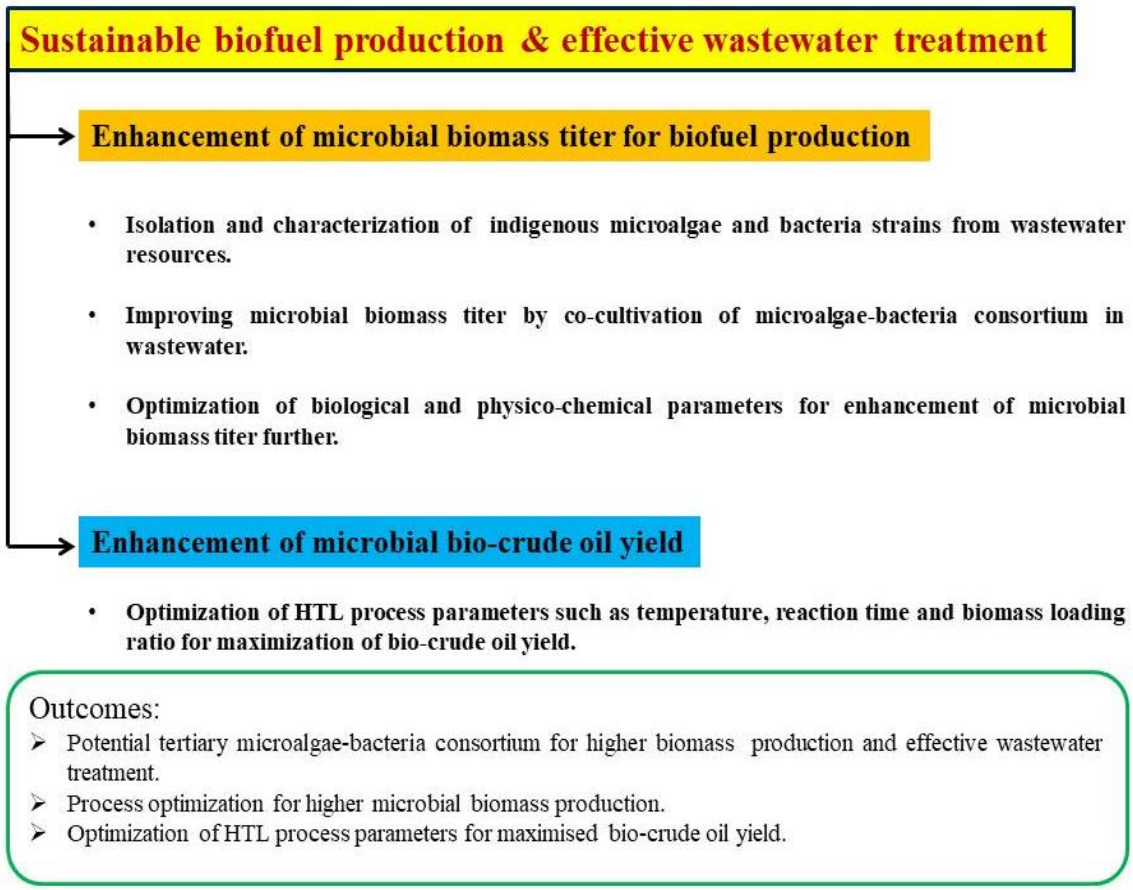

Fig.1.1 Two different approaches were employed for the sustainable production of bio-crude oil via enhancing microbial biomass feedstock through co-cultivation of microalgae-bacteria consortium and maximising the bio-crude oil yield through optimisation of the HTL process parameters.

The work was initiated by isolating six indigenous microalgal strains and three bacterial strains were isolated from oil refinery wastewater. In the first phase, potential strains grown in wastewater were further characterized in a synthetic

wastewater along with assessing the treatment efficiency. The characterization of the strains was performed in terms of biomass titer, percent removal of nutrients and chemical oxygen demand (COD). In the second phase, the best microalgae-bacteria consortium was selected amongst different combinations of selected microalgae and bacteria, on the basis of their performance in terms of total biomass titer, nutrient and COD removal efficiency. The total biomass titer for the best microalgae-bacteria consortium was further improved optimizing the process parameters. In the third phase, demonstration of a sustainable process for producing a biomass feedstock coupled with wastewater treatment was accomplished by selecting the best microalgae-bacteria consortium grown on a synthetic wastewater and raw dairy wastewater in an automated bioreactor. In the fourth phase, establishing the feasibility of application at industrial scale was accomplished by assessing the performance of the tertiary consortium based on the biomass titer and wastewater treatment efficiency. This consortium was evaluated by feeding them with the following wastewaters: paper industry wastewater (PWW), textile industry wastewater (TWW), leather industry wastewater (LWW) and municipal wastewater (MWW). Based on the microbial biomass titer and nutrient removal efficiency obtained from the different wastewaters, PWW was selected for subsequent experimentation. In the fifth stage, maximizing the microbial biomass titer was accomplished using an automated photobioreactor which was operated in fed-batch mode condition. The reactor was intermittently fed limiting nutrients contained in the PWW. The wet microbial biomass obtained from fed-batch cultivation was subjected to hydrothermal liquefaction (HTL) for the production of bio-crude oil. Further, optimization of process parameters of the hydrothermal liquefaction process parameters was performed to obtain a higher bio-crude oil yield. Characterization of the bio-crude oil

was conducted to assess its potential as an alternative to conventional fossil fuels. In the final stage, pilot scale demonstration of the process was conducted in an open pond to access the production of the microbial biomass feedstock and wastewater efficiency. The overall objective of the study was to develop an efficient process with a novel microalgae-bacteria consortium that can be scale-up for bio-crude oil production and can be used as a potential candidate for effective wastewater treatment.

1.3 Objectives

The present investigations are carried out on "**Sustainable biofuel production through effective wastewater treatment using indigenous algae-bacteria consortia**" with the following objectives:

- *Isolate and characterize indigenous microalgal and bacterial strains from wastewater resources.*
- *Evaluate various microalgal-bacterial consortia for the production of microbial biomass and effective wastewater treatment.*
- *Optimize process for enhancing the quantity of the microbial biomass feed-stock for biofuel production.*
- *Develop a process to enhance the quantity of microbial biomass feedstock for biofuel production by screening different wastewater sources.*
- *Optimize hydrothermal liquefaction process parameters for the production of higher bio-crude oil from microbial biomass.*
- *Demonstration large scale of the developed process for biomass production and subsequent bio-crude production through hydrothermal liquefaction.*

1.4 Organization of the thesis

The thesis has been presented in 9 chapters. Chapter 1 covers the background and motivation for the present study with a detail emphasis on the bottlenecks involved, approaches that can be taken up and the envisaged objectives to resolve the issues in the current state of art technology. Chapter 2 is a brief review of literature on the present energy crisis and environmental issues. The importance of microalgae in wastewater treatment and biofuel production is discussed. Moreover, bottlenecks of the microalgal based biofuel production were highlighted. The chapter also reviewed about the interaction within microalgae-bacteria consortium and its significant wastewater treatment efficiency. The advancement in the field of microbial bio-crude oil production was also included. Chapter 3 deals with the isolation of six microalgal and three bacterial strains from wastewater resources. Screening of each individual isolates was performed in terms of their growth potential in artificial wastewater (AWW) followed by the characterization of selected isolates in AWW. Chapter 4 investigates the performance efficacy of microalgae-bacteria consortium with respect to biomass generation and wastewater treatment in comparison to individual microalga. Chapter 4 also covers the characterization of different combinations of microalgae-bacteria consortia (e.g., primary consortia, secondary consortia and tertiary consortia) in AWW followed by the selection of the best consortia based on its performance efficacy. Molecular and morphometric identification of best consortia is included in this chapter. Chapter 5 covers the maximization of the total biomass titer by (i) the optimization of the inoculum size of microalga-bacteria tertiary consortium, and (ii) the statistical optimization of the fermentation medium. Chapter 6 is focused on developing of a process for increasing the production of microbial biomass feedstock and eventually the quantity of biofuel produced by screening of

different wastewaters. The work in chapter 7 examines optimizing the HTL process temperature (ºC), reaction time (min) and biomass loading rate (%, w/v) as a mean to maximize the bio-crude oil yield. Chapter 8 discusses the pilot-scale demonstration of the developed process for biomass production by intermittent feeding of industrial grade of nitrogen (urea) and phosphorus (single super phosphate). This chapter also covers the extensive characterization of the bio-crude oil as a mean to assess its potential as an alternative to conventional fossil fuels. Finally, chapter 9 covers an overview of the present study with the conclusions and future prospects.

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If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

Chapter 2

Literature Review

2.1 Energy outlook, salient environmental issues and alternative solutions

Population growth, industrial expansion and economic development have resulted in a global energy demand of 5.43×10^{20} joules $year^{-1}$ (British petroleum, 2018). This demand is fulfilled by petroleum oil (34%), natural gas (23%) and coal (28%) while energy production from coal (56%) and oil (30%) are major contributors to the Indian energy sector. Recent statistics states that the majority of the energy demand is fulfilled by fossil fuels and anticipated to be exhaust within next 50 years (). Furthermore, extensive utilization of fossil fuels has led to global warming due to the release of carbon dioxide and other anthropogenic greenhouse gases into the environment. Various sectors (agricultural, commercial and residential, industry, electricity and transportation) which are solely responsible towards carbon dioxide emission are shown in Fig. 2.1. As a result, the global temperature has been reported to increase at an alarming rate of 0.07 ºC per year coupled with an incremental carbon dioxide increase of 3 ppm per year (Status of biofuels, Knect, Energy Blog, 2018).

Numerous global challenges facing economical and sustainable development include developing renewable energy supplies and reducing environmental pollution. Renewable energy will play a vital role in dealing with energy security and environmental issues across the globe. Renewable energy technologies are a means of

providing a route for carbon-neutral power generation and hence, a reduction of greenhouse gases emissions.

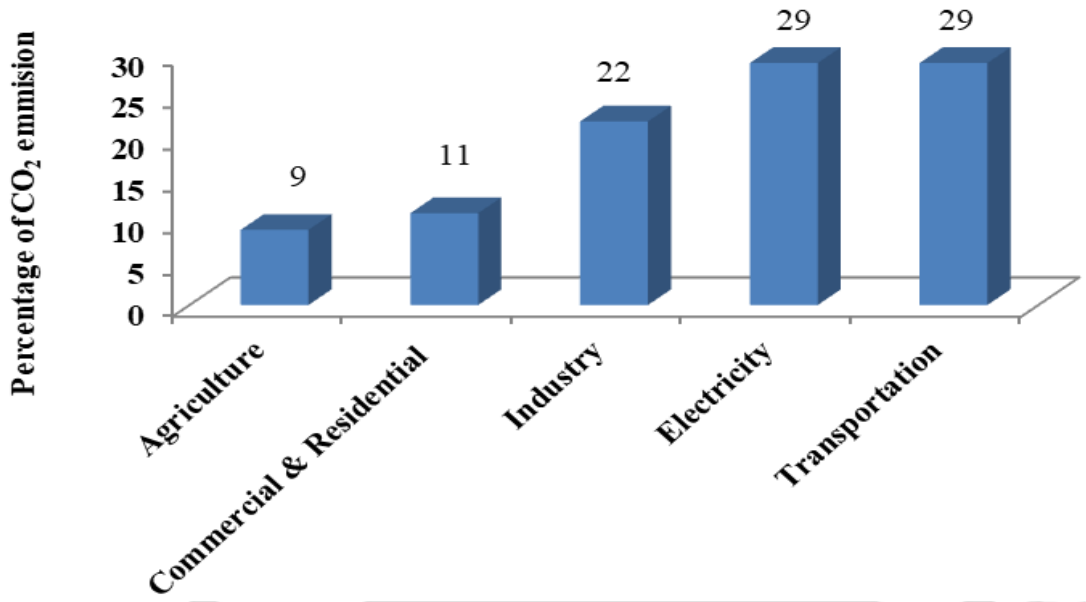

Fig.2.1 Percent carbon dioxide emission by different sectors

Alternative energy sources currently used across the globe include solar, wind, biomass, geothermal, wave, hydropower and nuclear have less of an environmental impact when compared to fossil fuels (Milano et al., 2016). Among these renewable energy resources, biomass based bioenergy is considered as a potential feedstock for developing a sustainable process to fulfill a fraction of the current energy demand due its high energy yielding efficiency and significant economic potential.

2.2 Microalgal biofuel

Biofuels are characterised as first, second, third and fourth generation fuels based on the biomass feedstock utilized (Fig. 2.2). Generally, first generation biofuels are derived from food crops, such as soybean, corn, maize, sugar beet, and sugar cane. These types of biofuels have created numerous concerns because of their negative

impacts on food security, global food markets, water scarcity, and deforestation (Medipally et al., 2015). In addition, second generation biofuels derived from nonedible oils (Jatropha curcas, Pongamia pinnata, Simarouba glauca, etc.), lignocellulose biomass, and forest residues require large areas of land for cultivation. Moreover, second generation biofuel production also lacks efficient technologies for the commercial exploitation of wastes as a source for biofuel production. Based on the drawbacks associated with the first and second generation biofuels, microalgae biomass based biofuels is classified as third generation biofuels because of the following: (1) An appropriate non-food feedstock for biofuel production; (2) Contains more lipid than terrestrial plants (40-80% of dry weight); (3) High reproduction and growth rate; (4) Atmospheric carbon dioxide is utilized for growth; and (5) Does not contain reduced sulphur chemicals which will produce sulphur dioxides upon combustion (Suganya et al., 2016). Biofuel production from Microalgae cultivation is considered as an effective strategy for producing biofuels because of the following properties: (1) Fast growth rate, (2) Elevated levels of oil production, (3) Low water utilization rates, (4) Ability to grow on non-arable lands (Winckelmann et al., 2015), (5) Tolerant to diverse environmental conditions, (6) Ability to grow under a wide range of cultivation conditions, (7) High rate of photosynthetic efficiency, and (7) Ability to grow in a low cost medium (Chisti et al., 2007). Microalgal biodiesel containing fatty acid methyl esters (FAME) is generally produced by the

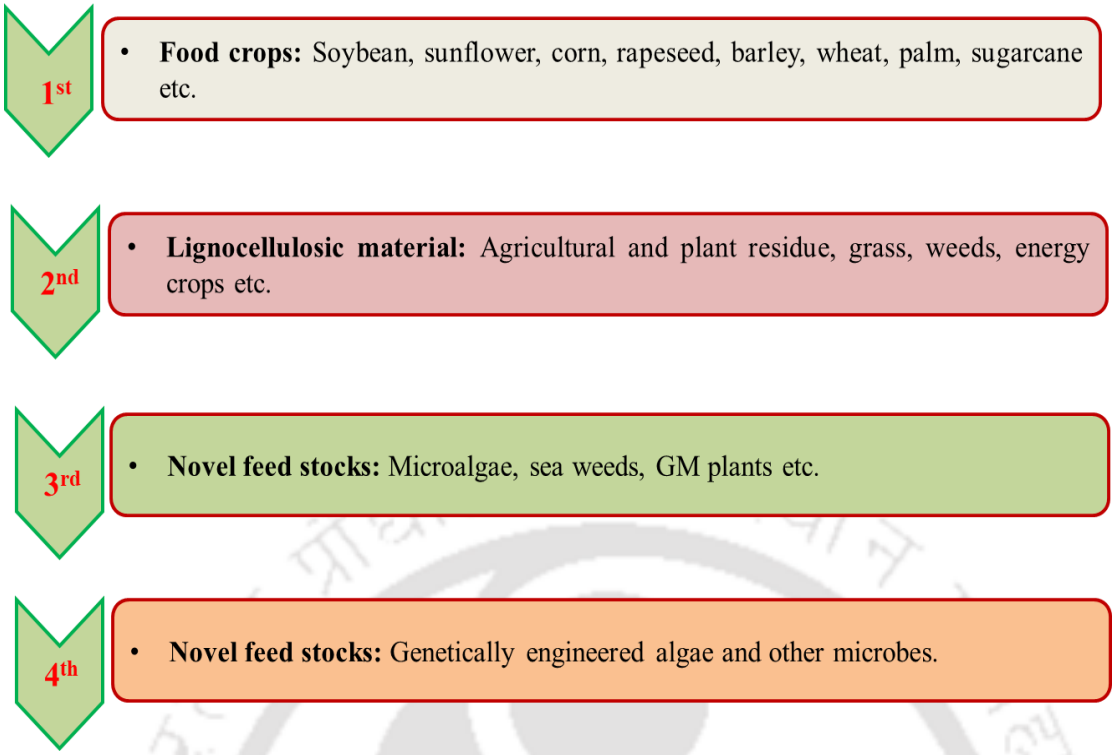

Fig.2.2 Different generations of biofuel and their corresponding feed stocks (Source: Dutta et al., 2014)

Biodiesel production from microalgae can be derived directly from the transesterification of algal biomass (Lewis et al., 2000). Alternately, biodiesel can also be produced by employing a two-step process wherein the lipids are extracted and subsequently trans-esterified by the processes involves lipid extraction through alcohols such as methanol, isopropanol and petroleum ether (Johnson and Wen, 2009; Mulbry et al., 2009). The direct transesterification process is fast and a cost-effective technology. Microalgae has been reported to produce biogas as a source of fuel, although the yield of biogas formation is quite low because of the sensitivity of algal cells to bacterial degradation and the low carbon and nitrogen (C:N) ratio, which leads to the formation of ammonia, an inhibitor of anaerobic microorganisms. In *Scenedesmus* sp., remaining biomass free from lipids and amino acids gives better biogas yield compared to raw biomass (Ramos-Suárez and Carreras, 2014). Many microalgal species are capable of producing hydrocarbons, which can further be

processed into diesel, kerosene and gasoline. For example, *Botryococcus braunii* has been produce hydrocarbons (n-alkanes, branched alkanes and naphthalene etc.) with an excellent oil yield (Hillen et al., 1982). Many microalgae species are able to produce hydrogen from water in presence of sunlight and the absence of oxygen (Veeravalli et al., 2019). The steam reformation of algae oil (Xiaohong et al., 2019), photo-fermentation (Kapdan and Kargi, 2006) and the photolysis of water mediated by photosynthetic algae (Ran et al., 2006). Biosyngas, a useful energy source rich in H_2 and CO, is produced by biomass gasification in presence of low oxygen levels, water vapour or air. Depending on the biomass type and reaction conditions, the quantity of H_2 and CO will vary (Ghasemi et al., 2012). Additional constituents in the gas may include methane, water, other hydrocarbons and ash. In the gasification process, high temperatures (800–1200°C) are essential, and the biomass feedstock water content should not be more than 20% (Ghasemi et al., 2012).

Bioethanol is produced by sugar fermentation. Microalgae species containing over 50% starch have been reported to produce bioethanol. Compared to the traditional use of woody biomass for bioethanol production, employing microalgae is a better option based on the following: (1) does not contain lignin, a complex recalcitrant chemical, (2) less complex cellular composition and can be readily utilized, (3) contain large quantity of polysaccharides, which can be converted into sugar, and (iv) can be genetically engineered to produce ethanol. Bio-oil, another biofuel, is produced by the thermochemical conversion of wet microalgal biomass (lipids, proteins and carbohydrates) by employing HTL (Goswami et al., 2019).

2.3 Problem associated with microalgal biofuel technology

Algal biofuel production is unsustainable and expensive because the growth requires a significant water source, nitrogen, phosphorus and CO_2 whether cultivation

is in open pond or closed bioreactor. Due to increasing demands for microalgal biomass and products, large-scale production systems are necessary. However, current microalgal production technologies for producing biofuels and its commercialization are not cost-effective due to various bottlenecks, one of which is the costly harvesting process (Branyikova et al., 2018). In commercial production systems with culture broths densities below 0.5 kg/m^3, large culture volumes are required for an economical oil extraction process (Chen et al., 2014). Mata et al (2010) reported 20–30% of the total production cost is associated with biomass harvesting. While other researchers have reported that the recovery process cost contributed approximately 50% of the oil production cost (Greenwell et al., 2009; Chisti, 2007). Based on these current cost issues, a key research objective is to develop effective and economic technologies for harvesting biomass from the suspended growth reactors. Moreover, the energy cost of algal extraction is ten-fold higher than the energy cost of soybean oil extraction (Kapoore et al., 2018). According to Savage et al (2012), the 2012 cost was US $27 per gallon (Savage et al., 2012).

The energy return on investment (EROI) is a tool used to assess the economics of producing a fuel. An EROI value is the ratio between the energy derived from an energy resource and the energy used to produce that resource. For example, easily recovered fossil fuels are characterized with high EROI values. In case of biofuels production, if the EROI < 1, the energy required to produce the fuel is greater than energy present in the fuel and co-products whereas, if the EROI > 3 then the fuel is considered as a sustainable energy source (Hall et al., 2011). The estimated EROI for algal biofuels produced in open ponds or photo-bioreactors is between 0.13 to 0.71 (Hall et al., 2011). EROI values for algal fuels less than 1 is unsustainable because the energy required to scale-up production include constructing, operate and maintain is

greater than the energy derived from the algal biofuels (Saad et al., 2019). High production cost, low biomass titer or productivity and cost intensive downstream process have been the major bottlenecks preventing the development of an economical technology for the production of low value bulk chemicals such as biofuel (Zhou et al., 2012).

2.4 Biological methods for municipal wastewater treatment

Over the past few decades, the large quantities of agriculture run-offs and municipal wastewaters produced are mainly due to anthropogenic activities such as increasing agricultural practices, urbanization and industrialization (Goncalves et al., 2017). Agriculture run-offs and the disposal of wastewaters without adequate treatment will lead to serious pollution problems. In many countries, municipal wastewaters are treated before discharge into fresh water bodies. The wastewater treatment process is broadly classified into the following three categories: physical, chemical and biological treatment. The unit operations included in each category is shown in Figure 2.3. Among the wastewater treatment methods, the aerobic biological treatment process is considered as eco-friendly, reliable, cost-effective and with a high potential process to remove a wide range of organic matters and inorganic nutrients by indigenous microorganisms such as bacteria, fungi, microalgae as well as protozoa.

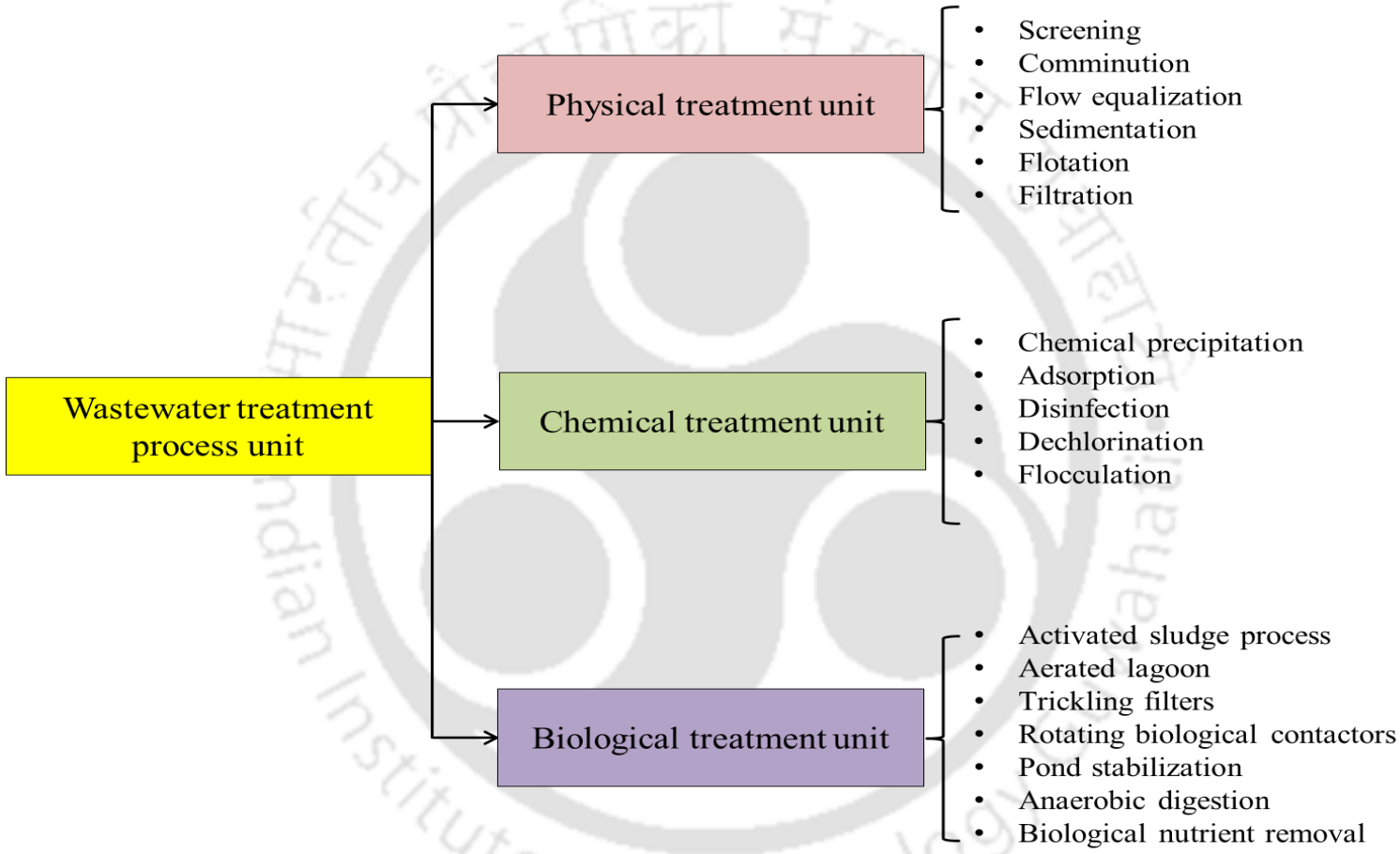

Fig.2.3 Various methods adopted for wastewater treatment process unit operation (Source: Rawat et al., 2011)

2.4.1 Microalgal cultivation in wastewater

Employing municipal wastewater as a growth medium for microalgal cultivation offers advantages such as nutrient removal and cultivation the production of microalgae which can be used to produce a biofuel. Wastewaters from domestic, agricultural, commercial or industrial sources generally contains an abundance of organic and inorganic matter, nutrients, and many toxic elements including heavy metals and if discharged directly to receiving water bodies without any prior treatment results in organics and nutrients loadings which leads to oxygen depletion and subsequent eutrophication. The end result is eutrophication causes the destruction of an ecosystem. Hence, wastewater treatment is an unavoidable practice as a means to maintain sustainable ecosystems. Phycoremediation is one class of biological treatment with a high potential for removing reduced carbon and nutrients as well as the production of microalgae biomass. This method is associated with lower capital costs and a simplified treatment process when compared to conventional treatment methods (Fito et al., 2019). Microalgae can be employed to replace the tertiary treatment component in conventional activated sludge municipal wastewater treatment. These organisms utilize carbon, nitrogen and phosphorus sources for growth and metabolism (Makut et al., 2019). They enhance the removal of nutrients, heavy metals, pathogens from wastewater and assist native heterotrophic bacteria to degrade organic matter by producing oxygen photo synthetically (Makut et al., 2019). Microalgal based system possesses a number of advantages over other processes for advanced nutrient removal from municipal wastewaters such as for example, filtration, advanced oxidation, adsorption, coagulation, electrocoagulation, ion exchange because of the following: (1) minimum mechanical equipment, (2) lower

land area than wet land, (3) no extra chemicals, (4) lesser energy for its operation thus reducing capital cost (Wang et al., 2017). Moreover, the algal biomass harvested after wastewater treatment can be converted into a wide range of products, such as biofuels, food, fine chemicals, animal feeds and fertilizers etc. The main applications of microalgae are summarized during wastewater treatment are (1) BOD removal, (2) nutrient removal, (3) heavy metal removal, (4) heterotrophic pollutant and pathogen removal, (5) biogas production, and (6) toxic monitoring Thus, microalgae cultivation in wastewater is an integrative approach which leads to a cost effective and eco-friendly process for the sustainable production of biofuel and bio-based chemicals because of reduced costs associated with a reduction in large volume of fresh water, nutrients, and trace elements for cultivation (Makut et al., 2019).

2.4.2 Mechanism of pollutant removal by microalgae

Eutrophication is a major problem associated to the discharge of untreated nutrients containing municipal wastewaters into fresh water bodies. In addition to nitrogen and phosphorus chemicals, the presence of organic pollutants are also responsible for the development of algal blooms, the growth of aquatic plants such as ???, oxygen depletion and the loss of many species such as fish which are sensitive to threshold levels of these organics Moreover, heavy metals discharge into the environment mainly through population growth, urbanization, industrialization, and agricultural practices, are toxic and non-biodegradable at low concentrations. The most common heavy metal contaminants which include Cd, Cr, Ni, Cu, Hg, Pb, and Zn persists and eventually accumulatie in the food chain, thus causing serious threats to the hemans and the environment (Kumar et al., 2015). To improve wastewater remediation processes using microalgae, it is very important to understand the

mechanisms involved in the removal of organic pollutants, carbon, pathogens, heavy metals and nutrients. The mechanism for organic and inorganic nutrient removal from wastewater by microalgae is shown in Fig. 2.4.

(A) Organic nutrient removal: : The presence of organic matters such as glucose, acetate and volatile fatty acids (VFAs) such as acetic acid, propionic acid, and butyric acid increase the chemical oxygen demand (COD) and biological oxygen demand (BOD) level of the wastewater. These organics are generally metabolised by the microalgae by different pathways shown in Fig. 2.4.

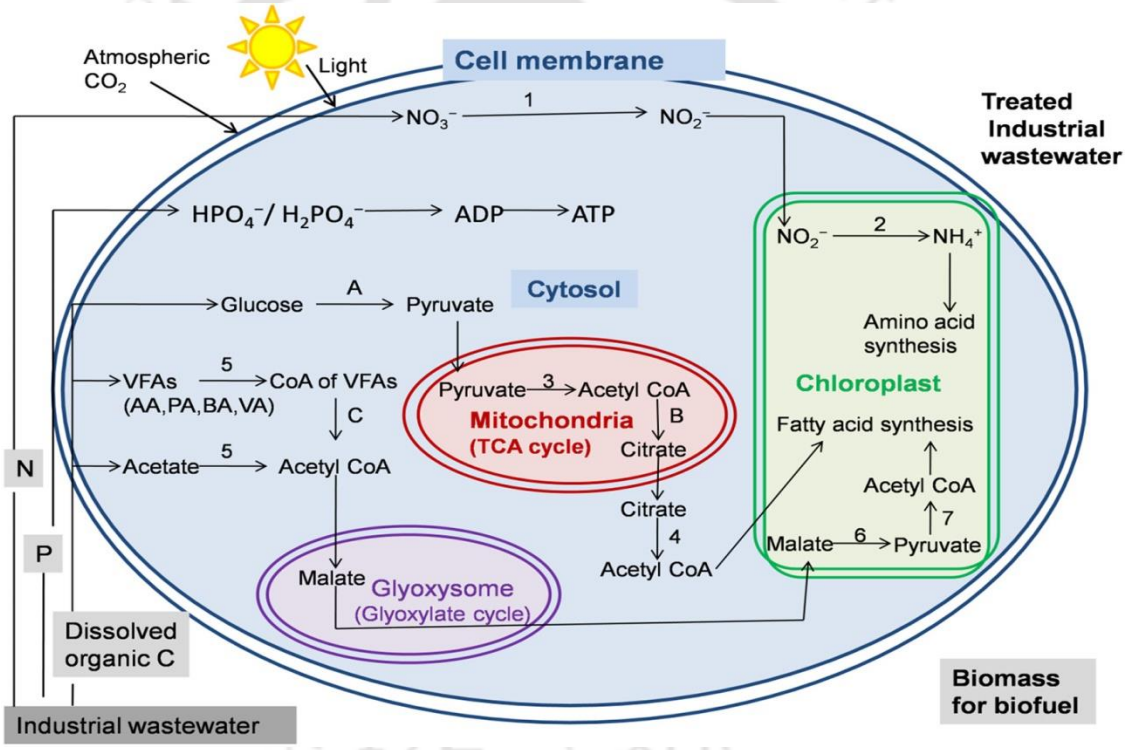

Fig.2.4 Mechanism of assimilation of organic and inorganic nutrient from industrial wastewater by microalgae. 1: Nitrate reductase enzyme; 2: Nitrite reductase enzyme; 3: Pyruvate dehydrogenase complex; 4: Citrate lyase enzyme; 5: Acetyl CoA synthetase enzyme; 6: malate dehydrogenase enzyme; 7: pyruvate formate lyase enzyme. A: Glycolysis cycle; B: Citric acid cycle; C: β- Oxidation. VFAs (volatile fatty acids); AA: acetic acid; PA: propionic acid; BA: butyric acid; VA: valeric acid. (Source: Gupta et al., 2019)

Glucose is transported across the microalgal cell membrane through transporter proteins known as hexose/H^+ symport system. Glucose is mainly metabolized through

glycolysis cycle in the cytosol to produce pyruvate as the end product (Gupta and Pawar,2019). In the next step, pyruvate dehydrogenase complex (PDH) converts pyruvate into acetyl-CoA in the mitochondria matrix. Then, acetyl-CoA enters into tricarboxylic acid cycle (TCA) to produce citrate, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) (Perez-Garcia et al., 2011; Bellou et al., 2014). Under nitrogen or phosphorus stress condition, citrate accumulates in the mitochondria matrix and it is subsequently transported into the cytosol to produce acetyl-CoA and oxaloacetate by ATP dependent citrate lyase which is present in cytosol (Bellou et al., 2014; Chalima et al., 2017). The acetyl CoA is utilized as a precursor for fatty acid biosynthesis (Perez-Garcia et al., 2011; Bellou et al., 2014). Similarly, acetate transported across the cell membrane by a monocarboxylic/proton transporter. The metabolism of acetate begins with the acetylation of acetate, where it produces acetyl CoA by acetyl CoA synthetase (Perez-Garcia et al., 2011; Gupta and Pawar, 2019). The metabolism of VFAs leads to the formation of acetyl CoA, propionyl CoA, butyryl CoA, and longer carbon chain CoAs by acetylation. Then co-enzymes of VFAs are converted into acetyl CoA by β-oxidation process in glyoxysome. The acetyl CoA is subsequently converted into citrate via the TCA cycle in the mitochondria or to produce malate via the glyoxylate cycle in the glyoxysome (Perez-Garcia et al., 2011; Chalima et al., 2017). Malate enters into the chloroplast to produce pyruvate using malate dehydrogenase enzyme. The pyruvate formate lyase enzyme converts pyruvate into acetyl CoA, which is a building block for fatty acid biosynthesis (Perez-Garcia et al., 2011).

(B) Inorganic nutrient removal: ***Nitrogen removal:*** The wastewater contains an abundant amount of nitrogen in the form of ammonium, nitrate, and nitrite. The assimilation of any form of nitrogen takes place across the cell membrane, where it is reduced to ammonium (Umamaheswari and Shanthakumar, 2016; Singh and Pandey, 2018). The transport of nitrate, nitrite, and ammonium across the cell membrane occurs through nitrate/nitrite transporter proteins and ammonium transporter protein; both belong to the ammonium transporter family. The assimilation of $NO^{3-} - N$ involves two-step process catalysed by the enzymes nitrate reductase and nitrite reductase (Barsanti et al., 2014). In the first step, nitrate reductase catalyses the reduction of NO^{3-}–N into NO^{2-}–N using nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agent. In the next step, NO^{2-}–N is reduced to NH_4–N by nitrite reductase using ferredoxin to catalyse the six-electron transfer reaction (Barsanti et al., 2014).

$$NO_3^- + 2H^+ + 2e^- + Nitrate\ reductase \rightarrow NO_2^- + H_2O \quad (2.1)$$

$$NO_2^- + 8H^+ + 6e^- + Nitrite\ reductase \rightarrow NH_4^+ + 2H_2O \quad (2.2)$$

NH_4^+-N resulting from NO_3-N and NO_2-N reduction is actively incorporated into microalgal cells and converted into amino acids via glutamine synthetase-glutamate synthase pathway, where glutamine synthase catalyses glutamine formation from glutamate and adenosine triphosphate (ATP) as follows:

$$Glutamate + NH_4^+ + ATP + glutamine\ synthase \rightarrow glutamine + ADP + P_i \quad (2.3)$$

Ammonium is the most preferred and energetically favoured nitrogen source for microalgae because no redox reaction is associated with its assimilation. Moreover, NH_4^+–N removal may occur in response to an increase of pH and temperature, where

large amounts of NH_4^+-N can be volatilized (Gonçalves et al., 2017). ***Phosphorus removal:*** Phosphorus is another inorganic nutrient present in high concentration in industrial wastewater which is required for microalgae growth and metabolism. The most abundant types of phosphorus present in wastewater are HPO_4^- (mono hydrogen phosphate), $H_2PO_4^-$ (di hydrogen phosphate), polyphosphate, and organic phosphate. Orthophosphate (mono and di hydrogen phosphate) is most readily usable phosphorus source that can be assimilated into organic components (phospholipids, nucleic acids, and protein) by the microalgae via phosphorylation process (Fig. 2.4). The phosphorylation can be a substrate level phosphorylation, oxidative phosphorylation or photophosphorylation. The process of phosphorylation forms ATP (adenosine tri-phosphate) from ADP (adenosine di phosphate) that needs some energy input. The required energy can be obtained through oxidation of substrate, mitochondrial electron transport chain, and transformation of light energy (Umamaheswari and Shanthakumar, 2016; Singh and Pandey, 2018). Another way to remove phosphorus from wastewater is the storage of excess phosphate in polyphosphate granules of microalgae cell when the wastewater contains a higher concentration of phosphate. The microalgae can utilize the stored phosphate when the external supply of phosphorous is limited (Umamaheswari and Shanthakumar, 2016; Singh and Pandey, 2018). **(C) Pathogen removal:** Microalgae photosynthetic activity can contribute to the deactivation of pathogens by increasing wastewater pH, temperature and dissolved oxygen concentration (Muñoz et al., 2006). **(D) Heavy metals:** Microalgae possess molecular mechanisms that allow them to discriminate, non-essential heavy metals from those which are essential for growth. The benefits of using microalgae for heavy metals removal include the following: rapid metal uptake capability, time and energy

saving, eco-friendly, low cost, faster growth rate, high efficiency, large surface to volume ratio, ability to bind with heavy metals up to 10% of their biomass, no toxic waste generation, no synthesis required, useful in both batch and continuous systems, and applicability to waters containing high metal concentrations or relatively low contaminant levels (Monteiro et al., 2012). The phenomenon of microalgae based heavy metal remediation is broadly categorised into two categories: (1) bioaccumulation by living cells, and (2) biosorption by non-living, non-growing biomass or biomass products. Bioaccumulation of heavy metals by living microalgae occurs in two steps: (i) the first step (i.e., passive removal) takes place rapidly and is essentially independent of cell metabolism, occurring in both living and non-living cells. In this process, heavy metal ions are adsorbed onto functional chemical groups present on the cell surface by electrostatic interactions. This process includes physical adsorption, ion exchange, chemisorption, coordination, complexation, chelation, micro-precipitation, entrapment in the structural polysaccharide network, and diffusion through the cell wall and membrane. In the physical adsorption mechanism, the heavy metal ion in solution binds to polyelectrolytes present in microbial cell wall through various electrostatic interactions such as Van der Waals forces, covalent bonding and redox interaction to achieve electro-neutrality. This process is pH dependent where with increasing pH, numerous sites such as for example, acetamide chitin, structural polysaccharides, phosphate and amino groups of nucleic acids, amino and carboxylic groups of proteins and hydroxyl groups of polysaccharides) are replaced by negative charges that increase the attraction of metal cations and their adsorption to the cell surface. In case of the ion exchange mechanism, functional groups on cell wall such as. carboxyl (–COOH), hydroxyl (–OH), phosphate (PO_4^{-3}),

di-phosphorus trioxide P_2O_3), amino ($–NH_2$), sulfhydryl (–SH), amide, primary amine-group, aromatic, halide-group and aliphatic alkyl-group) will result on negative charge to the cell surface. As a result, the heavy metal cations binding to the cell surface if the pH was within the range of ??? to ??? with a high binding affinity via counter ion interactions (Mehta et al., 2002; Monteiro et al., 2012; He et al., 2014). In the complexation process, the heavy metal ion forms coordination bonds with amino and carboxylic functional groups of the microalgae cell wall polysaccharide. Most of the microalgae synthesize class III metallothioneins which is a metal binding protein that assists in the bioaccumulation of heavy metal cations (Won et al., 2008). All higher plants and many microalgae synthesize phytochelatin synthase. Phytochelatins are small metal binding peptides activated in the presence of heavy metal ions (Ahner et al., 1995). Shanab et al. (2012) reported that the mechanism of metal accumulation inside the vacuoles or cytoplasm contributes towards heavy metal tolerance. The cytoplasmic metal concentrations are minimized by binding or complexing the metal ions with phytochelatin or in the form of metallo-sulfur, metallo-iron or metallo-phosphate complexes in the cytosol. They are carried into vacuoles where the acidic pH displaces the metal, allowing the peptide to return to the cytosol. In the vacuole, the metal would thereby be sequestered by organic acids that are usually present in high concentration. Dwivedi (2012) proposed the presence of polyphosphate bodies in microalgae that could sequester heavy metals and thus, provide a storage pool for heavy metals and detoxify them. Thus, detoxification of heavy metals in microalgal cells could be achieved either by (i) binding to specific intracellular compounds and/or transport to specific cellular compartments such as vacuoles or polyphosphate

bodies, or (ii) efflux of metals back into solution by active transport (Monteiro et al., 2012).

2.5 Problems associated with microalgal wastewater treatment

Though microalgae based wastewater treatments is an alternative to replace conventional wastewater treatment technology but as a technology, it cannot completely substitute conventional processes. Increasing the treatment efficiency of microalgae batch systems as a means to avoid process failure and improve the effluent quality as well as to minimize the cost of this technology by reducing land requirement currently face many challenges. It is reported that in conventional wastewater treatment plants, 1 ha area of land is required for the treatment of 30,000-50,000 p.e. (person equivalent), whereas in microalgae-based treatment processes up to 30-50 ha might be needed for the same treatment capacity (Acien et al., 2016).

This significant land requirement limits the application of these technologies to small towns, from 200 to 15,000 p.e., which often lack efficient wastewater treatment systems. However, key challenges for the phycoremediation technology and the production of biomass lies in finding microalgae species which have the ability to survive in different types of wastewater, wastewater composition, and the competition process between the microalgae strain and the indigenous organisms as well as increasing the yield and utilization of the biomass. Improving the treatment efficiency in the phycoremediation of wastewater, researchers used indigenous microalgae strains which have potential to remove various pollutants from wastewater with production of microalgae biomass. The utilization of indigenous microalgae strains is associated with environmental conditions which do mean that these microalgae strains may be effective in different locations. The lack of criteria for the

utilized microalgae in the phycoremediation process and the production of biomass has caused researchers to examine several microalgae strains. Therefore, specific criteria should be considered in terms of the potential of microalgae species to be used in different wastewater composition under different environmental conditions, as well as the ability of microalgae to survive and compete with other indigenous microorganisms in the wastewater.

2.6 Microalgae-bacteria interaction and their emergent application in biotechnology

Microalgae-bacteria based biotechnology has gained interest to produce a biomass feedstock which can be subsequently used for producing biofuel, agricultural fertilizers or animal feeds as well as an alternative method for a multi-step wastewater treatment process. Long-term biological impact between microalgae and heterotrophic bacteria can be divided into the following type of interaction: **(1) Mutualistic association:** Mutualistic association is the interaction in which two organisms behave as partners, where both species benefit, whereas a symbiotic association is a close and long term interaction between two different species. In nature, most aerobic and microalgae can survive in a micro-ecosystem where they influence the symbiotic growth of each other (Fukami et al., 1997; Subashchandrabose et al., 2011). Algae and bacteria synergistically affect each other physiology and metabolism. Although bacteria can negatively impact algae cultures, algae-bacteria interactions are promising in biotechnology application because recent studies have shown positive effect of these interactions on biomass production, wastewater treatment and the flocculation process. During wastewater treatment, the symbiotic relationship between microalgae and bacteria can be useful in improving the treatment efficiency. Their

mutual interactions include both positive roles and also negative mutual influence (Table 2.1).

Table 2.1 Positive and negative interactions between microalgae and bacteria

	Positive interactions	Negative interactions
Microalgae to bacteria	✓ O_2 supply ✓ CO_2 consumption	• pH increase • Temperature of the medium increase • Antibacterial effect • Dissolved oxygen concentration increases
Bacteria to microalgae	✓ Dissolved oxygen decrease ✓ CO_2 generation ✓ Growth stimulative effects	• Algicidal effects

Microalgae provide O_2 which is utilized by the aerobic bacteria as electron acceptor for the degradation of organic pollutants and in turn CO_2 released from bacterial respiration is utilised by microalgae for its growth and metabolism (Munoz and Guieysse, 2006). The cooperative interaction between microalgae and heterotrophic bacteria is shown in Fig. 2.5. However, microalgae and bacteria do not limit their interactions to CO_2/O_2 exchange. Microalgae can have a negative effect on bacterial activity by increasing the pH, the dissolved oxygen (DO) concentration or by excreting inhibitory metabolites (Oswald, 2003; Schumacher et al., 2003) which are especially toxic to gram-positive bacteria.

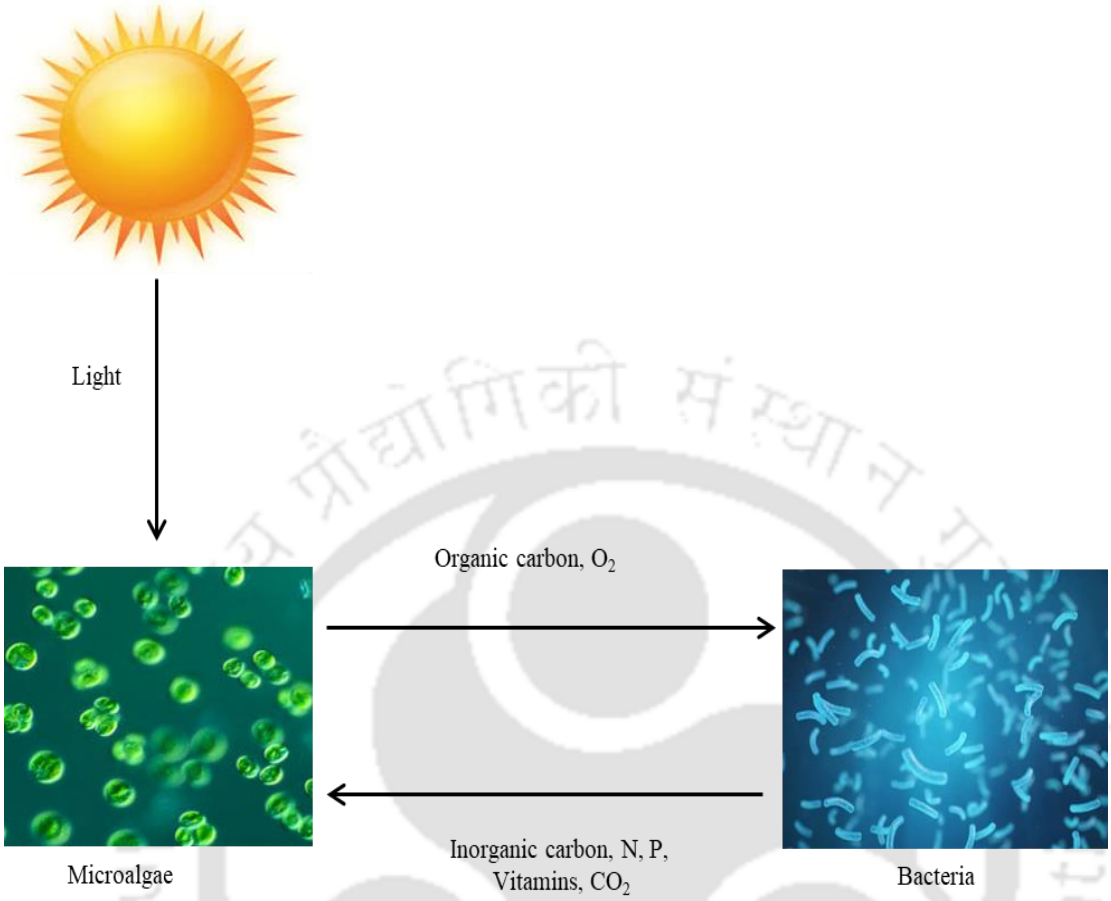

Fig.2.5 Cooperative interaction between microalgae and bacteria

Microalgae can augment bacterial activity by releasing extracellular compounds. Wolfaardt et al. (1994) reported that the removal of diclofop-methyl was increased by a bacterial consortium up to 36% when actively growing algae or their metabolites were added to the culture. Similarly, bacteria such as *Azospirillum brasilense* strain Cd excrete vitamins or algal growth-promoting substances which are used as a "helper" for microalgal growth (de-Bashan et al., 2004; Fukami et al., 1997). In comparison, two aerobic bacteria (*Pseudomonas diminuta* and *P. vesicularis*) can promote algal growth by reducing the photosynthetic oxygen tension within the microenvironment of the algal cells rather than releasing any growth promoting

substance (Mouget et al., 1995). The symbiotic cultures of microalgae and bacteria often result in complete elimination of contaminating bacteria in aquaculture systems. An illustrating example of a mutualistic relationship between *Emiliania huxleyi* and *Phaeobacter gallaeciensis*, wherein the bacterium produces antibiotic molecules to protect the host from other bacterial pathogens (Seyedsayamdost et al., 2013). **(2) Commensalistic association:** Commensalism is a relationship in which only one partner benefits. In this association, microorganisms present in the phycosphere represent a bacterial diversity dwelling on the algal surface (Cho et al., 2015). The interaction between *Chlamydomonas reinhardtii* and heterotrophic bacteria is another example of commensalism. *C. reinhardtii* uses vitamin B_{12} delivered by heterotrophic bacteria, although the bacteria do not make use of the organic carbon released by the alga (Kazamia et al., 2012). **(3) Parasitic association:** Parasitism involves two different species with the parasites benefiting but sacrificing the host's benefits. Zhang et al. (2012) has reported that *Chlorella pyrenoidosa* in wastewater can inhibit the growth of heterotrophic bacteria by competing for the available nutrients.

2.6.1 Current and promising applications of microalgae-bacteria interactions

1. Biomass production: Over the past decades, many research initiatives have focused on developing microalgal biomass production from axenic algal mono-cultures. Culturing microalgae with bacteria is recognised as a potential source of enhancing the production of microalgal biomass containing lipids and carbohydrates. In this respect, any bacterial partner affecting the growth of a microalga should consider the following: growth, include motility, chemotaxis, type IV secretion systems, quorum sensing systems and synthesis of growth promoters (Fuentes et al., 2016). Several reports have investigated

the stimulating effect of bacteria on algal growth. For example, the growth of alga *Asterionella glacialis* was improved because of glycoprotein production by *Pseudomonas* sp. (Riquelme, 1988). Two species of *Pseudomonas* (*P. diminuate* and *P. vesicularis*) increased the growth of *Chlorella* sp. and *Scenedesmus bicellularis* by creating more favourable environmental conditions, such as the reduction of photosynthetic oxygen tension (Mouget et al., 1995). Guo et al. (2013) reported the existence of *Pseudomonas* resulted in an approximately 40% higher cell concentration of *C. vulgaris* when compared to that in a single algal culture. The decomposing activities of bacteria can enhance the growth rate of microalgae (Du et al., 2013). Gonzalez and Bashan, (2000) reported an enhancement in the growth of *C. vulgaris* due to the synthesis of phytohormones by *Azospirillum brasilense.* Kazamia et al. (2012) co-cultured vitamin B_{12} dependent microalgae *Lobomonas rostrata* and the bacterium *Rhizobium loti* without supplementing with vitamin B_{12} and an organic carbon source in the culture medium. This study concluded no improvement in algal or bacterial growth. The results showed enhanced algal and bacterial growth with a balance in population density. This growth enhancement is possible because of the mutual growth regulation between the microalga and bacterium, where the bacterium provides vitamin B_{12} to the microalga and the microalga in turn provides organic carbon compounds to the bacterium. The bacterial communities associated with microalgae cultures can also regenerate or fix inorganic nutrients that would not be bioavailable to them. The bioaccesibility of iron for many species of microalgae such as for example, dinoflagellates and coccolithophores which are responsible for the

formation of blooms, is dependent on a close association with some bacteria species (Fuentes et al., 2016) reported an algal associated heterotrophic bacterium related to γ-proteobacteria *Marinobacter* is able to produce a siderophore, labelled as vibrioferrin, which binds Fe (III) and hence, making it available for microalgae and bacteria (Amin et. al., 2009). The microalga subsequently utilizes this iron in the photosynthetic mechanism of inorganic carbon fixation. Part of the fixed carbon is released back to the medium as organic molecules (dissolved organic matter (DOM)) which could be utilised for bacterial growth. Bacteria association with microalgae is pivotal role not only for growth but also in the composition of the microalgal biomass. The chemical composition of the microalgal biomass is a key factor in aquaculture; therefore, microalgae-bacteria interactions are important in aquaculture activities. Moreover, the interaction between microalgae and bacteria shows some positive effects on microalgal growth and accumulation of valuable compounds (Table 2.2). Besides positive effects on microalgal growth, there are some detrimental effects of bacteria on the biomass yield of microalgae cultures. In high cell density cultures, the presence of bacteria is expected to diminish light availability for microalgal cell growth. As a consequence, the algal biomass yields in non-axenic photoautotrophic cultures will likely decrease. Therefore, the increasing yield of target value molecules, including carotenoids and fatty acids, may require providing higher light irradiance because of the poor economics for by-product recovery which is due to low concentration of specific products in the cells extracts (Forjan et al., 2015). Therefore, control of algae-bacteria interactions is indispensable as a means to

avoid decreasing the yield of algal biomass as well as algal-derived products.

2. Biomass harvesting: Microalgae biomass harvesting is an expensive process and could account for one-fourth of the total biomass production cost (Mata et al., 2010). Several harvesting techniques in including ultrafiltration with membranes, electrocoagulation, flocculation and centrifugation are un economical at full-scale (Wijffels et al., 2010). When compared to other harvesting technology methods, bio-flocculation processes are cheap, environmental friendly and sustainable for the bulk harvesting of algal biomass. Bioflocculation could be an efficient microalgae harvesting method which might be promoted by any of the specific bacterial species associated to the cultivation of specific microalgae (Wang et al., 2012).

Table 2.2 Microalgae-bacteria interactions with various mediators on the production of valuable chemicals (Fuentes et al., 2016).

Microalga	**Bacterium**	**Microalgae mediators**	**Bacterial mediators**
		Algal growth improvement	
E. huxleyi	*P. gallaeciensis*	Dimethyl sulphonio propionate	Promoters and antibiotics
B. braunii	*Rhizobium sp.*		AHL
T. pseudonana CCMP1335	*R. pomeroyi* DSS-3	2,3-dihydroxy-propane-1-sulfonate	Vitamin B_{12}
S. trochoidea	*Roseobacter*	Organic molecules	Vibrioferrin
N. oleoabundans	*A. vinelandii*		Siderophore

Scenedesmus sp.	*A. vinelandii*	Siderophore
Accumulation of fatty acids and lipids		
C. vulgaris	*A. brasilense*	Siderophore mediated nitrogen fixation
Photoautotrophic and heterotrophic accumulation of starch and carbohydrates		
C. vulgaris	*A. brasilense*	Siderophore mediated nitrogen fixation
C. sorokiniana	*A. brasilense*	Siderophore mediated nitrogen fixation

During the bioflocculation process, the aggregation of bacteria and microalgae cells is triggered by the secretion of extracellular biopolymers by bacteria leads to the formation of large flocs. These flocculated cells are able to settle without adjusting the pH or using metal and chemical flocculants (Vandamme et al., 2013). The formation of microalgal bacterial flocs can be a promising approach for microalgae harvesting coupled with wastewater treatment. The formation of these aggregates between microalgae and bacterium is advatageous because of the faster settling when compared to microalgae alone (Van Den Hende et al., 2011). Many bacteria from the genera *Flavobacterium*, *Terrimonas* and *Sphingobacterium*, which are naturally associated with microalgal growth, have shown a combined role in *Chlorella vulgaris* harvesting (Ummalyma et al., 2017). Poly γ-glutamic acid a biopolymer secreted by *Bacillus subtilis* has been effective in harvesting both freshwater and marine microalgae (Branyikova et al., 2018). Using poly γ-glutamic acid from *B. subtilisis* which to harvest microalgae such as *Nannochloropsis oculata* LICME 002,

Phaeodactylum tricornutum, *C. vulgaris* LICME 001 and *Botryococcus braunii* LICME 003 has resulted in more than 90% flocculation efficiency (Ummalyma et al., 2017). Although there is the problem with high cost, bio-flocculants are novel methods for harvesting microalgae due to their uniqueness of being safe, biodegradable, eco-friendly and short span of time. Bacterial mediated flocculation of microalgae is seen in Table 2.3.

Table 2.3 Microalgae-bacterial mediated flocculation efficiency (%) (Ummalyma et al., 2017)

Bacteria	**Microalgae**	**Flocculation efficiency (%)**
Klebsiella pneumoniae	*Synecosystis*	95
Paenibacillus sp.	*Scenedesmus sp.*	95
B. subtilis γ-PGA)	*Nannochloropsis oculata* LICME002	96
B. subtilis γ-PGA)	*Phaeodactylum tricornutum*	97
B. subtilis γ-PGA)	*C. vulgaris* LICME001	90
B. subtilis γ-PGA)	*Botryococcus braunii* LICME 003	92
Solibacillus silvestris (proteoglycans)	*Nannochloropsis oceanic*	90
Escherichia coli	*Chlorella zofingiensis*	83
B. licheniformis CGMCC 2876 γ-PGA) F51	*Desmodesmus sp.*	92

3. **3. Cell disruption:** With an increasing interest for producing high-value chemicals from microalgal biomass, cost-effective cell disruption methods will be required in order to avoid high production costs of algal biomass and valuable products. The efficiency of cell disruption is competitive when using enzymes

cocktails to degrade intracellular components. Induced autolysis of microalgae has been proposed as a promising cell disruption method in order to avoid issues associated with using enzymes (Fuentes et al., 2016). To induce cell lysis, microalgal degradation by algicidal microorganisms such as bacteria, cyanobacteria, viruses and their algicidal molecules have been employed. Algicidal molecules produced by bacteria include derivatives of quinolones, pyrroles, alkaloids and enzymes. In this process the algicidal microorganism attacks microalgae and as a result, these cells are destroyed by morphological changes, pore formation in the cell membrane with loss of the cell ultrastructure organization, formation of radical oxygen species, loss of functionality of the antioxidant systems and inhibition of photosynthesis. The degradation of microalgae using algicidal microorganisms in co-cultures demonstrates its effectiveness as an advantageous disruption method which could facilitate and improve macromolecule recovery (Munoz et al., 2014).

4. 4. Energy production: Microalgae are capable of producing hydrogen or synthesizing lipids and carbohydrates which can be utilized to produce biofuels such as biodiesel bioethanol and biogas. However, there are some symbiotic bacteria and fermentative bacteria actively involved in microalgal biofuel production (Table 2.4).

(A) Biohydrogen: Microalgal-based hydrogen production represents a novel combination of fermentative and photolytic hydrogen generating processes. Efficient hydrogen production from microalgae strictly operated under anaerobic condition as the key enzyme hydrogenase is highly sensitive to oxygen. It has been reported that such anaerobic environment suitable for microalgal hydrogen production might be conferred by the bacteria which can consume O_2 generated photosynthetically by the microalgae, without damaging the photosynthetic apparatus (Fuentes et al., 2016).

The bacterial symbionts, *Brevundimonas* sp., *Rhodococcus* sp. and *Leifsonia* sp. were found to enhance hydrogen production in the microalga *Chlamydomonas* by consuming oxygen during respiration, which is essential for the activation of a Fe-dependent hydrogenase in *Chlamydomonas* (Lakatos et al. 2014). Hence, with the help of symbiotic bacteria that consume oxygen evolved during microalgal photosynthesis, microalgae can capture light energy and produce hydrogen simultaneously without further manipulation of the system. The solar energy can also be converted into electricity by synergistic cooperation between photosynthetic microalgae and heterotrophic bacteria in the form of microalgal fuel cells, without external input of exogenous organics or nutrients (He et al. 2009).

Table 2.4 Involvement of symbiotic and fermentative bacteria in microalgal biofuel production (Yao et al., 2019)

Microalgae	**Bacteria**	**Biofuel**
Chlamydomonas CC124	*Brevundimonas* sp., *Rhodococcus* sp., *Leifsonia* sp.	Hydrogen
Chlorella vulgaris	*Pseudomonas* sp.	Triglyceride
Tetraselmis striata	*Pelagibaca bermudensis*	Triglyceride
Auxenochlorella protothecoides	*Escherichia coli*	Triglyceride
Green microalgae NKG 120701	*Pseudoalteromonas undina*	Ethanol
Chlamydomonas reinhardtii	*Bacillus licheniformis*	Ethanol
Chlorella vulgaris	*Clostridium thermocellum*	Hydrogen, methane
Botryococcus braunii	*Aeromonas* sp., *Raoultella* sp.	Methane
Nannochloropsis gaditana	*Raoultella ornithinolytica*	Methane

(B) Biodiesel: Microalgae produce triacylglycerol and other lipids can be trans-esterified to produce fatty acid methyl esters. The microalgal growth associated with triacylglycerol production could be further enhanced by the bacteria symbionts. Higgins and VanderGheynsta, (2014) reported that growth and lipid content was

increased from two to six-fold while co-culturing the green microalga *Auxenochlorella protothecoides* with *E.coli* when compared to the axenic cultivation of microalga. **(C) Bioethanol and biogas:** The Residual microalgal biomass after extraction of useful products can be further fermented to ethanol or converted into biogas. Microalgae can produce up to 40% dry weight of starch granules which can be utilized by various bacteria to produce ethanol (Ramanan et al. 2015). Ethanol is produced by the saccharification of carbohydrates produced by marine microalgae. Ethanol is produced by saccharification of marine microalgae utilizing amylase from the marine bacterium *Pseudoalterimonas undina* (Matsumoto et al. 2003).

5. Nutrient Removal and Wastewater Treatment: Algal–bacterial systems based nutrient rich wastewater treatment have been used extensively since the 1950s to produce various value-added chemicals and remove nutrients. The use of co-culturing in wastewater treatment is an eco-friendly approach due to the use of CO_2/O_2 between the two symbionts and the performance of nutrients removal can also be increased in co-culture system when compared to single culture systems. This can be achieved by selecting appropriate microalgal and bacterial strains which could have positive effects on each other. Mujtaba et al. (2017) reported an 85% removal of NH_4-N, a 66% removal of phosphate, and a 86% removal of COD from municipal wastewater while co-cultivating immobilised *Chlorella vulgaris* and suspended *Pseudomonas putida*. Ren et al. (2015) reported an 88.7% removal of NH_4-N, 80.1% removal of phosphate and 80.5% of COD while co-cultivating *Scenedesmus sp.* and anaerobic sludge native bacteria in a containing starch wastewater. Hernandez et al. (2013) found the removal efficiency of NH_4-N, phosphate, and COD were 95%, 80.7% and

86.1%, respectively, while cultivating *Chlorella sorokiniana* and activated sludge native bacteria in a potato processing wastewater for 10 days.

6. Bioremediation: The cooperative interaction between algae-bacteria consortium can have a positive effect on metal bioremediation. Low levels of metal ions present in wastewater are required for microalgal growth and metabolism which, whereas higher metal ion concentrations are lethal to the cells. In this prospect, algal-bacterial community in mutualistic interactions can detoxify and assimilate metals from metal rich environments. This process includes physical adsorption, covalent bonding, ion exchange and chemisorption, surface precipitation, redox reactions or crystallization on the cell surface. The degradation of organic pollutants including black oil, acetonitrile, phenol, naphthalene, thiocyanate and benzopyrene and azo compounds have also been reported by several reporters (Subashchandrabose et al., 2013; Mahdavi et al., 2015; Ryu et al., 2015). Apart from this, the degradation of toxic pesticides such as monocrotophos, quinalphos, methyl parathion, DDT, atrazine and endosulphan was also reported by Subashchandrabose et al. (2013).

7. Potential of residual microbial biomass as animal/fish feed: The selection of appropriate microalgae-bacteria consortium could significantly enhance the productivity, efficiency and sustainability of aquaculture according to work by Natrah et al. (2011). Bacteria can stimulate algal growth, which is the key component of the diet in aquaculture of other organisms. Therefore, a healthy feed supply could encompass grazers, algae and their associated beneficial bacteria. The co-supplement of algae and bacteria has been reported to produce a healthier *Artemia* cultures, possibly through improved nitrogen assimilation (Toi et al., 2014). In addition, well-

selected consortia of microalgae and bacteria might also lead to a better shellfish larval populations (Natrah et al., 2011).

2.7 Biomass processing for biofuel production

Biomass to biofuel conversion technologies are broadly classified into three categories namely, chemical, biochemical and thermo- chemical conversion (Fig. 2.6). Thermochemical conversion generally implied to upgrade biomass by heating under high pressure in an anoxygenic enclosure. Production of bio-crude oil via hydrothermal liquefaction (HTL) of microalgae biomass has been gaining the attention of the researchers as it offers multiple advantages over other conventional process of chemical, biochemical and thermochemical conversion methods because of the following: (i) able to convert wet algal biomass into bio-crude oil and hence, bypassing the need for drying (Guo et al., 2015); (ii) high energy efficiency and low tar yield (Gollakota et al., 2018); (iii) use of catalyst is not essential; (iv) higher conversion rates (Vardon et al., 2012); (v) convert non-lipid fraction of the biomass into bio-crude; and (vi) nutrient rich aqueous phase collected after HTL can be recycled for growth of microalgae.

2.7.1 Effect of reaction conditions on bio-crude oil yield:

The physicochemical properties as well as the yield of the bio-crude strongly affected by the HTL process parameters such as process temperature, reaction time, biomass loading concentrations, biomass composition (Mathimani and Mallick, 2019). ***Reaction temperature:*** As temperature plays the most important role on bio-crude oil yield, its selection is essential for the safety and economical industrial operation. The maximal bio-oil yields occur in the temperature range of 250–375ºC. This phenomenon is mainly attributed to the competing reactions that dominate at

different temperature ranges. Fig. 2.8 shows the reaction types in different temperature regimes for microalgae HTL. The ionic character of water changes with temperature affecting a variety of reactions to be hastened. At low temperatures (<220 ºC), hydrolysis dominates, but as the temperature increases, repolymerization competes with hydrolysis. From 220 to 250 ºC, the hydrolysis products may be more water soluble causing a lower bio-crude oil yield. But, from 250 ºC to the critical point, the bio-crude oil yield reaches a maximum, while the organic content in the aqueous phase is reduced. It is due to the rapidly increasing ionic product of water near critical point. With the high ionic product, the dissociated H^+ and OH^- from hot liquid water catalyse both acid and base catalysed reactions. Under these conditions, carbohydrates, protein and lipids readily undergo isomerization, reforming, depolymerization and repolymerization reactions to produce bio-crude oil.

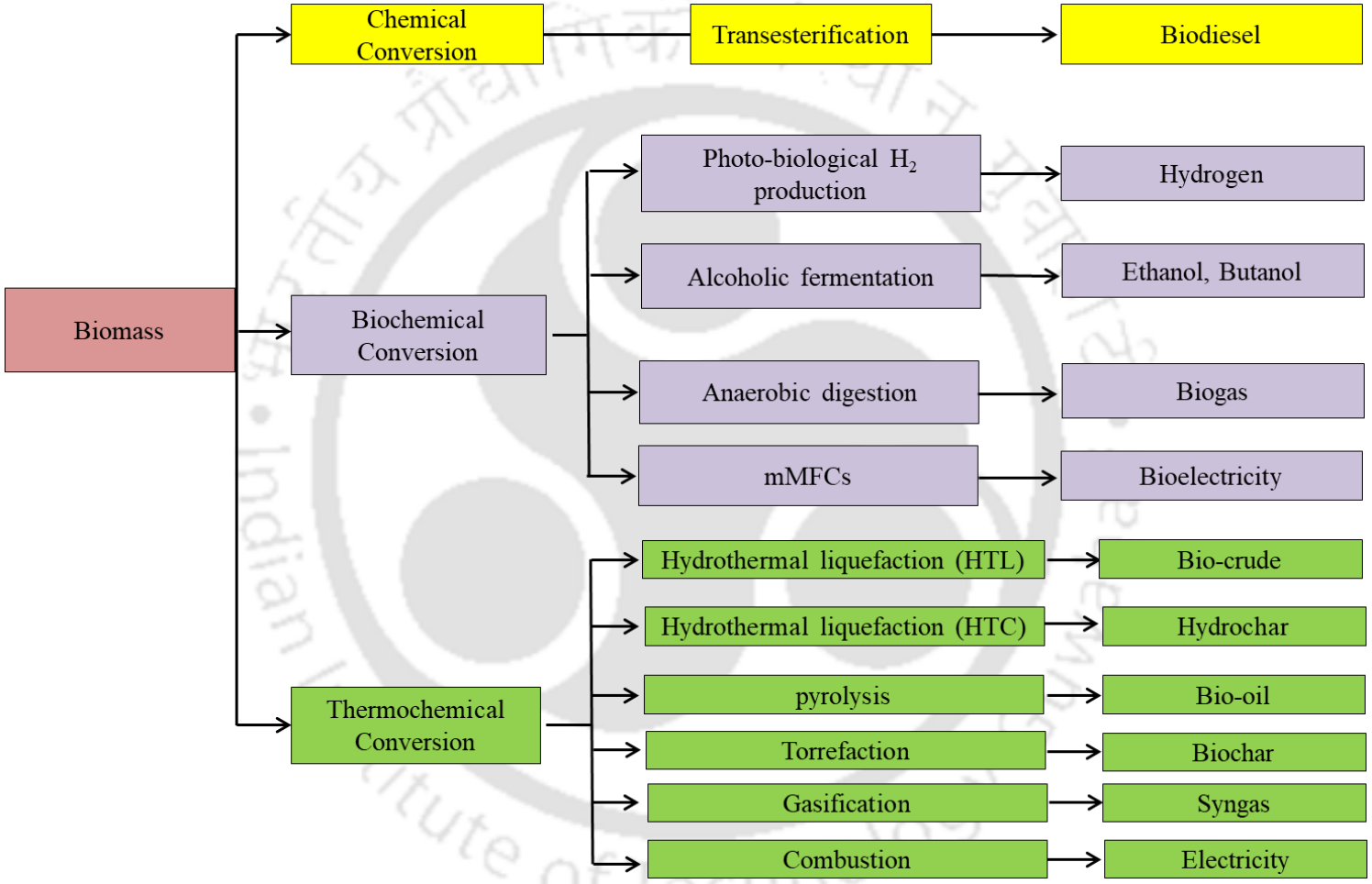

Fig. 2.6 Schematic of biomass to biofuel conversion technologies

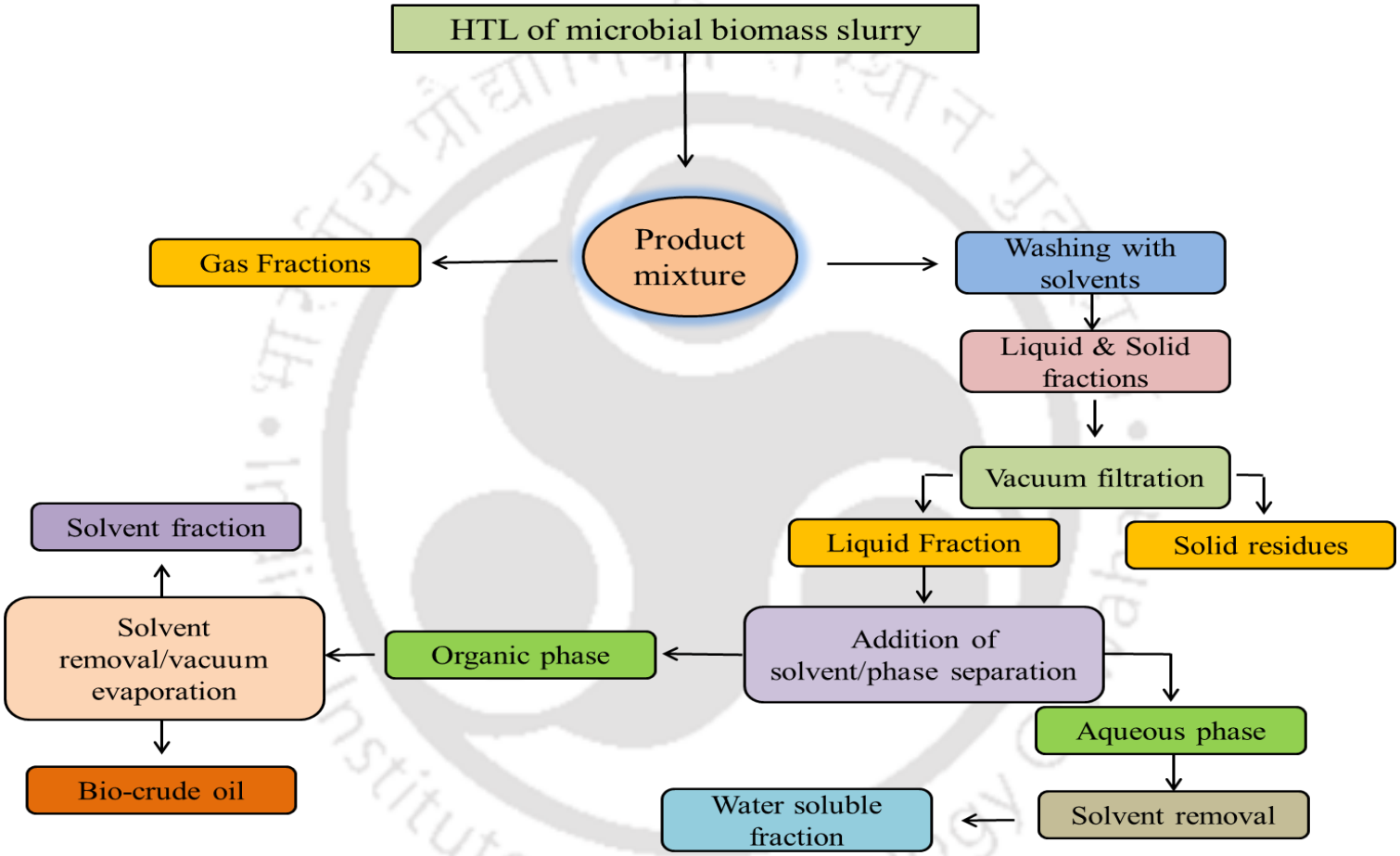

Fig. 2.7 Detailed HTL process for bio-crude oil extraction from microbial biomass.

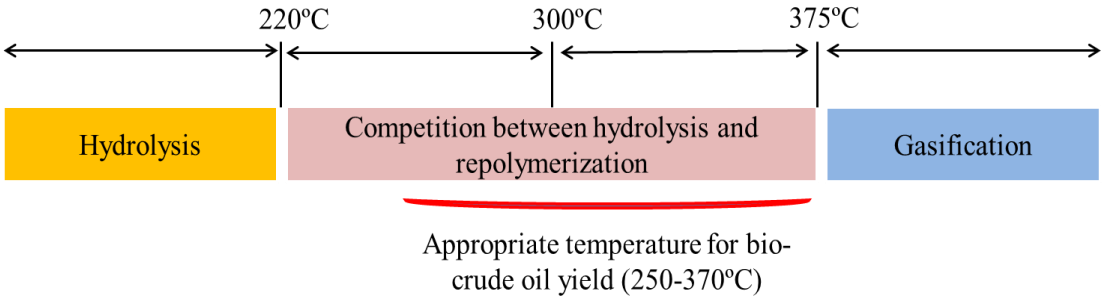

Fig. 2.8 Types of reaction in different temperature ranges of HTL (Source: Guo et al., 2015)

Above the critical point, higher gas yields resulted as supercritical conditions favour decarboxylation, cleavage, steam reforming and gasification reactions of intermediates (Guo et al., 2015). The carbon recovery of bio-crude oil enhances from 5% to 43.2% as the temperature increases to 240 ºC from 160 ºC (Yu et al., 2011). Yu et al., (2011) found that increasing temperature is favourable for decreasing the O/C ratio in bio-crude oil while enhancing the H/C ratio, but the sulphur content increases with temperature. ***Reaction time:*** Sufficient reaction time facilitates the maximum conversion of organics into bio-crude oil. Therefore, reaction time is a crucial factor for economic operation of HTL. On the other hand, extended reaction time may result in undesired re-polymerization which may result in the large production of gases, aqueous products, and char with lower bio-crude oil yield (Shuping et al., 2010). In initial short reaction time, biomass will decompose and depolymerise into smaller molecules. However, further increase in reaction time results the rearrangement of these molecules to form bio-crude oil through condensation and repolymerization (Guo et al., 2015). Ross et al., (2010) and Jena et al., (2011) found that reaction times longer than 60 min have negative impact on bio-crude oil yield of *Spirulina*. ***Biomass loading Concentration:*** The increase in biomass/water ratio to maximum may not have a positive influence on the yield and quality of the bio-crude oil, whereas low biomass feedstock concentration lead to poor economics. Peterson et al., (2008)

suggested that the target biomass concentrations should be 15-20 (wt. %, w/v) in order to achieve practical economy. Jena et al., (2011) found that 20 (wt. %, w/v) biomass loading is the optimum for *S. platensis* liquefaction and more microalgal concentrations show significant effect on product yields. Although, in this case, relatively low concentrations of microalgal biomass can increase bio-crude oil production in a limited range; the requirement of additional water results in increased expenses such as higher energy requirements for heating. These additional costs associated with increased water content make the whole process uneconomical (Guo et al., 2015).

2.8 Microalgal wastewater treatment in outdoor condition

Microalgae are cultivated in different systems including open ponds, closed photobioreactors and hybrid systems. Open ponds i.e. waste stabilization pond (WSP) and open raceways or high raceway algal pond (HRAP) are the most commonly used open algal systems for wastewater treatment. According to Oswald (1995), ponds with properly design and well maintain can produce consortia of algae and bacteria that can biodegrade organic wastes contained in wastewater and consequently produce energy-rich algal biomass. Open ponds have advantages over other microalgal wastewater treatment systems due to their low construction, operation and maintenance costs, negligible or absence of electrical energy requirement, high performance and ease of operation (Mara, 2008). However, the major disadvantages of these systems are huge land requirement, difficulty in controlling environmental conditions such as temperature and illumination, susceptibility to contamination by unwanted bacteria species, grazers, and other organisms, and water loss due to evaporation especially in tropical and semi-arid regions (Mara, 2008). WSP typically comprise of series of anaerobic, facultative and one or more maturation ponds.

Anaerobic ponds are sometimes omitted as microalgae are not able to grow in anaerobic condition. Facultative ponds are usually 1 to 2 m deep (Mara, 2006) where wastewater treatment is achieved through algal-bacterial symbiosis. They are considered as primary facultative pond when they receive their influent organic load directly from raw wastewater source or secondary facultative pond when they receive a pre-treated wastewater; for example, the effluent of anaerobic pond or wastewater from primary settling tank (Mara, 2006). Facultative ponds are designed for BOD/COD removal based on permissible areal (surface) organic loading with typical organic loading from 100 to 400 kg BOD $ha^{-1}.d^{-1}$ (Mara, 2003). Treatment efficiencies of facultative ponds in terms of filtered and unfiltered BOD/COD and TSS removals could be greater than 95%, 70% and 90% respectively which are comparable to those obtained by other wastewater treatment systems (Mara, 2006). Besides oxygen production from algal photosynthesis, these ponds receive additional oxygen from the atmosphere through the surface due to wind action. However, it is also reported that the oxygen produced by algal photosynthesis is more useful in waste stabilisation pond than that supplied by wind aeration (Shilton and Harrison, 2003). Maturation ponds are generally shallower than or as deep as facultative ponds with their depth ranging from 1 to 1.5 m (Mara, 2006) which receive the effluents from facultative ponds. They are designed for pathogens, NH_4-N removals and some level of BOD removal from the waste effluents. The main disadvantage of maturation ponds is large land area requirement though this is often overlooked where land is readily available at low-cost. However, maturation ponds are suitable for algal growth but are mainly used for pathogen removal (Mara, 2006). The pathogen removal in maturation ponds results from increase in temperature due to high solar radiation, elevated pH due to accumulation of hydroxide ions from aqueous dissociation of

carbonate-bicarbonate ions and photo-oxidation resulting from the combined effect of high irradiance and high DO concentration (Curtis et al., 1992). Raceway ponds (also synonymously called open raceways) are another type of open systems in which microalgae are commonly cultivated. HRAP systems are a 'quantum leap' over conventional ponds because they integrate ecological engineering principles and incorporate many different (physical, chemical and microbiological) natural treatment processes. The diversity of natural treatment processes that occurs in the different components of the system enables HRAP systems to be much more resilient and robust than mechanical treatment. HRAP systems essentially consist of four main unit processes arranged in series: 1. wastewater solids removal and subsequent ambient temperature anaerobic digestion; 2. aerobic treatment by sunlight-powered algal growth on the supernatant; 3. algal removal and subsequent conversion to biofuel; and 4. further polishing of the treated effluent as required. HRAP share some characteristics and advantages with facultative ponds but some of their disadvantages include long light path due to large volume per pond area leading to low algal biomass concentration which consequently increases harvesting cost, light shading, lack of control of environmental conditions, difficulty in achieving low flow velocity as turbulence is required for stirring the paddle wheel, loss of water through evaporation and difficulty in screening algal species for specific application (Tredici, 2004; Sheehan et al., 1998). According to Sheehan et al. (1998), the major problem in screening algal species grown in open ponds is the inability of the isolated strains to dominate in such systems as they are easily out-competed by contaminant native algal species in the vicinity. Furthermore, open raceway ponds are designed for maximum algal biomass productivity considering pond depth, water circulation velocity,

retention time, frequency of culture dilution, temperature, and pH as key design parameters (Sheehan et al., 1998).

2.9 Current challenges

1. Lack of robust microalgae and bacteria strains which can tolerate a wide range of chemicals present in different types of wastewater.

2. Lack of understanding about the microalgae-bacteria consortium strategy for higher biomass production coupled with effective wastewater treatment.

3. Direct conversion of wet microbial biomass (microalgae and bacteria) and improved bio-crude oil yield via optimization of HTL process parameters.

4. Large scale outdoor cultivation of microalgae-bacteria consortium to treat industrial wastewater as well as biomass production under fluctuating environmental conditions.

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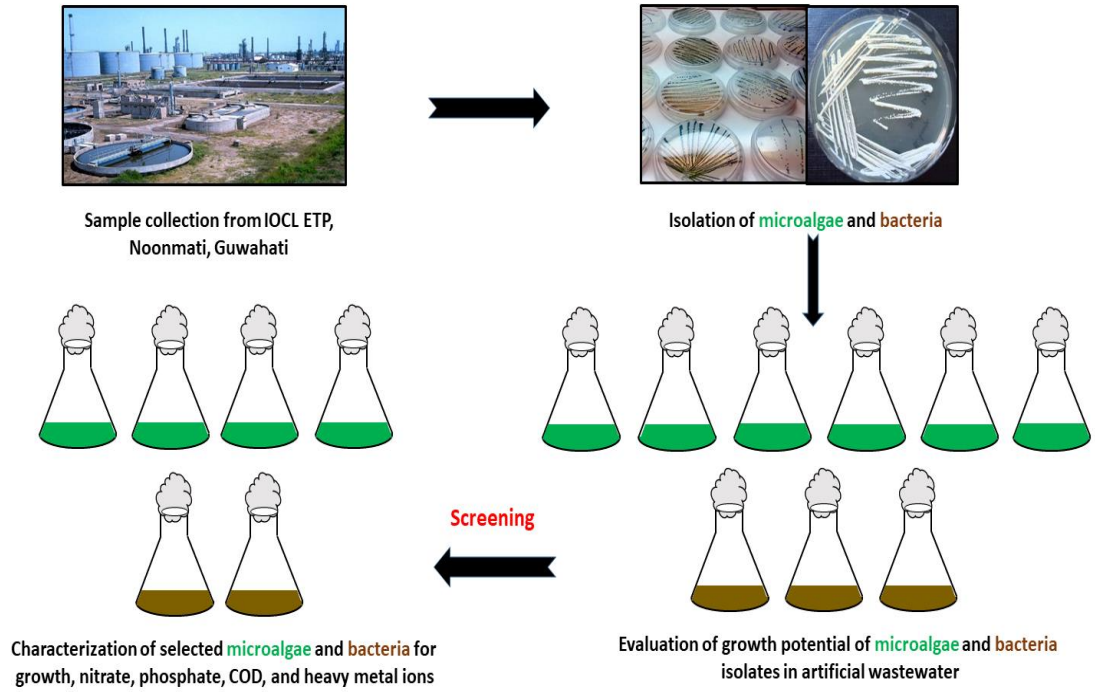
Somewhere, something incredible is waiting to be known.

Carl Sagan

Chapter 3

Isolation and characterization of indigenous microalgal and bacterial strains from wastewater resources



Sampling, isolation and screening of indigenous microalgae and bacteria strains for the evaluation of performance efficiency in terms of growth and wastewater treatment.

3.1 Background and motivation

Population growth, industrialization and urbanisation is increasing at the cost of increasing carbon dioxide (CO_2) emission, depleting energy and water resources, and contamination of water bodies with toxic pollutants (Khan et al., 2019). Water pollution caused by various anthropogenic activities as well as wastewaters released from paper and pulp, textile or tannery and pharmaceutical industries etc. have caused many human health problems in developed and developing countries (Shahid et al., 2019). Moreover, the extensive exploitation of fossil fuel for power generation and transportation is one of the major causes of energy issues. Global challenges facing many nations include energy security, reducing energy supplies, and environmental pollution. One approach to attain these goals is to utilize microalgae cultivation by employing wastewater nutrients. This integrative approach is cost effective and eco-friendly mode for the sustainable production of biofuel and bio-based chemicals because of the reduced costs associated with using large volumes of fresh water, nutrients, and trace elements. However, overcoming challenges impeding the cultivation of microalgae using wastewater such as a lack of robust microalgae strains which can tolerate a wide range of pollutants typically present in wastewater and competition between microalgae strain and other native micro-organism present in wastewater must be addressed (Makut et al., 2019). Microalgae-bacteria consortia based wastewater treatment coupled with biomass production have been gaining importance in recent years. Such microalgae-bacteria consortia can be used as robust biological systems with the ability to function under fluctuating growth environments and nutrient loads owing to their diverse metabolic activities and adaptation to different environmental conditions. The successful development of this technology

requires isolating robust microalgae and bacteria strains from wastewater resources which can grow in presence of different chemicals present in wastewater.

In the present study, microalgae and bacteria strains were isolated from an oil refinery wastewater located in Guwahati, Assam, India. With the aim of screening potential strains which can grow in wastewater along with considerable treatment efficiency, the isolates were characterized in formulated artificial wastewater (AWW). The characterization of the viable strains was performed based on the biomass titer, percent of nutrients removed and chemical oxygen demand (COD).

3.2 Materials and methods

3.2.1 Isolation and screening of microalgal and bacterial strains

Six microalgal strains and three bacterial strains were isolated from oil refinery wastewater collected from Indian Oil Corporation Limited, Noonmati (26.19 °N, 91.80 °E), Guwahati, Assam, India. In order to isolate microalgal strains, the collected oil refinery wastewater sample was inoculated into BG11 medium comprising of (g L^{-1}) $NaNO_3$, 1.5; $K_2HPO_4.3H_2O$, 0.4; $MgSO_4.7H_2O$, 0.075; $CaCl_2.2H_2O$, 0.036; Na_2CO_3, 0.02; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001 and A5 + Co solution (1 mL L^{-1}) that consists of (g L^{-1}) H_3BO_3, 2.86; $MnCl_2.H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $CuSO_4.5H_2O$, 0.079; $Na_2MoO_4.2H_2O$, 0.390; and $Co(NO_3)_2.6H_2O$, 0.049 (Barsanti et al., 2014) and incubated in an orbital shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 30°C under a 100 µE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. Pure cultures of microalgae strains were obtained by conventional streaking methods followed by serial dilution and spread plating techniques (Muthuraj et al., 2014). Bacterial strains were isolated by inoculating the sample in a nutrient broth (Hi Media, India) and incubated in an incubator shaker at 30°C and 150 rpm. Axenic bacterial strains were obtained by

conventional streaking methods followed by serial dilution and spread plate techniques. Isolated microalgae and bacterial strains were maintained in BG11 agar slants and 50% glycerol stocks respectively.

In order to evaluate their growth potential in wastewater, the isolates were grown in AWW comprising of (g L^{-1}) starch, 1.0; $NaNO_3$, 1.731; K_2HPO_4, 0.044; $KHCO_3$, 0.967; K_2CO_3,0.707; $NaHCO_3$, 0.714; $MgCl_2.6H_2O$, 0.317; $MgSO_4.7H_2O$, 0.160; $CaCl_2$,0.319 and trace metal solution (1 mL L^{-1}) that consists of $FeSO_4.7H_2O$, 7.940; $MnCl_2.4H_2O$, 0.525; $CuSO_4.5H_2O$, 0.0679; $CrCl_3.6H_2O$, 0.247; $CdCl_2.H_2O$, 0.013; $NiCl_2.6H_2O$, 0.186; $PbCl_2$, 0.124; $ZnSO_4$, 0.5; $AsCl_3$,0.5; $Al_2(SO_4)_3.16H_2O$, 0.459; and $Na_2Mo_4.2H_2O$, 0.015 (Yusof et al., 2010). The microalgal isolates were grown in an orbital shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 30°C under a 100 µE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. The bacterial strains were grown in incubator shaker at 30°C and 150 rpm. Microalgal growth was monitored by measuring chlorophyll-a, optical density (OD) at 690 nm, and dry cell weight (DCW). The bacterial growth profile was obtained by measuring OD at 600 nm and DCW. Based on their growth performance in AWW, four microalgae and two bacterial strains were selected for further characterization and process development.

3.2.2 Characterization of the selected individual isolates in AWW

In the present study, the aim was to develop a sustainable process for the generation of microbial biomass as a feedstock for biofuel production coupled with waste water remediation. Therefore, it was important to characterize these isolates (four microalgae and two bacteria) on the basis of their growth potential and waste water treatment efficiency. With this objective, the characterization was carried out in 500 mL Erlenmeyer flasks containing 200 mL of AWW media with 1% (v/v) inoculum. The microalgal isolate were grown in an orbital shaker (Multitron-Pro,

Infors HT, Switzerland) at 150 rpm, 30°C under a light intensity of 100 µE m^{-2} s^{-1} with a light:dark cycle of 16:8 h. The bacterial strains were grown in incubator shaker at 30°C and 150 rpm. All the microalgal and bacterial isolates were cultivated for seven days. Wastewater treatment efficiency of the individual isolate was evaluated by analysing the samples withdrawn at 24 h interval in order to obtain dynamic profile for nitrate, phosphate, COD, and heavy metal.

3.2.3 Analysis of growth, nutrient utilization, COD and heavy metal ion removal

Analysis of growth, nutrient utilization, COD, and heavy metal ion removal was carried out at every different sampling times. Growth of the microalgal strains was measured based on OD, chlorophyll-a, and DCW whereas growth of the bacterial strains was measured in terms of OD and DCW. Microalgal and bacterial OD was monitored by measuring the absorbance at 690 nm (A_{690}) and 600 nm (A_{600}) respectively using a UV–visible spectrophotometer (Cary 100, Varian, Australia). The absorbance values were converted into DCW through appropriate calibration equations. In the chlorophyll-a analysis, the sample was centrifuged at 13,000 rpm for 5 min and the pellet was extracted with 1.5 mL methanol 99.9% by incubating in dark at 45°C for 30 min. Next, the sample was again centrifuged at 13,000 rpm for 5 min and the absorbance spectra was obtained in the range of 400–750 nm using UV–visible spectrophotometer (Cary 100, Varian, Australia). The chlorophyll-a concentration was obtained using the Eq. 3.1 (Pruvost et al., 2011).

$$Chlorophyll - a\ (\mu g m L^{-1}) = 16.5169 \times A^{*}_{665} - 8.0962 \times A^{*}_{652} \qquad (3.1)$$

where, $A^{*}_{665}=A_{665}-A_{690}$ and $A^{*}_{652}= A_{652}-A_{690}$

The nitrate concentration in the supernatant was determined using the salicylic acid method (Cataldo et al., 1975) with a sodium nitrate as the standard. Phosphate estimation was carried out using the ascorbic acid method with potassium hydrogen

phosphate (dibasic) as a standard (Parsons et al., 2013). Samples for heavy metal ion analysis were centrifuged at 13,000 rpm for 5 min and the clear supernatant was collected for analysis using atomic absorption spectroscopy (Varian AA240, Australia). Standard procedures (APHA, 2017; Trivedi et al., 1986) were followed during analysis. COD analysis was performed using HACH COD reagents and quantified in a DR900 colorimeter (Hach, USA). All chemicals and reagents used were of analytical grade from Hi Media, India. All the experiments were conducted in triplicate and the data were represented as mean ± standard error.

3.3 Results and discussion

3.3.1 Isolation and screening of the microalgal and bacterial strains with the potential to grow in wastewater

Six microalgal (A1, A2, A3, A4, A5, & A6) and three bacterial (B1, B2, & B3) strains were isolated from oil refinery wastewater. While multiple microalgal and bacterial strains exist in the collected sample, only those microalgal and bacterial strains grown in BG11 and nutrient broth medium respectively were selected for further studies. Further, selection of suitable microalgal and bacterial strains were key towards formation of microalgae-bacteria consortium for the generation of biomass feedstock and effective wastewater treatment. All the isolated strains were characterized to evaluate their growth potential in AWW containing heavy metals, starch, nitrogen, and phosphorus. At the end of seven days of cultivation, four microalgal strains (A1, A2, A4, & A6) and two bacterial strains (B1 and B3) were found to grow substantially (Fig. 3.1). However, microalgal strains A3 & A5 and bacterial strain B2 inability to grow in AWW may be attributed to potential heavy metal toxicity. The results indicate species specific resistance of the microorganisms to heavy metal toxicity. For instance, the microalgae *C. pyrenoidosa* and *Spirogyra*

communis were shown to grow even in presence of Pb^{2+}, Cu^{2+}, and Cr^{4+} (Sati et al., 2016). Further, bacterial strains *Bacillus laterosporus* and *B. licheniformis* were resistant to Cd^{2+} and Cr^{4+} (Zouboulis et al., 2004). However, a set of other microalgal and bacterial strains were found to be intolerant towards different concentration of heavy metals (Baz et al., 2015; Sati et al., 2016). Based on their growth potential in AWW containing a wide variety of heavy metals, four microalgal strains (A1, A2, A4, & A6) and two bacterial strains (B1 and B3) were selected for further characterization with respect to their wastewater treatment efficiency and subsequent microalgae-bacteria consortium formation.

3.3.2 Characterization of the selected individual isolates in AWW with respect to growth and wastewater treatment efficiency

Four microalgal (A1, A2, A4, and A6) and two bacterial (B1 and B3) strains selected from the screening exercise were then characterized for their potential to treat wastewater based on the removal efficiency for nitrate (Fig. 3.2A and D), phosphate (Fig. 3.2B and E), COD (Fig. 3.2C and F), and heavy metals (Table 3.1) from AWW.

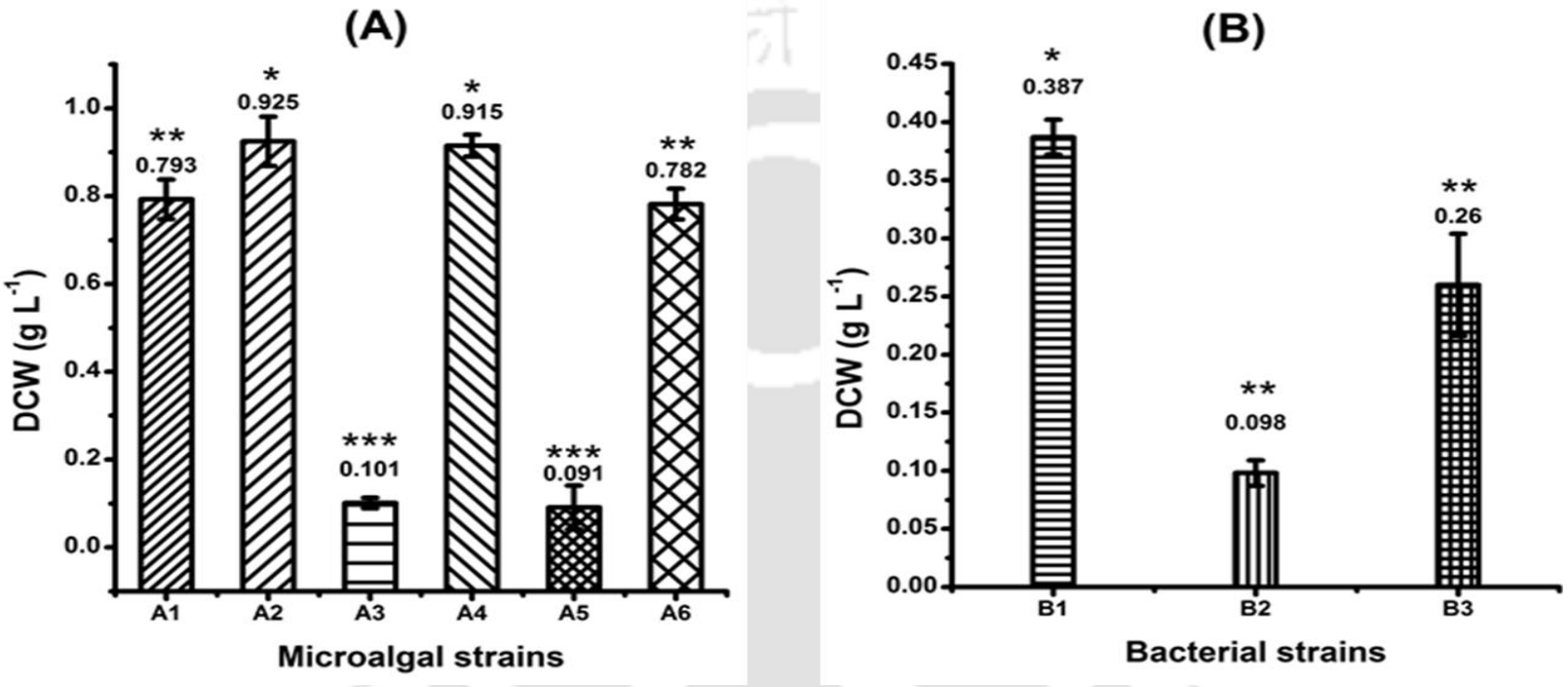


Fig. 3.1 Comparative growth profile (DCW, g L^{-1}) of (A) microalgal strains and (B) bacterial strains isolated from the oil refinery wastewater. Six microalgal (A1, A2, A3, A4, A5, and A6) and three bacterial (B1, B2, and B3) strains were grown on artificial wastewater. The microalgal strains were grown in an orbital shaker at 150 rpm, 30°C under a light intensity of 100 µE m^{-2} s^{-1} with a light:dark cycle of 16:8 h. The bacterial strains were grown in an incubator shaker at 30°C and 150 rpm. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. The asterisk sign represents the significant difference between the biomass titer obtained for different strains analysed using one-way analysis of variance based on the Tukey's method. Biomass titer that do not share a common symbol are significantly different

While microalgal strains contributed predominantly towards the removal of nitrate (45–62% efficiency) and phosphate (100% efficiency), COD removal was mainly mediated by the bacterial strains with an efficiency of 31−33%. Microalgae satisfy their critical requirement of nitrogen for growth by converting inorganic nitrogen sources (nitrate, nitrite, nitric acid, ammonium, and ammonia) into organic macromolecules such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP & ATP), and genetic materials (RNA & DNA) by assimilation (Barsanti et al., 2014). Phosphorous is important in energy metabolism and it is also found in nucleic acids, lipids, proteins, and the intermediates of carbohydrate synthesis and hence exhibit significant impact on microalgal growth (Cai et al., 2013). In the present study, adding starch, as a source of the organic carbon to the AWW at a concentration of 1 g L^{-1} increased the COD to 1200 mg L^{-1}. Characterization of the individual isolate revealed that the effective utilize of starch by the bacterial strains in comparison to the microalgal strains resulted in a higher COD removal efficiency (Fig. 3.2 and Fig. 3.3). COD removal by the bacterial strains is a result of aerobic biological treatment where the oxidative degradation of organic compounds leads to both energy and carbon utilization (Mujtaba et al., 2015). Numerous studies have reported that bacteria which are able to synthesize starch degrading enzymes enables them to utilize complex sugar molecules effectively (Alariya et al., 2013; Mishra et al., 2008). Dynamic profiling of the nutrient utilization by microalgal strains showed that growth cessation was likely due to discontinued nitrate utilization after day 5 of cultivation while phosphate was completely consumed by day 4 (Fig. 3.3B and C). The highest nitrate removal efficiency of 62.65% for the microalga A2 (Fig. 3.2A) might be attributed to the maximum growth amongst all the microalgal strains was based on using DCW (Fig. 3.1A) or Chlorophyll-a content (Fig. 3.3A). In similar

studies, a total nitrogen removal efficiency of 61% was reported by *Chlorella* sp. when grown on centrate wastewater (Min et al., 2011). A phosphate removal efficiency of 86.4% was reported for *C. vulgaris* when characterized on a simulated wastewater (Nagabalaji et al., 2019). The results indicate that the selected microalgal strains were highly efficient in removing nitrate and phosphate from SW. The uptake of nitrate and phosphate by both of the bacterial isolates, B1 and B3, ceased after day 3 of cultivation (Figs. 3.3F and G) resulted in lower efficiency of nitrate (22.9%) and phosphate (19.42%) removal. This lower removal efficiency for nitrate and phosphate by the bacteria could be attributed to the cessation of growth beyond day 3 of cultivation (Fig. 3.3E). In spite of the availability of key nutrients in sufficient amount, termination of growth of the bacteria which was unusual might be attributed to the heavy metal toxicity. In the present study, SW was supplemented with five heavy metals including Cu, Cr, Cd, Ni, and Pb.

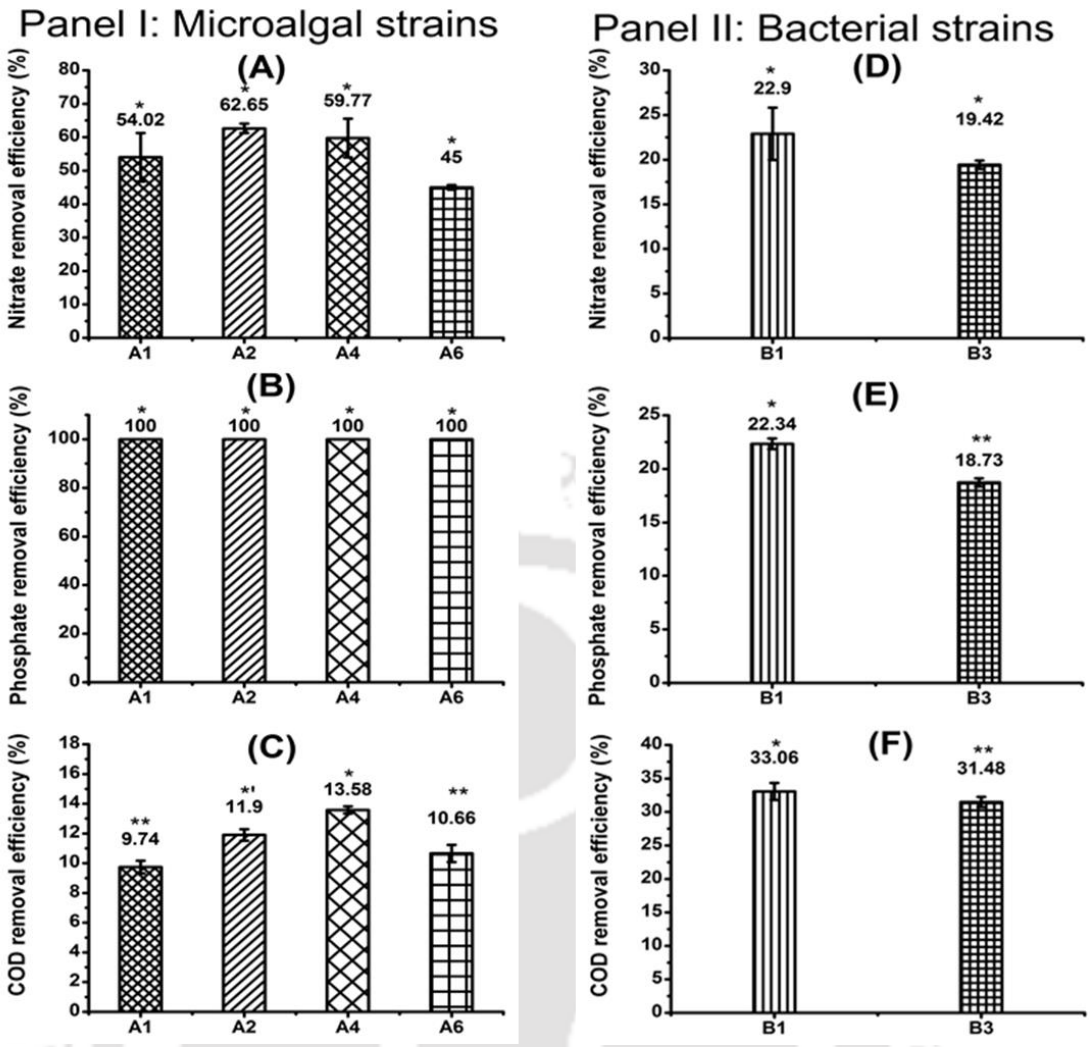


Fig. 3.2 Nitrate, phosphate, and COD removal efficiency (%) of the screened microalgal (A1, A2, A4, and A6) and bacterial (B1 and B3) strains grown on artificial wastewater. Panel I represent profiles for the microalgal strains and Panel II represents profiles for the bacterial strains. The microalgal strains were grown in an orbital shaker at 150 rpm, 30°C and a 100 μE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. The bacterial strains were grown in an incubator shaker at 30°C and 150 rpm. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. The asterisk sign represents the significant difference between the nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency obtained for different strains analysed using one-way analysis of variance based on Tukey's method. Nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency that do not share a common symbol are significantly different. In (C), *' signifies that the strain A2 has similar statistical significance to the group with two asterisks.

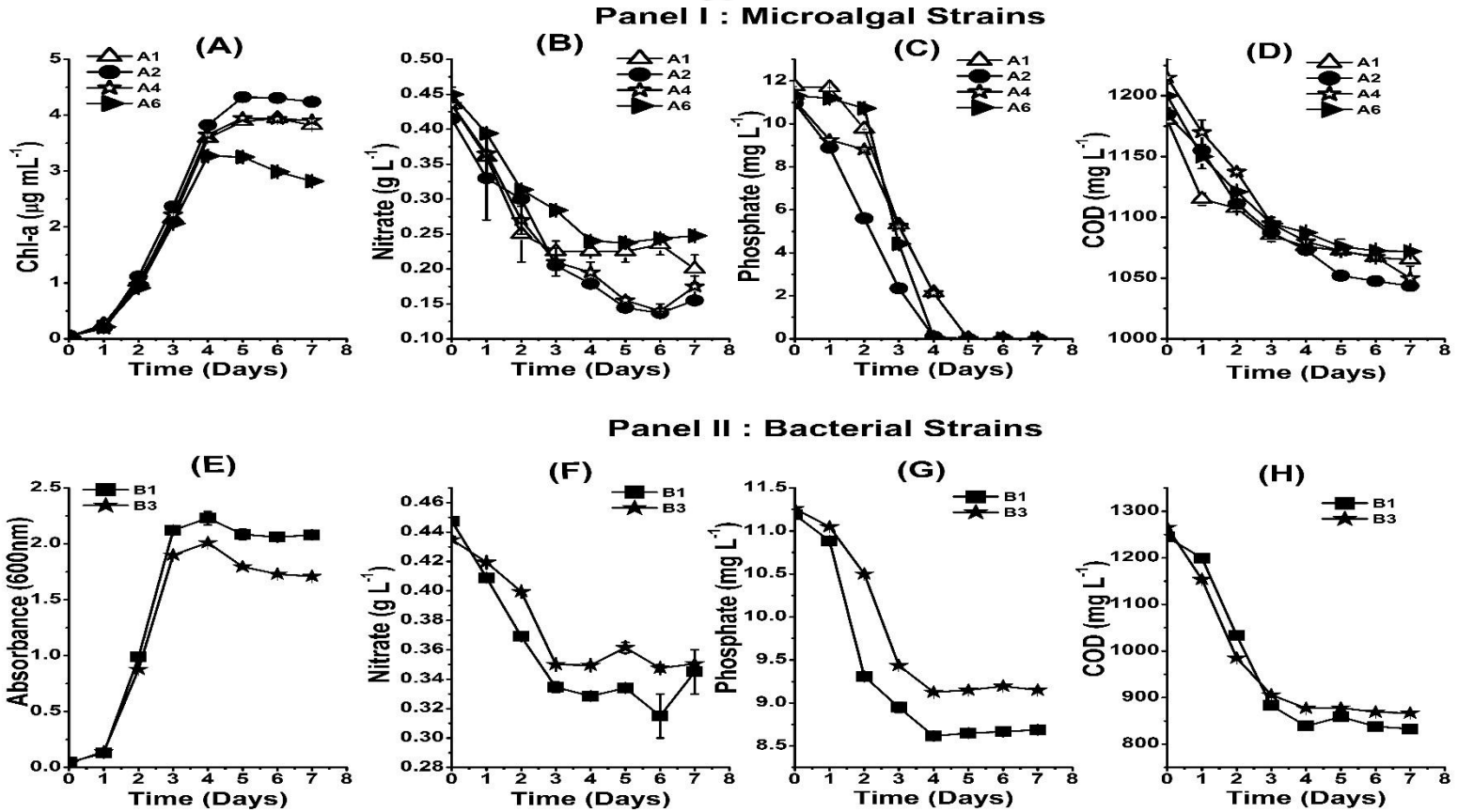


Fig. 3.3 Dynamic profiles of growth and utilization of nitrate, phosphate & COD by the screened microalgal (A1, A2, A4 and A6) and bacterial (B1 and B3) strains grown on artificial wastewater. Panel I represents profiles for the microalgal strains and Panel II represents profiles for the bacterial strains. The microalgal strains were grown in an orbital shaker at 150 rpm, 30°C under 100 µE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. The bacterial strains were grown in an incubator shaker at 30ºC and 150 rpm. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error.

Table 3.1 Heavy metal ion removal efficiency (%) of individual microalgal and bacterial strains. All the experiments were conducted in triplicate and the data were expressed as a mean ± a standard error

Individual strains	Copper (Cu)	Chromium (Cr)	Cadmium (Cd)	Nickel (Ni)	Lead (Pb)
A1	48.09±0.06	33.9±0.04	29.5±0.06	36.4±0.01	22.53±0.08
A2	51.41±0.01	38.54±0.05	31.69±0.07	38.55±0.05	37.22±0.07
A4	62.69±0.08	48.26±0.02	28.72±0.03	46.5±0.02	48.35±0.08
A6	33.95±0.05	44.69±0.02	34.69±0.09	21.83±0.09	33.76±0.05
B1	39.03±0.06	39.36±0.01	27.78±0.03	18.09±0.01	23.19±0.04
B3	27.11±0.07	33.34±0.03	19.8±0.04	23.03±0.1	31.13±0.02

From the profile of the heavy metal ion removal efficiency by the individual isolates (Table 3.1), it was observed that while the microalgal strains could remove majority of the heavy metal ions with higher efficiency, removal efficiency of many metal ions were lower in case of bacteria. For instance, the removal efficiency of Cd, Ni, and Pb by the bacterium B1 was 27.78%, 18.09%, and 23.19% respectively. B3 was able to remove Cu, Cd, and Ni with an efficiency of only 27.11%, 19.8%, and 23.03%, respectively. Past work have demonstrated the ability of different microalgal strains to remove a wide range of heavy metals with higher efficacy from mixed domestic-industrial wastewater (Hammouda et al., 2015) or centrate municipal wastewater (Wang et al., 2010). The results indicate that while selected microalgal strains are potential candidates to remove heavy metals along with nitrate and phosphate, bacterial strains are effective in removing COD with significant efficacy. Therefore, co-cultivation of microalgae and bacteria could be an effective strategy for biomass feedstock generation coupled with wastewater treatment.

3.4 Conclusions

Six microalgae and three bacteria strains were isolated and screened in AWW on the basis of their growth potential. Among these isolates, four microalgae and two bacteria strains were characterised individually in AWW to evaluate their performance efficacy in terms of biomass titer, percent nutrients remove and COD. To attain higher total biomass titer as well as wastewater treatment efficiency, these four microalgae and two bacteria strains were designed to configure a microalgae-bacteria consortium for further characterisation in AWW.

3.5 References

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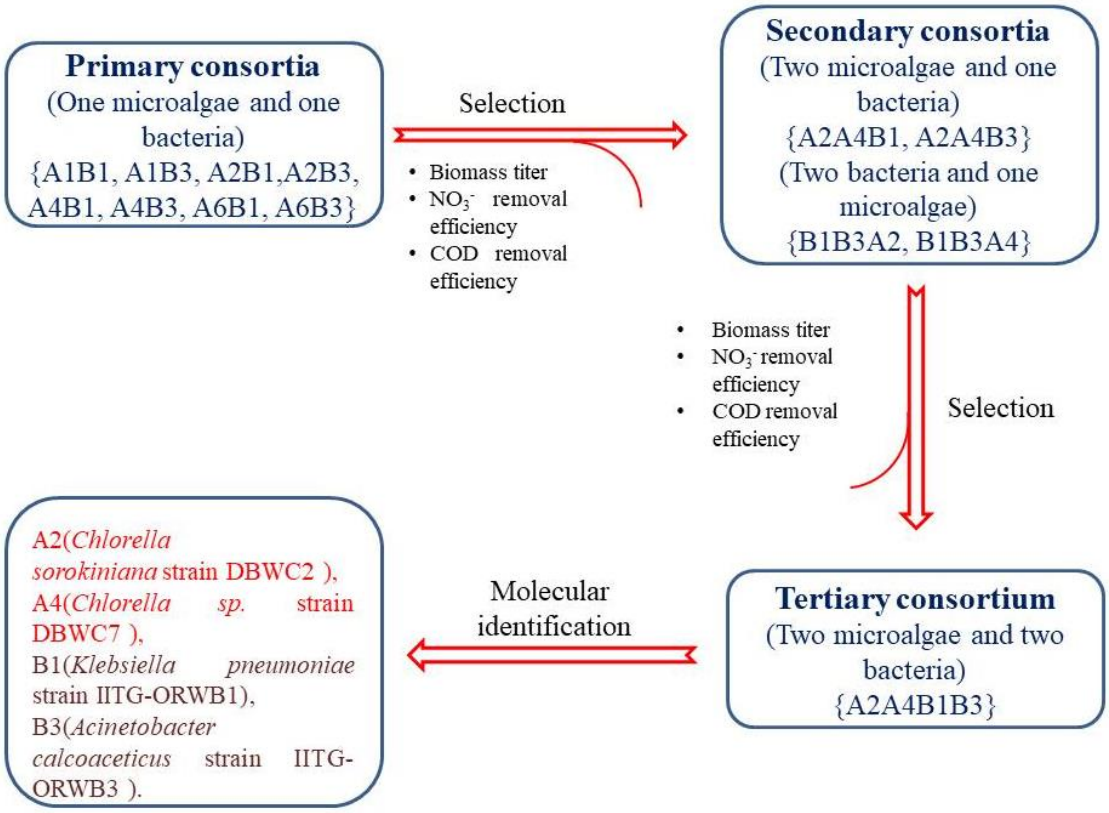
In much of society, research means to investigate something you do not know or understand.

Neil Armstrong

Chapter 4

Evaluation of various microalgal-bacterial consortia for the production of microbial biomass and effective wastewater treatment



Characterisation of different types of microalgae-bacteria consortia in AWW in order to evaluate their biomass titer, nitrate removal, phosphate removal, COD removal and heavy metal ion removal efficiency.

4.1 Background and motivation

Phycoremediation is one of the potential wastewater treatment methods which involve reduced capital costs, less energy for operation and lower technical inputs than conventional treatment methods (Fito et al., 2019). Moreover, microalgae support the removal of BOD, nutrients, heavy metals, pathogens, and heterotrophic pollutant from wastewater (Munoz et al., 2006). However, the primary challenges which are associated with the phycoremediation technology lie in the variability in wastewater composition, lack of robust microalgae strains, lower biomass titer, and competition between the microalgae strains and the native microorganisms prevailing in wastewater. Microalgae-bacteria consortium is considered as the most promising candidate in algal biotechnology which can offer enhanced biomass titer with better wastewater treatment efficiency as compared to microalgae or bacteria alone (Choi et al., 2017; Makut et al., 2019; Mujtaba et al., 2016). In nature, most of the microalgae and bacteria form micro-ecosystem where they develop symbiotic relationship by means of exchanging (i) nutrients, (ii) gases, and (iii) signalling molecules etc. (Subashchandrabose et al., 2011). The advantageous association of photosynthetic microalgae and heterotrophic bacteria have been attributed to various factors *e.g.* (i) enhanced microalgal growth by growth promoting substances secreted by bacteria (Dao et al., 2018; Gonzalez et al., 2000); (ii) bacteria mediated reduction in dissolved oxygen level of the medium resulting in alleviation of oxygen inhibition to microalgae (Mouget et al., 1995); (iii) exchange of O_2/CO_2, micro- or macro nutrients and signalling molecules etc. (De-Bashan et al., 2008; Du et al., 2013; Fuentes et al., 2016; Praveen and Loh, 2015) and (iv) secretion of dissolved organic matter by microalgae which serves as a source of carbon, nitrogen and energy for the co-existing bacteria (Dao et al., 2018). These studies affirm the need to construct

microalgae-bacteria consortium in order to increase total biomass titer and wastewater treatment efficiency.

The present study deals with the construction of different types of microalgae-bacteria consortium from the pool of selected microalgae and bacteria isolates. In the next step, these different types of microalgae-bacteria consortia were characterised in AWW to evaluate the performance in terms of biomass titer and wastewater treatment efficiency. The selection of best microalgae-bacteria consortium was done on the basis of (i) improved total biomass titer, and (ii) better wastewater treatment efficiency.

4.2 Materials and methods

4.2.1 Characterization and selection of the microalgae-bacteria consortia in AWW

With the aim of evaluating the performance of microalgae-bacteria consortia in terms of biomass titer and waste water treatment efficiency, in the first step, eight primary combinations of consortia were considered. Each primary combination was formed by considering one microalgal and one bacterial strain chosen from a pool of four microalgal and two bacterial isolates (as mentioned in previous chapter 3, section 3.2.2). Selection of appropriate primary combinations was guided by the bottom-up approach (Padmaperuma et al., 2018) which involves sequential steps of defining the desired objective function followed by short-listing the suitable combination(s). With the aim of demonstrating a sustainable process, the overall objective was broken down into two concurrent sub-objectives of (a) improved total biomass titer and (b) better wastewater treatment efficiency. Further, in the majority of literatures, evaluations of wastewater treatment efficiency have been performed based on the removal efficiency of COD, total nitrogen and phosphate by the microorganisms

(Liang et al., 2013; Mujtaba et al., 2015; Rasouli et al., 2018; Tricolici et al., 2014). Therefore, in the present study the better performing primary combination(s) of microalgae-bacteria consortia were selected on the basis of (i) total biomass titer (microalgae plus bacteria); (ii) nitrate removal efficiency; and (iii) COD removal efficiency. In the next step, similar performance evaluation was carried out for four secondary combinations of microalgae-bacteria consortia. Each secondary combination was formed by considering either one microalgal-two bacterial strains or two microalgal-one bacterial strain, chosen from a pool of the microalgal and bacterial isolates present in the selected better performing primary combination(s). Finally, characterization was performed for one tertiary combination of microalgae-bacteria consortium. The tertiary combination comprised of two microalgal-two bacterial strains chosen from better performing secondary combination(s) of microalgae-bacteria consortia. All the experiments were carried out for seven days in an orbital shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 30°C under 100 $\mu E\ m^{-2}\ s^{-1}$ light intensity with a light:dark cycle of 16:8 h using AWW medium. The characterization was performed in terms of dynamic profile of growth, total biomass titer, and removal efficiency of nitrate, phosphate, COD & heavy metal ions. Based on the total biomass titer (DCW), nitrate and COD removal efficiency (%), the tertiary combination of microalgae-bacteria was selected as the best consortium for further identification, optimization, and process development.

4.2.2 Identification of microalgal and bacterial strains present in the tertiary consortium

Two microalgal and two bacterial strains present in the tertiary consortium were identified through molecular analysis via 18S rDNA sequencing for microalgal strains and 16S rDNA sequencing for bacterial strains. For molecular analysis of

axenic cultures of the microalgal strains, single pass sequencing was performed on each template using 18S rDNA ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCG CTTATTGATATGC-3′) universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, USA). Similarity to sequences was determined using BLAST (Altschul et al., 1990). A phylogenetic tree was constructed from the 18S rDNA sequences of the isolated strains and related species using MEGA 7 software and neighbor-joining method with the Jukes-Cantor model. Fragments of 16S rDNA gene from axenic cultures of the bacterial strains was amplified by 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) universal primers. Forward and reverse DNA sequencing reactions of PCR amplicons were carried out with forward primer and reverse primers using BigDye® Terminator v3.1 Cycle Sequencing Kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence was used to carry out BLAST with the NCBI gene bank database. Based on maximum identity score, the first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

4.2.3 Analysis of growth, nutrient utilization, COD and heavy metal ion removal

Analysis of growth, nutrient utilization, COD, and heavy metal ion removal was carried out at every sampling time point. Growth of the microalgal strains was measured in terms of OD, chlorophyll-a, and DCW whereas growth of the bacterial strains was measured in terms of OD and DCW. Microalgal and bacterial OD was monitored by measuring the absorbance at 690 nm (A_{690}) and 600 nm (A_{600})

respectively using a UV-visible spectrophotometer (Cary 100, Varian, Australia). The procedure for chlorophyll-a, nitrate, phosphate, COD and heavy metal ions were described in section 3.2.3.

4.3 Results and discussion

4.3.1 Characterization and selection of best microalgae-bacteria consortium with respect to biomass titer and wastewater treatment efficiency

With the aim to investigate the performance efficacy of microalgae-bacteria consortium with respect to biomass generation and wastewater treatment in comparison to individual microalga, different combinations of microalgae-bacteria consortia were characterized in AWW. Further, it was intended to select best microalgae-bacteria consortium based on its performance efficacy. Eight combinations of primary consortia, four combinations of secondary consortia and one tertiary consortium were considered. In all primary combinations of microalgae-bacteria consortia, an increment in biomass titer in the range of 4−21% was observed when compared with the corresponding individual microalgal isolate (Fig. 4.1A). While improvement in nitrate removal efficiency was recorded to be in the range of 3−20% (Fig. 4.1B), improvement in COD removal efficiency was significantly higher within the range of 280−380% (Fig. 4.1D).

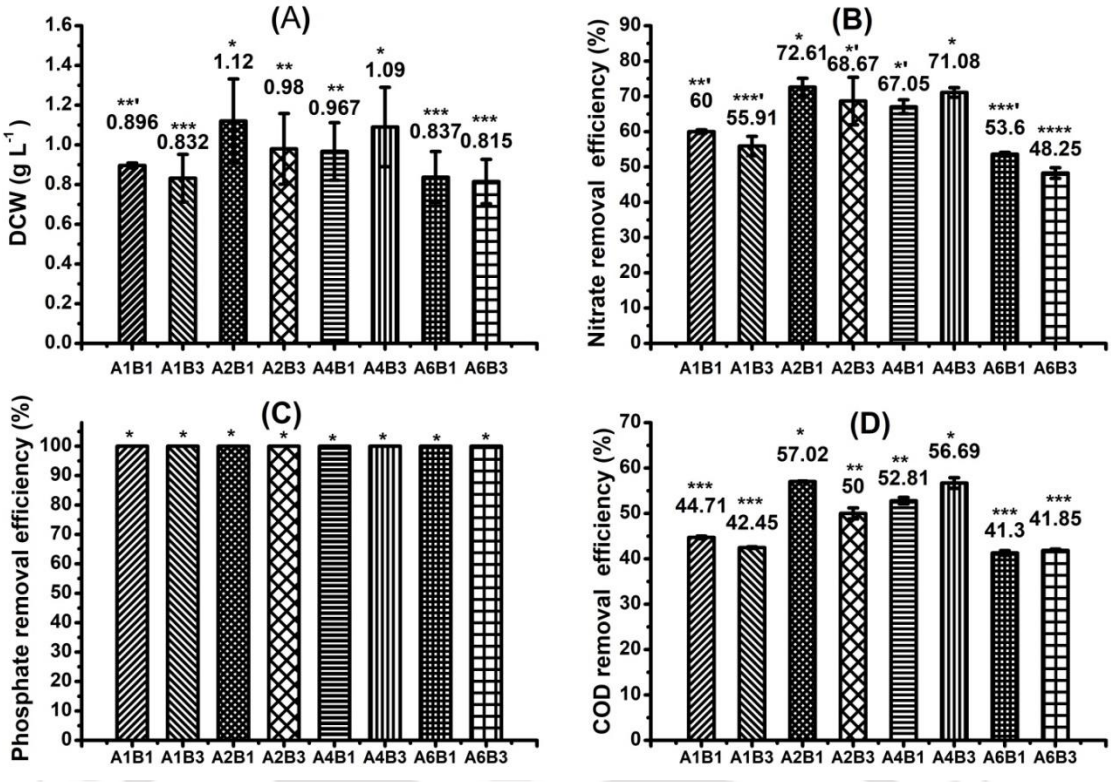


Fig. 4.1 (A) Growth (DCW, g L^{-1}), (B) nitrate removal efficiency (%), (C) phosphate removal efficiency (%), and (D) COD removal efficiency (%) of eight primary consortia of microalgae and bacteria grown on artificial wastewater. The experiments were carried out in an orbital shaker at 150 rpm, 30°C under 100 µE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. The asterisk sign represents the significant difference between the biomass titer or nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency obtained for different consortium analysed using one-way analysis of variance based on Tukey's method. Biomass titer or nitrate removal efficiency or COD removal efficiency that do not share a common symbol are significantly different. In (A), **' signifies that the combination A1B1 have similar statistical significance to the group with three asterisks. In (B), *' signifies that the combination A2B3 & A4B1 have similar statistical significance to the group with two asterisks, **' signifies that the combination A1B1 have similar statistical significance to the group with three asterisks and ***' signifies that the combination A1B3 & A6B1 have similar statistical significance to the group with four asterisks

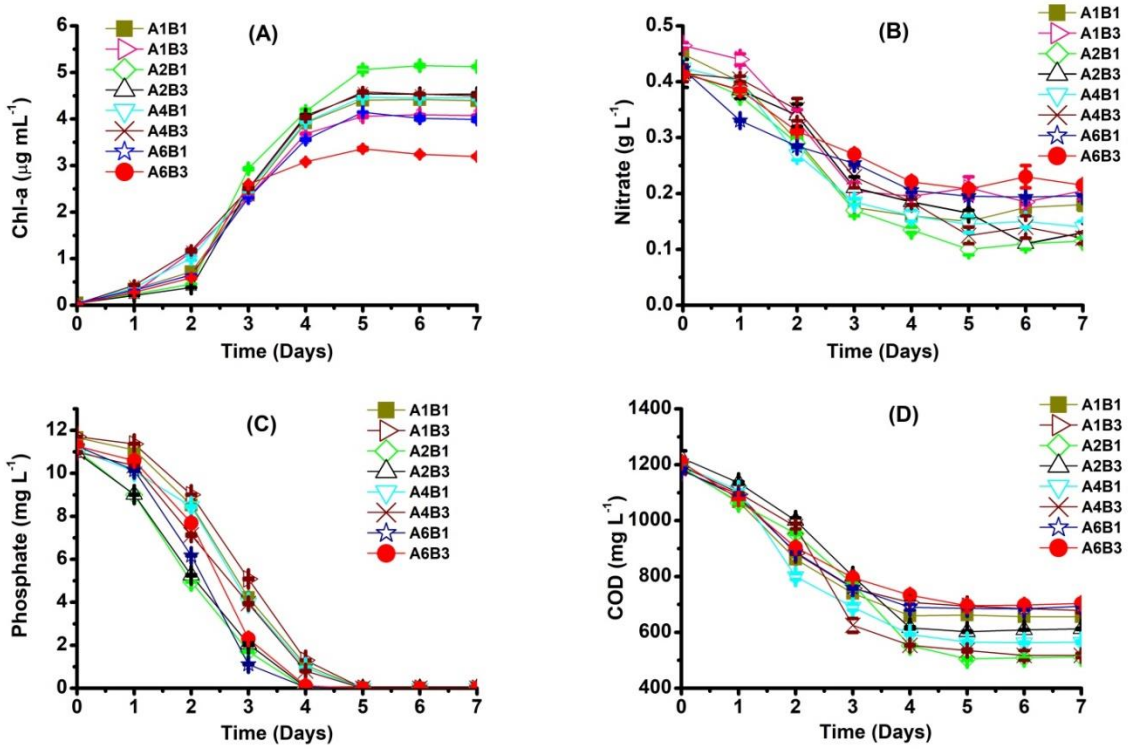


Fig. 4.2 Dynamic profile (A) Growth (DCW, g L^{-1}), (B) nitrate removal efficiency (%), (C) phosphate removal efficiency (%), and (D) COD removal efficiency (%) of eight primary consortia of microalgae and bacteria grown on artificial wastewater. The experiments were carried out in an orbital shaker at 150 rpm, 30°C under 100 μE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error

However, as observed for the individual microalgal isolates, phosphate was also completely utilized by the all primary combinations of microalgae-bacteria (Fig. 4.2 C). Various studies have reported similar improvement in growth and nutrient removal efficiency for co-cultivation of microalgae and bacteria as compared to microalgae alone. For instance, growth as well as nutrient removal efficiency of microalgae was reported to be enhanced by bacteria (Mujtaba and Lee, 2016). An increase in nitrogen removal efficiency by 76.5% and dry cell weight or chlorophyll-a content by 1.42 times was reported when *Scenedesmus dimorphus* was co-cultivated with nitrifying bacteria in comparison to the microalga alone (Choi et al., 2018). The possible reason for the enhancement of microbial biomass titer and in turn, nitrate or

COD removal efficiency could be due to the cooperative interaction between microalgae and bacteria. Under illuminated conditions, algae perform photosynthesis by using nutrients and produce O_2 which is used by the heterotrophic bacteria as an electron acceptor to degrade organic matter present in wastewater. CO_2 released as by-product of bacterial oxidative degradation is used as a carbon source in algal photosynthesis (Oswald and Gotass, 1957). Praveen and Loh, (2015) demonstrated the symbiotic exchange of CO_2 and O_2 between *C. vulgaris* and *Pseudomonas putida* when grown on wastewater in a bioreactor. However, the interaction between algae and bacteria is not only confined to gaseous exchange means. The algal growth, for instance, was shown to be enhanced by growth promoting factor indole-3-acetic acid produced by bacteria (De-bashan et al., 2008). Vitamin B_{12} produced by bacteria and bacterial siderophores are also known to promote faster growth of microalgae (Fuentes et al., 2016). The highest biomass titer was found to be 1.12 g L^{-1} in case of primary consortium A2B1 followed by A4B3 (1.09 g L^{-1}). The nitrate and COD removal efficiency for these two best performing primary consortia A2B1 and A4B3 were found to be 72.61% & 71.08% and 57.02% & 56.69% respectively (Fig. 4.1B & D). Mujtaba et al. (2015) reported 80% nitrogen removal and 60% phosphate removal from synthetic wastewater by co-culturing *C. vulgaris* and *P. putida*. Co-cultivation of *C. sorokiniana* and *Methylococcus capsulatus* in industrial wastewater from potato processing plant resulted in removal of ammoniacal nitrogen by 67%, phosphate by 43%, and COD by 91% (Rasouli et al., 2018). Similarly, 78% removal of NH_4^+ and 92% removal of phosphate were reported for mixed culture of *C. vulgaris* and *B. licheniformis* grown in municipal wastewater (Liang et al., 2013).

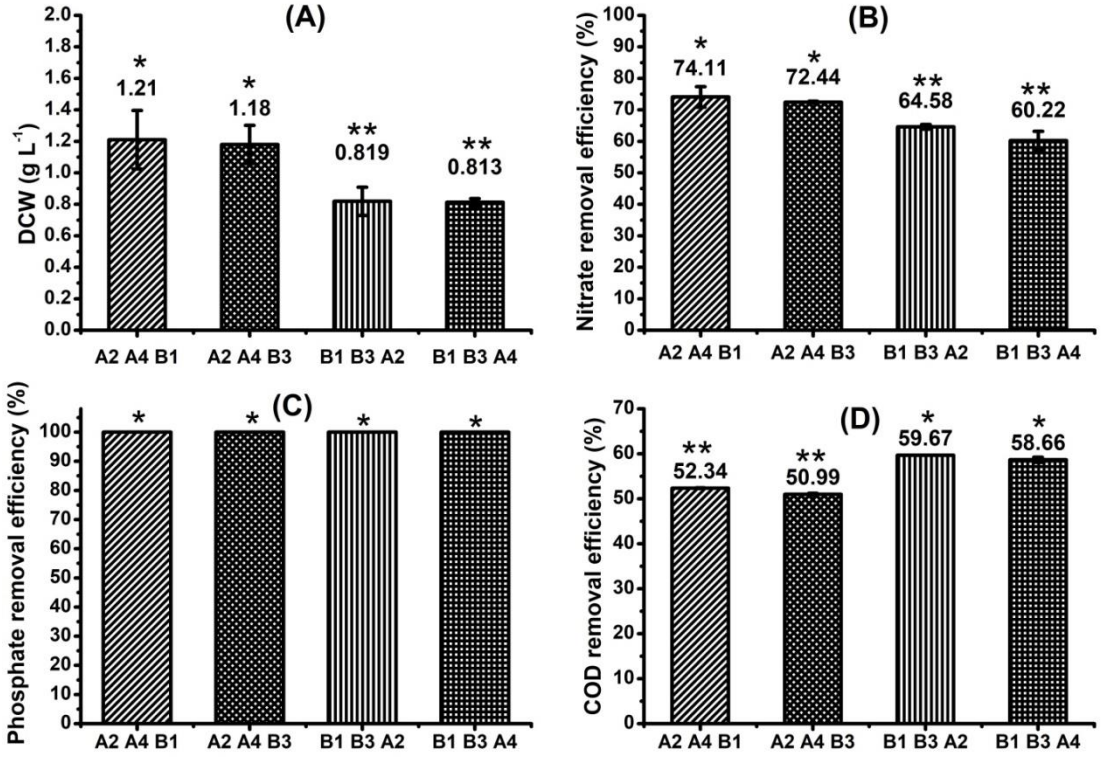


Fig.4.3 (A) Growth (DCW, g L^{-1}), (B) nitrate removal efficiency (%), (C) phosphate removal efficiency (%) and COD removal efficiency (%) of four secondary consortia of microalgae and bacteria grown on artificial wastewater. The experiments were carried out in an orbital shaker at 150 rpm, 30°C under 100 µE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. The asterisk sign represents the significant difference between the biomass titer or nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency obtained for different consortium analyzed using one-way analysis of variance based on Tukey's method

With the aim of improving the biomass titer and wastewater treatment efficiency further, four different combinations of secondary microalgae-bacteria consortia (A2A4B1, A2A4B3, B1B3A2, and B1B3A4) were formed using microalgal and bacterial strains present in two best performing primary consortia, A2B1 and A4B3. As observed from the figure, out of four secondary consortia, the ones which comprised of two microalgae and one bacterial system performed well in terms of biomass titer and nitrate removal efficiency (Fig. 4.3A & B). For instance, in case of A2A4B1 and A2A4B3, the biomass titer and nitrate removal efficiency was observed to increase further to 1.21 g L^{-1} & 1.18 g L^{-1} and 74.11% & 72.44% respectively.

However, the other two secondary consortia B1B3A2 and B1B3A4 comprising of two bacteria and one microalgal system exhibited improved performance in terms of COD removal efficiency (Fig. 4.3D). Phosphate removal efficiency for all four types of secondary consortium was found to be 100% which implies that the microalgae partner of the consortium completely utilised phosphate during the cultivation period (Fig. 4.3C). Table 4.2 describes the g-nitrate removal/g-biomass/hr; g-phosphate removal/g-biomass/hr; and g-COD removal/g-biomass/hr of four types of secondary consortia.

Table 4.2 g-nitrate removal/g-biomass/hr; g-phosphate removal/g-biomass/hr; and g-COD removal/g-biomass/hr of four types of secondary consortia

	g-nitrate removal/ g-biomass/hr	g-phosphate removal/ g-biomass/hr	g-COD removal/g-biomass/hr
A2A4B1	0.0015	0.04	3.077
A2A4B3	0.0016	0.05	3.02
B1B3A2	0.002	0.07	5.26
B1B3A4	0.0019	0.08	5.14

Biomass titer the key criteria for selection; the secondary consortium A2A4B1 was considered to be the best amongst all four combinations followed by A2A4B3. Though the COD removal efficiency of A2A4B1 was less yet the biomass titer was high. This may be attributed to the fact that while bacteria contribute mainly towards utilization of organic matter, microalgae predominantly contributes towards nitrate removal which in turn resulted in higher biomass titer. In the final step, the tertiary consortium A2A4B1B3 was formed using microalgal and bacterial strains present in the two best performing secondary consortia, A2A4B1 and A2A4B3. Characterization of the tertiary consortium was carried out in AWW and its performance was

compared with the best combinations of the primary consortia and secondary consortia (Fig. 4.4). Biomass titer, nitrate, and COD removal efficiency was found to be highest for the tertiary consortium with a value of 1.27 g L^{-1}, 79.78%, and 62.43% respectively. Removal efficiency of all the five heavy metals from AWW was also found to be highest for the tertiary consortium as compared to primary and secondary consortium (Table 4.1). Based on these results, the tertiary consortium was selected as the best microalgae-bacteria system enabling highest biomass feedstock generation coupled with efficient nutrient removal from AWW. Tertiary consortium was therefore considered for subsequent optimization experiments and process development.

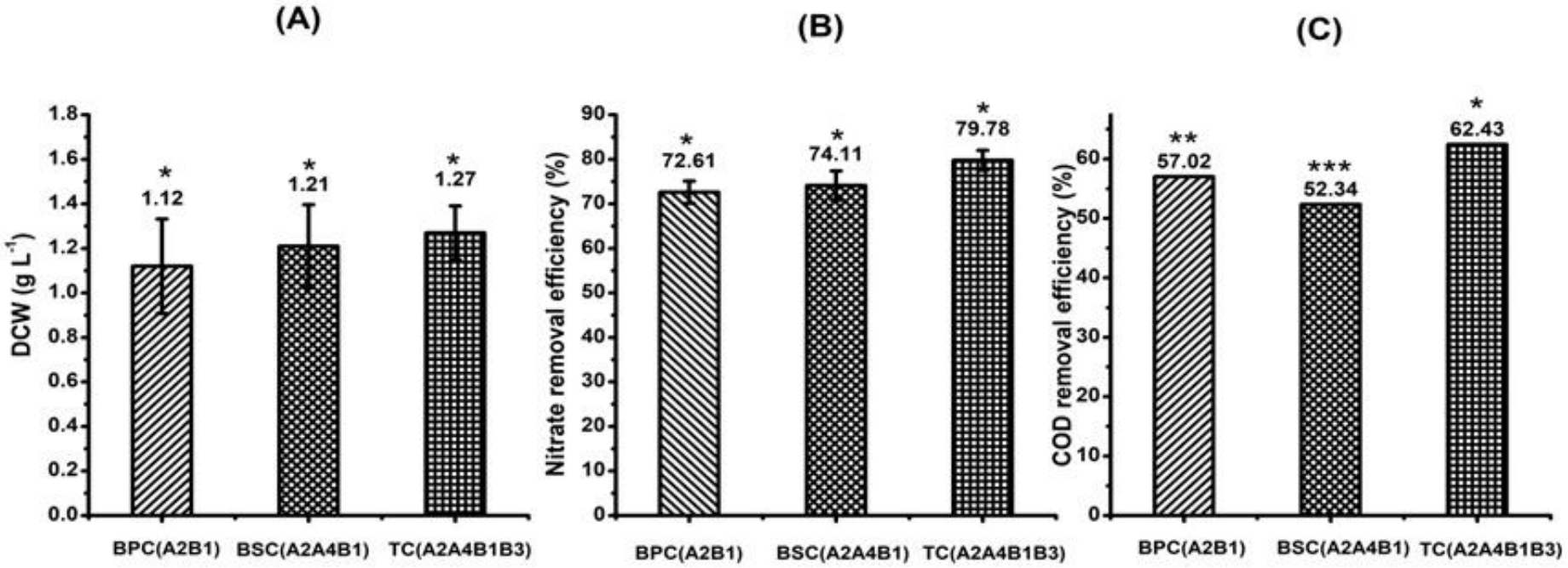


Fig. 4.4 Comparative performance evaluation of best primary consortium (BPC), best secondary consortium (BSC) and tertiary consortium (TC) in terms of (A) growth (DCW, g L^{-1}), (B) nitrate removal efficiency (%), and (C) COD removal efficiency (%). The experiments were carried out in an orbital shaker at 150 rpm, 30°C under 100 μE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. The asterisk sign represents the significant difference between the biomass titer or nitrate removal efficiency or COD removal efficiency obtained for BPC, BSC & TC analysed using one-way analysis of variance based on Tukey's method. Biomass titer or nitrate removal efficiency or COD removal efficiency that do not share a common symbol are significantly different

Table 4.1 Heavy metal ion removal efficiency (%) of different combinations of algae-bacteria consortia. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error

Types of consortium		Copper (Cu)	Chromium (Cr)	Cadmium (Cd)	Nickel (Ni)	Lead (Pb)
Primary consortium	A1B1	71.03±0.08	58.46±0.05	33.09±0.09	57.83±0.01	34.83±0.01
	A1B3	68.13±0.06	60.81±0.09	30.98±0.03	57.6±0.07	34.36±0.05
	A2B1	72.56±0.05	69.18±0.07	48.49±0.06	61.29±0.04	54.82±0.09
	A2B3	70.8±0.04	66.87±0.08	42.8±0.05	61.12±0.07	54.76±0.01
	A4B1	66.22±0.07	57.57±0.05	36.01±0.06	57.09±0.05	54.59±0.06
	A4B3	68.86±0.08	58.77±0.02	42.61±0.02	60.92±0.09	48.97±0.07
	A6B1	66.91±0.03	57.11±0.04	30.28±0.04	40.21±0.06	34.51±0.05
	A6B3	64.27±0.01	58.55±0.01	29.57±0.01	39±0.07	35.33±0.07
Secondary consortium	A2A4B1	60.39±0.01	63.41±0.02	49.91±0.05	55.9±0.09	56.7±0.01
	A2A4B3	58.79±0.01	51.69±0.04	44.85±0.01	53.96±0.02	55.92±0.05
	B1B3A2	57.74±0.02	49.55±0.05	41.56±0.09	51.32±0.01	57.73±0.06
	B1B3A4	56±0.08	53.21±0.03	39.41±0.08	49.91±0.03	56.6±0.09
Tertiary consortium	A2A4B1B3	78.1±0.07	76.34±0.02	55.19±0.07	69.78±0.04	65.12±0.08

4.3.2 Identification of the microalgal and bacterial strains present in the tertiary consortium

The partial 18S rDNA gene (353 bp in length for A2 and 353 bp in length for A4) sequences of the microalgal strains were submitted to GenBank (Accession Number: MG719942 for A2 and MG719943 for A4). BLAST analysis in the nucleotide database revealed that the microalgal strain A2 is the closest relative to *C. sorokiniana* with maximum similarity of 99%, whereas the other microalgal strain A4 is the closest relative to *Chlorella sp.* with a maximum similarity of 99%, whereas the other microalgal strain A4 is the closest relative to *Chlorella* sp. with a maximum similarity of 99%. Phylogenetic trees were constructed based on the 18S rDNA sequences of the strains which showed 99% similarity with the organism under the order Chlorellales (Fig. 4.5A and B). Hence, based on the molecular analyses, the microalgal isolates A2 and A4 were identified and designated as *Chlorella sorokiniana* strain DBWC2 and *Chlorella* sp. strain DBWC7 respectively.

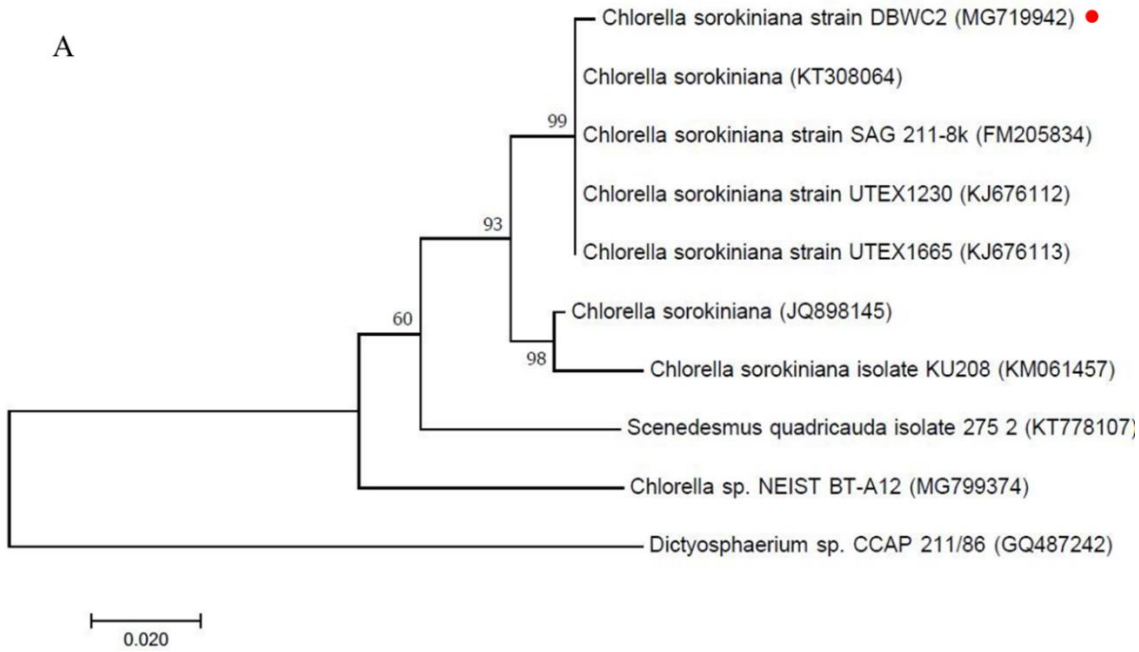


Fig.4.5 (A) Molecular analysis of *Chlorella sorokiniana* strain DBWC2. The phylogenetic tree was constructed using the neighbor-joining method with the Jukes-

Cantor model. Bootstrap test values are shown next to the branches. The isolated strain reported in the present study is marked with a solid red circle

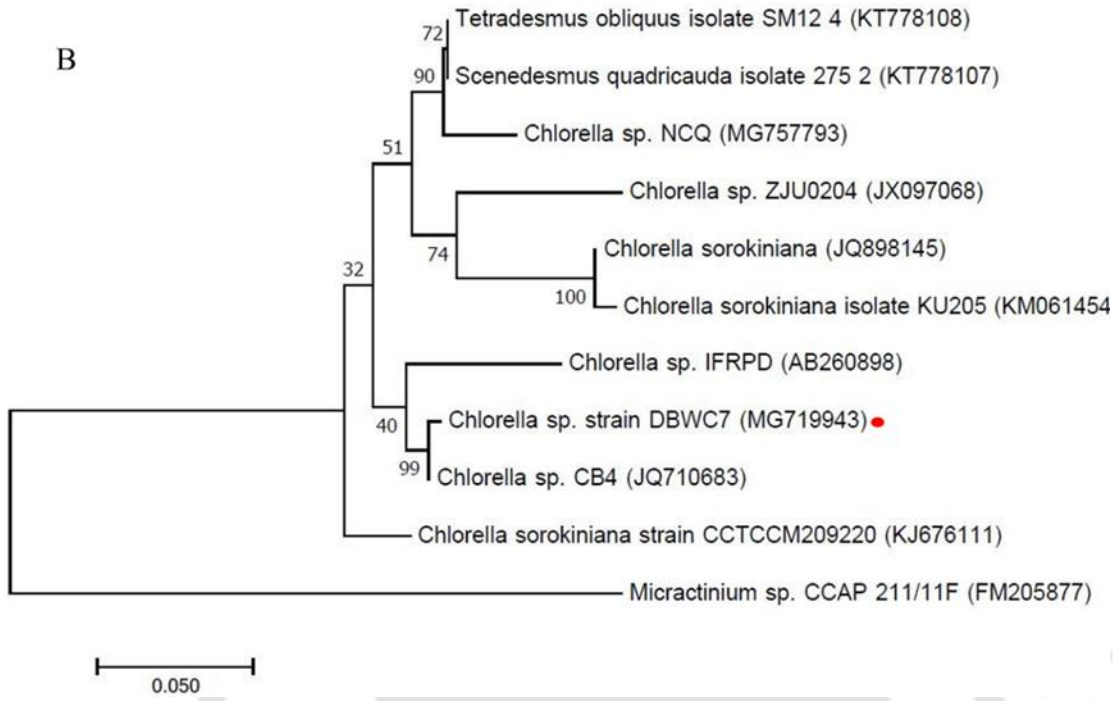


Fig.4.5 (B) Molecular analysis of *Chlorella* sp. strain DBWC7. The phylogenetic tree was constructed using the neighbor-joining method with the Jukes-Cantor model. Bootstrap test values are shown next to the branches. The isolated strain reported in the present study is marked with a solid red circle

The partial 16S rDNA gene sequences (1476 bp in length for B1 and 1474 bp in length for B3) of the bacterial strains were deposited in the GenBank and the accession numbers MH036335 and MH036346 were obtained for B1 and B3 respectively. BLAST analysis showed that B1 is the closest relative to *Klebsiella pneumoniae* with maximum similarity of 99% whereas B3 appeared to be the closest relative to *Acinetobacter calcoaceticus* with maximum similarity of 99%.

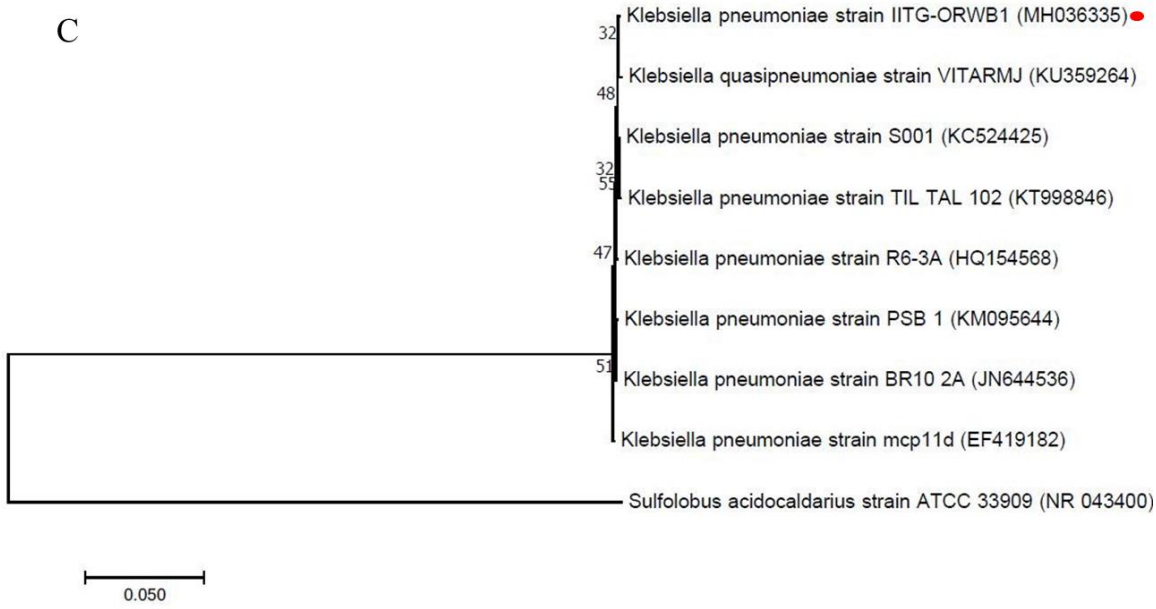


Fig.4.5 (C) Molecular analysis of *Klebsiella pneumoniae* strain IITG-ORWB1. The phylogenetic tree was constructed using the neighbor-joining method with the Jukes-Cantor model. Bootstrap test values are shown next to the branches. The isolated strain reported in the present study is marked with a solid red circle

The phylogenetic relation of the bacterial strains with other closely related bacteria is presented in the phylogenetic trees (Fig. 4.5 C and D). The nearest homolog species was found to be *K. pneumoniae* strain S001 for B1 and *A. calcoaceticus* strain B40 for B3. Based on the molecular analyses, the bacterial strains B1 and B3 were identified and designated as *Klebsiella pneumoniae* strain IITG-ORWB1 and *Acinetobacter calcoaceticus* strain IITG-ORWB3 respectively.

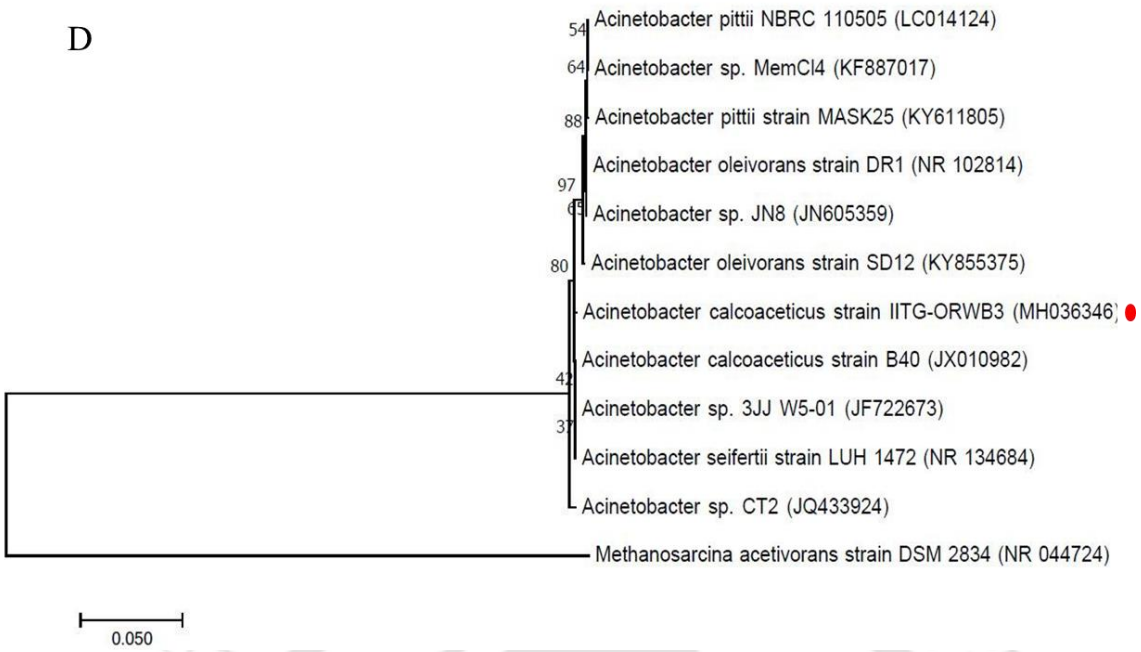


Fig.4.5 (D) Molecular analysis of *Acinetobacter calcoaceticus* strain IITG-ORWB3. The phylogenetic tree was constructed using the neighbor-joining method with the Jukes-Cantor model. Bootstrap test values are shown next to the branches. The isolated strain reported in the present study is designated with a solid red circle

4.4 Conclusions

Tertiary consortium was the best performing microalgae-bacteria consortium in terms of total biomass titer, nutrient and COD removal efficiency amongst different combinations of selected microalgae and bacteria. The total biomass titer of tertiary consortium was 1.27 g L^{-1} which is 4.95% and 13.39% higher than the total biomass titer of primary consortium and secondary consortium respectively. The nitrate removal efficiency of tertiary consortium was 79.78% which is 7.65% and 9.87% higher than the nitrate removal efficiency of primary and secondary consortium respectively. Moreover, significant COD removal was also observed higher in tertiary consortium as compared to primary and secondary consortium i.e. 19.24% and 9.48% higher COD removal efficiency than primary and secondary consortium respectively. Hence, the selected microalgae-bacteria consortium may be a potential platform

towards sustainable production of microbial biomass as feedstock for biofuel synthesis using wastewater.

4.5 References

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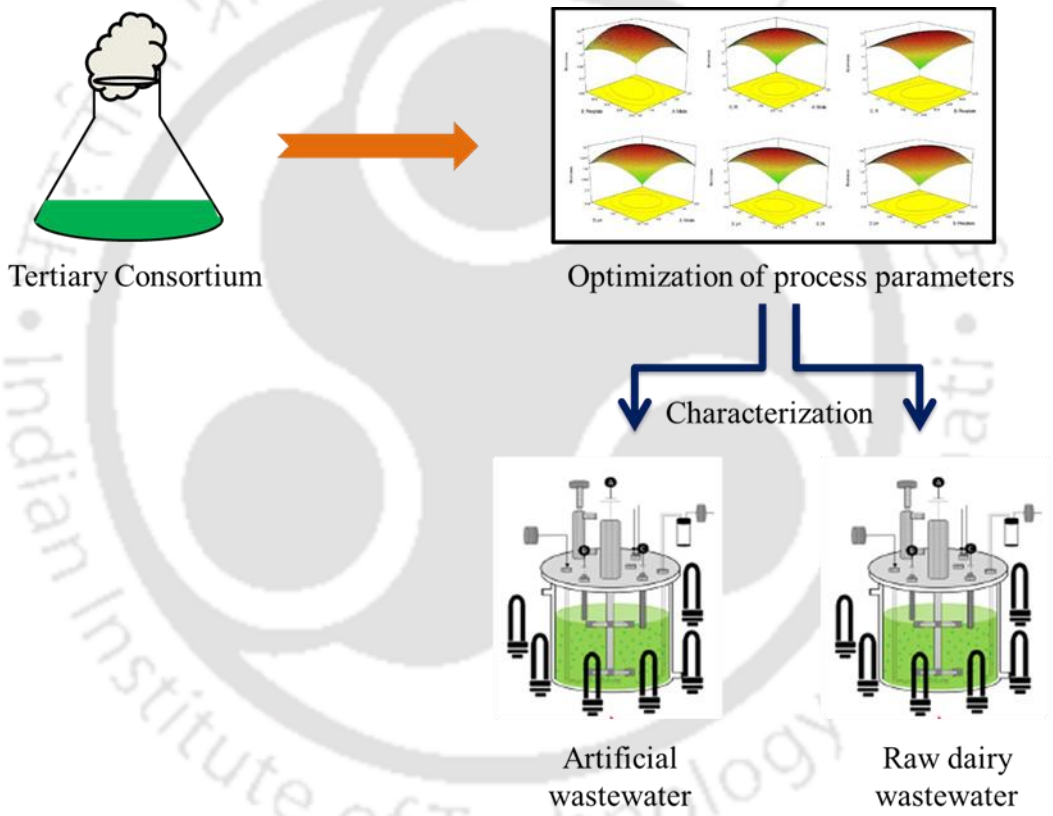
Look deep into nature, and then you will understand everything better

Albert Einstein

Chapter 5

Process optimization for enhancement of microbial biomass feedstock for biofuel production



Total biomass titer of the tertiary microalgae-bacteria consortium was further improved via statistical optimisation of process parameters. Finally, with the aim of demonstrating a process for sustainable production of biomass feedstock coupled with wastewater treatment, the tertiary consortium was grown on artificial wastewater and raw dairy wastewater in an automated bioreactor with optimised parameters.

5.1 Background and motivation

The designed microalgae-bacteria tertiary consortium resulted in improved biomass titer of 1.27 g L^{-1} as compared to best primary and secondary consortium as explained in the section 4.3.1. However, the total biomass titer of the tertiary consortium was lower when compared with other microalgae-bacteria co-cultivation studies reported in the literature (Wang et al., 2015; Xue et al., 2018). Therefore, in order to attain higher biomass titer an optimal growth medium with optimal microalgae-bacterial inoculum ratio and size need to be designed in a systematic way. Optimization of the process parameters can be performed using statistical methods such as response surface methodology (RSM) and factorial designs which can deal with large number of variables with very less number of experiments (Palabhanvi and Belur, 2013). Other mathematical tools such as artificial neural networks (ANN), simulated annealing and genetic algorithm (GA) are being used extensively to model and optimize complex non-linear functions in various fields of engineering (Sathish and Prakasham, 2010; Zafar et al., 2012; Whiteman and Kana, 2014).

In the present study, a Central Composite Design (CCD) was constructed to optimize the initial concentration of nitrate, phosphate, microalgae-bacteria inoculum ratio and initial pH of the medium and to study the effect of their interaction on biomass titer as model response. A 4^5 quarter factorial CCD was generated and was employed to optimize the total biomass titer. The results were analysed through RSM with the objective function as maximization of biomass titer.

5.2 Materials and methods

5.2.1 Maximization of total biomass titer via statistical optimization

With the objective of improvement in total biomass titer, various factors such as nitrate concentration, phosphate concentration, microalgae-bacteria inoculum ratio, and initial pH of the medium were optimized through Central Composite Design (CCD) based Response Surface Methodology (RSM). Table 5.1 states the actual values and the coded values of the variables employed. Coded values of +α, +1, 0, and -1, -α correspond to high, medium, and low levels of the variables respectively.

Table 5.1 Actual values and coded values of the variables employed in CCD-RSM based optimization

Variables		Range and level				
Code	Variables	-α	-1	0	+1	+α
X_1	Initial nitrate concentration (g L^{-1})	1.13	1.43	1.73	2.03	2.33
X_2	Initial phosphate concentration (mg L^{-1})	24	34	44	54	64
X_3	Microalgae-bacteria inoculum ratio	0.5	0.75	1	1.25	1.5
X_4	Initial pH of the media (units)	5.5	7	8.5	10	11.5

A 4^5 quarter factorial CCD was generated using Design-Expert® (Version 7, Stat-Ease Inc., USA) and was employed to optimize the total biomass titer. The CCD predicted thirty experiments which included sixteen factorial points, eight axial points and six replicates of the centre points (Table 5.2). Response surface methodology (RSM) is a mathematical modelling technique which utilizes a polynomial equation to model the interaction amongst the variables. Under RSM, the linear, quadratic, and interaction effects between the selected parameters and the total biomass titer of the mixed consortium were mathematically expressed in the form of a quadratic polynomial equation, Eq. (5.1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (5.1)$$

where, Y is the maximum biomass titer (DCW, g L^{-1}), X_i is the i^{th} parameter, k is the total number of parameters and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients. In the present study, a total of thirty experiments were conducted and the experimental response values are tabulated in Table 5.2. The inoculum size in all the experiments was kept at the optimum value of 18% (v/v).

Table 5.2 Full factorial central composite design matrix of four variables in coded and natural units along with the observed response, biomass yield. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error

Standard order	Nitrate (g L^{-1}) X_1	Phosphate (mg L^{-1}) X_2	Inoculum ratio X_3	pH (Units) X_4	Biomass (g L^{-1})	
					Observed	Predicted
1	1.43	34	0.75	7	0.81±0.08	0.74
2	2.03	34	0.75	7	0.64±0.01	0.65
3	1.43	54	0.75	7	0.757±0.07	0.83
4	2.03	54	0.75	7	1.16±0.04	1.12
5	1.43	34	1.25	7	0.955±0.06	0.98
6	2.03	34	1.25	7	0.89±0.05	0.8
7	1.43	54	1.25	7	0.7125±0.08	0.75
8	2.03	54	1.25	7	0.9825±0.03	0.95
9	1.43	34	0.75	10	0.8625±0.07	0.93
10	2.03	34	0.75	10	0.925±0.06	0.82
11	1.43	54	0.75	10	0.9625±0.05	0.98
12	2.03	54	0.75	10	1.235±0.09	1.25
13	1.43	34	1.25	10	1.05±0.02	1.02
14	2.03	34	1.25	10	0.8475±0.08	0.81
15	1.43	54	1.25	10	0.73±0.07	0.75
16	2.03	54	1.25	10	0.925±0.08	0.93
17	1.13	44	1	8.5	0.84±0.04	0.76
18	2.33	44	1	8.5	0.73±0.07	0.85
19	1.73	24	1	8.5	0.925±0.05	1.12
20	1.73	64	1	8.5	1.4±0.06	1.33
21	1.73	44	0.5	8.5	0.69±0.03	0.69
22	1.73	44	1.5	8.5	0.57±0.04	0.61
23	1.73	44	1	5.5	0.783±0.05	0.81
24	1.73	44	1	11.5	0.97±0.06	0.98
25	1.73	44	1	8.5	1.65±0.07	1.65
26	1.73	44	1	8.5	1.65±0.08	1.65
27	1.73	44	1	8.5	1.65±0.07	1.65

28	1.73	44	1	8.5	1.65±0.085	1.65
29	1.73	44	1	8.5	1.65±0.015	1.65
30	1.73	44	1	8.5	1.65±0.021	1.65

5.2.2 Sustainable production of microbial biomass coupled with wastewater treatment in bioreactor: comparative performance evaluation on AWW and raw dairy wastewater (RDWW)

A process has been demonstrated in an automated bioreactor by growing the selected microalgae-bacteria tertiary consortium in AWW under optimized conditions. The experiment was carried out in a 5 L automated bioreactor (Biostat B Plus, Sartorius, Germany) with a working volume of 3 L. The bioreactor was operated at 30 °C, agitator speed of 150 rpm, aeration at 1 vvm and light intensity of 250 µE m^{-2} s^{-1} for a light:dark cycle of 16:8 h. Finally, with the aim of demonstrating the prospective application of microalgae-bacteria consortium for feedstock generation coupled with wastewater treatment, the process was demonstrated on RDWW collected from Purabi Dairy (26.13 °N, 91.81 °E), Guwahati, India. The characterization of RDWW was carried out to obtain the concentration of phosphate (50 mg L^{-1}), total nitrogen content (30 mg L^{-1}), COD content (1260 mg L^{-1}), and pH (7.31). The experiment was conducted for ten days in an automated bioreactor by adjusting the initial pH, nitrate and phosphate content to their corresponding optimal values with the same cultivation conditions as mentioned in Section 2.5. Sampling was carried out at a regular interval of 24 h for the analysis of nitrate, phosphate, COD, and chlorophyll-a. Final biomass in terms of DCW was measured at the end of the cultivation period. Please note that, for characterization on both AWW and RDWW, two separate bioreactor experiments were conducted. While, in the first experiment microalgae-bacteria consortium was used as the inoculum; in the second experiment consortium of microalgae only was used as the inoculum (control batch).

For RDWW, one negative control experiment was performed without any external inoculation of algae-bacteria consortium to test growth ability of the native organisms.

5.2.3 Analysis of growth, nutrient utilization, COD and heavy metal ion removal

Analysis of growth, nutrient utilization, COD, and heavy metal ion removal was carried out at every sampling time point. Growth of the microalgal strains was measured in terms of OD, chlorophyll-a, and DCW whereas growth of the bacterial strains was measured in terms of OD and DCW. Microalgal and bacterial OD was monitored by measuring the absorbance at 690 nm (A_{690}) and 600 nm (A_{600}) respectively using a UV-visible spectrophotometer (Cary 100, Varian, Australia). The procedure for chlorophyll-a, nitrate, phosphate, COD and heavy metal ions were described in section 3.2.3.

5.3 Results and discussion

5.3.1 Optimization of inoculum size for the maximization of total biomass titer

With the aim of maximizing the total biomass titer, the inoculum size of the tertiary consortium was optimized by varying the same from 2 to 20 (%, v/v). For any inoculum size, a ratio of 1:1:1:1 was maintained for A2, A4, B1 and B3 respectively. With the increase in inoculum size, biomass titer and nutrient removal efficiency was found to increase linearly till 18 % and beyond which no improvement was observed (Fig. 5.1). Maximum biomass titer, nitrate and COD removal efficiency at 18% inoculum size was found to be 1.35 g L^{-1}, 85.13% and 73.7% respectively. With the optimization of inoculum size, while increase in biomass titer and nitrate removal efficiency was marginal, the increment in COD removal efficiency was significant at 18% inoculum size as compared to the un-optimized inoculum size.

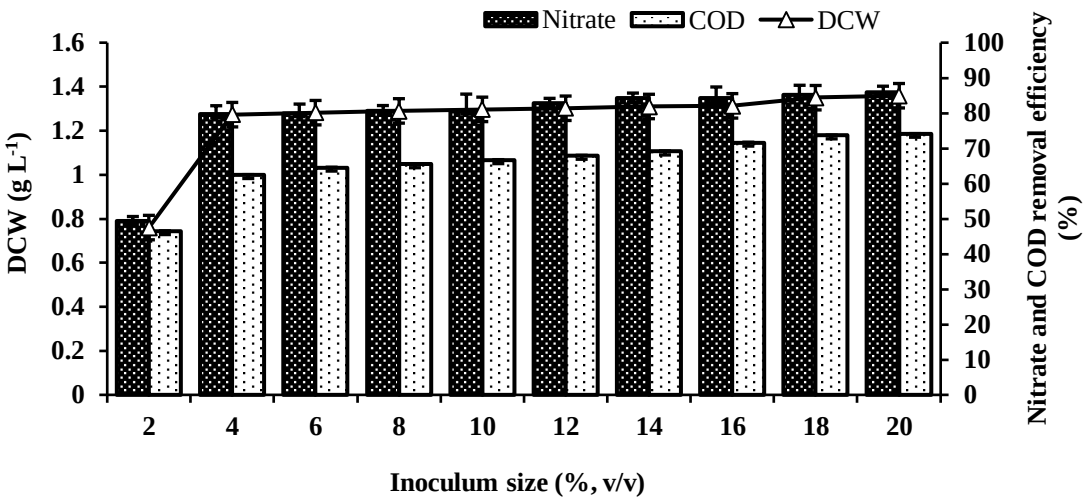


Fig.5.1 Optimization of inoculum size (%, v/v) of the tertiary consortium. The experiments were carried out in an orbital shaker at 150 rpm, 30°C under 100 μE m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. All the experiments were conducted in duplicate and the data were expressed as mean ± standard error

5.3.2 Statistical optimization of the medium towards maximization of the biomass titer

With the aim of maximizing the total biomass titer, statistical optimization of the fermentation medium was carried out. In the current study, a CCD was constructed to optimize the initial concentration of nitrate, phosphate, microalgae-bacteria inoculum ratio and initial pH of the medium and to study the effect of their interaction on biomass titer as model response. The experiments were conducted in duplicates and the results were analysed through RSM with the objective function as maximization of biomass titer. The experiments resulted in a wide variation in the biomass titer from 0.57 g L^{-1} to 1.65 g L^{-1} depicting the importance of optimization (Table 5.2). Model construction yielded a second order polynomial equation, Eq. (5.2), which correlated the process parameters with the predicted response of biomass titer.

$$R = 1.65 + 0.023X_1 + 0.060X_2 - 0.021X_3 + 0.042X_4 + 0.095X_1X_2 - 0.023X_1X_3 - 6.906x\,10^{-3}X_1X_4 - 0.079X_2X_3 - 9.344x\,10^{-3}X_2X_4 - 0.038X_3X_4 - 0.21{X_1}^2 - 0.12\,{X_2}^2 - 0.25{X_3}^2 - 0.19{X_4}^2 \quad (5.2)$$

where, R is the predicted biomass titer (g L^{-1}) while, Xi (i=1−4) represents the initial concentration of nitrate (g L^{-1}), phosphate (mg L^{-1}), microalgae-bacteria inoculum ratio, and initial pH of the medium respectively. Experimental data were analysed with the help of ANOVA and the results have been represented in Table 4. The model Fischer 'F' test showed a significant value of 35.78 with $p < 0.05$. This can be interpreted as the interaction between the individual processes variables resulted in significant increase in the biomass formation. Further the correlation coefficient (R^2) was found to be 0.97 indicating that only 3% of the 30 experiments performed could not be fitted into the model. p values of linear, square, and interactive terms of individual process variables are shown in Table 4. Based on the p values, phosphate concentration, initial pH of the medium, interaction between nitrate and phosphate, interaction between phosphate and inoculum ratio, and quadratic effects of all the parameters were found to be significant for biomass titer. The model was validated by comparing model predicted biomass titer (1.65 g L^{-1}) with the corresponding experimental value (1.73 g L^{-1}) at optimized concentration of nitrate (1.79 g L^{-1}), phosphate (49.55 mg L^{-1}), microalgae-bacteria inoculum ratio (0.97), and initial pH of the medium (8.65). CCD-RSM based optimization of the process variables resulted in 28.14% increment in biomass titer as compared to the biomass obtained from the un-optimized condition.

Table 5.3 Analysis of variance (ANOVA) for the selected quadratic model of biomass yield

Source	Sum of squares	Degree of freedom	Mean square	*F* Value	*p* value Prob > *F*
Model	3.5	14	0.25	35.78	< 0.0001
A-Nitrate	0.012	1	0.012	1.77	0.203
B-Phosphate	0.086	1	0.086	12.26	0.0032
C-IR	0.01	1	0.01	1.49	0.2416
D-pH	0.042	1	0.042	6.01	0.027
AB	0.14	1	0.14	20.52	0.0004
AC	8.58E-03	1	8.58E-03	1.23	0.2855
AD	7.63E-04	1	7.63E-04	0.11	0.7457
BC	0.1	1	0.1	14.4	0.0018
BD	1.40E-03	1	1.40E-03	0.2	0.6614
CD	0.023	1	0.023	3.28	0.0904
A^2	1.21	1	1.21	172.43	< 0.0001
B^2	0.36	1	0.36	52.15	< 0.0001
C^2	1.69	1	1.69	242.04	< 0.0001
D^2	0.96	1	0.96	136.86	< 0.0001
Residual	0.1	15	7.00E-03		
Lack of Fit	0.1	10	0.01		
Pure Error	0	5	0		
Cor Total	3.61	29			
R^2	0.9709	Adjusted R^2	0.9438	Predicted R^2	0.8325

5.3.3 Comparative performance evaluation of tertiary microalgae-bacteria consortium on AWW and RDWW in bioreactor

With the aim of demonstrating a process for sustainable production of biomass feedstock coupled with wastewater treatment, the tertiary microalgae-bacteria consortium was grown on AWW in a 5 L automated bioreactor. Finally, assessment of prospective application potential of selected microalgae-bacteria consortium was

carried out via cultivation in RDWW in bioreactor. Comparative performance evaluation of the consortium in both AWW and RDWW was carried out at the respective optimum values of inoculum size, nitrate concentration, phosphate concentration, microalgae-bacteria inoculum ratio, and initial pH of the medium. As observed from Fig. 5.2, when grown on AWW, a significant improvement in the growth of microalgae and COD removal efficiency (111%) was observed for microalgae-bacteria consortium as compared to the control batch (only microalgae). The total biomass titer in case of consortium was 2.84 g L^{-1}, an increase by 77.5% when compared to the control batch (Fig. 5.3A). However, the improvement in nitrate removal efficiency (11.81%) was comparatively marginal (Fig. 5.3 B) albeit almost complete utilization was observed (Fig. 5.2). Further, cultivation of the consortium using AWW in a bioreactor resulted in significant improvement (41.9%) in biomass titer as compared to shake flask. This may be attributed to the notable elevation in the utilization of organic carbon source in presence of oxygen supplied by means of aeration in the bioreactor which in turn resulted in increased bacterial growth. The increase in the utilization of organic carbon source in the reactor was also evident from the significantly improved COD removal efficiency. On the other hand, the released CO_2 as a by-product of oxidative degradation of organic carbon source, was utilized by the microalgae as carbon source resulting in improved biomass titer (Wang et al., 2015). A similar improvement in microalgae growth, total biomass titer, nitrate and COD removal efficiency was observed when grown in RDWW (Fig. 5.2 and Fig. 5.3). Interestingly, comparative performance evaluation in terms of growth of microalgae, total biomass titer, and wastewater treatment efficiency of the selected microalgae-bacteria consortium was precisely similar in both AWW and RDWW.

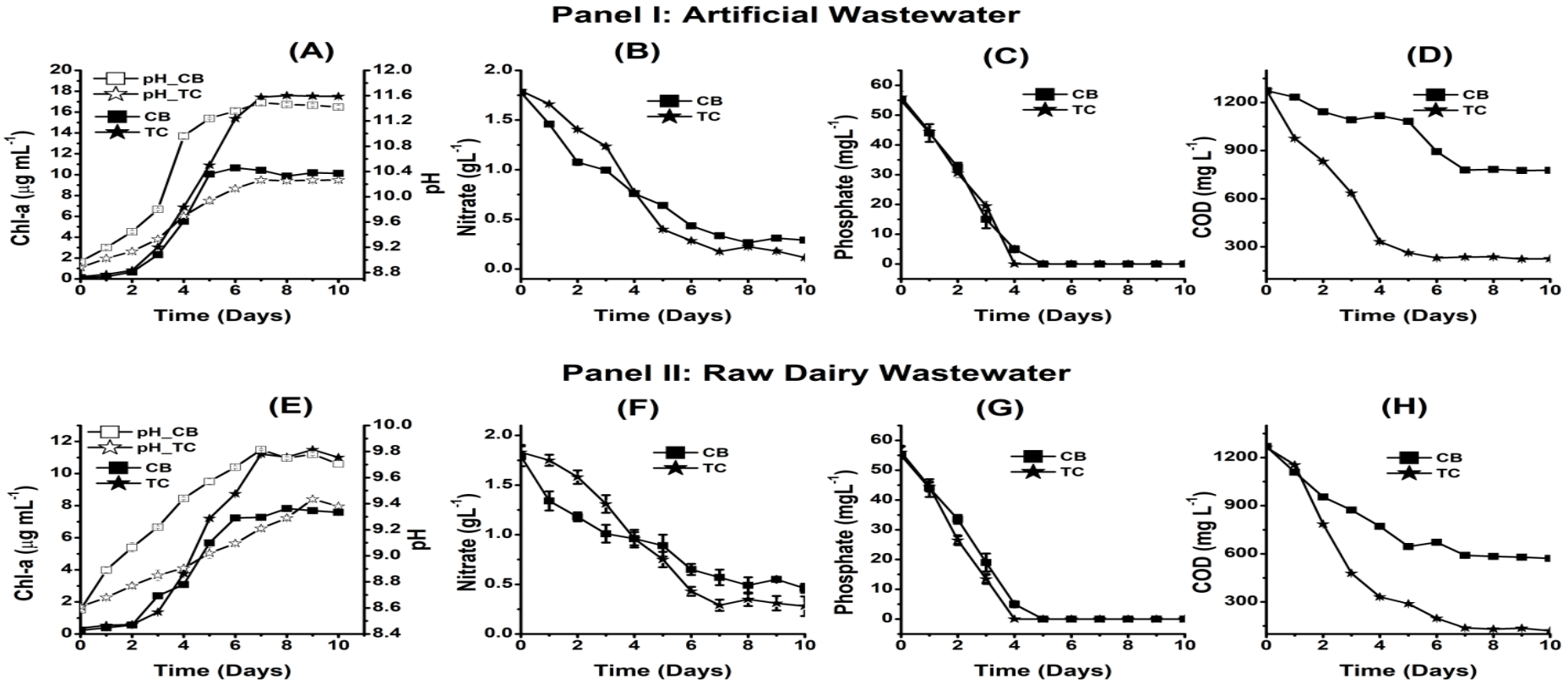


Fig. 5.2 Dynamic profiles of growth (Chl-a), pH and utilization of nitrate, phosphate & COD by the tertiary consortium (TC) and consortium of microalgae alone (control batch, CB) grown on artificial wastewater (Panel I) and raw dairy wastewater (Panel II). The experiment was carried out in a 5 L automated bioreactor with a working volume of 3 L. The bioreactor was operated at 30 °C, agitator speed of 150 rpm, aeration at 1 vvm and light intensity of 250 μE m^{-2} s^{-1} for a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error.

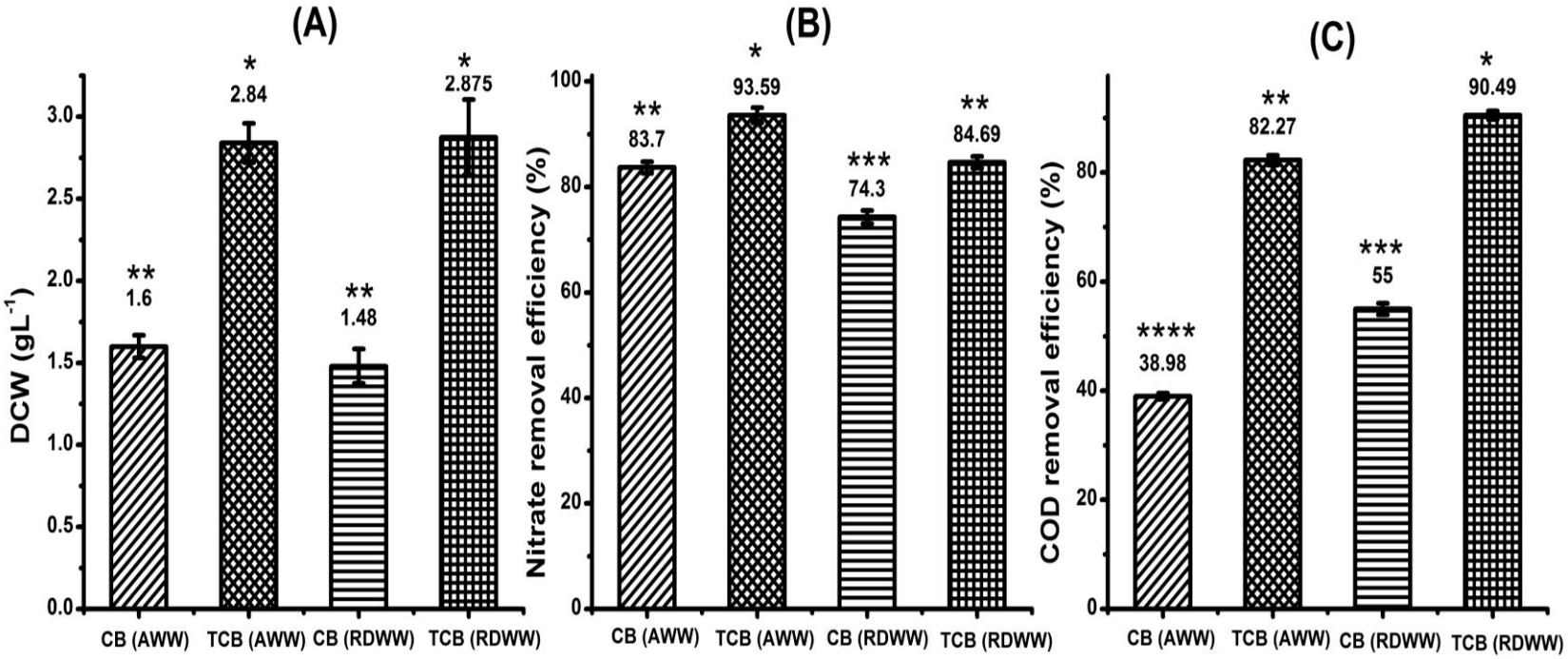


Fig. 5.3 Comparative performance evaluation of tertiary consortium (TC) and consortium of microalgae alone (control batch, CB) in terms of (A) growth (DCW, g L^{-1}), (B) nitrate removal efficiency (%), and (C) COD removal efficiency (%). The experiment was carried out in a 5 L automated bioreactor with a working volume of 3 L. The bioreactor was operated at 30 ºC, agitator speed of 150 rpm, aeration at 1 vvm and light intensity of 250 µE m^{-2} s^{-1} for a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error. AWW represents artificial wastewater and RDWW represents raw dairy wastewater. The *asterisk sign* represents the significant difference between the biomass titer or nitrate removal efficiency or COD removal efficiency obtained for CB and TC analysed using one-way analysis of variance based on Tukey's method. Biomass titer or nitrate removal efficiency or COD removal efficiency that do not share a common symbol are significantly different

For instance, total biomass titer and biomass productivity in AWW and RDWW was found to be 2.84 g L^{-1} & 2.87 g L^{-1} and 284 mg L^{-1} d^{-1} & 287 mg L^{-1} d^{-1} respectively. Biomass titer and productivity in the range of 2.8−3.5 g L^{-1} and 309−394 mg L^{-1} d^{-1} respectively was reported when *Scenedesmus obliquus* was co-cultured with different combinations of bacteria in BG11 medium (Wang et al., 2015). Co-cultivation of *Chlorella vulgaris* with different combinations of six bacterial strains resulted in biomass titer of 9.38− 12.31 g L^{-1} with productivity of 411− 505 mg L^{-1} d^{-1} (Xue et al., 2018). Further, maximum biomass productivity of 26.3 mg L^{-1} d^{-1} and 18.8 mg L^{-1} d^{-1} was reported when combination of *Chlorella sorokiniana* and aerobic bacteria was grown on wastewater from a potato processing industry and treated liquid fraction of pig manure respectively (Hernandez et al., 2013). Therefore, biomass titer and productivity achieved in the present study was found to be comparable with other microalgae-bacteria consortia reported in the literature. While nitrate removal efficiency was estimated to be 93.59% & 84.69%, COD removal efficiency was recorded to be 82.27% & 90.49%, in AWW and RDWW respectively. Complete phosphate utilization was observed for both microalgae- bacteria consortium and control batch (Fig. 5.2). In a previous study, cultivation of the microalgae-bacteria consortium in dairy wastewater resulted in COD, total nitrogen, and total phosphorus efficiency of 91%, 68%, and 38% respectively (Tricolici et al., 2014). For both AWW and RDWW, a concomitant increase in pH of the broth was observed with growth of microalgae (Fig. 5.2 A and E). This gradual increase in broth pH may be attributed to the utilization of dissolved CO_2 by photoautotrophic or mixotrophic growth of microalgae (Delgadillo-Mirquez et al., 2016). Further, uptake of nitrate (NO_3^-) by microalgae has been reported to increase alkalinity of the culture medium (Brewer et al., 1976). In the present study, a similar correlation between increase in broth alkalinity and nitrate removal (Fig. 5.2 B and F) was observed for both tertiary consortium (TC) and control batch (CB) grown on AWW and

RDWW. However, in case of TC increase in pH of the broth was found to be lower in comparison to CB, albeit higher growth of microalgae. This may be attributed to the combinatorial effect of higher growth of microalgae and higher nitrate removal in TC in comparison to CB. The results point towards scope for possible application of the selected microalgae-bacteria consortium towards sustainable production of microbial biomass as feedstock for biofuel synthesis using wastewater. In view of the industrial application, it is important to note that one of the key problems using batch culture system for wastewater treatment is the higher hydraulic retention time required for nutrient removal (An et al., 2003; González et al., 1997) and hence, it is difficult to keep pace with the rate of production of wastewater by the treatment plant. Further, persistent contamination in batch cultivation system may be attributed in part due to this longer retention time, which in turn results in lower overall biomass productivity despite higher yield. The issue of upper limit of biomass productivity due to longer hydraulic retention time may be overcome by using a continuous cultivation system at a higher dilution rate. A chemostat will provide scope for continuous processing essential for efficient removal of nutrient from wastewater using best tertiary combination of microalgae and bacteria screened in the present study. Further, in order to realize optimal growth of the microbes in chemostat, various parameters e.g., initial concentration of nitrate and phosphate in wastewater, microalgae-bacteria inoculum ratio and initial pH of the media can be adjusted to their respective optimal values as found in the present study.

5.4 Conclusions

The present study has made an attempt to optimise the inoculum size as well as inoculum ratio of the tertiary consortium, and the medium components (nitrate and phosphate concentration, initial culture pH) for the maximization of total biomass titer. Total biomass titer has increased to 1.73g L^{-1} which is 36.22% higher than the biomass titer obtained from

un-optimized parameters. These results signify the importance of media optimization on the enhancement of total biomass titer. Finally, a process was demonstrated for sustainable production of biomass feedstock coupled with wastewater treatment by co-culturing the best consortium in AWW and RDWW in bioreactor with optimised parameters.

5.5 References

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Research is creating new knowledge.

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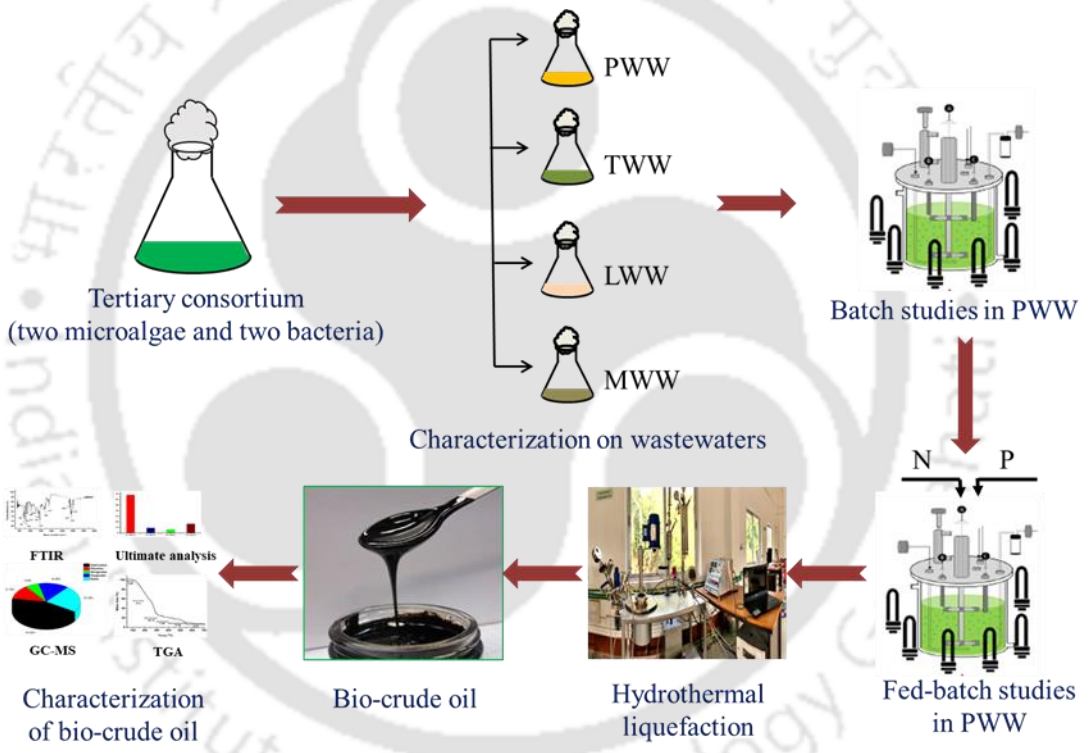
Chapter 6

Process development for the enhancement of microbial biomass feedstock for biofuel production via the screening of various wastewaters



Tertiary consortium was characterized on four different types of wastewater: paper industry wastewater (PWW), textile industry wastewater (TWW), leather industry wastewater (LWW) and municipal wastewater (MWW). Based on the total microbial biomass titre and nutrient removal efficiency, PWW was selected for subsequent experimentation. A process engineering strategy was implemented in an automated photobioreactor under fed-batch mode of cultivation to maximize the microbial biomass titre. The wet microbial biomass obtained from fed-batch cultivation was further subjected to HTL for direct conversion into bio-crude oil. Finally, extensive characterization of the bio-crude oil was carried out.

6.1 Background and motivation

The continuous discharge of inadequately treated wastewater into the ambient environment, especially in developing countries is the major cause of environmental pollution. However, the effort was made to minimize this problem using conventional wastewater treatment methods that require high capital and operational costs are not affordable (Fito and Alemu, 2018). Microalgae cultivation in municipal as well as various industrial wastewaters is an emerging, highly promising approach for resource recovery and concomitant bioenergy generation. However, the recovery performance of photosynthetic green algae is strongly dependent on the associated bacterial partners present in the effluents (Shetty et al., 2019). In recent years microalgae-bacteria consortia have been gaining momentum in generation of biomass feedstock coupled with wastewater treatment. This may be attributed to various advantages which microalgae-bacteria consortia might offer over mixed-culture algal system: (i) ability to function under fluctuating environmental conditions and nutrient loading owing to their diverse metabolic activities and adaptation ability to different growth conditions; (ii) microalgae and bacteria can form micro-ecosystem where they can positively influence the growth of each other in different ways (Goswami et al., 2019). High cost and energy intensive downstream processing (harvesting and drying) of microbial biomass obtained from large scale cultivation remains another major obstacle towards commercialization of microalgae based biofuel. Production of bio-crude oil via hydrothermal liquefaction (HTL) of microalgae biomass has been gaining attentions of the researchers as it offers multiple advantages over conventional process of biochemical and thermochemical conversion methods.

Hence, the present study demonstrates a sustainable process for production of biofuel using microbial biomass as potential feedstock. Sustainability was aimed to be

achieved through a combinatorial approach of: (i) cultivation of a microbial consortium comprising of two microalgae and two bacteria; (ii) utilization of wastewater as a cheaper source of nutrients and replacement of fresh water; (iii) improving biomass productivity through intermittent feeding of limiting nutrients and mutualistic growth of the microbes; (iv) direct conversion of wet biomass into bio-crude oil and (v) biomass generation coupled with wastewater treatment.

6.2 Materials and methods

6.2.1 Sampling and characterization of different wastewaters

Four different types of wastewater samples were collected from effluent treatment plants of the Guwahati Municipal Corporation (GMC) and different industries across Guwahati (26.1445 °N, 91.7362 °E), Assam, India. These consisted of samples from (i) paper industry, (ii) textile industry, (iii) leather industry and (iv) GMC. The wastewater samples were allowed to settle down in order to eliminate the interference of total suspended particles. Subsequent to physical separation of the suspended particles, the pH, total nitrogen, phosphorus, chemical oxygen demand (COD) and concentrations of two heavy metals, namely Chromium (Cr) and Nickel (Ni) present in the wastewater samples, were measured.

6.2.2 Microalgae and bacteria consortium

In the previous section 4.2.1, the tertiary consortium was designed (two microalgae and two bacteria) towards generation of biomass feedstock utilizing wastewater as cheaper source of nutrients. These organisms were isolated from oil refinery wastewater sample collected from Indian Oil Corporation Limited, Noonmati (26.19 °N, 91.80 °E), Guwahati, Assam, India. The microalgae were identified as *Chlorella sorokiniana* strain DBWC2 & *Chlorella* sp. strain DBWC7 and the bacteria were identified as *Klebsiella pneumoniae* strain ORWB1 & *Acinetobacter*

calcoaceticus strain ORWB3. In the present study, this tertiary consortium was used for development of a sustainable process for production of bio-crude oil using microbial biomass as potential feedstock.

6.2.3 Characterization of growth and wastewater treatment efficiency of the tertiary consortium on different types of wastewater

With the aim of assessing the performance of the tertiary consortium in terms of biomass concentration and wastewater treatment efficiency, the pH values of all the four wastewater samples were adjusted to the optimal value of 8.65 as per section 5.3.2. For TWW and LWW, the concentration of total nitrogen was adjusted to the optimal value of 0.29 g L^{-1} as per section 5.3.2. The phosphorus concentration of only LWW was adjusted to the optimal value of 49.5 mg L^{-1} as per section 5.3.2. The composition of PWW and MWW was kept unaltered. Thereafter, the wastewater samples were inoculated with 18% (v/v) inoculum (Section 5.3.1) in which microalgae and bacteria were extant in the ratio of 1:1 as per section 5.3.2. The inoculated wastewater samples were incubated for 7 days in an orbital shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 30 °C under 100 μE m^{-2} s^{-1} light intensity with a photoperiod of 16:8 h light and dark cycle. All the experiments were carried out in duplicate in 500 mL shake flasks. At the end of the incubation period, the wastewater samples were characterized and screened on the basis of total biomass concentration along with removal efficiency of nutrients and heavy metals. The selected wastewater was then considered for subsequent process development. Dynamic profiles for growth of microalgae, utilization of total nitrogen, phosphate and COD were obtained by analysing the samples at every 24 h interval. The total biomass (microalgae plus bacteria) concentration achieved at the end of each batch was determined in terms of dry cell weight (DCW). Heavy metal removal efficiency

was calculated by analysing the fermentation broth at the end of the cultivation. The removal efficiency of total nitrogen, phosphate, COD and heavy metals was calculated using Eq. (6.1):

$$Removal\ efficiency\ (\%) = \left(\frac{S_i - S_f}{S_i}\right) \times 100 \quad (6.1)$$

where, S_i and S_f are the concentrations of a specific nutrient or heavy metal before and after cultivation respectively.

6.2.4 Batch and fed-batch mode of cultivation of tertiary consortium in automated bioreactor utilizing paper industry wastewater

Based on the characterization of tertiary consortium on different wastewater samples (detailed in the previous section), PWW was selected as the best culturing medium supporting maximum growth and nutrient removal efficiency. With the aim of evaluating the performance of tertiary consortium on PWW at a larger scale of operation, further experiment was performed in a 7.5 L automated photobioreactor (Biojenik Engineering, India) with a working volume of 4 L in batch mode of cultivation wherein the wastewater was inoculated (inoculum size of 18%, v/v) with (i) two bacteria only (designated as bacterial batch, BB); (ii) two microalgae only (designated as microalgal batch, MB) and (iii) two microalgae & two bacteria (designated as tertiary consortium batch, TCB) present in the tertiary consortium. The photobioreactor was operated at 30 °C with an agitation of 150 rpm, aeration and light intensity being 1 vvm and 250 μE m^{-2} s^{-1} respectively for a light:dark cycle of 16:8 h. The BB was conducted for 5 days while the MB and the TCB were run for 10 days. For all the three batches, the initial pH value, total nitrogen and phosphate concentrations were adjusted to their corresponding optimal values as detailed in section 5.3.2. Sampling was carried out at regular intervals of 24 h for the analysis of total nitrogen, phosphate and COD. Dynamic profiles for growth of microalgae (in

case of MB and TCB) and bacteria (only for BB) were obtained by measuring chlorophyll-a content and DCW respectively. Final microbial biomass (microalgae plus bacteria) concentration in terms of DCW was measured at the end of each batch. Dynamic profiles of dissolved oxygen (dO_2) and dissolved carbon dioxide (dCO_2) concentrations were obtained for all the three individual batches. Removal efficiency of the nutrients and heavy metals was estimated as described in Eq. (6.1). In the next step, a fed-batch strategy was implemented to maximize the microbial biomass concentration. The fed-batch was operated for a period of 18 days under same cultivation conditions as detailed for the batch mode. An intermittent feeding of the key nutrients, nitrogen source and phosphate, was performed in order to maintain their concentrations at their respective optimal values of 0.29 g L^{-1} and 49.5 mg L^{-1} throughout the entire course of fermentation. The concentrations of these key nutrients in the culture broth were monitored every 8 h. The growth of microalgae (in terms of chlorophyll-a content) and COD were estimated every 24 h. At the end of the experiment, the biomass was collected, centrifuged at 8000 rpm and processed for HTL. The total biomass (microalgae plus bacteria) concentration achieved was determined in terms of DCW.

6.2.5 Analysis of growth, nutrient utilization, COD and heavy metal ion removal

Analysis of growth, nutrient utilization, COD, and heavy metal ion removal was carried out at every sampling time point. Chlorophyll-a was estimated following the protocol described in section 3.2.3. The total nitrogen content was determined as the summation of the individual concentrations of nitrate, nitrite and ammoniacal nitrogen. Estimation of nitrate was followed by the protocol described in section 3.2.3. The mole fraction and the corresponding nitrogen concentration in nitrite and ammonia was measured and calculated according to the Standard Methods for

Examination of Water and Wastewater (APHA, 2017). Phosphate, COD and heavy metal concentration were estimated based on the procedures described in section 3.2.3. All chemicals and reagents procured from Hi Media, India and were of analytical grade.

6.2.6 Hydrothermal liquefaction of microbial biomass and sample preparation

HTL experiments were carried out for all three types of biomass feedstock obtained from TCB, MB and BB. The HTL experiment was performed using a stainless steel stirred tank batch reactor (Amar Equipments Pvt. Ltd., India) with a capacity of 750 mL. The biomass loading was kept at 15% (w/v) of the total working volume of 200 mL. The reaction temperature was maintained at 310 °C for 55 min at corresponding pressure with a heating rate of 10 °C min^{-1} (Boens et al., 2016). The reaction residence time was calculated from the time point the reactor reached the desired temperature. Supply of heat to the reactor was stopped on attaining the desired holding time. Dichloromethane (DCM) (99%, Hi-media) was added into the reactor once the reaction was complete and subsequently cooled, in order to separate the reaction mixture into the water soluble phase and DCM soluble phase. Then this mixture was filtered using Whatman ash less grade-41 filter paper (20 µm) to separate the bio-char. The extraction and separation of the organic phase and the aqueous phase was successively carried out using DCM in the separating funnel. The solvent from the organic phase was removed by evaporation under vacuum in a rota evaporator unit (R-300 digital, Buchi, Switzerland) at 60 °C to obtain the bio-crude oil. The bio-crude yield was calculated using the following Eq. (6.2):

$$Yield\ of\ bio-crude\ oil\ (wt.\%) = \frac{Weight\ of\ the\ bio-crude}{Weight\ of\ the\ dry\ microbial\ biomass} \times 100 \quad (6.2)$$

6.2.7 Analysis of bio-crude oil

6.2.7.1 Gas chromatography-Mass spectrometry

The composition of the bio-crude oil was analysed by Gas chromatography-Mass spectrometry (GC-MS) (Agilent Technologies, USA) using HP5-MS capillary column (30 m, 0.25 mm id, 0.25 mm film thickness). The inlet temperature and split ratio were maintained at 300 °C and 20:1 respectively. 2 µL of the sample was injected into the GC–MS system consisting of an Agilent 7890B gas chromatograph and Agilent 5977B mass selective detector. The temperature of the column was initially held at 50 °C for 5 min and then ramped to 300 °C at a rate of 10 °C min^{-1}. On attaining 300 °C, the temperature was maintained isothermally for 4 min, thereby amounting to a total run time of 37 min. Helium was used as the carrier gas with a constant flow rate 1.6 mL min^{-1}. Data acquisition of the chromatogram peaks was carried out using the Mass Hunter WorkStation and the probable compounds were identified using NIST Mass Spectral Database (NIST 14).

6.2.7.2 Thermogravimetric analysis

Thermogravimetric analysis (TGA) of the bio-crude oil was performed using a thermogravimetric analyzer (STA7200, Hitachi, Japan) in an inert atmosphere of nitrogen. The flow rate of nitrogen was maintained at 50 mL min^{-1}. For TGA analysis of the bio-crude oil, 10 µL of the sample was taken in the alumina crucible and heated from room temperature to 700 °C at a heating rate of 20 °C min^{-1}. The sample weight loss with respect to rise in temperature was recorded to estimate the boiling point distribution.

6.2.7.3 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis of the bio-crude oil was conducted using IR-Affinity-1 (Shimadzu, Japan) in order to analyze the

functional groups present in the sample. The infrared spectrum range was between 400 cm^{-1} and 4000 cm^{-1} and scanning was executed at the rate of 20 with a step size of 4 cm^{-1}.

6.3 Results and discussion

6.3.1 Characterization of tertiary consortium on different wastewater samples

With the aim of assessing its potential to be exploited at commercial scale, the tertiary consortium has been characterized in terms of its growth and wastewater treatment efficiency on different wastewater samples. Four different types of wastewater were considered in the present study: PWW, TWW, LWW and MWW. These wastewaters differed in terms of concentration of the nutrients and pH. Major nutrients such as total nitrogen, phosphate and COD content were found to be abundant in case of PWW and MWW (Table 6.1). However, these nutrients were present in lesser amount in case of TWW and LWW. All the wastewater samples contained significant amount of heavy metals, chromium and nickel, with the exception of MWW (Table 6.1).

Table 6.1 Analysis of wastewater samples. *ND: Not detected

Wastewater type	Parameters					
	pH	Total Nitrogen (mg L^{-1})	Phosphate (mg L^{-1})	COD (mg L^{-1})	Chromium (mg L^{-1})	Nickel (mg L^{-1})
Paper Industry	6.29±0.09	490±0.02	77.70±0.073	5250±0.27	0.98±0.02	0.53±0.04
Textile Industry	7.74±0.01	230±0.07	53.16±0.012	735±0.31	0.27±0.02	0.43±0.07
Leather Industry	7.53±0.04	250±0.04	48.30±0.061	400.5±0.14	0.69±0.06	1.01±0.03
Municipal	7.42±0.04	540±0.02	69.20±0.049	1650±0.25	*ND	*ND

Figure 6.1 elucidates the comparative performance of the tertiary consortium in different wastewater samples with respect to growth and removal efficiency (%) of total nitrogen, phosphate and COD. Maximum total microbial biomass titre of 2.97 g L^{-1} was achieved when tertiary consortium was grown in PWW, followed by 2.14 g L^{-1} in MWW. Growth performance of the consortium was inferior in both LWW and TWW (Fig. 6.1 A). Further, an improved microalgal growth was observed in case of PWW and MWW with a final chlorophyll-a concentration of 31.79 µg mL^{-1} and 21.87 µg mL^{-1} respectively (Fig. 6.2). In spite of the wide variation in the initial concentrations of total nitrogen, phosphate and COD, these nutrients were exhausted within similar cultivation period for all wastewater samples (Fig. 6.2), depicting higher utilization rates of these nutrients by the microbes when grown on PWW and MWW and in turn higher growth rate of the microbes. A biomass titre of 826 mg L^{-1} was reported when *C. vulgaris* was co-cultivated with indigenous bacteria in municipal wastewater (Ryu et al., 2014). Das et al., (2018) has reported chlorophyll-a concentration of 12.5 µg mL^{-1} when a marine species of *Chlorella* was grown in consortium with *Phormidium* sp. in tannery wastewater. Better growth performance of the consortium in PWW and MWW may be attributed to higher amount of inorganic nutrients (total nitrogen and phosphate) primarily supporting growth of the microalgae (Choi et al., 2012; Pathak et al., 2014) and higher amount of COD primarily favouring growth of the bacteria (Rasouli et al., 2018) in comparison to TWW and LWW. Microalgae assimilates inorganic nitrogen sources into organic macromolecules and genetic materials and hence, critically essential for their growth (Barsanti et al., 2006).

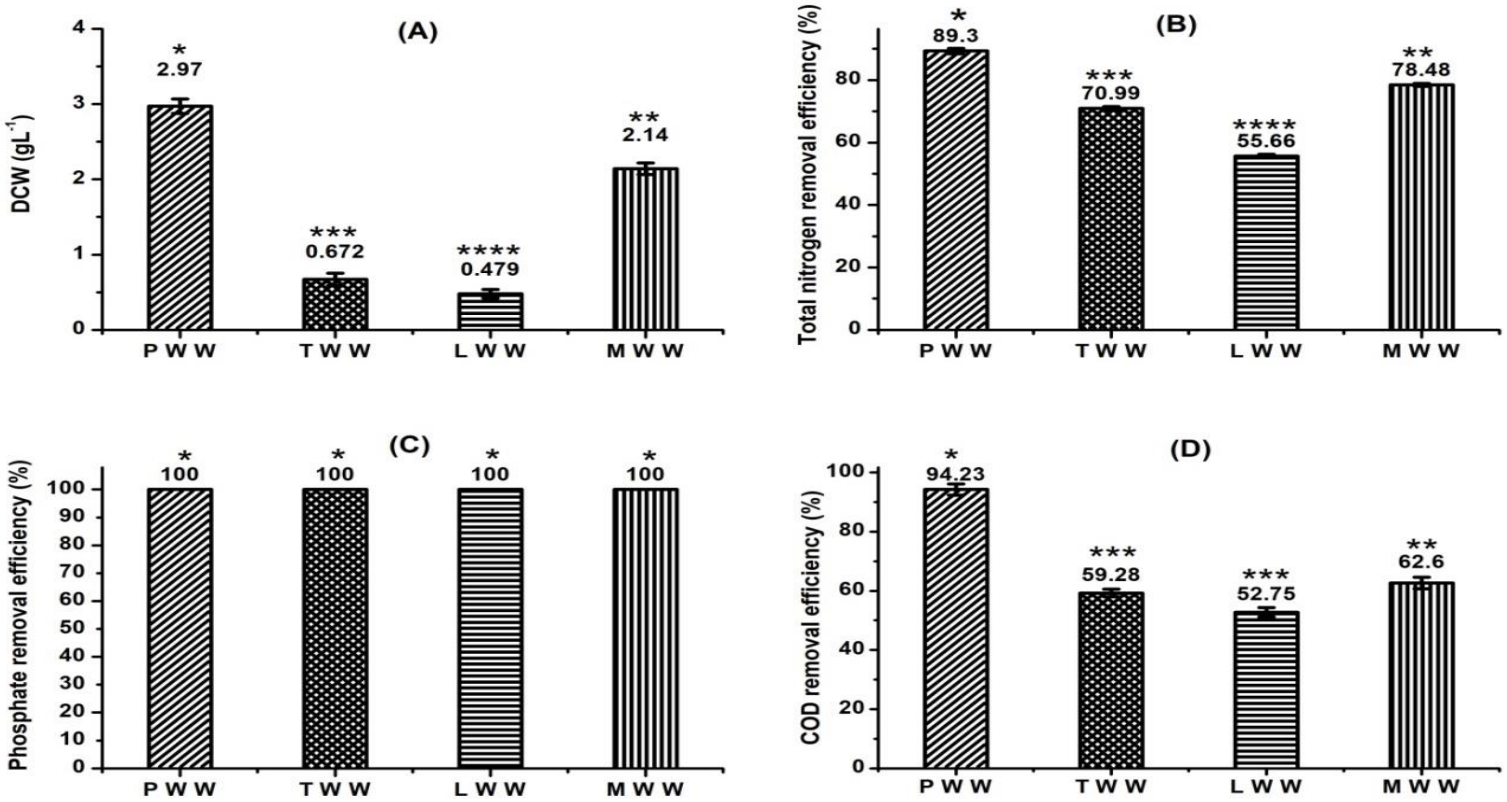


Fig. 6.1 (A) Total biomass titre (DCW, g L^{-1}), (B) total nitrogen removal efficiency (%), (C) phosphate removal efficiency (%) and (D) COD removal efficiency (%) of the tertiary consortium grown on paper industry wastewater (PWW), textile industry wastewater (TWW), leather industry wastewater (LWW) and municipal wastewater (MWW). The *asterisk sign* represents the significant difference between the biomass concentrations or nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency obtained for different wastewater samples analysed using one-way analysis of variance based on Tukey's method. Biomass concentration or nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency that do not share a common symbol are significantly different

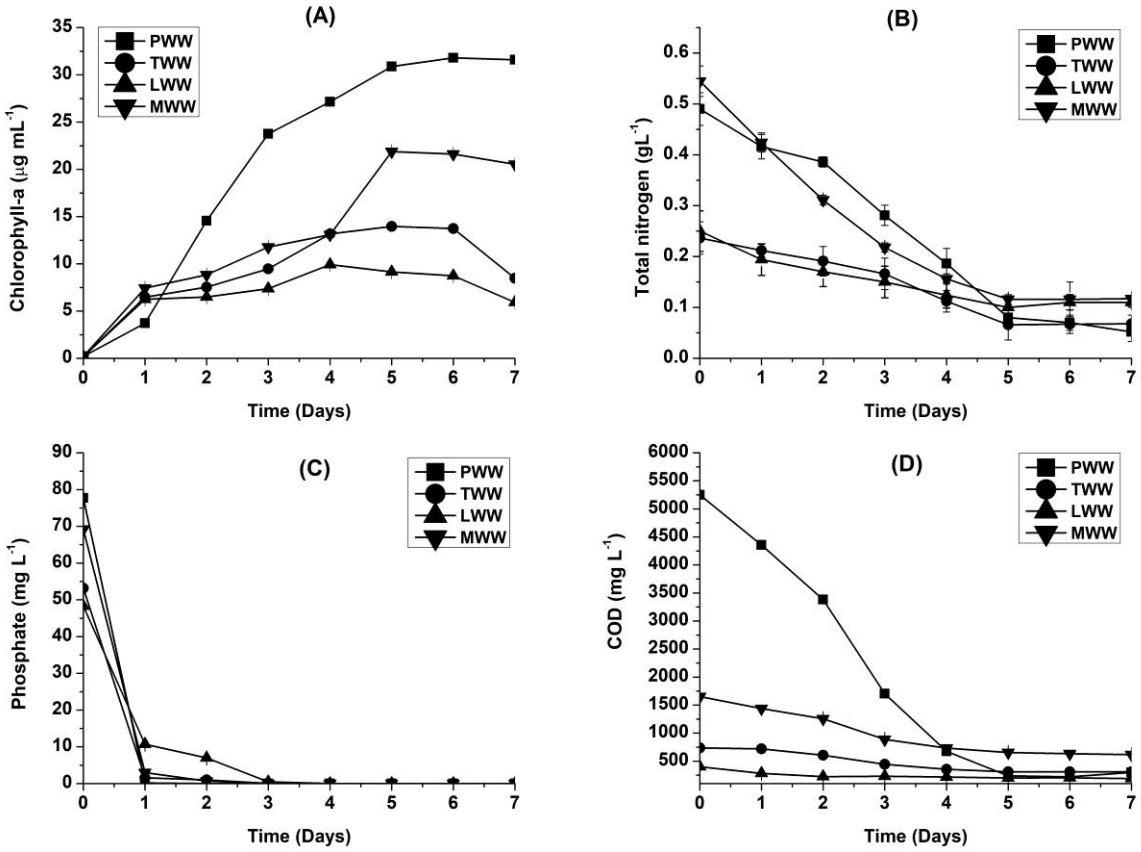


Fig. 6.2 Dynamic profiles of growth (Chl-a), utilization of total nitrogen, phosphate & COD by the tertiary consortium grown on paper industry wastewater (PWW), textile industry wastewater (TWW), leather industry wastewater (LWW) and municipal wastewater (MWW). The experiments were carried out in an orbital shaker at 150 rpm, 30°C under 100 μE $m^{-2}s^{-1}$ light intensity with a light:dark cycle of 16:8 h. All the experiments were conducted in triplicate and the data were expressed as mean ± standard error

Phosphorous also play crucial role in microalgal energy metabolism via synthesis of nucleic acids, lipids, proteins and the intermediates of carbohydrate (Cai et al., 2013). In a microalgae-bacteria consortium, bacterial strains remove COD through aerobic biological treatment involving oxidative degradation of the organic compounds for both energy and carbon usages (Mujtaba et al., 2015). This improved growth performance of the consortium in PWW and MWW correlated well with the removal efficiency of the key nutrients *viz*., total nitrogen, phosphate and COD. The highest

nitrogen removal efficiency of 89.3% was observed in case of PWW followed by MWW (78.48%) and TWW (70.99%) (Fig. 6.1B). The nitrogen removal efficiency was the lowest at a value of 55.66% when grown on LWW. Phosphate was completely exhausted for all wastewater samples as seen in Fig. 6.1C. While, COD removal efficiency was maximum at a considerably higher value of 94.23% in case of PWW, the same was merely in the range of 52–62% for rest of the wastewater samples (Fig. 6.1D). Total nitrogen and phosphate removal efficiency of 95.9% and 94.4% respectively was achieved from cultivation of *C. vulgaris* with indigenous bacteria in municipal wastewater (Ryu et al., 2014). An algae-bacterium combined system of *C. vulgaris* and *Bacillus licheniformis* when grown in municipal wastewater resulted in removal of ammoniacal nitrogen and phosphate with an efficiency of 78% and 92% respectively (Liang et al., 2013). Bioremediation of tannery wastewater by a consortium of marine *Chlorella* sp. and *Phormidium* sp. resulted in 91.16% nitrogen and 88.88% phosphate removal (Das et al., 2018). COD removal efficiency of ~91% was achieved when microalgae-bacteria consortia was cultivated in wastewater from potato processing plant (Rasouli et al., 2018), dairy industry (Tricolici et al. 2014) and municipality (Su et al., 2012). While, a commendable removal efficiency of both the heavy metals (61.95% for Ni and 45.71% for Cr) was observed in PWW, the same was lower in LWW (Table 6.2). However, an effective Cr removal (56.38%) was observed in case of TWW. Das et al., (2018) has reported 94.45% removal of Cr from tannery wastewater employing a consortium of *Chlorella* sp. and *Phormidium* sp. Based on the maximum total biomass concentration and nutrient removal efficiency, PWW was selected for subsequent characterization and process development in the bioreactor.

Table 6.2 Heavy metal ion removal efficiency (%) of tertiary consortium grown in different types of wastewater. All the experiments were performed in triplicate and the data were expressed as mean ± standard error. *ND: Not Detected

Wastewater Type	Nickel (Ni)	Chromium (Cr)
Shake flask experiments		
Paper Industry	61.95 ± 0.07	45.71 ± 0.09
Textile Industry	30.00 ± 0.04	56.38 ± 0.02
Leather Industry	35.50 ± 0.05	22.07 ± 0.04
Municipal Wastewater	*ND	*ND
Bioreactor		
Paper Industry	74.54 ± 0.04	80.00 ± 0.07

6.3.2 Batch and fed-batch mode of cultivation of tertiary consortium in the bioreactor utilizing paper industry wastewater

In order to evaluate the performance of the tertiary consortium in PWW at a larger scale of operation, characterization was carried out in 7.5 L automated bioreactor with a working volume of 4 L. A condescending performance in terms of growth and nutrient removal efficiency was exhibited by the tertiary consortium (TCB) in comparison with bacteria (BB) and microalgae (MB) when cultivated individually in batch mode of operation (Fig. 6.3). For instance, an increment in total biomass titre of 211% and 31% was achieved for the TCB (3.17 g L^{-1}) compared to BB and MB respectively (Fig. 6.3A). For TCB, the final chlorophyll-a concentration was evaluated to be 32.50 µg mL^{-1} which was 1.2 folds higher than the chlorophyll-a concentration obtained (27.30 µg mL^{-1}) in the MB. Cho et al., (2015) reported a biomass titre of 3.31 g L^{-1} when *C. vulgaris* was co-cultivated with four different growth enhancing bacteria.

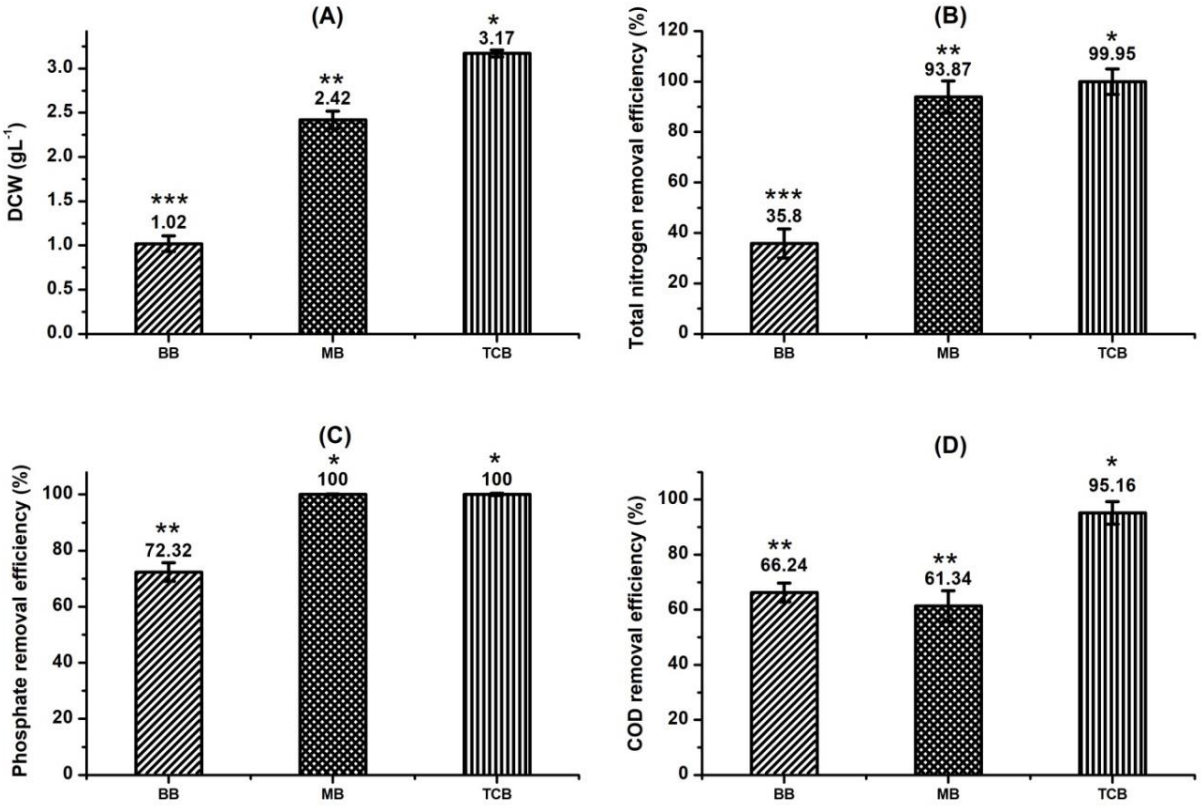


Figure 6.3 (A) Total biomass concentration (DCW, g L^{-1}), (B) total nitrogen removal efficiency (%), (C) phosphate removal efficiency (%) and (D) COD removal efficiency (%) of the tertiary consortium grown on paper industry wastewater in photobioreactor under batch mode of cultivation. BB represents bacterial batch involving two bacteria; MB represents microalgal batch involving two microalgae and TCB represents tertiary consortium batch involving two microalgae and two bacteria. The *asterisk sign* represents the significant difference between the biomass concentration or nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency obtained for BB, MB and TCB analysed using one-way analysis of variance based on Tukey's method. Biomass concentration or nitrate removal efficiency or phosphate removal efficiency or COD removal efficiency that do not share a common symbol are significantly different

However, removal efficiency for total nitrogen was found to be analogous for both TCB (99.95%) and MB (93.87%), albeit a reduction by 2.7 folds in case of BB (35.8%) (Fig. 6.3B). Similar trend was observed in case of phosphate removal. While, phosphate was completely exhausted for both TCB and MB, 72.32% removal was observed in case of BB (Fig. 6.3C). Interestingly, an uppermost COD removal by 95.16% was recorded for TCB, significantly higher than both MB and BB (Fig. 6.3D).

The total nitrogen, phosphate and COD removal efficiency of TCB, achieved in the present study, was found to be better than majority of literatures reported till date. For instance, phosphate and nitrogen removal efficiency of 47% and 62% respectively was reported for co-cultivation of immobilized *C. sorokiniana* and *Azospirillum brasilense* on unsterile municipal wastewater (Covarrubias et al., 2012). Phosphate, total nitrogen and COD removal efficiency of 72.8%, 71% and 46% respectively was achieved for combined growth of *C. sorokiniana* and *Pseudomonas* H4 in wastewater (Chen et al., 2017). However, a complete removal of both nitrogen and phosphate along with 97% removal of COD was observed on co-cultivation of *C. vulgaris* FACHB-30 and *P. putida* in municipal wastewater (Shen et al., 2017). Removal efficiency of Ni and Cr was observed to be significantly enhanced to 74.54% and 80% respectively as compared to growth in shake flasks utilizing PWW (Table 6.2).

With the aim of improving the biomass titre, cultivation of tertiary consortium was conducted through intermittent feeding of nitrogen source and phosphate in order to maintain their respective optimal concentrations throughout the entire course of fermentation. As observed from the figure (Fig. 6.4 B, C), concentration of total nitrogen and phosphate in the culture broth was maintained at approximately 0.29 g L^{-1} and 49.5 mg L^{-1} respectively. Total biomass titre was evaluated to be 4.1 g L^{-1}, an increment by 29.3% when compared to the batch mode of cultivation (Fig. 6.4A). A total biomass titre in the range of 2.23–3.15 g L^{-1} was obtained when cyanobacterium *Arthrospira platensis* and microalga *C. vulgaris* were cultivated in fed-batch mode in wastewater derived from the anaerobic digestion of poultry litter depending on different concentrations of ammoniacal nitrogen (Markou et al., 2015). The maximum concentration of chlorophyll-a was observed to be 46.92 µg mL^{-1} when the tertiary

consortium was cultivated in PWW in fed-batch mode of operation. This chlorophyll-a concentration was found to be 1.44 times higher than that obtained in batch mode.

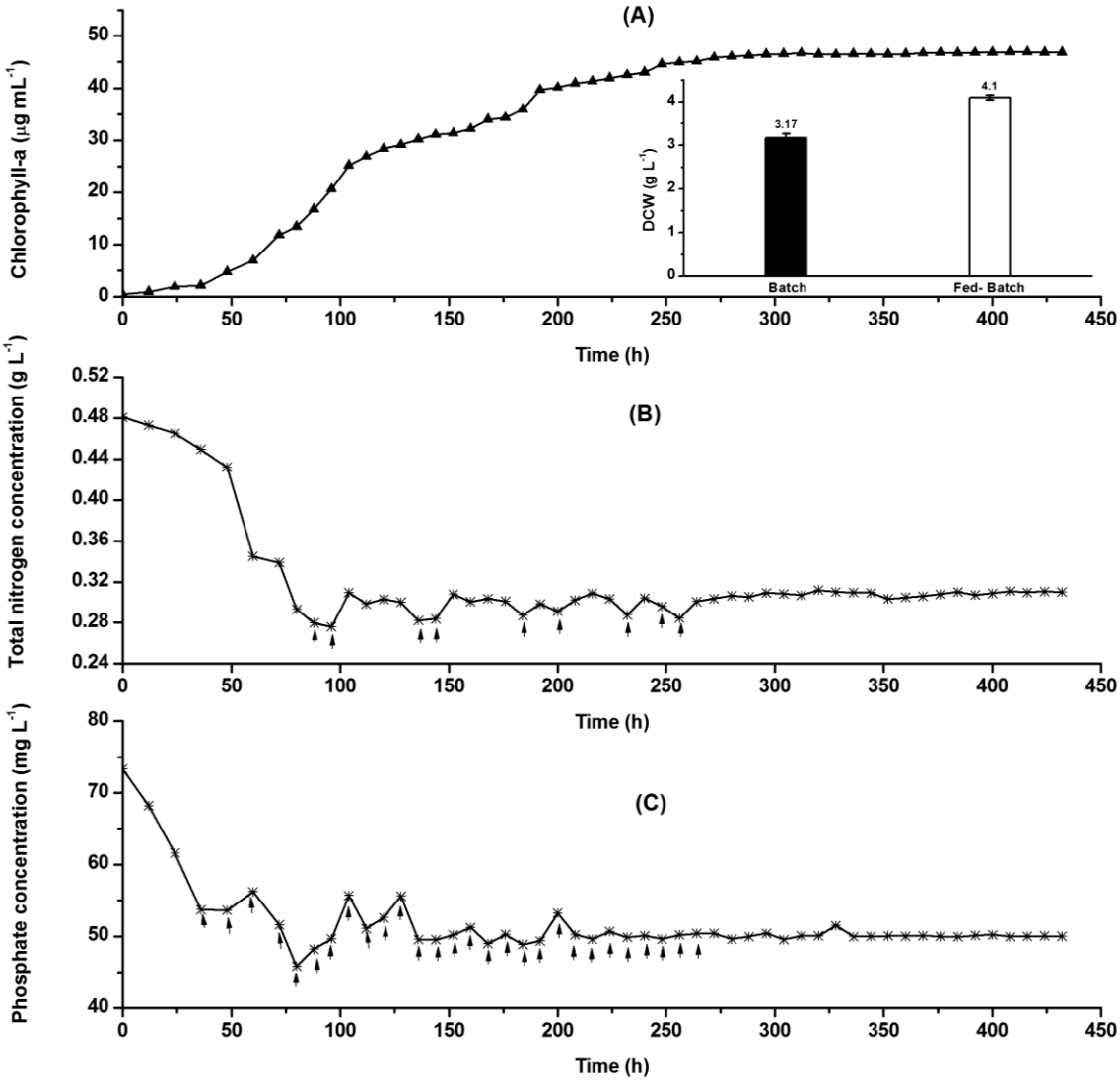


Fig. 6.4 Dynamic profiles for (A) growth of microalgae (Chlorophyll-a, μg mL^{-1}); (B) total nitrogen (g L^{-1}) and (C) phosphate (mg L^{-1}). The tertiary consortium was grown on paper industry wastewater in a photobioreactor under fed-batch mode with intermittent feeding of nitrogen source and phosphate. The graph in the inset of panel A compares total biomass concentration obtained in batch and fed-batch cultivation

6.3.3 Symbiotic growth of microalgae and bacteria

In the present study, an enhancement in total biomass titre and in turn, nutrient removal efficiency was observed when a consortium of two microalgae and two

bacteria were grown together (TCB) as compared to growth of two bacteria (BB) or two microalgae (MB) alone. The possible reasons for this improved biomass titre and nutrient removal efficiency may be attributed to various mutualistic interactions between microalgae and bacteria *e.g.*, symbiotic exchange of CO_2 and O_2 (Praveen and Loh, 2015) and supply of micronutrients and growth promoting factors from bacteria (Cho et al., 2015; Droop et al., 2007; Hernandez et al., 2009). An inhibition in the growth of the bacteria in BB was expected post 40 h of cultivation as the dO_2 level dipped significantly below saturation value and in turn, resulted in limitation to the oxygen supply (Fig. 6.5A). However, in this batch the peak dCO_2 concentration was recorded to be ~1.5% during exponential phase of the bacterial growth (Fig. 6.5A). Contrary to the BB, in the MB an initial drop in the dO_2 level was marked till 20 h followed by a sharp increase and its maintenance close to the saturation value till ~140 h (Fig. 6.5B). While the initial drop in the dO_2 level may be due to the consumption of oxygen by the native bacteria present in the wastewater, increase in the dO_2 level can be attributed to the initiation of exponential growth of the microalgae after 20 h which continued till 120 h (Fig. 6.5B). A concomitant drop in dO_2 level was observed with the attainment of stationary phase of microalgal growth. Nevertheless, in this batch, rate of CO_2 supply appeared to be the rate controlling step as evident from lower dCO_2 level (maximum of <1%) as compared to the BB. Interestingly, in case of TCB while dO_2 level above 40% was maintained for entire cultivation period except the initial hours where a sudden decrease in the dO_2 level was due to combined growth of the native bacteria present in the wastewater and the bacteria present in the consortium (Fig. 6.5C). The maintenance of dO_2 level above 40% post 50 h of cultivation corroborated well with the growth of microalgae contributing to continuous release of oxygen in the culture broth (Fig. 6.5C). A higher

dissolved carbon dioxide tension in the range of 1–4% was maintained in TCB for a prolonged cultivation period of 120 h, which might be attributed to the sustained growth of the bacteria in the consortium (Fig. 6.5C). While the bacteria consumed oxygen produced by microalgae during photosynthesis as an electron acceptor to degrade organic matter (COD) present in the wastewater, the microalgae fixed CO_2 released as by-product of bacterial oxidative degradation. This mutualistic interaction between microalgae and bacteria in terms of exchange of CO_2 and O_2 promoted the individual growth of these organisms present in the tertiary consortium, which resulted in improved total biomass concentration and better nutrient removal efficiency. In a previous study, glucose removal efficiency was reported to increase from 50% to 100% in batch mode of operation and 73% to 100% in continuous mode of operation of the bioreactor when immobilized microalgae *C. vulgaris* was co-cultivated with *P. putida* (Praveen and Loh, 2015). This improvement in glucose utilization was attributed to a symbiotic CO_2/O_2 gas exchange between these two microbes. Further, an exponential growth of the microalgae was observed during the symbiosis when compared to un-aerated condition (Praveen and Loh, 2015). However, in the present study existence of any other possible interaction may not be ruled out for growth upliftment.

6.3.4 Characterization of bio-crude oil produced via hydrothermal liquefaction of microbial biomass

The yield of bio-crude oil obtained from HTL of microbial biomass generated by growing tertiary consortium in PWW was 15% (w/w). The bio-crude oil yield obtained for TCB was found to be significantly higher than BB (4.4%, w/w) and MB (8.2%, w/w). This better bio-crude oil yield in case of TCB might be due to difference in composition of biomass feedstock as compared to BB and MB.

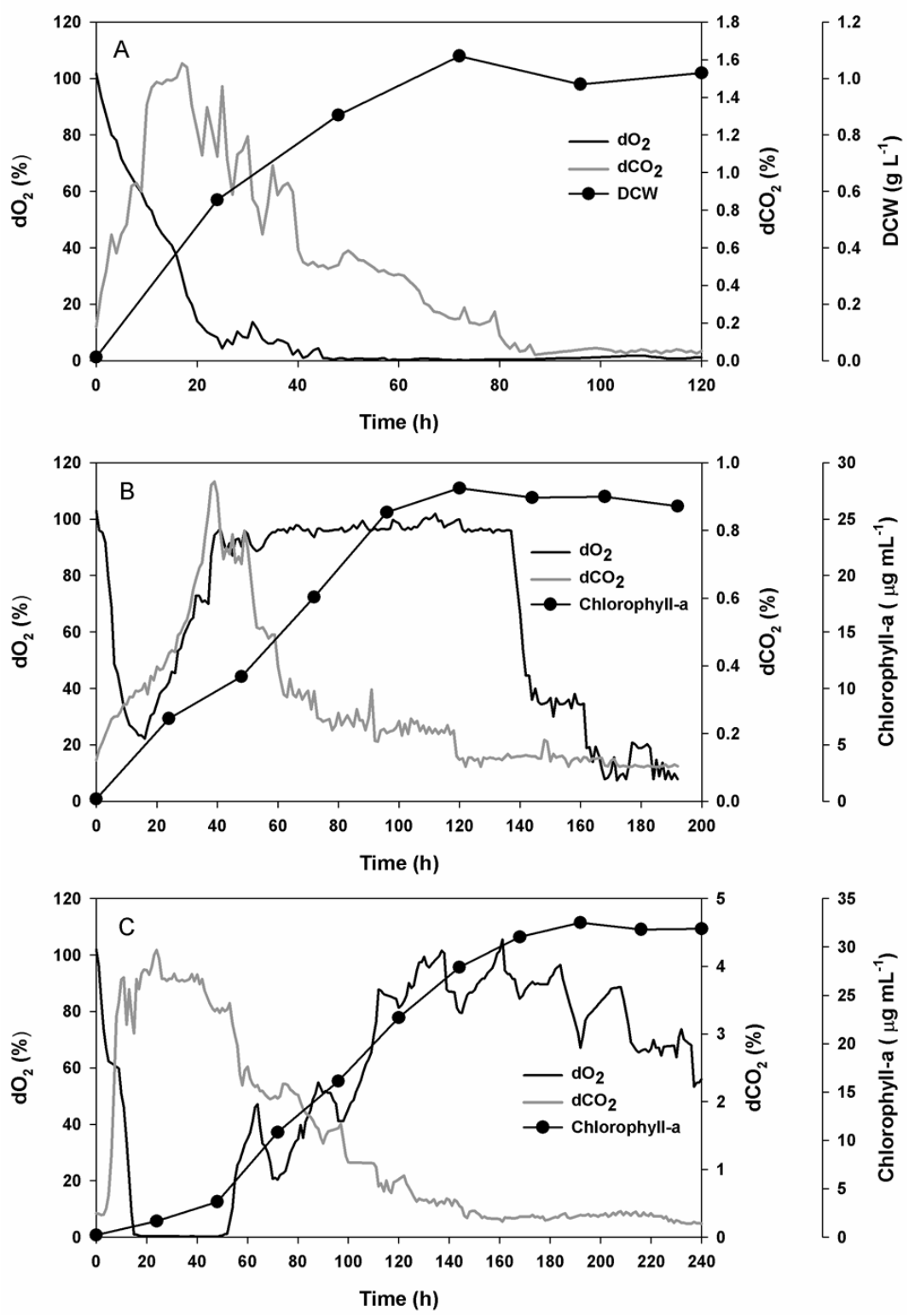


Fig. 6.5 Dynamic profiles for: (A) dO_2 (%), dCO_2 (%) & growth of bacteria (DCW, g L^{-1}) in case of bacterial batch (BB); (B) dO_2 (%), dCO_2 (%) & growth of microalgae (Chlorophyll-a, µg mL^{-1}) in case of microalgal batch (MB); and (C) dO_2 (%), dCO_2 (%) & growth of microalgae (Chlorophyll-a, µg mL^{-1}) in case of tertiary consortium batch (TCB). The experiment was carried out in a photobioreactor under batch mode of cultivation

However, the bio-crude oil yield obtained from TCB of the present study was found to be 41.69% and 39.02% lower than that reported for mixed culture of microalgae (Chen et al., 2014) or microalgae-bacteria consortia (Boens et al., 2016), respectively. This inferior yield might be due to the un-optimized process parameters and quality of the biomass feedstock. The lower yield of bio-crude oil from TCB can be improved by optimizing the HTL parameters e.g., biomass loading, residence time and reaction temperature (Chiaramonti et al., 2017). Further, *in-situ* catalytic treatment is expected to increase the bio-crude oil yield (Nam et al., 2017). Elemental composition of the bio-crude oil in terms of C, H, N, S and O (%) was found to be 63.16, 8.11, 5.37, 0.21 and 23.15 respectively. This elemental composition of the bio-crude oil obtained in the present study was found to be comparable with the bio-crude obtained via HTL of other microalgae (Biller et al., 2015). However, heavy metals and inorganic phosphorous was not found to be present in the bio-crude oil.

The FT-IR spectra of bio-crude oil exhibited characteristic absorption peaks reflecting presence of various functional groups and in turn, corresponding compounds (Fig. 6.6A). The band at 3265 cm^{-1} refers to the O-H stretching indicating presence of water or alcohol in the bio-crude oil. The bands at 2965, 2930, 2852, 1450 and 1377 cm^{-1} depict C-H vibrations, indicating the presence of alkanes. The C=O stretching vibration at 1652 cm^{-1} attributed to the presence of ketones, aldehydes and carboxylic acids. C-O stretching at 1265, 1170, 1060 and 970 cm^{-1} represents presence of primary, secondary and tertiary alcohols in the bio-crude oil. Existence of phenols, esters, ethers and aromatic compounds are represented by the presence of O-H bends at 872, 735 and 700 cm^{-1}. These results corroborate with the findings reported by Shuping et al., (2010).

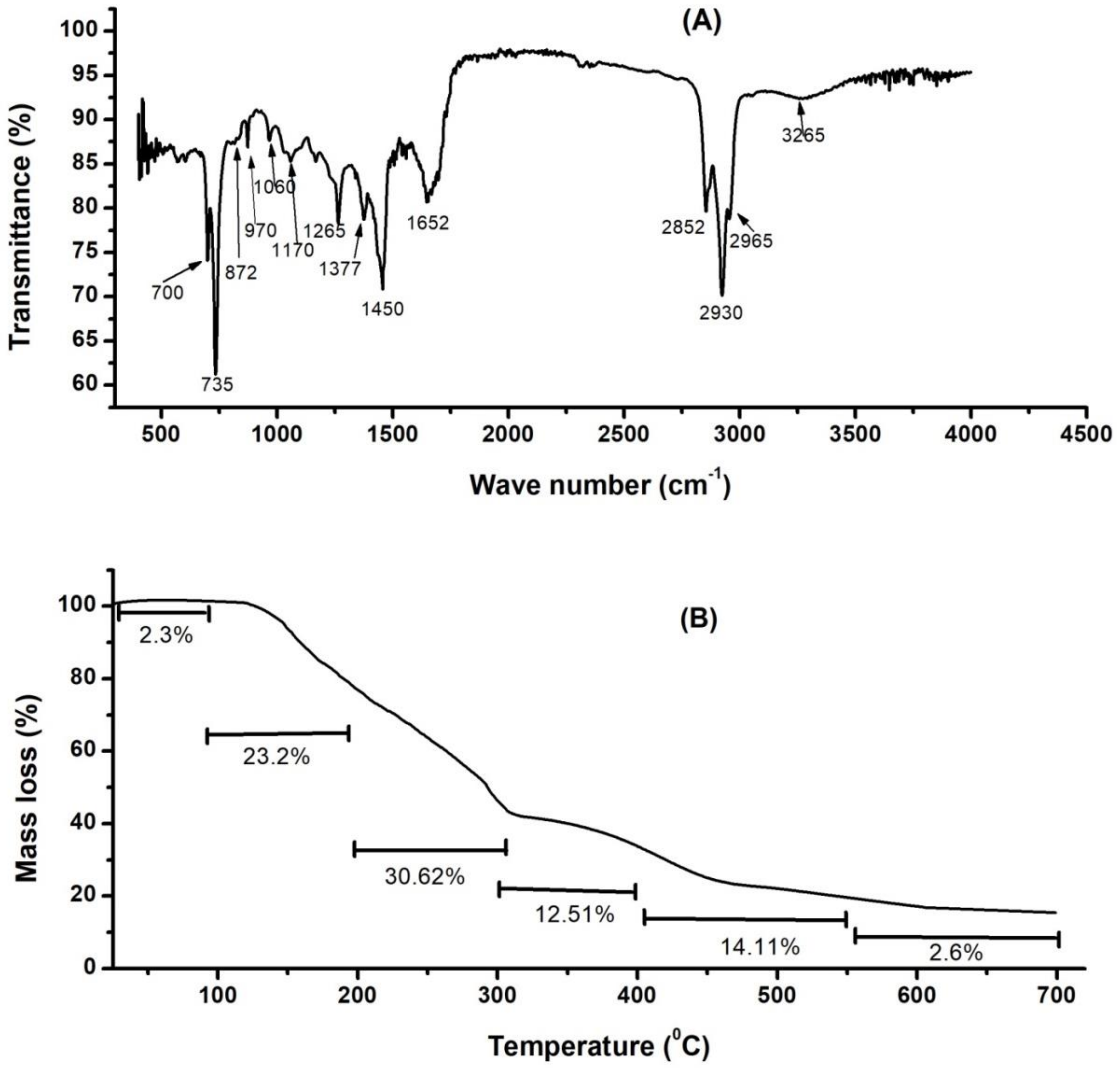


Figure 6.6 (A) FTIR spectra and (B) TGA curve of bio-crude oil obtained via hydrothermal liquefaction of microbial biomass

GC-MS analysis (Table 6.3) revealed that more than 82% of the bio-crude oil was composed of hydrocarbons, alcohols, amines, amides, aldehydes and ketones. These findings complement the results obtained from FTIR analysis. The lists of all major compounds with a score of 75 on a scale of 100 have been enlisted in Table 6.3. Percentage of hydrocarbon content (1, 2, 4, 6, 7, 8, 11, 12, 13, 14, 17, 18, 19, 20, 25, 26 and 28) in the bio-crude oil was found to be high, depicting good oil quality. The presence of nitrogenous compounds (5, 9 and 10) in bio-crude oil indicates thermal

conversion of the protein fraction present in the biomass. The oxygen content in the bio-crude oil may be attributed to the presence of oxygen containing functional groups such as ketones, aldehydes, alcohols and esters produced by the decomposition of proteins and polysaccharide fractions of the microbial biomass. This oxygen and nitrogen content could interfere with the quality of the bio-crude oil and hence, additional up gradation in terms of deoxygenation and denitrogenation is essential prior to its application as fuel oil.

The boiling point distribution of the bio-crude oil has been estimated using TGA in nitrogen atmosphere (Fig. 6.6B). As per the analysis, the maximum distillate fraction of 30.62% lies within the boiling point range of 200–300 °C (Table 6.4) depicting suitability of the bio-crude oil for conversion into diesel oil, jet fuel and fuel for stoves. It is interesting to note that N, P and chromium was completely absent in HTL aqueous phase with presence of nickel in trace amount (0.04 mg L^{-1}). Therefore, this post HTL aqueous phase can be recycled for subsequent cultivation of microalgae or can be used as utility in other industrial applications.

Table 6.3 Major chemical compounds present in the bio-crude oil obtained from hydrothermal liquefaction of tertiary consortium biomass at 310 °C and 55 min

Sl. No.	Retention time (min)	Name of the compound	Area (%)
1	3.11	Undecane	1.16
2	3.21	Benzene, 1-ethyl-3-(1-methylethyl) -	1.20
3	3.25	Bicyclo [2.2.1] heptan-2-ol, 4-chloro-1, 7, 7-trimethyl-, exo-	1.48
4	7.51	Pyrene, 1, 3-dimethyl-	1.95
5	8.01	benzene acetonitrile, 4, 4'-[1, 2-ethenediyl] bis-	1.51
6	8.73	Tetracosane	1.44
7	9.49	1, 4-Dimethyl-6-phenyl-naphthalene	1.90
8	9.80	Phenanthrene, 2, 3, 5-trimethyl-	3.78
9	10.73	2-Phenazinecarbonitrile, 7-amino-	5.37
10	27.00	Dibenzepin	0.50
11	27.10	Fluoranthene	1.39
12	27.39	n-Heptadecene	0.99
13	27.59	Octadecane, 2, 6, 10, 14-tetramethyl-	0.49
14	27.70	Phenanthrene, 4, 5-dimethyl-	0.61
15	27.84	10-Hydroxynortriptyline	10.72
16	28.18	3, 7-Dimethyldibenzothiophene	1.92
17	28.44	5-Methoxy (5H) dibenzo [a, d] cycloheptene	0.57
18	28.65	Bicyclo [2.2.1] heptane, 3-methylene-2-(3-phenylprop-1-en2yl)-	10.55
19	28.69	Naphthalene, 1-(phenylmethyl) -	2.60
20	28.76	Phenanthrene, 3, 6-dimethyl-	5.80
21	29.18	3-(3-Indolyl)-5-oxo-3-pyrazoline-4-carbonitrile	0.56
22	29.26	Normethadone	3.05
23	29.37	Benzo[b]naphtha [2, 3-d] thiophene, 7, 8, 9, 10-tetrahydro-	2.16
24	30.03	3-Naphthalen-2-yl-3-piperidin-1-yl-propan-1-ol	2.09
25	30.18	Phenanthrene, 3, 6-dimethyl	0.61
26	30.45	Phenanthrene, 2, 5-dimethyl-	3.82
27	30.67	Isobenzofuran, 1, 3-dihydro-1, 1-dimethyl-3-phenyl-	0.83
28	30.92	Pyrene, 4, 5, 9, 10-tetrahydro	3.20
29	31.08	4-(1-Methyl-1-siletanyl) phenol	9.90
30	31.61	Benzo[b]selenophene-3-carboxaldehyde, 2-methyl-	0.50
Total area (%)			82.65

Table 6.4 Distillate range of different fractions of bio-crude oil obtained via thermogravimetric analysis

Distillate Range (ºC)	Typical applications	Fraction of bio-crude oil (%)
25-100	Bottle gas and chemicals	2.33
100-200	Gasoline	23.20
200-300	Jet fuel, fuel for stoves & diesel oil	30.62
300-400	Lubricating oil for engines, fuel for ships & machines	12.51
400-550	Lubricants & candles	14.11
550-700	Fuel for ships, factories	2.60

6.4 Conclusions

A sustainable process was demonstrated towards production of bio-crude oil from microbial biomass feedstock via combinatorial approach of: (i) improved biomass concentration through intermittent feeding of limiting nutrients and mutualistic growth of microalgae-bacteria; (ii) utilization of wastewater as cheap source of nutrients and water; (iii) direct conversion of wet biomass into bio-crude oil via hydrothermal liquefaction and (iv) simultaneous remediation of wastewater. The process may be a potential technology platform towards sustainable production of bio-crude oil from microbial biomass utilizing wastewater.

6.5 References

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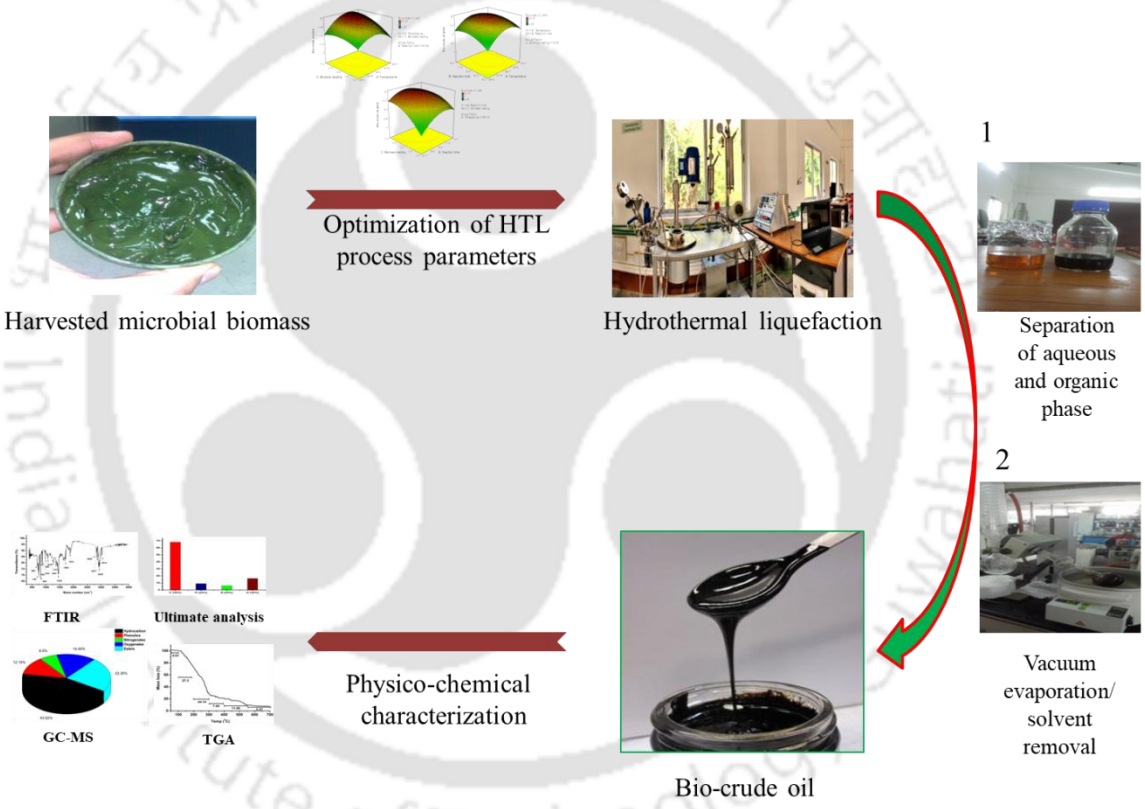
The environment is where we all meet ; where all have a mutual interest; it is the one thing that all of us share.

Lady Bird Johnson

Chapter 7

Optimization of process parameters of hydrothermal liquefaction for bio-crude oil production from microbial biomass



Statistical optimization of HTL process parameters such as temperature (ºC), reaction time (min) and biomass loading (%, w/v) for maximisation of bio-crude oil yield.

7.1 Background and motivation

The bio-crude oil yield obtained from HTL of tertiary consortium grown in paper industry wastewater under un-optimised process parameters was 15% as seen in section 6.3.4 which was quite lower than that reported for mixed culture of microalgae (Chen et al., 2014) or microalgae-bacteria consortia (Boens et al., 2016). This inferior yield might be due to the un-optimized process parameters and quality of the biomass feedstock. The lower yield of bio-crude oil from TCB can be improved by optimizing the HTL parameters e.g., biomass loading, reaction time and temperature (Chiaramonti et al., 2017). Moreover, the physicochemical properties as well as the yield of the bio-crude strongly affected by the HTL process parameters such as process temperature, reaction time, biomass loading concentrations, biomass composition (Mathimani and Mallick, 2019). In view of the high costs of refining and up-gradation of bio-crude, it is essential to evaluate its physico-chemical properties and carry out composition analysis before bio-crude can be considered further for subsequent processing for transportation fuels. However, analyses of bio-crude oil derived from microalgal biomass are limited in the literature (Arun et al., 2018; Cheng et al., 2019; Wei et al., 2018). To the best of our knowledge, no study has been reported for evaluation of bio-crude obtained via hydrothermal liquefaction of mixed culture of microalgae and bacteria grown in large scale open raceway pond using industrial wastewater.

Present study demonstrates a large scale process for sustainable production of bio-crude oil from microbial biomass as feedstock. With the aim of maximizing bio-crude oil yield, statistical optimization of key HTL parameters *e.g.* biomass loading, temperature and reaction time was carried out. Finally, a detail physico-chemical

characterization of bio-crude oil was carried out to assess it suitability for future refining and up-gradation in order to be used as petroleum fuel.

7.2 Materials and methods

7.2.1 Hydrothermal liquefaction of mixed culture of microalgae and bacteria

The mixed culture biomass of microalgae and bacteria grown in paper industry wastewater was used as feed stock for hydrothermal liquefaction. The HTL reaction was carried out in a stainless steel stirred tank batch reactor (Amar Equipment Pvt. Ltd., India) of 750 mL capacity with a magnetic drive and removable reaction vessel. The reaction was carried out at different combinations of temperature, time, and biomass loading. The reaction time did not include the time required to attain desired reaction temperature. After each reaction, the reactor was rapidly cooled down to room temperature by circulating tap water through cooling coils located outside the reactor. The reaction mixture was extracted using dichloromethane (DCM) (99%, Hi-media) followed by filtration using Whatman ash less grade-41 filter paper (20 μm) to separate the bio-char from the liquid fraction. Separation of the organic phase and the aqueous phase was successively carried out using DCM in the separating funnel. The solvent from the organic phase was removed by evaporation under vacuum in a rota evaporator unit (R-300 digital, Buchi, Switzerland) at 60 °C to obtain bio-crude oil. The bio-crude oil yield was calculated using Eq. (7.1):

$$\text{Yield of bio} - \text{crude oil (wt.\%)} = \frac{\text{Weight of the bio-crude oil}}{\text{Weight of the dry microbial biomass}} \times 100 \qquad (7.1)$$

7.2.2. Maximisation of bio-crude oil yield via statistical optimization

In order to enhance in the yield of bio-crude oil, three HTL process parameters such as temperature (ºC), reaction time (min), and biomass loading (%, w/v) were optimized through Central Composite Design (CCD) based Response Surface Methodology (RSM). The actual values and the coded values of the variables

employed are shown in Table 7.1. Coded values of +α, +1, 0, and -1, -α correspond to high, medium, and low levels of the variables respectively. A 4^4 quarter factorial CCD was generated using Design-Expert® (Version 7, Stat-Ease Inc., USA) and was employed to optimize the bio-crude oil yield.

Table 7.1 Actual values and coded values of the variables employed in CCD-RSM based optimization

Variables		Levels code and corresponding values				
Code	Variables	-α	-1	0	+1	+α
X_1	Temperature (ºC)	249.59	268	295	322	340.4
X_2	Reaction time (minute)	9.5	32	65	98	120.49
X_3	Biomass loading (%, w/v)	4.93	8	12.5	17	20.06

The CCD predicted twenty experiments which included eight factorial points, six axial points and six replicates of the centre points as shown in Table 7.2. RSM is a mathematical modelling technique which utilizes a polynomial equation to model the interaction amongst the variables. Under RSM, the linear, quadratic, and interaction effects between the selected parameters and the bio-crude oil yield of the microbial biomass were mathematically expressed in the form of a quadratic polynomial equation Eq. (7.2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (7.2)$$

Where, Y is the maximum bio-crude oil yield (wt. %), X_i is the i^{th} parameter, k is the total number of parameters and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients. In the present study, a total of twenty experiments were conducted and the experimental response values are shown in Table 7.2.

Table 7.2 Full factorial central composite design matrix of four variables in coded and natural units along with the observed response, bio-crude oil yield

Standard order	Temperature (ºC)	Reaction time (min.)	Biomass loading (%, w/v)	Bio-crude oil yield (%)	
				Observed	Predicted
1	268	32	8	8.6	7.13
2	322	32	8	7.44	7.51
3	268	98	8	13.1	12.69
4	322	98	8	8.2	10.84
5	268	32	17	11.3	10.39
6	322	32	17	16.82	18.97
7	268	98	17	10	11.65
8	322	98	17	14.8	17.99
9	249.59	65	12.5	4.25	5.74
10	340.40	65	12.5	15.35	11.39
11	295	9.50	12.5	11.12	12.04
12	295	120.49	12.5	19.29	15.90
13	295	65	4.93	10.8	11.12
14	295	65	20.06	22.66	19.87
15	295	65	12.5	21.77	21.50
16	295	65	12.5	21.32	21.50
17	295	65	12.5	21.47	21.50
18	295	65	12.5	21.6	21.50
19	295	65	12.5	20.93	21.50
20	295	65	12.5	21.53	21.50

7.2.3 Evaluation of physico-chemical properties of bio-crude oil

The chemical and physical properties of the bio-crude oil obtained from the microbial biomass at optimized HTL process parameters, were analysed to assess its suitability for exploitation as petroleum fuel.

7.2.3.1 Elemental analysis and heating value

The elemental composition of bio-crude oil in terms of C, H and N content was carried out by CHN elemental analyser (Perkin Elmer series ii). Before elemental analysis, the bio-crude oil was dried at room temperature in a fume hood. The oxygen content was calculated by using Eq. (7.3):

$$\mathrm{O}(\%) = 100 - (\mathrm{C} + \mathrm{H} + \mathrm{N})\% \qquad (7.3)$$

The higher heating value (HHV) of bio-crude oil was calculated based on elemental composition using Dulong formula (Brown et al., 2010) as per Eq. (7.4):

$$\text{HHV} = 0.3383 \times \text{C} + 1.422 \times (\text{H} - \frac{\text{O}}{8}) \quad (7.4)$$

where C, H, and O are the carbon, hydrogen, and oxygen mass percentages of dry bio-crude oil.

Energy recovered through HTL process was calculated using Eq. (7.5) as follows:

$$\text{Energy recovery (\%)} = \frac{\text{HHV of biocrude} \times \text{mass of bio-crude}}{\text{HHV of microbial biomass} \times \text{mass of biomass}} \times 100 \quad (7.5)$$

HHV of microbial biomass was calculated based on elemental analysis as explained for bio-crude oil.

7.2.3.2 Chemical composition analysis

The chemical composition of the bio-crude oil was analysed by gas chromatography with mass spectroscopy (GC-MS) (Agilent Technologies, USA) using HP5-MS capillary column (30 m, 0.25 mm id, 0.25 mm film thickness). The inlet temperature and split ratio were maintained at 300 °C and 20:1, respectively. 2 µL of the sample dissolved and diluted in dichloro methane was injected into the GC–MS system consisting of an Agilent 7890B gas chromatograph and Agilent 5977B mass selective detector. The oven was programmed at an initial temperature of 50 °C and then heated at a constant rate of 10 °C min^{-1} until a temperature of 300 °C reached. On attaining 300 °C, the temperature was maintained isothermally for 4 min; thereby amounting to a total run time of 37 min. Helium was used as the carrier gas with a constant flow rate 1.6 mL min^{-1}. Data interpretation of the chromatogram peaks was carried out using the Mass Hunter WorkStation and the probable chemical compounds were identified using NIST Mass Spectral Database (NIST 14). Fourier transform infrared spectroscopy (FTIR) analysis of the bio-crude oil was conducted using IR-Affinity-1 (Shimadzu, Japan) in order to analyse the functional groups present in the sample.

The infrared spectrum range was between 400 cm^{-1} and 4000 cm^{-1} and scanning was done at the rate of 20 with a step size of 4 cm^{-1}. The reflectance bands give the information about the presence of main functional groups.

7.2.3.3 Physical property analysis

The physical property of the bio-crude oil was analysed in terms of dynamic viscosity, density, and total acid number (TAN). Density of the bio-crude oil was measured by density meter (Antonpar DMA 4500M). TAN is the number of milligrams of potassium hydroxide (KOH) required neutralizing the acidic functions present in one gram of oil. It is expressed as mg KOH per gram of the sample. To determine the TAN value, 50 mg of bio-crude oil sample was dissolved in 10 mL of isopropyl alcohol and then titrated by 20 mM KOH solution, using phenolphthalein as a colour indicator. The concentration of bio-crude oil in isopropyl alcohol was kept low to guarantee a certain transparency and good solubility of nonpolar compounds. Dynamic viscosity of bio-crude oil was measured at different temperatures by using an interfacial viscometer (Antonpar physica MCR 301 rheometer) with sample volume of 0.5 mL.

7.2.3.4 Thermal property analysis

The thermal characteristic of bio-crude oil was determined by quantitative thermo gravimetric analysis (TGA) method. TGA data of the bio-crude oil was obtained using a thermo-gravimetric analyser (STA7200, Hitachi, Japan) in an inert atmosphere of nitrogen. The flow rate of nitrogen was maintained at 50 mL min^{-1}. 10 µL of the sample was taken in the alumina crucible and heated from room temperature to 700 °C at a heating rate of 20 °C min^{-1}. The sample weight loss with respect to rise in temperature was recorded to estimate the boiling point distribution.

7.3 Results and discussion

7.3.1 Optimization of bio-crude oil yield obtained via hydrothermal liquefaction of mixed culture biomass

The present study reports an efficient method for production of bio-crude oil via one-step direct hydrothermal liquefaction of mixed culture biomass of microalgae and bacteria. With the aim of maximizing bio-crude oil yield, CCD was constructed to optimize three HTL process parameters e.g. temperature, reaction time and biomass loading and to assess the effect their interaction on bio-crude oil yield as model response. The results obtained from these experiments were analysed through RSM with the objective function as maximization of bio-crude oil yield. A wide variation in the bio-crude oil yield from 4.25 % to 22.66 %, w/w resulted from CCD designed experiments depicts importance of optimization of HTL process parameters (Table 7.2). Model construction yielded second order polynomial equations Eq. (7.6) which correlates the process parameters with the predicted response of bio-crude oil yield.

$$Y = 21.51 + 1.68 \times A + 1.15 \times B + 2.60 \times C - 0.56AB + 2.05AC - 1.07BC - 4.57A^2 - 2.66B^2 - 2.12C^2 \quad (7.6)$$

where, Y is the predicted bio-crude oil yield (%, w/w). A, B and C represents the temperature (ºC), reaction time (min), and biomass loading (%, w/v) respectively. ANOVA was carried out to analyse the experimental data and the results have been represented in Table 7.3. The model Fischer 'F' test showed a significant value of 10.05 with $p < 0.006$. This result indicates significant positive effect of the interaction between the individual processes variables on increment in bio-crude oil yield. The high value of correlation coefficient (R^2 = 0.9) indicates that only 10% of the 20 experiments performed could not be fitted into the model. Based on the *p* values of linear, square, and interactive terms of individual process variables, temperature,

biomass loading, interaction between temperature and biomass loading, and quadratic effects of all the parameters were found to be significant for bio-crude oil yield (Table 7.3).

Table 7.3 Analysis of variance (ANOVA) for the selected quadratic model of bio-crude oil yield

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*p*-value Prob > F
Model	598.077	9	66.453	10.0514	0.0006
A-Temperature	38.492	1	38.492	5.822228	0.0365
B-Reaction Time	18.003	1	18.003	2.723118	0.1299
C-Biomass Loading	92.415	1	92.415	13.97831	0.0039
AB	2.486	1	2.486	0.376089	0.5534
AC	33.538	1	33.538	5.072816	0.0480
BC	9.202	1	9.202	1.391861	0.2654
A^2	301.330	1	301.330	45.57795	< 0.0001
B^2	102.100	1	102.100	15.44324	0.0028
C^2	64.925	1	64.925	9.820416	0.0106
Residual	66.113	10	6.611		
Lack of Fit	65.695	5	13.139	157.191	< 0.0001
Pure Error	0.417	5	0.083		
Cor Total	664.191	19			
R^2	0.900	0.810	Predicted R^2	0.2461	

The optimum value for temperature, biomass loading and reaction time was found to be 299.73 ºC, 16.14 (%, w/v) and 64.95 min, respectively. The model was validated by comparing model predicted bio-crude oil yield (22.66%) with the corresponding experimental value (21.73%) at optimized process parameters. CCD-RSM based optimization of the process variables resulted in 44.86% increment in bio-crude oil yield as compared to the bio-crude oil yield obtained from the previous study (Section 6.3.4) under un-optimized condition. A similar bio-crude oil yield of 24.6% was reported for HTL of mixed culture biomass of *Chlorella* sp. and native bacteria present in wastewater (Boëns et al., 2016). At the above optimized HTL process parameters, the bio-crude oil yield of bacteria batch (only two bacterial partner of the tertiary consortium) and microalgae batch (only two algal partner of the tertiary

consortium) was found to be (5.3%, w/w) and MB (11.2%, w/w). This better bio-crude oil yield in case of tertiary consortium might be due to difference in composition of biomass feedstock as compared to bacteria batch and microalgae batch.

7.3.2 Effect of process parameters of hydrothermal liquefaction on bio-crude oil yield

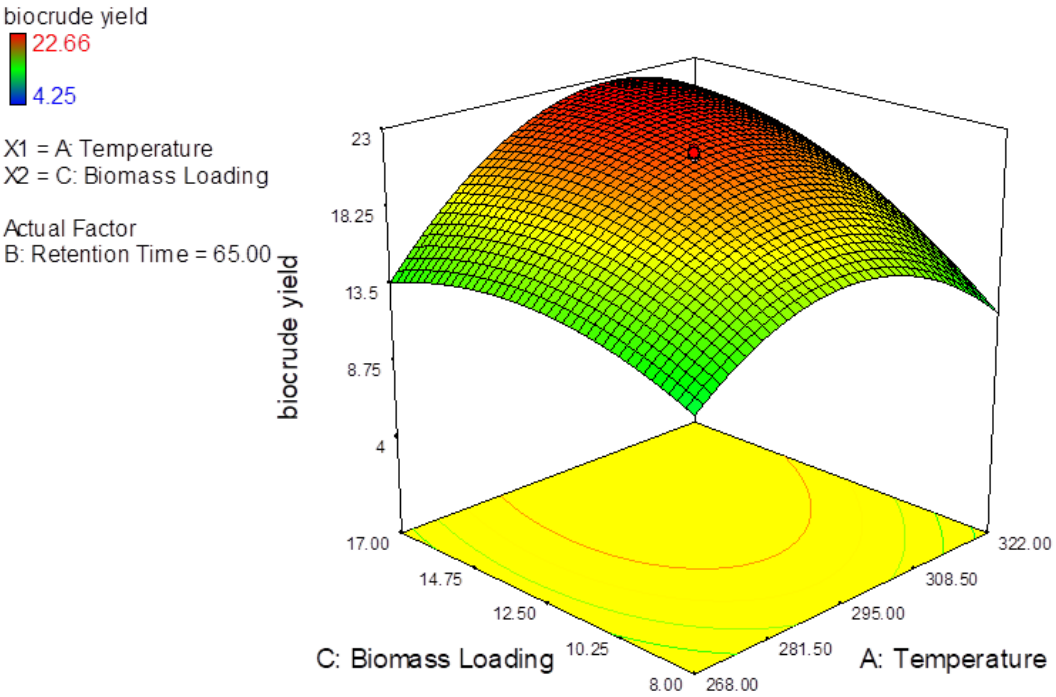


Fig.7.1 Response surface plot of bio-crude yield as function of temperature (ºC) and biomass loading (%, w/v)

Fig. 7.1 represents 3D response surface plot for the interactive effect of temperature, biomass loading on bio-crude oil yield. The effect of biomass loading and reaction temperature on bio-crude oil yield at a constant reaction time of 65 min was shown in Fig. 7.1. It was observed from the figure that the bio-crude oil yield increases linearly with the increase in temperature till 299 ºC, followed by gradual decrease with further increase in temperature. A similar profile of variation in bio-crude oil yield with

variation in biomass loading was observed with highest bio-crude oil yield predicted at a biomass loading of 15.5 %, w/v.

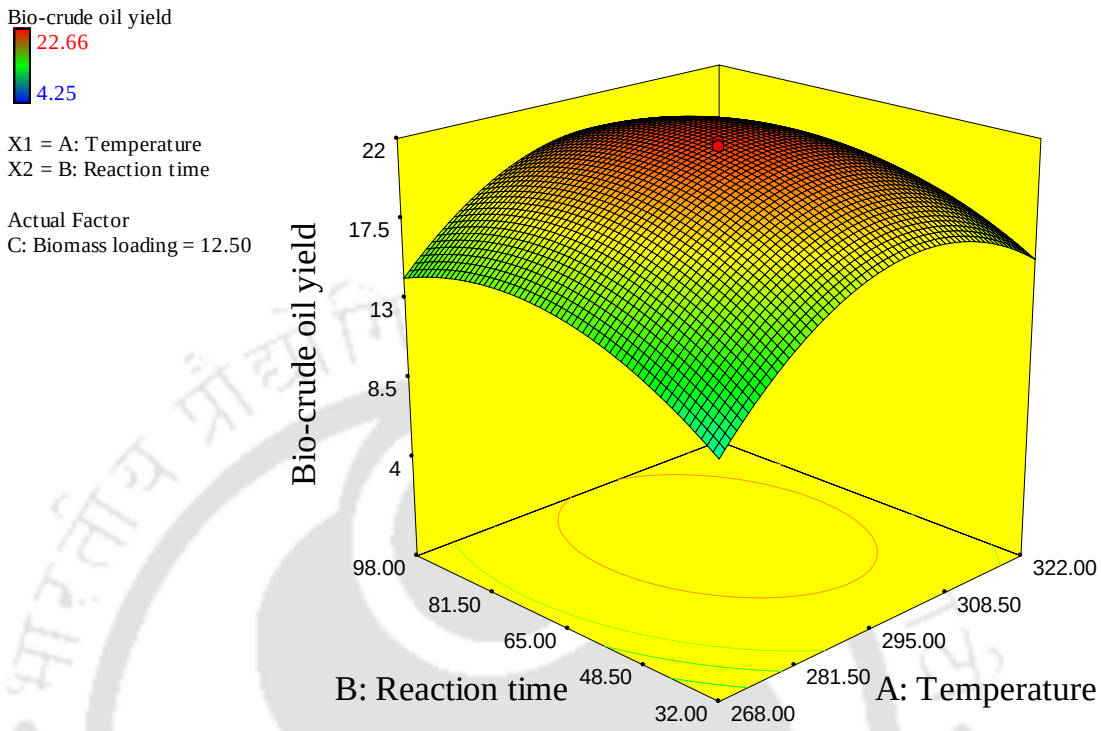


Fig.7.2 Response surface plot of bio-crude yield as function of temperature (ºC) and reaction time (min.)

Both, temperature and reaction time were shown to exhibit positive combinatorial effect on bio-crude oil yield till 299 ºC and 74 min, respectively at a constant biomass loading of 12.5%, w/v (Fig. 7.2). However, Bio-crude oil yield was negatively correlated with the further increase in both temperature and reaction time.

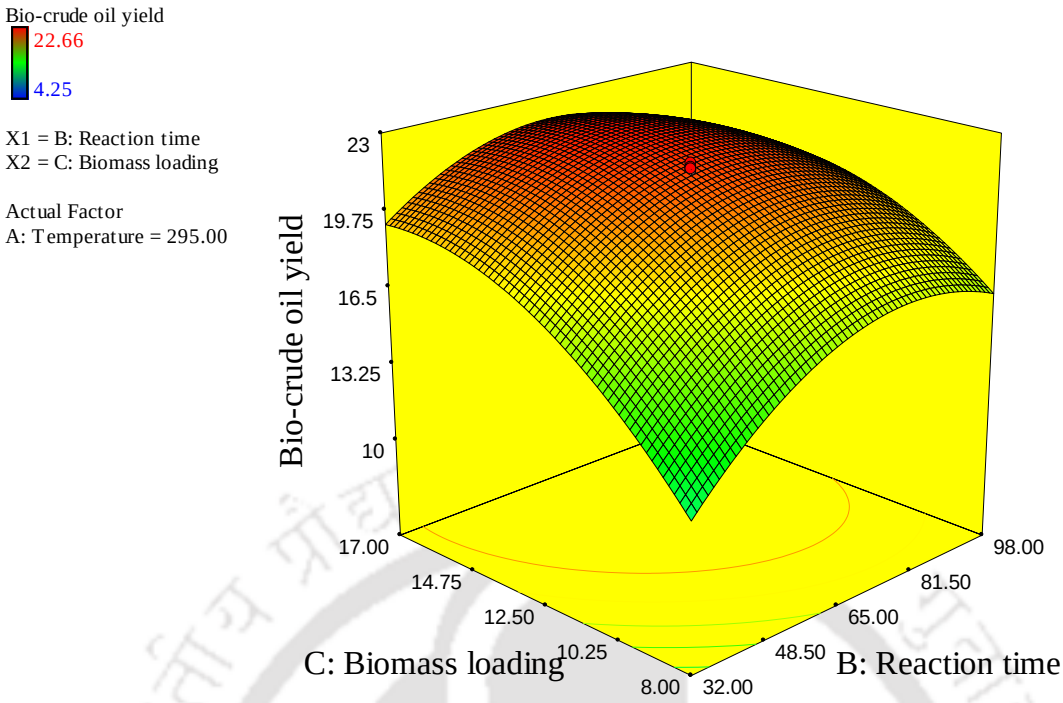


Fig.7.3 Response surface plot of bio-crude yield as function of biomass loading (%, w/v) and reaction time (min.)

Fig. 7.3 represents the plot of bio-crude oil yield as a function of biomass loading and reaction time at a constant temperature of 295 ºC. Under this condition, the bio-crude oil yield first increases and then decreases with the increase in biomass loading or reaction time. The bio-crude oil yield was predicted to be highest at biomass loading and reaction time of 15.54% (w/v) and 73.1 min, respectively. HTL process typically consists of two reaction phases. In the initial phase of HTL, the biomass is decomposed and depolymerized to small compounds which may rearrange through condensation and re-polymerization in the subsequent phase (Xu et al., 2014). However, extended reaction time may result in undesired re-polymerization which might have negative influence on the bio-crude oil yield (Shuping et al., 2010) as also observed in the present study where bio-crude oil yield was found to decrease beyond reaction time of 73.1 min. It has been reported that the conversion rate of microbial biomass to bio-crude oil increases during the first phase of liquefaction process until

approximately 300 °C due to the breaking of chemical bonds by de-polymerization reaction of the polymers present in biomass. However, further increase in reaction temperature beyond 300 °C promotes re-polymerization reaction between activated small intermediate liquid molecules leading to the formation of lower molecular weight char and gas molecules and in turn, results in the reduction in bio-crude oil yield (Xu et al., 2014).

7.3.3 Evaluation of physico-chemical properties of bio-crude oil

As Physico-chemical characterization of bio-crude oil was carried out to assess requisite refining and up-gradation requirement for possible blending with the petroleum fuel.

7.3.3.1 Elemental analysis and energy content of the bio-crude oil

The elemental analysis of bio-crude oil was carried out to evaluate the energy density and its potential as a substitute for fossil fuel. From the ultimate analysis of bio-crude oil, the amount of carbon (C), hydrogen (H), nitrogen (N), and oxygen (O) was found to be 68.21%, 9.12%, 6.18%, and 16.49%, respectively. Elemental analysis of the microbial biomass represents C, H, N and O content of 43.49%, 6.37%, 10.68%, and 39.46%, respectively. HHV of the bio-crude oil was calculated to be 33.11 MJ kg^{-1}. HHV of the bio-crude oil obtained in the present study was found to be comparable with the heating value of the bio-crude obtained from various lignocellulosic biomass (28−36 MJ kg^{-1}) and microalgae (30−48 MJ kg^{-1}) (Ramirez et al., 2015). A heating value of 33.3 MJ kg^{-1} was reported for bio-crude by HTL of mixed culture microalgal biomass at 300 ºC with 1 h retention time (Chen et al., 2014). However, HHV of bio-crude oil was found to be 18.2% and 26.58% lower than that of biodiesel and diesel, respectively (Table 7.4).

Table 7.4 Ultimate analysis and physical property analysis of the bio-crude oil

Properties	HTL bio-crude oil	Biodiesel (Ramirez et al., 2015)	Diesel (Ramirez et al., 2015; Koley et al., 2018)
C (wt%)	68.21	-	83-87
H (wt%)	9.12	-	10-14
N (wt%)	6.18	-	0.1-2.0
O (wt%)	16.49	-	0.05-6.0
H/C (mol/mol)	0.80	1.87	1.79
O/C (mol/mol)	0.18	0.11	0
HHV (MJ kg^{-1})	33.11	40.5	45.1
Viscosity (mPa.s)	10.15	1.7-5.3	1.1-3.5
Density (kgm^{-3})	1.14	0.88	0.85
TAN (mg of KOH per g of oil)	0.7	-	0.57
Energy recovery (%)	42.95	-	-

HHV takes into account heat of vaporization of the water during combustion and is directly correlated with the ultimate and proximate analyses (Ramirez et al., 2015). For instance, heating vale is directly proportional to the carbon and hydrogen fraction of bio-crude, while oxygen and nitrogen content shows a negative effect on HHV (Ramirez et al., 2015). Oxygen to carbon ratio (O/C = 0.18) of the bio-crude oil obtained in the present study was found to be similar to biodiesel (O/C = 0.11), albeit absence of oxygen in petroleum diesel is recorded (Table 7.4). This significant oxygen content resulted from the de-polymerization of the biomass components into various oxygenated compounds such as alcohols, ketones, aldehydes, furans, phenols and other oxygenates. Higher oxygen content in the bio-crude is not desirable owing

to various disadvantages such as poor miscibility with fossil fuels, low thermal stability and highly reactive nature, rendering side reactions during transport and storage (Panahi et al., 2019). However, enrichment of diesel fuel with oxygen containing chemicals has been considered to be an effective strategy for reduction in smoke emission with small penalties on NO_x and fuel consumption (Ramirez et al., 2015). Hydrogen to carbon ratio (H/C) of the bio-crude was calculated to be 0.8, significantly lower than that reported for biodiesel and petro diesel (Table 7.4). Lower H/C ratio is regarded as unfavourable fuel properties hindering direct application of bio-crude as transport fuel. Further, presence of nitrogen in the bio-crude may lead to the formation of solid deposits via interaction with degradation products (Ramirez et al., 2015). Energy recovery using HTL process under optimal condition was calculated to be 42.95%. A wide variation (19.3 %− 66.1 %) in the energy recovery from HTL process was reported for different microalgal biomass in presence or absence of catalyst (Biller et al., 2011). While, these properties are not regulated for biofuels, it is prudent to produce biofuels with the properties similar to conventional fuels to ensure minimal modifications to engines. To that end, it will be essential to carry out standard crude oil refining process (catalytic up-gradation) of the bio-crude before it can be realized for blending at commercial scale.

7.3.3.2 Chemical composition of the bio-crude oil

Major chemical constituents of the bio-crude oil determined through GC-MS analysis is shown in Table 7.5. These compounds were classified into different groups such as hydrocarbons (37.84%), esters (20.3%), oxygenates (12.56%), phenolics (10.6%) and nitrogenates (5.65%) which, in total represent 86.95% of the bio-crude oil composition. The main chemical groups for bio-crude oil obtained via hydrothermal liquefaction of different feedstock have been reported as phenolic: 6% −

65% (Chumpoo et al., 2010; Karagöz et al., 2005; Kosinkova et al., 2015); esters: 2%−44% (Chumpoo et al., 2010; Kosinkova et al., 2015); aromatics and heterocyclics: 6% − 35% (Chumpoo et al., 2010; Kosinkova et al., 2015); aldehydes: 0%− 18% (Chumpoo et al., 2010; Karagöz et al., 2005); Carboxylic acids: 2%−40% (Karagöz et al., 2005; Li et al., 2010; Jena et al., 2011); ketones: 0%−38% (Karagöz et al., 2005; Li et al., 2010; Jena et al., 2011); alkanes: 9% − 13% (Li et al., 2010; Jena et al., 2011) and nitrogenates: 12%− 23% (Li et al., 2010; Jena et al., 2011). Effect of varying compositions of such different groups of compounds on diesel and biodiesel have been elucidated in the literature. However, for HTL bio-crude oil these relationships have not been established yet. The higher percentage of hydrocarbon content in the bio-crude oil indicates good oil quality. Further, hydrocarbon fraction of the bio-crude oil comprises of normal alkanes (19.47%), branched alkanes (15.67%), normal alkenes (12.18%), branched alkenes (32.13%), and aromatic hydrocarbon (12.68%). Normal alkanes positively influence Cetane number of the fuel the most, followed by branched alkanes, normal alkenes, branched alkenes, cycloalkanes, and aromatics. Higher Cetane number signifies good ignition quality, good cold start properties, reduction in emission of smoke, CO and unburnt hydrocarbon (UHC). In case of diesel, presence of aromatics was found to offer high heating value and density, but at the cost of ignition quality (Ramirez et al., 2015). While, presence of nitrogenates such as amines may be attributed to the thermal conversion of the protein fraction present in the microbial biomass, the oxygen content of the bio-crude oil is mainly due to the presence of ketones, aldehydes, alcohols and esters produced by decomposition of carbohydrate fractions of the microbial biomass. Presence of esters in the bio-crude oil might offer resemblance to

biodiesel which is more oxygenated and comprised of fatty acids methyl or ethyl esters (Ramirez et al., 2015).

Table 7.5 Major chemical compounds present in the bio-crude oil obtained from optimised HTL process parameters of a tertiary consortium cultivated in a paper industry wastewater

Sl. No.	Retention time (min)	Name of the compound	Classification of compound	Area (%)
1	18.160	Naphthalene	Hydrocarbon (Aromatic)	4.80
2	22.773	Tetradecane, 2,6,10-trimethyl	Hydrocarbon (Branched alkane)	5.93
3	26.89	(8E)-8-Heptadecene	Hydrocarbon (Liner alkene)	2.87
4	27.02	3-Heptadecene	Hydrocarbon (Linear alkene)	1.04
5	27.39	n-Heptadecene	Hydrocarbon (Linear alkene)	0.99
6	29.17	(2E)-3,7,11,15-Tetramethyl-2-hexadecene	Hydrocarbon (Branched alkene)	1.24
7	30.955	8-Heptadecene	Hydrocarbon (Linear alkene)	2.58
8	31.758	Heptadecane	Hydrocarbon (Linear alkane)	7.47
9	32.844	1-Undecene, 5-methyl	Hydrocarbon (Branched alkene)	1.66
10	33.223	7-Heptadecene,17-chloro-	Hydrocarbon (Branched alkene)	2.04
11	36.372	2-Hexadecene,3,7,11,15-tetramethyl-	Hydrocarbon (Branched alkene)	7.22
12	7.409	n-Octyl phenyl ketone	Oxygenates	1.12
13	8.735	Norephedrine	Oxygenates	2.20
14	11.393	2,6,8-Trimethyl bicyclo[4.2.0]oct-2-ene-1,8-diol	Oxygenates	9.24
15	3.69	Methylpent-4-enylamine	Nitrogenates	5.65

16	5.036	Phenylephrine	Phenolics	6.59
17	9.864	Phenol,4-ethyl-	Phenolics	4.01
18	23.956	Chloroacetic acid, pentadecyl ester	Ester	10.73
19	33.514	2-phenylethyl propyl ester	Ester	5.2
20	33.737	N,N'-Bis(carbobenzyloxy)-lysine methyl ester	Ester	2.04
21	42.22	Hexadecanoic acid, 2-methylpropyl ester	Ester	2.33
Total area				86.95

Various types of functional groups traced out through FT-IR analysis complemented the findings of GC-MS analysis (Fig. 7.4A). The FT-IR transmittance of bio-crude oil reveals presence of hydrocarbons, alcohols, phenols, esters, ethers, carboxylic acids, and ketones. The band at 3255 cm^{-1} represents O-H stretching vibrations, may be due to the presence of water or alcohol in bio-crude oil. The bands observed at 3014, 2935, 2829, 2877, 1445 and 1373 cm^{-1} confirms C-H vibrations, indicating the presence of alkyl groups. The C=O stretching vibrations at 1695 cm^{-1} indicates the presence of aldehydes, ketones or carboxylic acids. Presences of primary, secondary and tertiary alcohols were correlated with the presence of C-O stretching at 1261, 1070, and 985 cm^{-1}. O-H bends at 875, 854,800, 740 and 696 cm^{-1} depict presence of phenols, esters, and aromatic compounds.

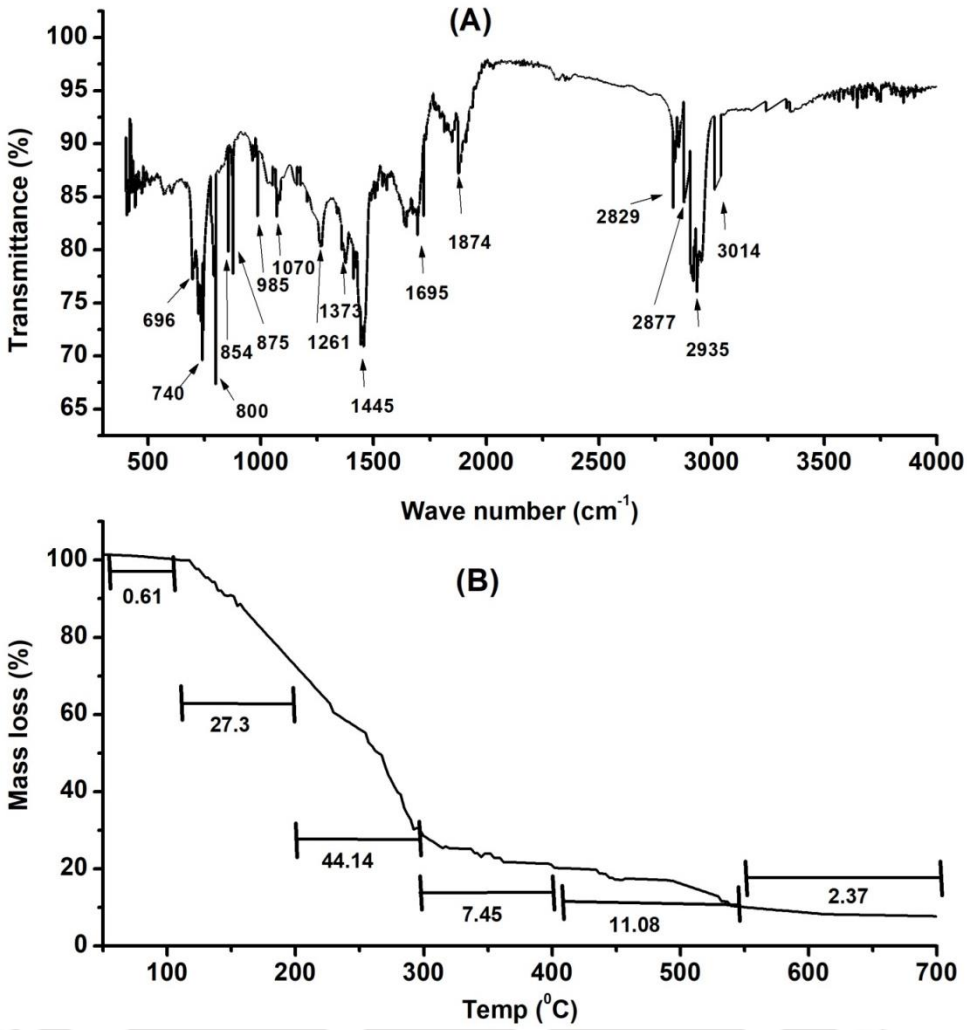


Fig.7.4 (A) FTIR spectra and (B) TGA curve of bio-crude oil obtained via hydrothermal liquefaction of microbial biomass

7.3.3.3 Physical properties of the bio-crude oil

The dynamic viscosity of bio-crude oil was found to be 10.15 mPa.s at 40 ºC, which is significantly higher as compared to the viscosity of diesel oil (1.1−3.5 mPa.s) and biodiesel (1.7−5.3 mPa.s) (Ramirez et al., 2015). The higher viscosity of bio-crude oil obtained in the present study may be due to the presence of straight chain hydrocarbons (Boelhouwer et al., 1951) and alcohols. High viscosity of the fuel results in poor atomisation, poor combustion (Lee et al., 2002) and high energy requirement for fuel pumping (Alptekin et al., 2008). However, higher density of bio-crude oil (1.14 Kg m^{-3}) in comparison to diesel (0.85 Kg m^{-3}) and biodiesel (0.88 Kg

m^{-3}) may be advantageous with respect to higher power output from combustion of a larger fuel mass (Alptekin et al., 2008). The TAN number of the bio-crude oil (0.7) was found to be in similar range with that of diesel oil (0.57).

7.3.3.4 Thermal property the bio-crude oil

Table 7.6 Distillate range of different fractions of bio-crude oil obtained via thermogravimetric analysis

Distillate Range (°C)	Typical Applications	Percentage fraction of bio-crude oil (%)
25-110	Bottle gas and chemicals	0.61
110-200	Gasoline	27.3
200-300	Jet fuel, fuel for stoves and diesel oil	44.14
300-400	Lubricating oil for engines, fuel for ships and machines	7.45
400-550	Lubricants and candles	11.08
550-700	Fuel for ships and factories	2.37

The bio-crude oil sample was analysed using TGA in nitrogen atmosphere to determine its boiling point distribution (Fig. 7.4B). An initial mass loss of 0.61% was due to dehydration when temperature of bio-crude was raised to 110 ºC. Following dehydration, 27.3% mass loss was observed in the temperature range of 110 ºC−200 ºC which might be attributed to the presence of gasoline fraction in bio-crude oil. Maximum distillate fraction of 44.14 % lies within boiling point range of 200 ºC−300 ºC, depicting suitability of bio-crude oil for conversion into jet fuel, fuel for stoves and diesel oil (Table 7.6). Small shares of 7.45%, 11.08% and 2.37% may be fractioned in to lubricating oil for engine, lubricants and fuel for ships in the temperature range of 300 ºC−400 ºC, 400 ºC−550 ºC and 550 ºC−700 ºC, respectively. Less amount of ash content of 7.05% was observed above 700 ºC. Therefore, as per

the TGA study 71.44 % of the bio-crude oil may be fractionated in to transportation fuels within the temperature of 110 ºC− 300 ºC. In a previous study, TGA of bio-crude oil obtained from HTL of mixed culture microalgal biomass showed approximately 60% of the bio-crude oils could be distilled in the range of 200 ºC–550 ºC (Chen et al., 2014) which is lower as compared to the present study.

7.4 Conclusions

The study demonstrates a sustainable approach for production of bio-crude oil via one step direct hydrothermal liquefaction of mixed microbial biomass generated through co-cultivation of tailored designed microalgae-bacteria consortium in paper industry wastewater. Suitability of bio-crude oil as potential replacement of petroleum fuel was evaluated in terms of energy density, elemental composition, chemical composition and physical properties. While, analyses indicate good oil quality, catalytic up-gradation of bio-crude oil is needed for deoxygenation and denitrogenation before it can be blended with petroleum fuel.

7.5 References

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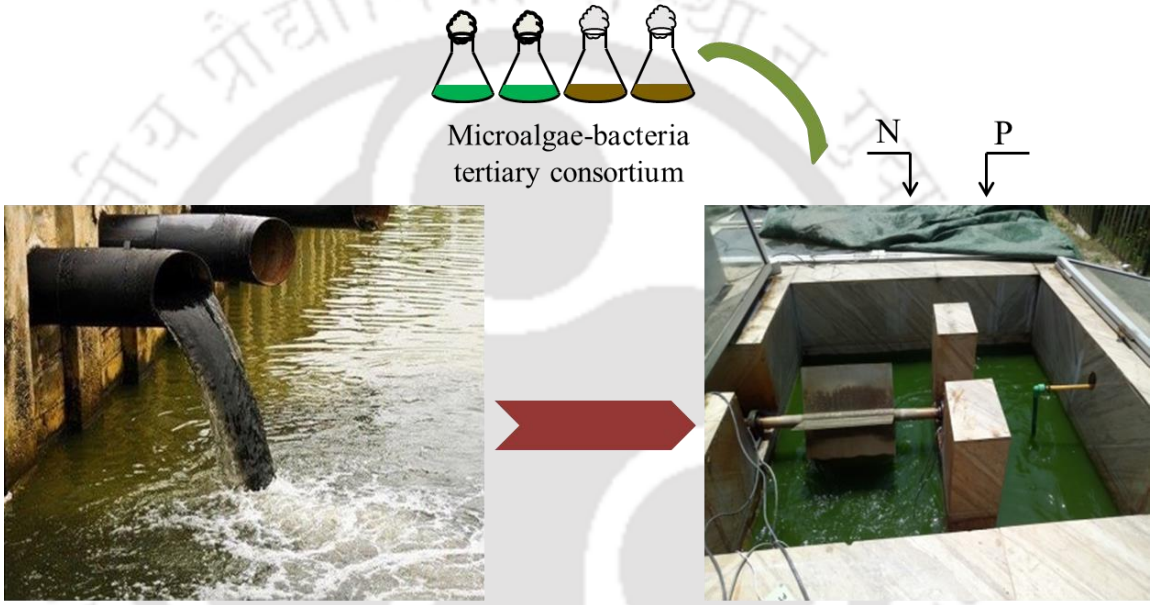
When I see people throwing away things we could use, I feel angry.

Mother Teresa

Chapter 8

Large scale demonstration of the developed process for biomass production



Large scale cultivation of microalgae-bacteria tertiary consortium in open raceway pond using paper industry wastewater.

8.1 Background and motivation

During the last couple of years, the search for alternative environmentally neutral fuels had brought back the interest in microalgae and bacteria systems such as high raceway algal pond (HRAP) (Chisti, 2007). As the biofuel production from microalgae is currently too expensive, the interest is focused towards the development of wastewater treatment systems with a neutral energy footprint which can produce both marketable products and effluent water for reuse. In comparison to conventional wastewater treatment systems, the potential of costs savings, including electrical power, are great enough to promote HRAPs independently from biofuels production (Suganyaa et al., 2016). In the previous study (Section 6.3.2) the performance of microalgae-bacteria tertiary consortium in terms of growth and wastewater treatment efficiency was evaluated via fed batch mode of cultivation in a photobioreactor under controlled growth condition using paper industry wastewater. The satisfactory performance of the consortium was marked by high biomass titer of 4.1 g L^{-1}. However, for the process to be realized at commercial scale, it was authoritative to assess the performance of the consortium in large scale outdoor condition under fluctuating environmental conditions such as illumination and temperature. To that end, the tertiary microalgae-bacteria consortium was grown in 500 L capacity open raceway pond using paper industry wastewater.

8.2 Materials and methods

8.2.1 Microalgae-bacteria tertiary consortium

Microalgae-bacteria tertiary consortium, considered in the present study was earlier demonstrated to exhibit superior performance in terms of growth and nutrient removal efficiency from different types of wastewater (Section 6.3.1). The consortium consists of two microalgae *Chlorella sorokiniana* strain DBWC2 &

Chlorella sp. strain DBWC7 and two bacteria *Klebsiella pneumoniae* strain ORWB1 & *Acinetobacter calcoaceticus* strain ORWB3 (Section 4.2.1).

8.2.2 Generation of microbial biomass in large scale open raceway pond utilizing paper industry wastewater

With the aim of assessing the growth performance and nutrient removal efficiency in large scale and fluctuating environmental condition, the consortium was grown in 500 L open raceway pond (Length: 144 cm, width: 144 cm, and depth: 30 cm) with a working volume of 350 L under outdoor condition (Spectrochem Instruments Pvt. Ltd., India). The consortium was grown in paper industry wastewater collected from effluent treatment plant of Eco Tech Papers Factory, Kamalpur, Guwahati, Assam. Initial characterization of the wastewater sample was carried out in order to access its physico-chemical properties (Table 8.1). The pH value of the wastewater sample was adjusted to the optimal value of 8.65, while the concentration of total nitrogen and phosphorus was kept unaltered as per section 5.3.2. Inoculation of the pond was carried out with 18% (v/v) inoculum of tertiary consortium (Section 5..31) in which microalgae and bacteria were present in 1:1 ratio (Section 5.3.2). For microalgae and bacteria, inoculum preparation was carried out in 20 L transparent plastic bottles and culture from mid log phase was transferred to the pond. Rotational speed of the paddle wheel was maintained at 150 rpm and evaporation loss was compensated on regular interval.

Table 8.1 Physico-chemical property of paper industry wastewater (PWW)

Parameters	**Values**
Colour	Light brown
pH (Unit)	6.3
NO_3^--N (mgL^{-1})	111
NO_2^--N (mgL^{-1})	0
NH_4^+-N (mgL^{-1})	271
TN (mgL^{-1})	382
TP (mgL^{-1})	69.5
COD (mgL^{-1})	5850
TSS (mgL^{-1})	210
TDS (mgL^{-1})	851
TS (mgL^{-1})	1061
Electrical Conductivity (dSm^{-1})	2

With the aim of maximizing the biomass titer and productivity, intermittent feeding of industrial grade urea and single super phosphate (SSP) was performed in order to maintain concentration of total nitrogen and phosphorous at their respective optimal values of 0.29 g L^{-1} and 49.5 mg L^{-1}, respectively throughout the cultivation period (section 5.3.2). The batch was run for 35 days with regular sampling at an interval of 24 h to obtain dynamic profile of growth, pH, and utilization of total nitrogen, phosphate and COD. Heavy metal removal efficiency was calculated by analysing the sample at an interval of every 5 days. The removal efficiency of nutrients and heavy metals was calculated using Eq. (8.1):

$$Removal\ efficiency\ (\%) = \left(\frac{S_i - S_f}{S_i}\right) \times 100 \qquad (8.1)$$

where, S_i and S_f are the initial and final concentration of a specific nutrient or heavy metal at the beginning and end of the batch, respectively.

8.2.3 Analysis of growth, nutrient utilization, COD and heavy metal ion removal

Analysis of growth, nutrient utilization, COD, and heavy metal ion removal was carried out at every sampling time point. Chlorophyll-a was estimated following the protocol described in section 3.2.3. The total nitrogen content was determined as the summation of the individual concentrations of nitrate, nitrite and ammoniacal nitrogen. Estimation of nitrate was followed by the protocol described in section 3.2.3. The mole fraction and the corresponding nitrogen concentration in nitrite and ammonia was measured and calculated according to the Standard Methods for Examination of Water and Wastewater (APHA, 2017). Phosphate, COD and heavy metal concentration was estimated using the procedure described in section 3.2.3. All chemicals and reagents were procured from Hi Media, India and were of analytical grade.

8.3 Results and discussion

8.3.1 Generation of microbial biomass feedstock in large scale open raceway pond utilizing paper industry wastewater

In an earlier study (Section 6.3.2), the performance of microalgae-bacteria tertiary consortium in terms of growth and wastewater treatment efficiency was evaluated via fed batch mode of cultivation in a photobioreactor under controlled growth condition using paper industry wastewater. The satisfactory performance of the consortium was marked by high biomass titer of 4.1 g L^{-1} (Section 6.3.2). However, for the process to be realized at commercial scale, it was imperative to assess the performance of the consortium in large scale outdoor condition where microbes are be subjected to fluctuating environmental conditions such as

illumination and temperature. To that end, the tertiary microalgae-bacteria consortium was grown in 500 L capacity open raceway pond using paper industry wastewater. The maximum biomass titer of 3.96 g L^{-1} was achieved within 19 days of cultivation followed by insignificant change in titer till the end of the batch (Fig. 8.1A). The maximum biomass productivity was estimated to be 208.4 mg L^{-1} day^{-1}. Maximum biomass productivity of 213 mg L^{-1} day^{-1} was obtained from co-cultivation of *Chlorella Saccharophila* and *Scenedesmus* sp. in 600 L HRAP utilizing dairy wastewater over cultivation period of 10 days (Hena et al., 2015). On contrary to the sharp increase in total dry cell mass, the rate of growth of microalgae was found to be comparatively lower during first 10 days of cultivation and thereafter, the microalgal growth was found to increase noticeably (Fig. 8.1B). Hence, while first phase of microbial growth was mainly driven by the bacterial part, the second phase of the biomass generation was contributed by the microalgal part of the consortium. This microbial growth pattern was found to be corroborated well with the change in culture pH (Fig. 8.1C). While the sharp decrease in culture pH in the initial phase of cultivation may be attributed to formation of CO_2 by means of metabolic activity of the bacteria partners, the gradual increase in broth pH in the later phase of the cultivation might be a combinatorial effect of CO_2 utilization by the microalgae partners present in the tertiary consortium during their growth and excretion of basic metabolites from the biodegradation of organic compounds (Delgadillo-Mirquez et al., 2016). The results point towards uncompromised growth performance of the tertiary consortium in large scale open raceway pond even under fluctuating sun light intensity from zero (in the night) to a maximum of 2237 μE m^{-2} s^{-1} and temperature range of 23 ˚C to 39 ˚C (Fig. 8.1D).

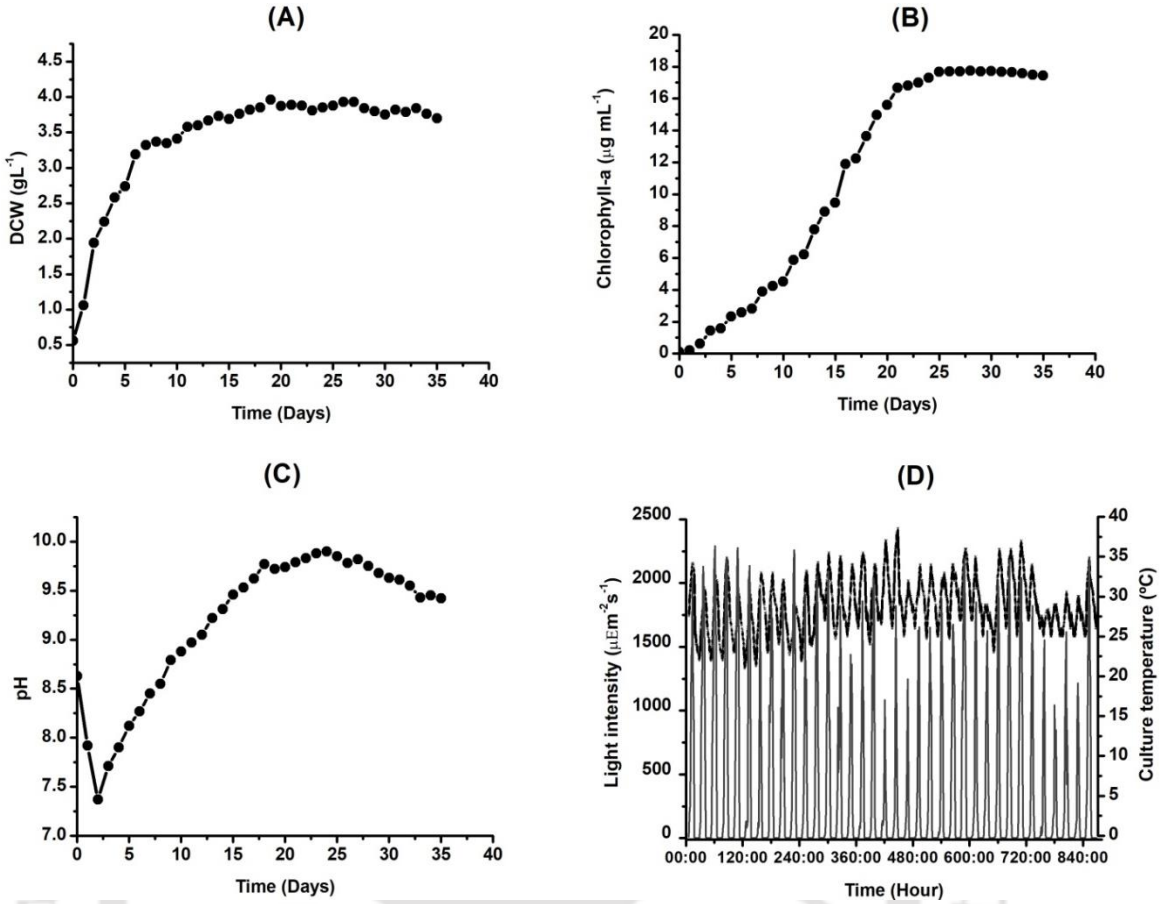


Fig. 8.1 Dynamic profiles of (a) total biomass concentration (DCW, g L^{-1}); (b) growth of microalgae (Chlorophyll-a, µg mL^{-1}); (c) pH and (d) fluctuating environmental parameters light intensity (continuous grey line) and temperature (continuous black line)

In the present study, COD removal efficiency of the tertiary consortium was also found to be as high as 90% in large scale operation under uncontrolled growth condition (Fig. 8.2A). COD removal efficiency of 85.44% was reported for co-cultivating of *Chlorella* sp., *Scenedesmus* sp. and *Stigeoclonium* sp. in HRAP containing 60 L municipal wastewater (Kim et al., 2014). The removal efficiency of two heavy metals Nickel and Chromium was found to be 68.85% and 52.87%, respectively (Table 8.2). Rapid utilization of native sources of nitrogen and phosphorous from the wastewater by the consortium was evident from the dynamic profile of the nutrients (Fig. 8.2B and C). The concentration of total nitrogen came

down from 0.38 g L^{-1} to 0.29 g L^{-1} within three days of cultivation, while concentration of phosphate was reduced from 69.5 mg L^{-1} to 49.5 mg L^{-1} within four days of cultivation. In the present study, with the aim of achieving higher microbial biomass titre, concentration of total nitrogen and phosphate was maintained at 0.29 g L^{-1} and 49.5 mg L^{-1} via intermittent feeding of industrial grade urea and single super phosphate, respectively (Fig. 8.2B and C).

Table 8.2 Percentage of heavy metal ion removal of tertiary consortium

Days	Heavy metal ion	
	Nickel (Ni)	Chromium (Cr)
5	8.19	8.04
10	27.86	20.68
15	47.54	36.78
20	54.09	43.67
25	59.01	47.12
30	67.21	48.27
35	68.85	52.87

8.4 Conclusions

Large scale demonstration of the developed process was carried out in open raceway pond under fluctuating environmental condition to evaluate of the performance efficiency of tertiary consortium in terms of total biomass titer and wastewater treatment. High biomass titer of 3.96 g L^{-1} with 90% of COD removal efficiency was achieved, depicting robust performance of the microalgae-bacteria consortium in industrial wastewater and under fluctuating environmental condition.

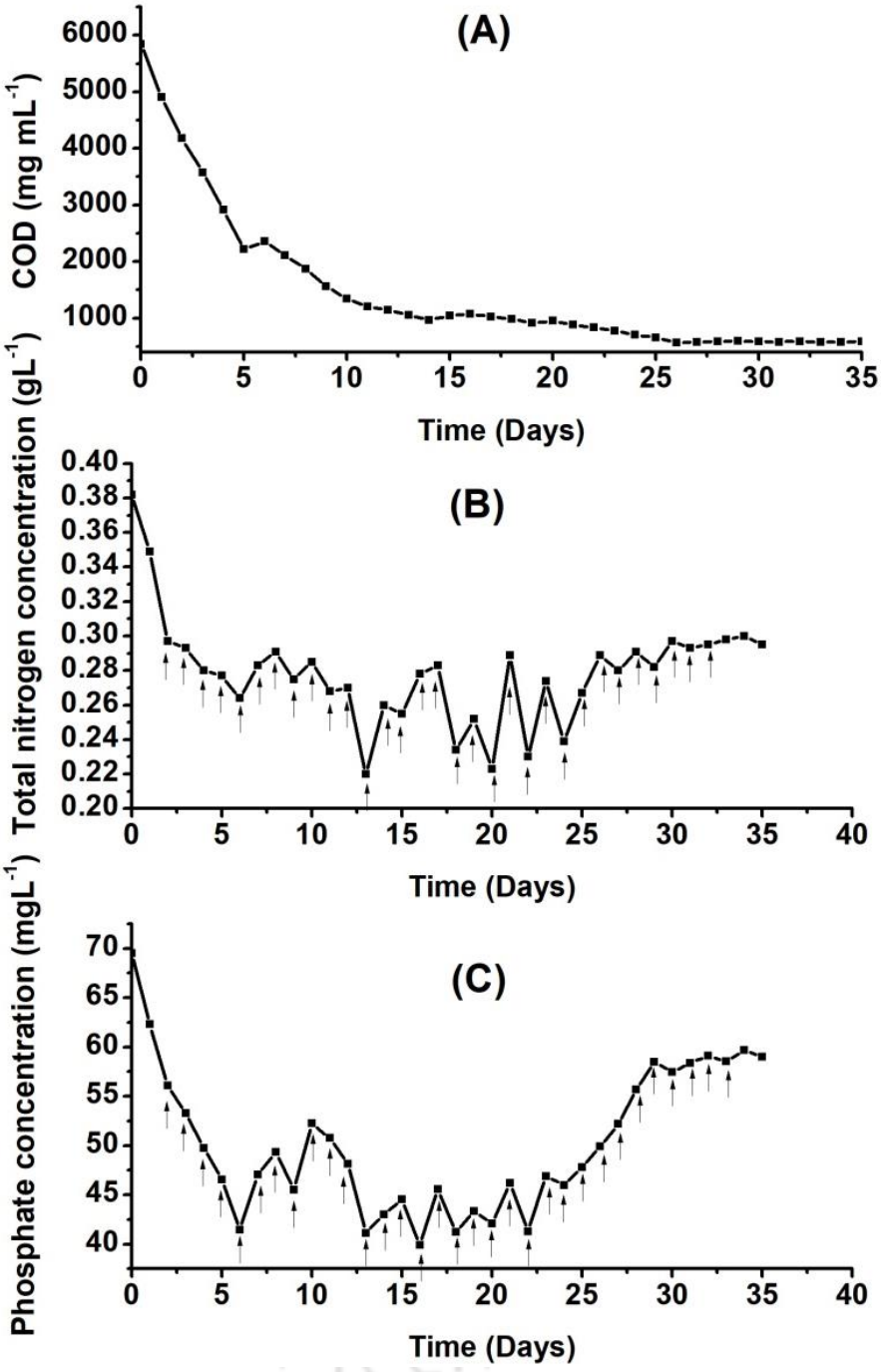


Fig. 8.2 Dynamic profiles for (a) COD; (b) total nitrogen (g L^{-1}) and (c) phosphate (mg L^{-1}). The tertiary consortium was grown on paper industry wastewater in open raceway pond under fed-batch mode with intermittent feeding of nitrogen and phosphorus source

8.5 References

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Progress is impossible without change, and those who cannot change their minds cannot change anything

George Bernard Shaw

Chapter 9

Conclusions

Six microalgal and three bacterial strains were isolated from oil refinery wastewater and screened in formulated artificial wastewater (AWW) to check their growth potential. In screening process, the microalgal strains (A1, A2, A4 and A6) and bacterial strains (B1, B2 and B3) showed significant growth which were further characterized in AWW to evaluate their growth potential as well as nutrient removal efficiency. With the aim of evaluating the performance in terms of biomass titer and waste water treatment efficiency, eight primary combinations of microalgae-bacteria consortia (combinations of one microalgal and one bacterial strain chosen from a pool of four microalgal and two bacterial isolates) were characterised in AWW. Among the primary consortia; A2B1 and A4B3 were selected on the basis of total biomass titer (microalgae plus bacteria), nitrate and COD removal efficiency. In the next step, performance evaluation was carried out for four secondary of microalgae-bacteria consortia by considering either one microalgal-two bacterial strains or two microalgal-one bacterial strain, chosen from a pool of the microalgal and bacterial isolates present in the selected better performing primary combination(s). Among other secondary consortia, A2A4B1 and A2A4B3 have showed improved biomass titer as well as nitrate and COD removal efficiency. Finally, characterization was performed for one tertiary combination of microalgae-bacteria consortium which comprised of two microalgal-two bacterial strains chosen from A2A4B1 and A2A4B3. The biomass titer, nitrate removal efficiency and COD removal efficiency

of the tertiary consortium was found to be 1.27gL^{-1}, 79.78% and 62.43% respectively. To enhance the biomass titer of the tertiary consortium; biological parameters (inoculum size and inoculum ratio) and physico-chemical parameters (initial pH of the media, initial nitrate concentration, and initial phosphate concentration) were optimised via statistical method. The biomass titer of 1.73 $gL^{-1}$was resulted at optimised parameters having inoculum size of 18%, initial pH of 8.65, initial nitrate concentration of 1.79 $gL^{-1}$and initial phosphate concentration of 49.5 mgL^{-1} and inoculum ratio of 0.97. In the next step, with the aim of establishing the feasibility of application at industrial scale, performance of this tertiary consortium in terms of biomass titer and wastewater treatment efficiency was evaluated on four different types of wastewater: paper industry wastewater (PWW), textile industry wastewater (TWW), leather industry wastewater (LWW) and municipal wastewater (MWW). The performance of tertiary consortium in terms of biomass titer, nitrate and COD removal efficiency was 2.97 gL^{-1}, 89.3%, and 94.23% in PWW which is quite remarkable as compared to other wastewaters. Therefore, PWW was selected for further batch and fed-batch mode of cultivation in an automated photo-bioreactor. The total microbial biomass titer obtained from fed-batch cultivation is 4.1 gL^{-1} which is 29.33% more than the biomass titer obtained in batch cultivation (3.17 g L^{-1}). The wet microbial biomass thus obtained from fed-batch cultivation was further subjected to HTL. The bio-crude oil yield obtained at un-optimized HTL condition was 15%. To attain maximized bio-crude oil yield, the HTL process parameters such as temperature, reaction time and biomass loading ratio were optimized further via statistical method. 21.73% bio-crude oil yield was resulted at optimized temperature of 299.73 ºC, reaction time of 64.95 min and biomass loading of 16.14 (%, w/v) which is 44.86% higher than the yield at un-optimized conditions. Bio-crude oil with

energy recovery of 42.95% and heating value of 33.11 MJ kg^{-1} reflects 81.7% and 73.4% heating value of biodiesel and diesel, respectively indicates its potential as an alternative to conventional fossil fuels. High percentage of hydrocarbon content in bio-crude oil indicates good oil quality; presence of significant esters fraction might offer resemblance to biodiesel. Finally, with the aim to demonstrate large scale cultivation for sustainable production of biomass feedstock coupled with wastewater treatment, the tertiary microalgae-bacteria consortium was grown in 500 litre capacity open pond containing 350 litre of PWW as culture medium in fed-batch mode. The total biomass titer was found to be 3.96 g L^{-1} with COD removal efficiency of 90% during 19 days of cultivation under diurnal condition. The final outcome of the present study was to develop an efficient process with the tertiary consortium that can be industrially scalable for bio-crude oil production as well as effective wastewater treatment.

Engineering Significance

- ✓ Development of novel and robust microalgal and bacterial strains.
- ✓ Microalgae-bacteria consortium able to significantly remove pollutants from wastewater.
- ✓ An optimized HTL process has been demonstrated for enhanced bio-crude oil yield from microbial biomass.
- ✓ Demonstration of a scalable technology for wastewater bioremediation using novel microalgae-bacteria consortia.

Future Prospects

- ✓ Scale up of the bioprocess involving tertiary consortium towards high cell density cultivation and wastewater treatment in outdoor conditions.
- ✓ Coupling of large scale production with continuous HTL of harvested biomass.
- ✓ Catalytic up-gradation of bio-crude oil for improved yield and quality.
- ✓ Evaluation of bio-crude oil distillate as a potential alternate to transportation fuel.
- ✓ Techno-economic assessment of the proposed bioprocess strategy.

List of Publications

Published Manuscripts

1. **Makut, B. B.,** Das, D., Goswami, G. (2019). Production of microbial biomass feedstock via co-cultivation of microalgae-bacteria consortium coupled with effective wastewater treatment: A sustainable approach. Algal research, 37, 228-239.
2. Goswami, G.[1]**, Makut, B. B.[1]**, Das, D. (2019). Sustainable production of bio-crude oil via hydrothermal liquefaction of symbiotically grown biomass of microalgae-bacteria coupled with effective wastewater treatment. Scientific reports, 9(1), 1-12.

   (**1** ***represents equal authorship***)
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List of Conferences/Workshops/ Symposia

1. Bidhu Bhusan Makut, Debasish Das (2019). 'Sustainable production of bio-crude oil through effective wastewater treatment using indigenous microalgal strain' international conference on Algal Biomass, Bio products and Biofuels organised by Elsevier at CO, US.
2. Bidhu Bhusan Makut, Debasish Das, Gargi Goswami (2017). 'Development of a sustainable process for generation of microbial biomass as a feedstock for biofuel production.' BPI international conference conducted by IIT Guwahati.
3. Bidhu Bhusan Makut, Basavaraj Palabhanvi, Debasish Das (2017). 'National conference on Biodiversity, Biotechnology and Biology of Algae' organized by University of Madras.
4. Bidhu Bhusan Makut, Debasish Das (2016). 'Construction of microalgal-bacterial consortium for effective wastewater treatment and microalgal biomass production'. "Recycle" international conference organized by ACE IIT Guwahati.
5. Work shop on "Algal biotechnology" 2015 organized by IIT Bombay.
6. Work shop on "FERIAP" 2015 organized by Centre for Energy, IIT Guwahati.

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Vitae

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