BIOENGINEERED SYSTEMS FOR THE BIODEGRADATION AND TOXICITY REMOVAL OF ENDOCRINE DISRUPTING PHTHALATES (EDPs)

A Thesis

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for the award of the degree of

DOCTOR OF PHILOSOPHY

by

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January 2023

Dedicated to my Parents and Family members

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DECLARATION

I, hereby declare that the research findings embodied in this thesis entitled **"Bioengineered systems for the biodegradation and toxicity removal of endocrine disrupting phthalates (EDPs)"** is the result of investigations carried out by me under the supervision of Prof. Kannan Pakshirajan, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy.

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

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Bioengineered systems for the biodegradation and toxicity removal of endocrine disrupting phthalates (EDPs)"** by Dipak Kumar Kanaujiya for the award of the degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India. This work has not been submitted for the award of any other degree.

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ABSTRACT

ABSTRACT

Phthalic acid esters (PAEs) are released into the environment from a wide variety of sources. Due to the adverse effect on human health, PAEs-containing wastewater need be treated before discharge. Several conventional physicochemical methods have been extensively studied for PAEs degradation. However, owing to one or more disadvantages of these methods, biological treatment using suitable microorganisms is of recent interest. Numerous bacteria and fungi can degrade these recalcitrant organic pollutants, but they often fail at high initial concentrations due to their high toxicity. Moreover, for industrial application of these microorganisms, bioprocess development with efficient bioreactor systems is highly essential.

The current study demonstrated the complete degradation of various PAEs and their mixture even at a high initial concentration using different treatment systems, viz. continuous stirred tank bioreactor (CSTB), two-phase partitioning bioreactor (TPPB) and continuous with biomass recycle system followed by microfiltration under aerobic condition. In the initial screening study involving three bacteria *(Rhodococcus opacus, Cellulosimicrobium funkei and Ochrobactrum sp.*), *C. funkei* was identified to be the best for the biodegradation of PAEs as a single substrate. Complete biodegradation of dimethyl phthalate (DMP) and diethyl phthalate (DEP) as a single substrate for *C. funkei* was achieved up to 2500 and 1500 mg/L, respectively, in batch shake flasks. In the mixture study, more than 94 % total degradation of the EDPs was achieved at initial DMP and DEP concentrations of 2000 and 1500 mg/L, respectively. Intermediates formed during the biodegradation of DMP and DEP further confirmed phthalic acid as the central metabolite. The mixture study showed that an increase in the DMP concentration resulted in a high degradation of DEP, whereas DMP degradation reduced with an increase in the DEP concentration. Due to the low molecular weight and a short side chain of DMP, its specific degradation rate was more than that of DEP, which is a high molecular weight compound containing a long side chain.

A continuous stirred tank bioreactor (CSTB) was further examined for the biodegradation of DMP and DEP by *C. funkei*. Complete degradation was achieved even up to 3000 and 2000 mg/L initial concentrations of DMP and DEP, respectively, using the CSTB, which is attributed to the controlled conditions of aeration, agitation and pH in the bioreactor. Stoichiometric and mass balance analyses carried out in this batch bioreactor study clearly established the effectiveness of the biodegradation process using the CSTB. Degradation of a mixture of DMP and DEP was also found to be enhanced using the CSTB. Fed-batch operation with the reactor further revealed 82.87 % degradation efficiency of the EDPs mixture even at their high initial concentrations. Under the continuous mode of operation using the CSTB, a high degradation rate (178.37 mg/L·h) was achieved even at high total inlet loading rate (ILR) of 218.75 mg/L·h. The degradation rate under the continuous mode of operation was further enhanced (218.68 mg/L·h) by recycling the biomass into the bioreactor following microfiltration for biomass separation from the effluent using a tubular ceramic membrane. High germination index and low brine shrimp mortality with the phthalate degraded samples demonstrated the potential of the CSTB integrated with microfiltration for treating EDPs containing wastewater.

Another novel bacterium, *Gordonia sp*., was studied for the complete degradation of a mixture of six phthalates in the CSTB system. Complete degradation (100%) of phthalates, even at high total ILR (61.67 mg/L·h), was achieved using CSTB under continuous with biomass recycle mode, suggesting that the integrated biodegradation-microfiltration approach is best suited for efficient degradation of phthalates in treating EDPs containing wastewater. In addition, toxicity analysis of the degraded phthalates revealed very high GI and low mortality of brine shrimps, further confirming its potential for treating such wastewater. Hence, the bioengineered system consisting of CSTB with the degrading bacterium demonstrated successful biodegradation of different phthalates in synthetic wastewater under different operation modes of batch, fed-

batch, continuous and continuous with biomass recycle. The major mechanism of the biodegradation of EDPs by *Gordonia sp.* involved hydrolysis and/or esterification of the alkyl side chain of phthalates.

A novel bioengineered system, a two-phase partitioning bioreactor (TPPB) system, was evaluated for the biodegradation of DMP and DEP, which showed very high biodegradation efficiency of phthalates even at 3000 (2000 DEP and 1000 DMP) mg/L initial concentration and within a short duration (60 h). In this TPPB system, the non-aqueous phase liquid (NAPL) silicone oil used was found to be biocompatible, non-bioavailable and suitable for DEP delivery to the degrading bacterium. The mechanism of phthalate degradation in the TPPB system involved the slow release of the EDPs from the non-aqueous phase (silicone oil) to the aqueous phase as per real-time demand of *C. funkei*, which also aided in overcoming the substrate inhibition effect of the EDPs. Besides, a very high volumetric oxygen mass transfer coefficient due to the added silicone oil in the TPPB was observed for enhancing the EDPs biodegradation in this bioengineered system. Very high biodegradation efficiency values of the compounds by *C. funkei* were reported using the TPPB system. A high GI value of 86.17% of chickpea seeds soaked in phthalate degraded sample taken from the TPPB system along with a low mortality value of 13.33% of brine shrimps, demonstrated the utility of the TPPB-based bioengineered system in toxicity removal and treatment of phthalates from wastewater.

ORGANIZATION OF THESIS

The present thesis is divided into six chapters. **Chapter 1** provides a general introduction to phthalates, available literature on the sources, exposures, and adverse effects of EDPs on the environment, animal, and human health. Different remediation methods, including physical, chemical, biological, and advanced treatment technologies, to treat EDPs-containing wastewater are presented along with the aim and objectives of this thesis work. In **Chapter 2**, screening of potential bacteria for biodegradation of DMP and DEP, characterization and identification of the screened bacteria, biodegradation of DMP and DEP at different initial concentrations in shake flask, kinetic modelling, phthalate degradation in mixture and elucidation of phthalate biodegradation pathway are reported. **Chapter 3** describes the performance evaluation of the CSTB reactor operated under different modes of operation - batch, fed-batch, continuous and continuous with biomass recycle followed by microfiltration using an integrated tubular ceramic membrane for biodegradation and toxicity removal of DMP and DEP. **Chapter 4** reports the biodegradation of a mixture of 6 priority phthalates by using *Gordonia sp.* in a CSTB operated under different operation modes - batch, fed-batch, continuous and continuous with biomass recycle followed by microfiltration using an integrated tubular ceramic membrane along with toxicity assessment of the degraded phthalates. **Chapter 5** establishes the importance of the TPPB system over the conventional single-phase system for achieving high biodegradation efficiency of phthalates even at very high initial concentrations and toxicity removal assessment by seed germination and brine shrimp mortality assays. **Chapter 6** provides a summary and appropriate conclusion based on this thesis work. Some recommendations for future research in this field are also made in this chapter.

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Glossary of Acronyms

ASP	Activated sludge process	**DCW**	Dry cell weight
AOPs	Advanced oxidation process	**DTG**	Derivative thermogravimetric
ARR	Aquifer recharge and recovery	**ESI**	Electrospray ionization mode
BBP	Benzyl butyl phthalate	**EDC**	Endocrine - disrupting chemical
BOD	Biochemical oxygen demand	**EU**	European union
BCF	Bioconcentration factor	**FE-SEM**	Field emission - scanning electron microscopy
BH - MSM	Bushnell Haas - minimal salt medium	**GI**	Germination index
BHA	Butylated hydroxy anisole	**GAC**	Granular activated carbon
BLAST	Basic local alignment search tool	**HMW**	High molecular weight
COD	Chemical oxygen demand	**HPLC**	High - performance liquid chromatograph
CW	Constructed wetland	**HRT**	Hydraulic retention time
CSTB	Continuous stirred tank bioreactor	**ILR**	Inlet loading rate
DEHP	Di(2-ethylhexyl) phthalate	**IPA**	Isophthalic acid
DDT	Dichloro diphenyl trichloroethane	**IPM**	Isopropyl myristate
DEP	Diethyl phthalate	**LMW**	Low molecular weight
DMP	Dimethyl phthalate	**LB**	Luria Bertani
DBP	Di-n-butyl phthalate	**MBR**	Membrane bioreactor
DnOP	Di-n-octyl phthalate	**MBBR**	Moving bed biofilm reactor
DOM	Dissolved organic matter	**MTCC**	Microbial type cell culture
DO	Dissolved oxygen	**MF**	Microfiltration

NAPL	Non - aqueous phase liquid	**PCPP**	Personal care products and pharmaceuticals
NCBI	National centre for biotechnology information	**PAE**	Phthalic acid ester
nH	n-Hexadecane	**TA**	Terephthalic acid
OD	Optical density	**TGA**	Thermogravimetric analyzer
PA	Phthalic acid	**TPPB**	Two - phase partitioning bioreactors
PVC	Poly vinyl chloride	**US -EPA**	United states - environmental protection agency
PCB	Polychlorinated biphenyl	**UASB**	Up - flow anaerobic sludge blanket
PHA	Polycyclic aromatic hydrocarbon	**UPGMA**	Unweight pair group method with arithmetic mean
RBF	Riverbank filtration	**VP**	Vapor pressure
SBR	Sequencing batch reactor	**WWTP**	Wastewater treatment plant
SO	Silicone oil	**WHO**	World health organization
SRT	Sludge retention time	**XRD**	X - ray diffraction
SSE	Sum of square error		

List of Notations

K_{AW}	Air-water partition coefficient	K_{OW}	Octanol-water partition coefficient
ΔP	Applied pressure	**pa**	Pascal
$Y_{X/S}$	Biomass yield	**%**	Percentage
R^2	Coefficient of determination	**r**	pore size
°C	Degree centigrade	ε	Porosity
ρ_w	Density of water	α	Recycle ratio
g	Gram	**rpm**	Rotations per minute
g/L	Gram per liter	**s**	Second
K_s	Half saturation constant	**q**	Specific biodegradation rate
h	Hour	μ	Specific growth rate
K_I	Inhibition constant	**A**	Surface area of membrane
°K	Degree Kelvin	**t**	time
kPa	Kilo pascal	τ	Tortuosity factor
q_{max}	Maximum specific biodegradation rate	**vvm**	Vessel volumes per minute
μ_{max}	Maximum specific growth rate	μ	Viscosity of water
m	Meter	**v/v**	Volume/volume
mg/L	Milligram per liter	**kLa**	Volumetric oxygen mass transfer coefficient
Min	Minute	J_w	Water flux
MW	Molecular weight	L_h	Water permeability of membrane
K_{OA}	Octanol-air partition coefficient	**w/v**	Weight/volume

Chapter 1

Introduction and Review of literature

1.1. Introduction

Water is a valued resource essential for sustaining all living beings and is associated with significant human activities, such as agriculture, industry and domestic uses. However, many contaminants, such as daily household products, personal care products and pharmaceuticals (PCPP), industrial chemicals, pesticides, flame retardants, amitrole, atrazine, phthalic acid esters (PAEs), benzenehexachloride (BHC), butylated hydroxyanisole (BHA), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbon (PAH), etc., are released from different sources (Behera et al., 2011; Gao and Wen, 2016; Hu et al., 2021a; Kanaujiya et al., 2019; Luo et al., 2014a; Schriks et al., 2010; Yoon et al., 2010). Pollution due to these contaminants in groundwater, open water bodies and soil environment is interrelated, and leads to adverse effects on aquatic organisms and human health distress by becoming a part of the ecosystem (Daughton, 2010; Kanaujiya et al., 2019). Among these contaminants, phthalic acid esters (PAEs) are termed as emerging environmental pollutants due to their widespread occurrence in various ecological systems (Figure 1.1) (Das et al., 2021; Zarean et al., 2019).

Since the 1920s, PAEs have been widely used as plasticizers in manufacturing and processing a broad range of plastic products such as household stuff (children's toys, cosmetics, clothing, furnishings, nutritional supplements/food packaging, etc. (Das et al., 2021; Kang et al., 2012; Pang et al., 2021). They are also used for agricultural purposes (fertilizers, pesticides, insecticides, mulch plastic), as building and industrial materials (adhesives, cleaning materials, electronics, lubricants, paints and varnishes, waxes, inks, etc.), and for other applications (e.g., medical devices, pharmaceuticals, etc.) (Hu et al., 2021a; Kang et al., 2012; Net et al., 2015; Tuan Tran et al., 2022). Phthalates are generally used as plasticizers to improve the flexibility, processability, durability and strength of polymer materials. The demand for PAEs is

anticipated to increase further owing to their low production costs and the absence of appropriate and affordable substitutes for use in plastic products (Boll et al., 2020)

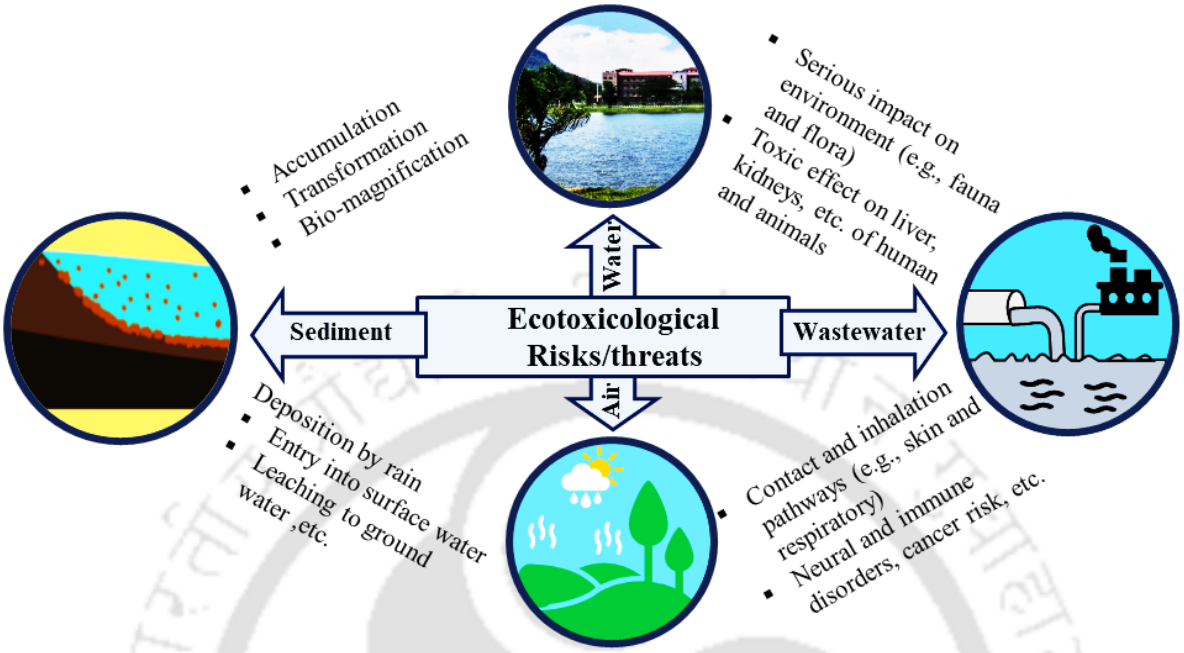


Figure 1.1: Effect of PAEs on the environment and associated health and ecotoxicological risks.

Despite the many advantages of PAEs, they pose numerous negative impacts on the environment and health, worldwide. When PAEs are used as plasticizers, they can slowly release and leach from the host polymers into the environment since they are not chemically bonded to the polymer molecules (Akhbarizadeh et al., 2020; J. Zhang et al., 2018; Zhu et al., 2022). As a result, PAEs are detected in diverse environments, such as the hydrosphere (water, wastewater, surface water, etc.), lithosphere (soil, sediment, etc.) and the atmosphere (Barreca et al., 2014; Blair et al., 2009; Dargnat et al., 2009; Liou et al., 2014). A more striking indication of widespread PAE occurrence is that PAE residues are detected in drinking water, and long-term exposure to PAEs may lead to dysfunctions of the human reproductive, nervous and immune systems (Andersen et al., 2018; Benjamin et al., 2017; Matsumoto et al., 2008). As a consequence, the United States Environmental Protection Agency (USEPA), the European Union (EU) and other pollution monitoring agencies have listed six PAE compounds as the

priority pollutants, including dimethyl phthalate (DMP), benzyl butyl phthalate (BBP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP), di-n-octyl phthalate (DOP) and di(2-ethylhexyl) phthalate (DEHP) (Gao et al., 2017; Kanaujiya et al., 2022; Net et al., 2015).

In recent years, there is a widespread concern about the presence of PAEs in the aquatic environment and their potential impact on humans as well as the entire ecosystem. However, it is very difficult to guess which environmental and communal health complications may arise from the existence of PAEs in surface water ecosystems as in most cases the adverse effects occur over a prolonged time period. Also, their specific concentrations typically observed in the environment are lower than the concentrations able to cause direct adverse effects, but due to bioaccumulation and biomagnification their concentrations in living systems may get amplified significantly (Benjamin et al., 2017; Quinn et al., 2009). Hence, there is a dire need for novel treatment technologies for PAEs at their source before they are released into the environment.

Over the last decade, a variety of physical, chemical and biological approaches have already been examined to remove or degrade the residues of PAEs to protect the environment, human and animal health (Abdel daiem et al., 2012; Benjamin et al., 2017; Gao and Wen, 2016; Ghosh and Sahu, 2022). Among these different approaches, physicochemical processes have certain limitations, such as high-energy consumption, ineffective micropollutant removal, difficulty in treating large volumes of wastewater, generation of hazardous sludge in huge amounts, corrosion and recontamination issues, etc. On the other hand, different biological treatment technologies are used for PAEs degradation, including aerobic and anaerobic microbial remediation, activated sludge processes (ASP), constructed wetland (CW), membrane bioreactors (MBRs), conventional ASP, sequencing batch reactor (SBR) and up-flow anaerobic sludge blanket (UASB) (Das et al., 2021; Gani and Kazmi, 2020; Ghosh and Sahu, 2022; Hu et al., 2021a; Tuan Tran et al., 2022; Zhu et al., 2022). Different degradation pathways and

reactions are reported to degrade and transform phthalate esters to reduce their environmental impact (Ahuactzin-Pérez et al., 2016; Tao et al., 2019a). However, being highly toxic and recalcitrant to biodegradation, PAEs removal using the conventional physicochemical systems still remains a challenge. Moreover, the treatment efficiencies of the conventional biological systems using mixed culture of organisms are lower than that of the physicochemical processes. In addition, the technical aspects required to implement engineered bioremediation processes to treat PAEs under environmental conditions are still lacking. Hence, there is a need to study PAEs degradation by novel microorganisms using bioengineered systems that are highly efficient and economical at a larger scale.

1.2. Phthalic acid esters

Phthalates or phthalic acid esters (PAEs) are chemically synthesized compounds and are mainly produced from phthalic anhydride and a suitable alcohol (Hu et al., 2021a). PAEs consist of a benzene ring to which two carboxylic groups are attached. Generally, three isomeric forms, viz. ortho, para and meta, of phthalates are possible depending on the position of the carboxylic groups. Phthalic acid (PA) is an ortho-isomer, the most abundant phthalate manufactured worldwide, and is mainly used as a plasticizer. Terephthalic acid (TPA) is known as a para-isomer and its esters are primarily utilized as monomers in the production of polyethylene terephthalate (PET) and a variety of polyester fibres. Isophthalic acid (IPA) is known as a meta-isomer and its esters are utilized for synthesizing resin, dope, and different industrial polymers (Benjamin et al., 2015; Das et al., 2021). These isomers of PAEs play a vital role in evolving various medical, industrial and commercial applications.

1.2.1. Physicochemical properties of PAEs

PAEs exhibit different physicochemical characteristics depending on their chemical structure and alkyl side chain length. They are chemically stable, odourless, tasteless, colourless and liquid at ambient temperature (Tran et al., 2021). Depending on the size of their alkyl chain,

their melting point is in between -25 and -55 °C and their boiling point is >250 °C. Additionally, as the carbon number of PAEs increases, the partition coefficients for air-water (K_{AW}), octanol-water (K_{OW}) and octanol-air (K_{OA}) also rise. Different physicochemical properties, viz partition coefficient, solubility, alkyl chain length, vapour pressure, etc., of PAEs are listed in Table 1.1. These characteristics of PAEs play a significant role in their behaviour, fate/transport, as well as degradation in the environmental matrix, including the atmosphere, hydrosphere, biosphere and lithosphere. For instance, PAEs contamination propensity in aquatic organisms and their dispersion in environmental matrices can be forecasted by their K_{OW} value.

PAEs became ubiquitous environmental pollutants due to their extensive applications, and compounds such as dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), diisononyl phthalate (DiNP) and diisobutyl phthalate (DiBP) are often detected in the environment. PAEs are grouped into: low-molecular-weight (LMW) phthalates with 3 to 6 carbon alkyl chains and high-molecular-weight (HMW) phthalates with 7 to 13 carbon alkyl chains. For example, DMP ($C_{10}H_{10}O_4$), DEP ($C_{12}H_{14}O_4$), and DBP ($C_{16}H_{22}O_4$) belong to the LMW group, whereas DEHP ($C_{24}H_{38}O_4$) and DnOP ($C_{24}H_{38}O_4$) belongs to the HMW group. The chemical structure of these different phthalates are depicted in Figure 1.2.

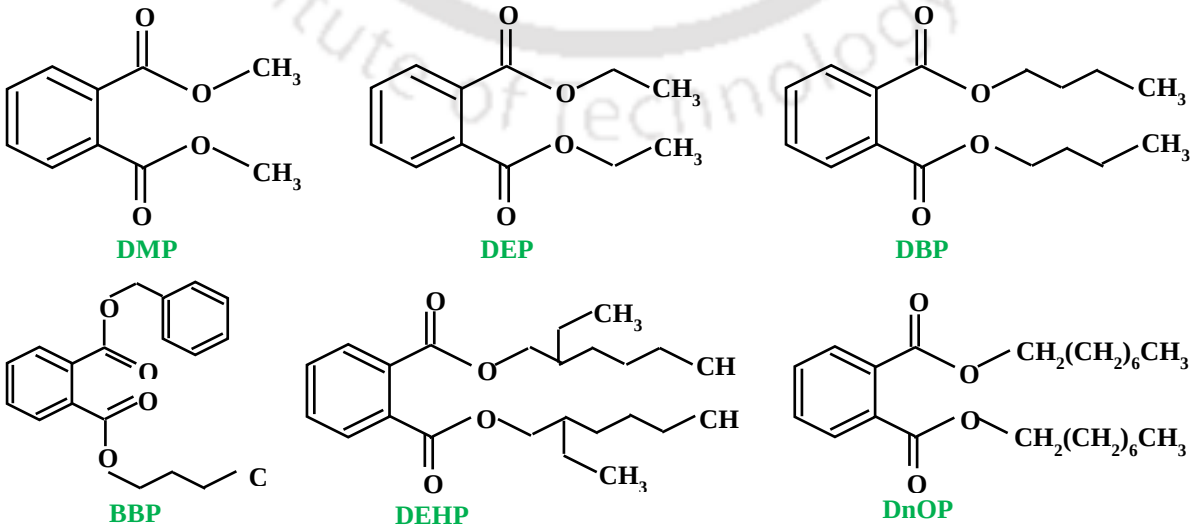


Figure 1.2: Chemical structure of different PAEs.

Table 1.1: Physiochemical properties of PAEs used in this study (Source: Ghosh and Sahu, 2022).

Phthalate ester	Formula	MW (g/mol)	Alkyl chain length	S_W (mg/L)	log K_{OW}	log K_{OA}	log K_{AW}	Vp (pa)	H ($Pa \cdot m^3$ /mol)	Boiling point: (°C)	Melting point (°C)	Specific gravity (20°C)
Dimethyl phthalate	$C_{10}H_{10}O_4$	194.18	1	5220	1.61	7.01	-5.40	0.263	0.0098	283	5.50	1.19
Diethyl phthalate	$C_{12}H_{14}O_4$	222.24	2	591	2.54	7.55	-5.01	0.065	0.0244	302	-40	1.12
Di-n-butyl phthalate	$C_{16}H_{22}O_4$	278.34	4	9.9	4.27	8.54	-4.27	0.005	0.133	340	-35	1.04
Butyl benzyl phthalate	$C_{19}H_{20}O_4$	312.37	4 & 6	3.8	4.70	8.78	-4.08	0.003	0.205	370	-35	1.11
Di (2 ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.57	8	0.003	7.73	10.53	-2.80	0.00003	3.95	385	-40	0.99
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.57	8	0.003	7.73	10.53	-2.80	0.00003	3.95	385	-25	0.98

MW: Molecular weight; S_W: Water solubility; Vp: Vapour pressure; K_{OW}, K_{OA} and K_{AW}: partitioning coefficient of Octanol-water, Octanol-air and Air-water, respectively; H: Henry's constant;

1.2.2. Application of PAEs

Since the 1930s, PAEs have been used as plasticizers in various plastic products, and DEHP contributes to 25% share of the total plasticizers produced. The high molecular weight PAEs, such as DEHP, DnOP, DiNP, BBP, etc. are mainly used in construction materials and several poly vinyl chloride (PVC) products such as children's products (toys, modelling clay, grip bumpers, etc.), clothing (raincoats, footwear, etc.), food packaging products (Hahladakis et al., 2018), and medical products containing PAEs include tubing, blood bags, single-use plastic gloves, and dialysis equipment (Koch et al., 2006; Zhou et al., 2019). The addition of a plasticizer to PVC makes it flexible and less fragile despite the fact that PVC is a hard plastic with a high glass transition temperature. The lower molecular weight PAEs, such as DMP, DEP, DBP, etc., are used in the production of pharmaceuticals and personal care products, adhesives, solvents, lubricants, coatings, insecticides, varnishes, wax and ink (Pang et al., 2021; Schettler et al., 2006).

Currently, there are nearly 60 different types of PAEs in use for various applications like lubricants, insecticides, material packaging, paints, additives, cosmetics, adhesives, etc. (Eichler et al., 2019). The most commonly utilized PAEs for manufacturing of different products are listed in Table 1.2. Due to their extensive application in various sectors, phthalates have become ubiquitous environmental contaminants. Around the world, plasticizer usage is estimated to be 7.5 million tonnes per year, with PAEs having up to 60-65 percent of that total (Hu et al., 2021a). Due to low manufacturing costs and a lack of alternatives, the demand for PAEs is continuously growing, which causes their accumulation in the environment. Despite all of PAEs applications and advantages, they pose numerous negative impacts on the environment and health, worldwide.

Table 1.2: Commonly used PAEs in different industrial products (Source: Das et al. (2021)).

PAEs	Application
DMP	Insect repellent
DEP	Cosmetics, fragrance, plasticizers, aerosols sprays, etc.
DBP	Pharmaceutical coating, cosmetics, wrapping materials, car care products, home furnishing, sealants and printing inks
BBP	Automotive products, conveyor belts, wrapping materials, adhesives, traffic cones, artificial 3D alphabets, perfumes and vinyl gloves
DEHP	Gloves, blood bags, dialysis bags, intravenous tubing, children dolls, diapers, shoes, tiles, air tubes and flexible PVC products
DnOP	Bottle cap liners, floorings, garden hoses, tarps, pool liners and conveyor belts

1.2.3. Sources of PAEs

The primary sources of PAEs in the environment are industrial wastewater, agricultural runoff, hospitals effluent, sewage, etc. Wastewater generated from large-scale plastics, pharmaceuticals, printing ink, adhesive and other chemical industries contributes majorly to environmental pollution by PAEs (Ji et al., 2010; Kanaujiya et al., 2019; Sun et al., 2013). Furthermore, many PAEs and their transformation products can reach the fields when they are irrigated with treated wastewater. As a result, the receiving waters get contaminated by these substances (Barbosa et al., 2016). Other sources of PAEs include sewage treatment facilities and leakage from landfills, industrial waste streams, and septic tanks (Schaider et al., 2016; Sui et al., 2015). Domestic wastewater is another primary source of many of the PAEs such as pharmaceutical products, personal care products and household products containing PAEs (Luo et al., 2014b). Also, small amounts of these compounds are contributed through their applications in various day-to-day use products. The various sources of PAEs in the urban water cycle are shown in Figure 1.3.

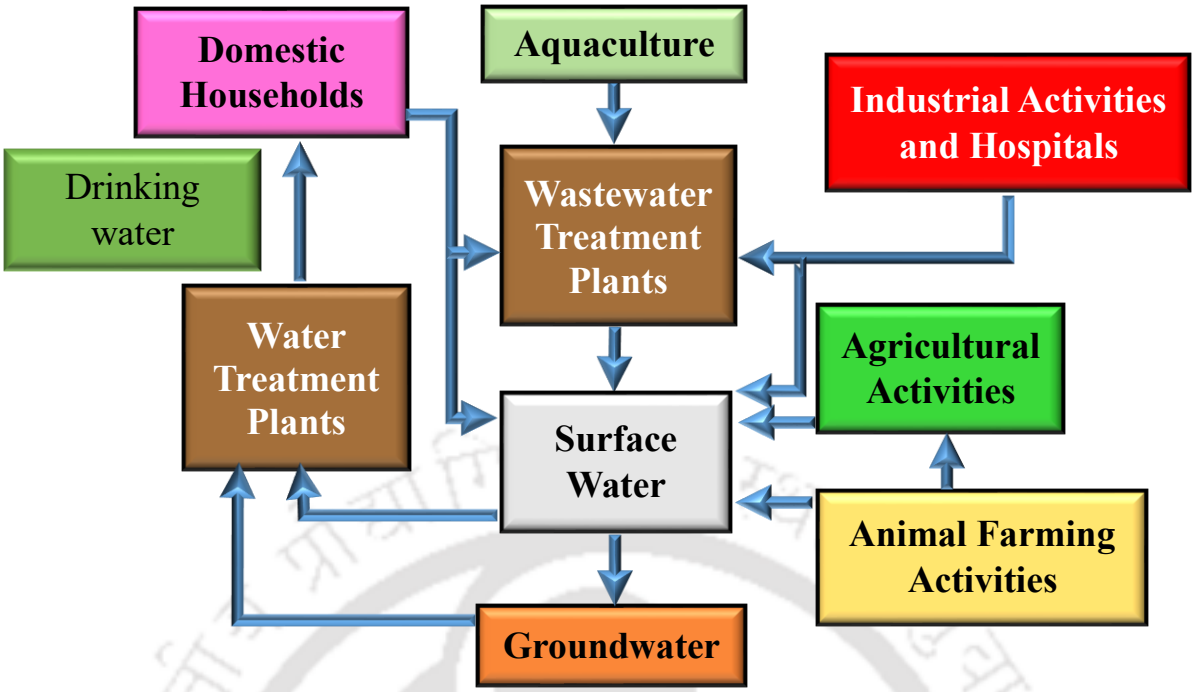


Figure 1.3: Release of EDPs into the environment from different sources.

1.2.4. Fate and occurrence of PAEs in the environment

PAEs enter the environment through different routes such as losses from production processes, weathering, leaching, or volatilization from finished products. They often get adsorbed on to particulate matter after getting released into the environment, and are transported through a number of pathways, including atmospheric transport, agricultural runoff, leaching from landfills, discharge from wastewater treatment plants, before finally reaching even the most remote environments (Gao et al., 2014). Moreover, their occurrence are not confined within a particular environmental compartment which is governed by their physicochemical properties and environmental factors such as vapor pressure (VP), octanol-water (K_{OW}), octanol-air (K_{OA}), and air-water (K_{AW}) partition coefficients (Kashyap and Agarwal, 2018). The emission rate of a specific phthalate into the environment is influenced by its equilibrium gas-phase concentration, which is governed by its concentration in the source and its vapour pressure (Liang and Xu, 2014). The K_{OA} of PAEs indicates their partitioning between the air and organic phase in atmospheric aerosols, plants and soil. The potential of PAEs to partition between the

two phases causes their dynamic dispersion leading to their exposure to humans through many routes, including dust inhalation, ingestion and dermal absorption. The K_{OA} value of PAEs increases with the alkyl chain length or molar volume. Higher K_{OA} value of PAEs reveals their high tendency to partition to soil, plants and aerosols. The K_{OW} of PAEs is an indicator of their partitioning between water and sediment/ animal/ plant lipids/ soil organic matter. The K_{OW} of PAEs increases with alkyl chain length, resulting in high hydrophobicity, which causes higher sorption to organic matter. The K_{AW} value of PAEs represents their volatility from water. The high solubility of PAEs in water and their low volatility are observed with their low K_{AW} values (Blanchard et al., 2014).

Thus, concentrations of PAEs vary among different compartments of the environment and depends not only on their physicochemical properties but also influenced by a variety of factors, including their degree of usage, the extent of degradation resistance and the characteristics of the environmental matrices. Thus, the most widely used PAEs with HMW are frequently found at high concentrations in the environment; for e.g., DEHP has been detected at very high concentrations (Salaudeen et al., 2018). The half-lives of PAEs range from about three years to as long as 2000 years for DMP and DEHP, respectively, in aqueous media (Staples et al., 1997). Hence, a considerable amount of EDPs has been found to have accumulated over time in water, atmosphere, sediments, soil and biota, leading to human and animal exposure through different environmental matrices (Table 1.3).

Table 1.3: Occurrence of PAEs in different compartments of the environment.

Sources / PAEs	DMP	DEP	DBP	BBP	BEHP	DnOP	Reference
Air (Concentration in ng/m^3)							
Berlin, Germany	919	722.7	1081.4	26.60	155.50	N/A	(Weschler et al., 2008)
Xi'an, China	501	N/A	590	N/A	470	N/A	(Wang et al., 2014)
Indoor air, industrialized area, Delhi, India	18311	12368	13909	11927	14995	11833	(Das et al., 2014)
Outdoor air, industrialized area, Delhi, India	6366	9761	2345	2584	7503	383	
Houses, indoor air, Norway	69	496	233	N/A	N/A	N/A	(Sakhi et al., 2019)
Bedrooms, indoor air, Japan	42	74	257	N/A	323	N/A	(Yoshida et al., 2020)
Homes, indoor air, Vietnam	26.5	66.5	84.3	N/A	14.2	3.79	(Anh et al., 2021)
Water (Concentration in µg/L)							
Malaysia surface water	7.1	28.6	108.9	22.1	130.9	2.3	(Santhi and Mustafa, 2013)
Wastewater, France	N/A	9.480	1.290	1.600	63.000	N/A	(Bergé et al., 2014)
Drinking water, Delhi, India	0.38	0.198	0.317	0.633	0.257	0.248	(Das et al., 2014)

Kaveri river, India	0.02	0.24	0.03	0.04	0.51	0.25	(Selvaraj et al., 2015)
Wastewater, Saudi Arabia	0.228	0.182	0.748	0.388	0.468	0.195	(Al-Saleh et al., 2017)
Surface water, Korea	0.180	0.050	0.340	N/A	0.134	0.020	(Lee et al., 2019)
Landfill leachate, Poland	7.320	2.930	1.860	N/A	75.600	N/A	(Kotowska et al., 2020)
Soil and Sediments (Concentration in µg/kg dry weight)							
Gomti river, India	316	137	155	N/A	947	312	(Srivastava et al., 2009)
Jukskei river, South Africa	12.80	44.80	N/A	N/A	3660	57.1	(Sibali et al., 2013)
Kaveri river, India	1.60	16.50	2.50	2.60	278	35.50	(Selvaraj et al., 2015)
Urban and rural soil, France	01.25	93.00	092.5	2.600	310.0	3.400	(Tran et al., 2015a)
Sewage sludge, South Africa	1080	4840	27990	76360	N/A	5000	(Salaudeen et al., 2018)
Top soil, Russia	470	680	42400	N/A	17200	N/A	(Brodskiy et al., 2019)
Sewage sludge, Korea	1200	72	5900	N/A	92000	N/A	(Lee et al., 2019)
Yangtze river delta, China	2.18	4.14	96.3	69.5	1510	74.8	(Wei et al., 2020)
Sediment, Gulf of Lion, Mediterranean Sea	2.83	1.27	14.06	N/A	66.90	5.36	(Alkan et al., 2021)

1.2.5. Exposure to PAEs

Most of the population gets exposed to different phthalates on a daily basis via dermal contact, ingestion and inhaling of PAEs contaminated food, water, air, dust and soil (Figure 1.4). According to several studies, DEHP is the most commonly encountered phthalate worldwide, and indoor contaminated dust is a potential source for PAEs exposure (Becker et al., 2004; Das et al., 2014; Net et al., 2015). The major routes for direct exposure to PAEs in humans are inhalation and dermal contact of phthalates containing cosmetics, perfumes, scents, personal care products, sanitary napkins, textiles, yoga pads, modelling clay and toys etc. (Berger et al., 2018; Hahladakis et al., 2018; Harley et al., 2016; Tang et al., 2020). Exposer to phthalates like DEP and DBP, often present in various cosmetics, including nail paint, deodorant, perfume, hair gels, hair sprays, body lotion, etc., is through inhalation and dermal contact (Eichler et al., 2019; Pang et al., 2021).

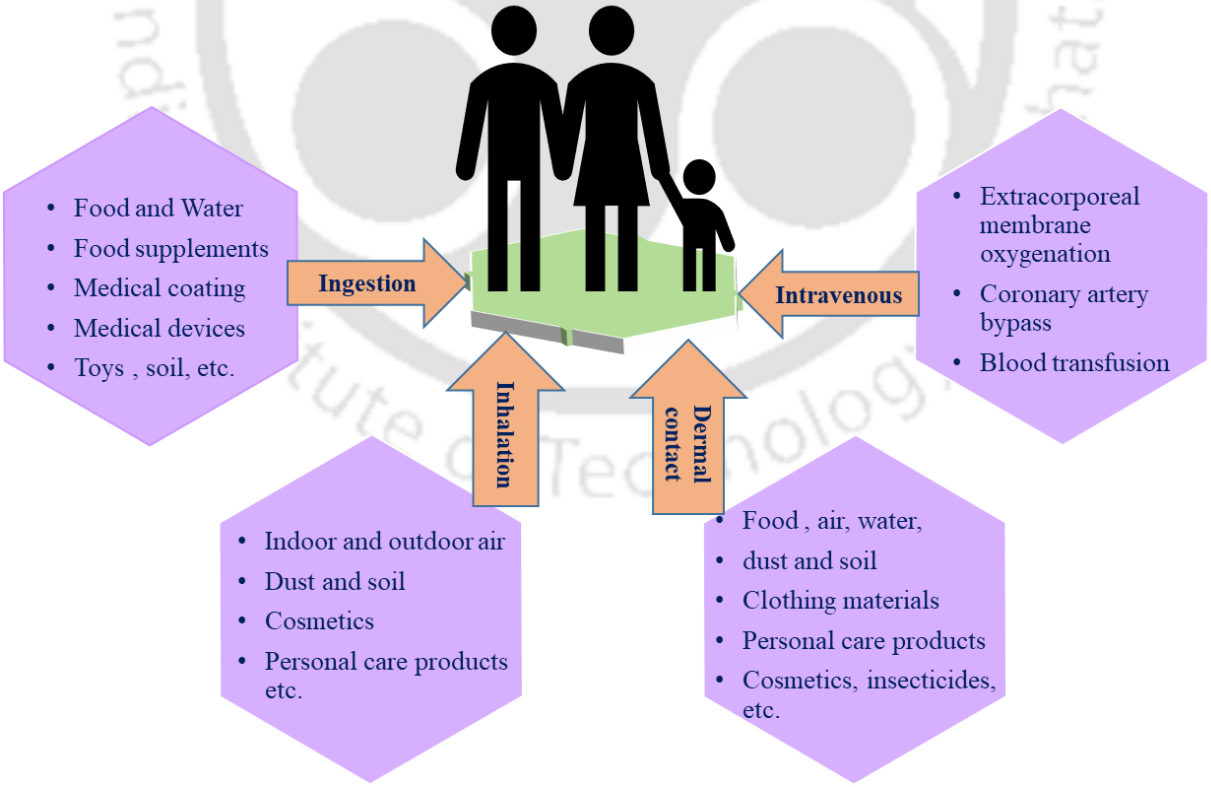


Figure 1.4: Routes of human exposure to EDPs.

Similarly, the major routes for indirect exposure to PAEs are the consumption of PAEs contaminated food, including vegetables, cereals, pulses, editable plants, etc. Naturally, plants may uptake and accumulate PAEs from the contaminated soil, causing health concerns for people through dietary consumption. In order to assess the potential of plant uptake, translocation and metabolism of DBP and DEHP and their metabolites (mono butyl phthalate (MBP) and mono (2- ethylhexyl) phthalate (MEHP)) a cultivation study using strawberry, carrot and lettuce plants was carried out. All the four PAEs were detected in the plant tissues, and the bioconcentration factors (BCFs) ranged from 0.16 ± 0.01 to 4.78 ± 0.59 (Sun et al., 2015). Furthermore, people get exposed to PAEs indirectly through the consumption of contaminated drinking water, alcoholic and non-alcoholic beverages, milk and dairy products, fish, chicken, eggs, processed meat, etc. that may get contaminated from packaging or through environment (Figure 1.5) (Clark et al., 2003; Fierens et al., 2012; Tsumura et al., 2002; Wen et al., 2020).

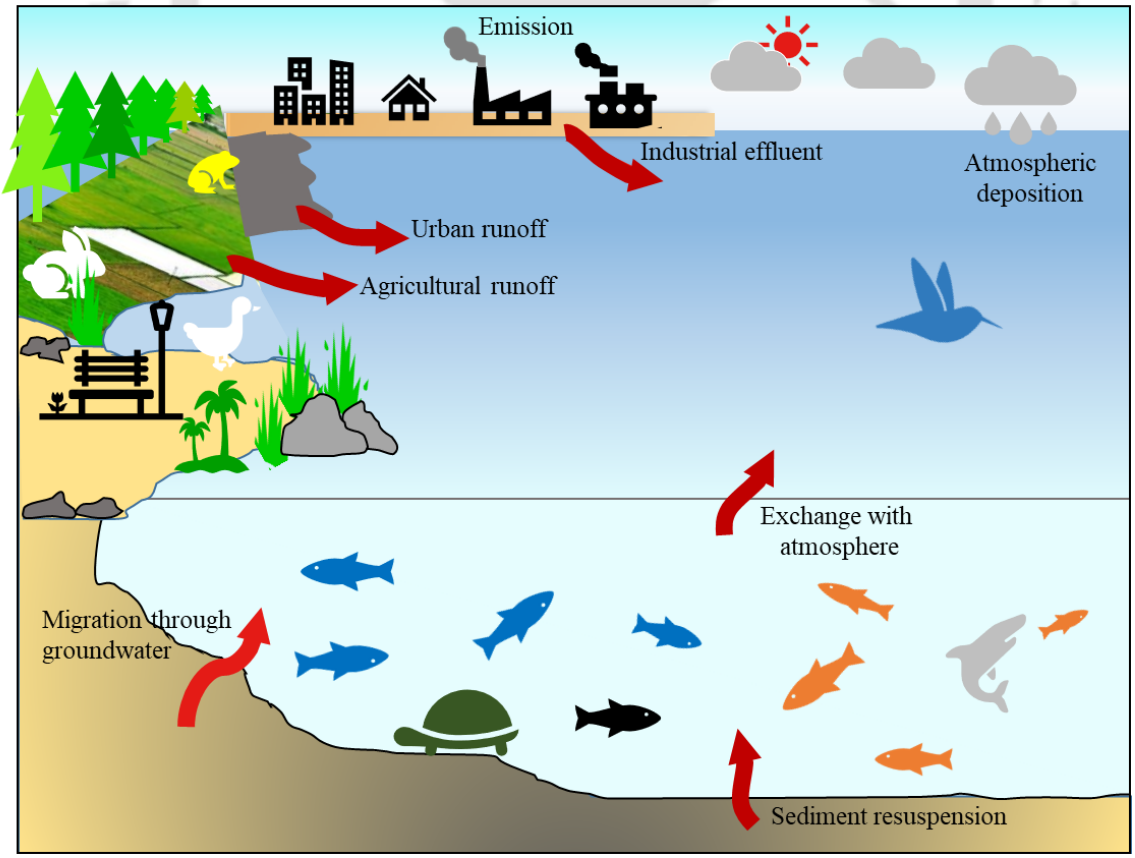


Figure 1.5: EDCs exposure to aquatic organisms.

Pharmaceutical medications also can potentially be a source of PAEs exposure through ingestion. Eudragit polymer is used to encapsulate medications for the general gastrointestinal system; it is a copolymer coating of ammonia methacrylate that breaks down in the lower intestine at higher pH. Generally, plasticizers viz. DBP and DEP are used in eudragit for effective drug delivery in the gastrointestinal tract of humans (Thakral et al., 2012). Additionally, exposure to medical treatments utilizing plasticized PVC tubing, such as hemodialysis and blood transfusions, can also lead to considerable doses of PAEs in humans (Calafat et al., 2004). Kaestner et al. (2020) studied the levels of MEHP and DEHP in the blood of patients on ECMO (extracorporeal membrane oxygenation) therapy and reported that their concentration in patient blood is 4.29 times higher than the concentration in control patient. The increased concentration of MEHP and DEHP in the ECMO therapy patients is associated with the PVC tubing system used for treatment.

PAEs have been found in the amniotic fluid of pregnant women and the breast milk of lactating mothers, which act as a secondary source of exposure for the foetus and breastfed infants (Filardi et al., 2020; Wittassek et al., 2009). In addition to ingestion exposure, children may also consume phthalates orally by putting PAEs containing toys and teethers in their mouths. Numerous studies throughout the world have discovered high concentrations of DEHP, DINP, and other PAEs in children's items, including toys and teething rings, etc. (Bouma and Schakel, 2010; Johnson et al., 2011). Weiss et al. (2018) reported that children under the age of two consume PAEs 12 times more than adults on a daily basis via ingestion.

1.2.6. Toxic effect of PAEs

Several studies have reported that PAEs mainly act as an endocrine disrupter in humans and animals (Andersen et al., 2018; Björvang and Damdimopoulou, 2020; Gao et al., 2017; Matsumoto et al., 2008). A potential endocrine disruptor is an exogenous substance or mixture

that modifies the functions of the endocrine system by mimicking the activities of natural hormones and consequently causes adverse health effects in an intact organism or its progeny or (sub) populations (Benjamin et al., 2017; Careghini et al., 2015; McLachlan et al., 2006). The potential health impact of PAE is shown in Figure 1.6. There is growing public concern about the toxicity of PAEs not just in animal tests but also in recent human investigations. PAEs can cause human health risks with lethal dosage 50 (LD50) values of just 1-30 g/kg of body weight (Tuan Tran et al., 2022). Studies conducted by the U.S. EPA have also demonstrated the high carcinogenic risk associated with even trace amounts of DEHP (low levels). PAEs exposure leads to adverse health problems, including developmental delays in children, abnormalities in the reproductive system, precocious puberty, allergies and asthma, obesity, neural and immune disorders, infertility and cancers (Bølling et al., 2020; Filardi et al., 2020; McLachlan et al., 2006). Moreover, it has been claimed that PAEs have adverse effects on the liver and testicles in addition to their effects on kidneys and thyroid (Tuan Tran et al., 2022).

In a study, increased concentration of PAEs, including DMP, DEP, DBP, BBP, DnOP and DEHP were detected in a group of infertile men compared to controls. The study also demonstrated a significantly high correlation between normal sperm morphology and PAEs exposure and the percentage of DNA damage in the sperm (Pant et al., 2010; Tranfo et al., 2012; Zhang et al., 2006). The early onset of puberty in girls has been linked to urinary levels of PAEs along with phenols and other phytoestrogens (Wolff et al., 2010). Zhang et al. (2015) also reported that exposure to phthalates such as DMP, DEP, DBP and DEHP can cause accelerated breast development and early menarche onset among young girls. Moreover, exposure to phthalates is linked to cardiovascular risk factors such as high blood pressure, atherosclerosis, and coronary heart disease (Mariana et al., 2016; Olsén et al., 2012).

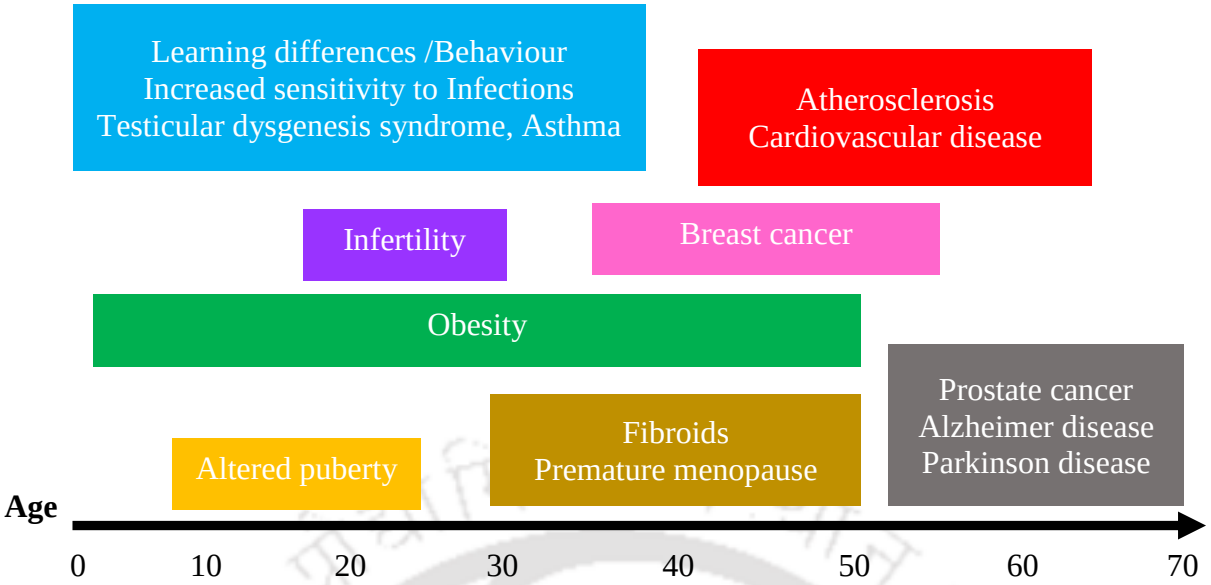


Figure 1.6: Examples of potential diseases and dysfunctions originating from early exposures to EDPs.

According to some studies, foetal exposure to PAEs like DEP, DBP, and DEHP causes intrauterine inflammation, which may contribute to a shorter gestation time and the birth of premature newborns (Filardi et al., 2020; Grindler et al., 2018; Radke et al., 2020). Moreover, exposure to phthalates during pregnancy may cause negative effects on diastolic blood pressure, which can raise the risk of pregnancy-related hypertension disorders (Werner et al., 2015). Main et al. (2006) found mono butyl phthalate (MBP) in breast milk samples collected 1-3 months after birth that adversely linked with free testosterone in the baby's serum samples. Prenatal exposure to DEP, DMP, and DBP may also negatively impair the development of the fetus's brain, leading to low IQs and aberrant behaviour in young children. (Jankowska et al., 2019; Miodovnik et al., 2011; Tanner et al., 2020). Prenatal and postnatal exposure to DEHP, DBP, BBP and DEP are linked to the development of asthma, eczema, wheezing, and allergy symptoms in children (Braun et al., 2013; G et al., 2019; Jøhnk et al., 2020). The preceding studies comprehend the necessity for adopting effective remediation strategies for PAEs removal.

In the last few years, concern regarding the occurrence of PAEs in the ecosystem has considerably increased due to the confirmation of adverse effects on human, animal and aquatic organisms. The hazardous nature of PAEs on diverse biotic components of ecosystems has therefore necessitated the removal of phthalates from wastewater which act as the main stream of PAEs pollution in the environment.

1.3. Treatment of PAEs containing wastewater

Most of the conventional wastewater treatment plants (WWTPs) are inefficient for PAEs removal (Kanaujiya et al., 2019). Therefore, upgrading treatment technologies and strategies for effluents generated by conventional WWTPs might reduce the release of PAEs into the receiving water bodies. It can also improve the overall quality of effluents for possible reuse. The design improvement of WWTPs includes advanced and innovative treatment technologies, which aim to transform organic pollutants into less harmful compounds or even to non-harmful compounds. Innovative water treatment processes include adsorption using granular activated carbon (GAC), membrane separation technologies and advanced chemical/oxidation technologies (Sudhakaran et al., 2013a). Other options, which use natural passive treatment systems to treat PAEs, such as riverbank filtration (RBF), aquifer recharge and recovery (ARR) and constructed wetlands (CWs), have also been used for the treatment of PAEs contaminated water.

1.3.1. Physicochemical treatment processes

Commonly used physicochemical methods such as adsorption, coagulation, flocculation and advanced oxidation processes (AOPs), including hydrogen peroxide, chlorine, ozone along with the combination of transition metals and metal oxides-based catalysts are reported to be able to efficiently remove PAEs. Moreover, some hybrid systems with combinations of two or more systems have recently been applied to enhance the removal of a wide range of PAEs.

1.3.1.1. Sorption process

One of the most simple and common treatment processes available for the removal of organic substances from wastewater is sorption, which uses biochar, activated carbon, etc., as sorbents. The first choice is activated carbon, which is a promising sorbent due to its vast surface area and chemical composition. Sorption of organics may involve absorption as well as adsorption. Sorption depends on the physicochemical properties of organic compounds (e.g., molecular weight, pK_a, K_{OW} and polarity) and the sorbent (e.g., polarity, surface properties, etc.) (Kanaujiya et al., 2019). To achieve high removal efficiency of PAEs, numerous innovative materials including modified activated carbon, activated sludge, microbial cultures, chitosan, and seaweed, were employed (Tuan Tran et al., 2022). For instance, Shaida et al. (2018) reported DEP removal by adsorption using an innovative low-grade coal sorbent modified by chitosan, i.e. coal-chitosan.

Graphene and its derivatives have proven to be a promising adsorbent material for PAEs removal; particularly, more than 80% removal of DEHP and DBP were obtained with 0.1 g/L graphene and 12 h adsorption time (Yang and Tang, 2016). Six different phthalates, including DMP, DEP, DBP, BBP, DEHP and DOP, were completely adsorbed on mesoporous carbon material that was made using the soft-templating process in the presence of citric acid (Jedynak et al., 2017). Pepper straw, a vegetable waste, was pyrolyzed at 500 °C to produce pepper straw biochars (PBs), which have the potential to be highly effective PAEs sorbents (Yao et al., 2019). Extracellular polysaccharides or activated sludge biomass might be a possible source of biomass for PAE adsorbents because of their steady performance, affordability, and availability. Xu et al. (2021) reported 67.87 % removal of DEP by adsorption using immobilized *Zoogloea sp.* on walnut shell biochar. One of the major drawbacks of the adsorption process is the production of hazardous sludge containing micropollutants, which could leak into the environment if disposed directly.

1.3.1.2. Coagulation/flocculation

In wastewater, the colloidal particles remain suspended owing to Brownian movement, which can be minimized using coagulation and floatation processes, destabilizing the particle movement and resulting in a particle collision. The instability of colloids, ions, macromolecules, bacteria, or fibres occurs during the coagulation reactions aided by a coagulant, a chemical reagent that forms flocs. These flocs settle quickly and are easily removed through sedimentation (Rubio et al., 2002). In order to remove PAEs and dissolved organic matter (DOM) from landfill leachate, Zheng et al. (2009) employed the coagulation (aluminum chloride as a coagulant) and flocculation processes and reported 30% removal of the PAEs from fresh landfill leachates, whereas in case of stabilized landfill leachate the value was 50%. PAEs in marine sediment was reported to degrade by 86% at pH 2 using a bimetallic catalyst that was synthesized with persulfate as the oxidation agent (Dong et al., 2019). The same study showed that electron transfer vacancies and the synergistic catalytic effects of the redox couples Fe^{3+}/Fe^{2+} and Ce^{4+}/Ce^{3+} aid in the degradation of PAEs.

1.3.1.3. Advanced oxidation processes

Advanced oxidation processes (AOPs) are promising alternative remediation technologies that employ different oxidizing species such as hydroxyl (•OH) or sulphate radicals (SO_4^{2-}) for converting hazardous organic contaminants to non-harmful compounds or even complete mineralization to CO_2, H_2O and salts. AOPs were first suggested in the 1980s for potable water treatment; since then, they have been widely used for treating different types of wastewater, such as sewage, pharmaceuticals, and other industrial wastewaters. The strong oxidants can degrade a wide range of organic pollutants (Mansouri et al., 2019; Sudhakaran et al., 2013b). AOPs can degrade any carbon-based pollutants (non-selectively) and hence can even be applied before or after a biological treatment process. Due to inefficiency of the conventional oxidation processes (i.e., Cl_2, HClO, H_2O_2, $KMnO_4$ and ClO_2), AOPs such as Fenton and

photo-Fenton processes, electro-Fenton processes, ozonation (catalytic), wet peroxide/air oxidation (catalytic), heterogeneous photocatalysis, electrochemical oxidation or a combination of these AOPs are of interest (Zhang and Li, 2014). The main disadvantages of these catalytic processes are high operating costs, energy requirements and generation of toxic by-products. In addition, these processes are prone to damage caused by different radical scavenging compounds present in complex wastewater.

1.3.2. Biological treatment processes

In order to overcome the drawbacks of physicochemical methods for organic pollutant removal, biological treatment systems are given a high priority as it is proving to be more sustainable, environment friendly, low cost and highly suitable for developing countries in meeting the environmental standards.

1.3.2.1. Microbiological aspects of PAEs degradation

Numerous species of bacteria, few fungi, yeast and algae, capable of degrading PAEs, are reported in the literature (Barbosa et al., 2016; Coday et al., 2014; Hu et al., 2021a; Kim et al., 2007). Microbial degradation of organic pollutants is associated with the catabolic activity of microbes and during the process pollutants get assimilated as growth substrates (Villegas et al., 2016). Microbial growth on PAEs depends on various operating conditions such as temperature, pH, doubling time, light requirement, agitation, aeration, etc., which indirectly affect the PAEs degradation. PAEs properties such as surface properties, aqueous solubility, structure and alkyl side chain length, partition coefficient, charge, etc., are important factors determining their treatment efficiency.

Among all the microbes, bacteria have been reported extensively to degrade different organic pollutants. As per the reports, more than 60 bacterial strains have been isolated from diverse habitats and shown the potential to degrade various PAEs (Hu et al., 2021a; Ren et al., 2018).

According to phylogenetic analysis, these 60 isolates from 31 genera belonged to a variety of bacterial species, including Actinobacteria (40%), Bacteroidetes (1.5%), Deinococcus-Thermus (1.5%), Firmicute (12%) and Proteobacteria (45%). *Rhodococcus* sp contribute a significant number of the reported isolates (15%) among the 31 taxa, followed by *Gordonia* (10%), *Arthrobacter* (8%) and *Bacillus* (8%) (Hu et al., 2021a). A detailed list of different bacteria and fungi capable of organic pollutant degradation along with their culture condition and treatment efficiency are presented in Table 1.4. Theoretically, the diversity of PAE-degrading bacteria in the natural habitats was far from being represented by the reported isolates from six phyla (Lewis et al., 2020; Song et al., 2019).

From the biochemical perspective, different isolates have distinct degrading abilities. *Rhodococcus* isolates demonstrated a high degradation efficiency towards a broad range of PAEs as substrates (Table 1.4). For instance, Zhao et al. (2018) reported that *Rhodococcus* sp. 2G is able to degrade seven different kinds of PAEs in just five days, with degradation efficiency values of 95%. Whereas *Bacillus subtilis* No. 66 and *Delftia* sp. TBKNP-05 was reported to completely mineralize PAEs even at very high concentrations, whereas other isolates, including, *Paenibacillus* sp. S-3 and *Gordonia* sp. JDC-2 were unable to mineralize PAEs into CO_2 and H_2O as the end products due to the lack of a responsible gene for PAEs intermediates metabolization (Jin et al., 2014; Wu et al., 2010; Zhang et al., 2018). Moreover, the degradation efficiency of PAEs varied with their molecular weight and alkyl chain length. Compared with HMW PAEs with long alkyl chain, such as BBP, DEHP and DnOP, LMW PAEs with short alkyl chain viz. DMP, DEP and DBP were easier to be degraded by such isolates (Chen et al., 2015).

Table 1.4: Potential microbes used for EDPs degradation.

Strain	Phthalates in mixture	Total initial concentration (mg/L)	Degradation %	Degradation time	References
Bacterial strain					
Sludge (microbial consortia)	DEP, DBP, BBP, DEHP	1000	100	30 days	(Chang et al., 2007)
***Rhodococcus ruber* L4**	DMP, DEP, DBP	300	100	97-100 h	(Lu et al., 2009)
***Arthrobacter sp.* ZH2**	DMP, DBP, DOP	600	8-100	40 h	(Wang et al., 2012)
Variovorax sp.	DEP, DMP, DBP	300	100	30 h	(Prasad and Suresh, 2012)
Microbial consortium (HD-1)	DMP, DEP, DBP, DnOP	1200	1-75	48 h	(He et al., 2013)
***Rhodococcus ruber* DP-2**	DBP	1200	100	60 h	(He et al., 2014)
***Arthrobacter strain* C21**	DMP, DEP, DBP, DEHP	320	50-99	3 days	(Z.-D. Wen et al., 2014)
***Mycobacterium sp.* YC-RL4**	DEHP, DMP, DCHP, DBP, DEP	250	86.3-100	5 days	(Ren et al., 2016)
***Raoultella* sp. ZJY**	DMP, DEP, DBP, DEHP	500	10-98.5	74 h	(Ebadi et al., 2017)
Bacillus thuringiensis	DMP, DEP, DPP, DBP	1600	82-96	80 h	(Surhio et al., 2017)
Rhodococcus sp. 2G	DMP, DEP, DBP, BBP, DEHP, DnOP, DiNP	1400	95	120 h	(Zhao et al., 2018b)
***Paracoccus kondratievae* BJQ0001**	DMP, DEP, DIBP, DBP, DEHP	1000	50-100	24-120 h	(Y. Xu et al., 2020)
Fungal strain					
Fusarium culmorum	DBP	500-1000	95	168-228 h	(Ahuactzin-Pérez et al., 2018a)
Pleurotus ostreatus	DEHP	1000	98	504 h	(Ahuactzin-Pérez et al., 2018b)
Stropharia rugosoannulata*, *Trichosporon porosum*, *Stachybotrys chlorohalonata	DBP, BPA	50 µM	53 - 95	24 h	(Carstens, 2018)

Most of these biodegradation studies were carried out under aerobic conditions, and a limited number of literatures are available on anaerobic biodegradation of PAEs. However, some recent studies reported the biodegradation of PAEs by different isolates under anaerobic conditions (Junghare et al., 2016; Zhao et al., 2017). Figure 1.7 depicts phthalate's common aerobic degradation pathway followed by bacteria such as *Rhodococcus pyridinivorans*, *Arthrobacter sp.*, *Pseudomonas putida*, etc. (Guo et al., 2010; Kumar Singh, 2018; Liu et al., 2020; Zhao et al., 2018a). In the case of phthalates, the long-chain phthalate esters are first broken into mono esters, followed by a series of enzymatic reactions to produce acetyl-CoA that enters the TCA cycle. The pathway includes a series of chain reactions that lead to the formation of acetyl-CoA pyruvate or acetyl-CoA succinate, an intermediate of the Krebs cycle (Fabbrini et al., 2002).

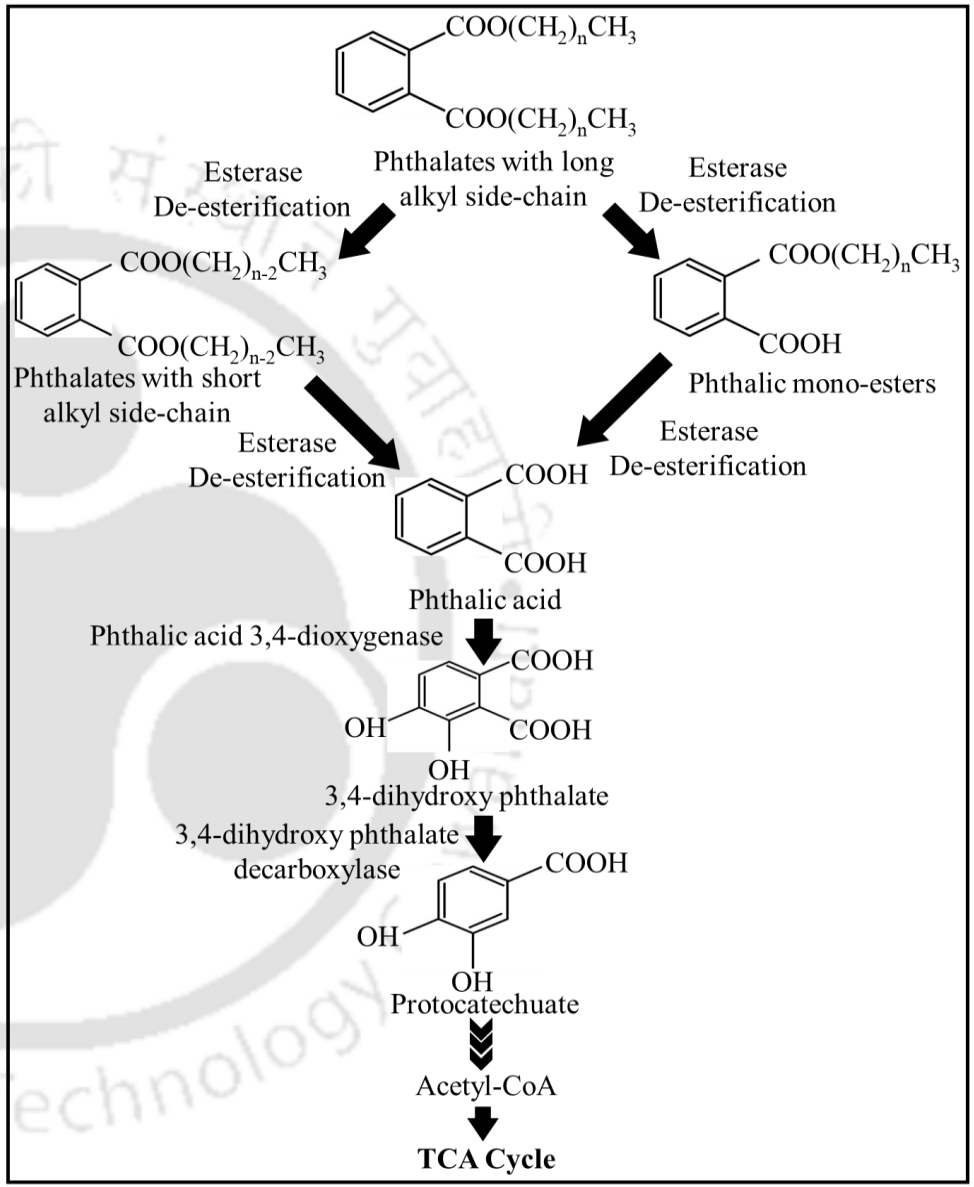


Figure 1.7: Common metabolic pathway of PAEs degradation.

Biological treatment systems involving these degrading microorganisms include conventional approaches, such as activated sludge process, packed bed and trickling bed bioreactor systems as well as advanced treatment systems, such as membrane-based bioreactor, two-phase portioning bioreactor, cell immobilized systems, etc. An overview of different biological treatment systems is provided in Figure 1.8, 1.9 and Table 1.5.

1.3.2.2. Conventional biological treatment systems

1.3.2.2.1. Activated sludge process

Activated sludge process (ASP) is a conventional technique used for treating both industrial and domestic wastewater. ASP relies on microbial activity for nutrient degradation and oxidizing carbon-rich compounds. The conventional ASP method involves the mixing of industrial effluent with microbial culture in an aerated reactor, followed by recycling of activated sludge to the aeration tank before the next cycle (Figure 1.8a) (Gonzalez-Gil et al., 2017). The main removal mechanisms in ASP includes microbial degradation or sorption on to microbial flocs. Hydrophobic compounds get adsorbed quickly owing to their surface properties (Sarkar et al., 2013). Co-metabolism of different pollutants by a microbial consortium plays a crucial role in ASP.

An activated sludge treatment process was used in sewage treatment, 60 to 70% of the phthalates present were biodegraded under oxygen and nitrate reduction cycle (Fauser et al., 2003). Stasinakis et al. (2008) reported 58.70±5.70% of DEHP could be degraded by the activated sludge treatment process. In sludge wastewater treatment plants that used aerobic activation, the removal efficiencies of DMP, DEP, DBP, BBP and DEHP ranged from 73 to 87% (Balabanič and Klemenčič, 2011). In another study, Tran et al. (2015) reported 93.9% degradation of DEHP during wastewater treatment by activated sludge treatment process (Table 1.5). Recently, Salaudeen et al. (2018) investigated the influent and effluent of three different wastewater treatment plants which adopted the activated sludge technology for PAEs

removal and reported that the removal efficiency of these plants varied between 27.3 and 99.5%.

ASP system has certain drawbacks; for example, it usually can't accomplish the discharge limit standards for micropollutants and, generally, a shallow quality of treated effluent is obtained. ASP process requires a comparatively large area for its setup. However, the addition of a polishing step or a tertiary treatment system with ASP can improve its micropollutants removal efficiency.

1.3.2.2.2. Up-flow anaerobic sludge blanket (UASB)

Among various anaerobic processes up-flow anaerobic sludge blanket (UASB) bioreactor has been widely used as an effective technology for the treatment of various wastewaters, including industrial, municipal, and food-processing (Kleerebezem and Macarie, 2003; Lettinga and Hulshoff Pol, 1991). For optimal degradation efficiency, UASB needs the formation of granular sludge with a function-coordinated microbial population to ensure biomass retention and toxin degradation. Additionally, it is well recognised that syntrophs, methanogens, and fermenters must interact ecologically in a balanced way for organic contaminants to be mineralized into CH_4 and CO_2 (Abbasi and Abbasi, 2012; Sekiguchi, 2006; Sekiguchi et al., 1999). However, because UASB reactor has been examined from a macro perspective of the reactor ecology, many of the interactions are still unexplained. In reality, these reactors are made up of a variety of granules, each of which functions as a separate micro-ecosystem for wastewater treatment. Conventional methods, may, therefore oversimplify the intricacy of the microbial ecology and granular growth.

Moreover, the UASB reactor's effluent does not meet discharge criteria, thus necessitating post-treatment or final polishing units (Khan et al., 2011). Most developing nations, including India, use UASB technology with polishing system as a post-treatment (Khan et al., 2014). Gani and

Kazmi, (2016) studied phthalate-containing sewage treatment using a USAB reactor and reported 53% degradation, whereas the degradation efficiency enhanced up to 74% when the system was coupled with a polishing pond. Recently, co-treatment of purified terephthalate and dimethyl terephthalate manufacturing wastewater using a mesophilic UASB reactor was studied by Kuroda et al. (2022). 76-100% COD removal by degradation of aromatic compounds and organic acids present in the wastewater was reported.

1.3.2.2.3. Biological trickling filter and biofilm reactor

Another widely used process for treating organic pollutants is a biological trickling filter with biomass immobilized over a support material (Figure 1.8b). The wastewater containing pollutants is slowly percolated through the support material, where the immobilized biomass degrades them. The other biofilm-based bioreactor systems, such as the packed bed bioreactor, work on the same principle. Although these types of systems were most commonly used in wastewater treatment plants (WWTPs) for removal of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) (Barbosa et al., 2016), their use in treating PAEs containing wastewater is negligible due to poor treatment efficiency. However, Kasprzyk-Hordern et al. (2009) evaluated the removal of more than 50 pharmaceuticals and EDCs by trickling filter and ASP process for over five months, but the removal efficiency was only 50% for most of these compounds.

1.3.2.2.4. Biological nitrification and denitrification processes

Biological nitrification/denitrification is another bioprocess reported in the literature for the removal of organic pollutants from wastewater with the aid of nitrifying and denitrifying conditions (Phan et al., 2014). Nitrification is a process where the biological oxidation of ammonium to nitrite and nitrate takes place, whereas in denitrification reduction of nitrate/nitrite to nitrogen gas occurs. Ahmed et al. (2017) reported very high removal (up to

100%) of many EDCs, such as 17β estradiol, bisphenol A, galaxolide, salicylic acid, etc., at the μgL^{-1} level by denitrification process. Suarez et al. (2010) studied the fate of certain EDCs and pharmaceutical compounds during the denitrification process. This particular study found that nitrification was more effective for removing galaxolide, ibuprofen, erythromycin, etc.

1.3.2.3. Advanced biological treatment systems

1.3.2.3.1. Membrane-based bioreactor

Membrane bioreactors (MBRs) work primarily in side stream and submerged configurations. In the case of the side-stream configuration, the membrane module is kept outside the working bioreactor unit. Side stream configuration requires a recirculation pump for circulating effluent from the bioreactor to the connected membrane module, which is an energy-intensive and high-cost process. Hence, to overcome the shortcomings of the side stream configuration, the concept of a submerged MBR system was considered in which the membrane module is kept inside the bioreactor that allows the feed/effluent to pass through and simultaneously treat or remove the organic pollutants (Figure 1.8c). Sludge retention time (SRT) and hydraulic retention time (HRT) are the two critical parameters governing MBR performance. SRT also depends upon the organic load and food-to-microorganism (F/M) ratio for treating specific wastewater (Judd, 2010).

Compared to conventional biological treatment procedures, combining biological and membrane filtration processes in an MBR increased the removal of organic pollutants (de La Torre et al., 2015). At the laboratory scale, MBRs have been investigated for PAEs removal as an additional step to traditional activated sludge processes and reported outstanding removal efficiency (Balabanič et al., 2012; Pirsaheb et al., 2009). In wastewater treatment plants, MBR revealed higher DEHP removal efficiency (70%) than the values (3%) obtained in the conventional treatment process during one year of operation (Camacho-Muñoz et al., 2012). Whereas, another study on DEHP removal using MBR showed 85% removal (Boonyaroj et al.,

2012) (Table 1.5). The mass balance analysis of the PAE degradation using MBRs revealed that the removal mechanism of phthalate esters involves physical retention, air stripping and biodegradation (Sakiti et al., 2013). However, because of the hydrophilic nature ($logD_{pH=7.8}$ < 3.2) of PAEs and other compounds, such as carbamazepine and diclofenac, these compounds could not be removed efficiently in MBRs. Furthermore, similar to the performance of other biological treatment systems, pH and temperature strongly influenced organic pollutant removal using the MBR system (Sanguanpak et al., 2015).

1.3.2.3.2. Moving bed biofilm reactor

MBBR is a biofilm-based reactor system in which biofilms are grown on small sized (1 to 4 cm) biomass carriers such as kaldness biosupport materials (Figure 1.8d). These specially designed biosupport material provides greater surface area for biofilm formation and can be used in biological wastewater treatment systems. The MBBR system, introduced nearly 30 years ago, is one of the best alternatives to the conventional suspended ASP owing to advantages such as simple and robust design, small tank volume, compact design, increased solid retention time for slow growing organisms, possibility of achieving aerobic and anaerobic organism growth in the same reactor, reduction of hydraulic head loss, etc. (Zupanc et al., 2013). Due to its high efficiency and affordable cost, the MBBR system is one of the most promising treatment methods for the removal of organic pollutants from wastewater. Casas et al. (2015) reported 20 % higher degradation efficiency of 21 different compounds using MBBR in comparison with the MBR.

Moreover, a study conducted for the removal of DEP and diallyl phthalate (DAP) from synthetic wastewater using MBBR reported 94.96% and 93.85% efficiency, respectively (Ahmadi et al., 2015) (Table 1.5). Luo et al. (2015) used sponge pieces as biosupport material in MBBR to remove four different pharmaceutical and personal care products (PPCPs)

(ibuprofen, salicylic acid, primidone and naproxen) with a removal efficiency value of 93.7, 91.1, 83.5 and 81.1%, respectively. The study also reported a moderate removal (50 to 70 %) for ketoprofen, metronidazole, acetaminophen and gemifibrozil. The major drawback of the MBBR system is plugging of aeration line and the requirement of periodic monitoring of the biomass activity.

1.3.2.3.3. Two-phase partitioning bioreactor

Two-phase partitioning bioreactors (TPPBs) are characterized by having two distinct phases, one immiscible and a biocompatible organic phase containing organic pollutants (target substrate) and the other aqueous phase with microbes (Figure 1.8e). The high-speed agitation in this bioreactor system creates tiny droplets of the organic phase with pollutants in the aqueous media inside the bioreactor. The partitioning of the two immiscible liquid phases is based on the equilibrium consideration and real-time demand of the growth of microorganisms (Daugulis, 2001a). Consumption of substrate by microbes causes disequilibrium, thereby inducing more substrate partitioning into the aqueous phase to maintain the equilibrium. A driving force that is induced by an imbalanced partitioning ratio between the two phases and substrate consumption by microbes in this self-regulated system directs the delivery of the substrate to the aqueous phase (Mahanty et al., 2008).

In TPPB systems, the availability of a high concentration of hydrophobic substrate for microbes and a large surface area of the hydrophobic substrate are achieved by dissolving it in the dispersed organic phase. Déziel et al. (1999) suggested that there are mainly three mechanisms for utilizing organic substrates by microorganisms in TPPB systems: uptake of dissolved organic substrate from the aqueous phase, aqueous-organic interfacial uptake of substrate and uptake of organic substrate by direct contact with the organic phase. However, interfacial uptake of the substrate is found to be the principal uptake mechanism, in which microbes

present in the interface take up the substrate and, therefore, the degradation rate of substrate is limited by agitation rate and phase volume (MacLeod and Daugulis, 2005). However, the mass transfer rate of the organic substrate from the organic to aqueous phase is mainly dependent on the interfacial area between the two phases, which strongly depends on the bioreactor agitation rate.

TPPBs are generally used for degrading those compounds which are hydrophobic and toxic to microorganisms at high concentrations. This system has been successfully utilized for the biodegradation of different xenobiotics (toxic organic compounds), such as polycyclic aromatic hydrocarbons, phenols, etc. from synthetic wastewater (Daugulis, 2001b; San-Valero et al., 2018a; Z. D. Wen et al., 2014). Mahanty et al. (2008) achieved complete pyrene mineralization even at a high initial concentration of 1000 mg/L using a TPPB reactor. Rodriguez Castillo et al. (2016) employed the TPPB system to degrade hydrophobic volatile organic compounds by using different hydrophobic ionic liquids as the organic phase. In another study, more than 90 % degradation of styrene was reported by using the TPPB system operated as a biotrickling filter (San-Valero et al., 2018b). Tomei et al. (2018) achieved more than 96% removal of 2,4-dimethylphenol (initial concentration: 1200 mg/L) and NaCl (initial concentration: 100 g/L) by using a TPPB system. In the last decade, TPPB systems have been widely studied for the degradation of different types of organic pollutants at different concentration levels, demonstrating the promising prospect of this system for the biodegradation of contaminants. The main drawback of this system is that it requires high agitation speed for efficient mass transfer of pollutants to the aqueous phase from the non-aqueous liquid phase, which is energy intensive and may lead to damage of the bacterial cell.

1.3.2.3.4. Immobilized bioreactor

Immobilization is the process of restriction of microbial cell mobility within a defined area. Based on immobilization process it can be classified under two broad categories: active

immobilization and passive immobilization. The most widely used active immobilization method is physical entrapment of microbial cells within porous matrices, such as alginate, agar, polyacrylamide, chitosan, collagen and gelatine. In active immobilization, the microbial cells are entrapped or bound to a support material by physical or chemical forces. Whereas passive immobilization is due to the multilayer growth of microbial cells on solid support material (biologically active or inert) to form a biological film. Passive immobilization is commonly used in a biofilm-based bioreactor system, MBBR, biotrickling filter, etc., discussed earlier. Cell immobilization, active or passive, offers many technical and economic advantages over the freely suspended cell system owing to its high cell concentration, the potential reuse of biomass, prevention of cell washout, improved genetic stability and protection against shear damage. It also reduces the cost of cell separation and recycling, which keeps overall cost of a bioprocess low (Figure 1.8f).

Immobilized systems have been reported previously for pollutant removal from wastewater. Sarma and Pakshirajan (2011) investigated pyrene biodegradation using immobilized *M. frederiksbergense* and found >90 % degradation. In this study, the *M. frederiksbergense* was entrapped using 5% sodium alginate and 3.5% $CaCl_2$ beads, and the pyrene was presented in encapsulated form using 3% alginate, 3% polyvinyl alcohol and 100 g/L Brij 30 as the surfactant. Pyrene encapsulation successfully releases the pollutant at a slow rate to reduce its toxic effect on the degrading microorganism. The active cell immobilization method is an excellent alternative technology for micropollutant degradation but the literature on bioreactors with immobilized cells is limited. Hence more such bioreactor systems need to be explored in great detail to make it industrially scalable.

Table 1.5: PAEs removal using different biological treatment processes.

Treatment Processes	Sample	EDPs	Concentration (µ/L)	Removal (%)	Reference
Activated sludge	Wastewater	DEHP	33	93.90	(Tran et al., 2015b)
Moving bed biofilm reactor	Synthetic wastewater	DEP	3×10^5	94.56	(Ahmadi et al., 2015)
Membrane bioreactor	Municipal solid waste leachate	DEP	12.50	81	(Boonyaroj et al., 2012)
		DBP	35.40	87	
		BBP	21.50	77	
		DEHP	65	96	
		BPA	5	94.2	
		OP	1.7	70.2	
		NP	1.5	98.7	
Combined UASB-constructed wetland	Urban wastewater	DEHP	8.5	90	(Reyes-Contreras et al., 2011)
		DiBP	2.7	80	
		DEP	2.7	70	
		DBP	0.4	90	
Aerobic digester	Wastewater sludge	DEHP	31.4 mg/kg	72	(Pham et al., 2011)
Anaerobic–anoxic–oxic (A^2O)	Synthetic wastewater	DBP	257	95	(Huang et al., 2010)
Fixed film bioreactor	Synthetic wastewater	DMP	$1\times10^5 – 5\times10^5$	81.4-100	(Pirsaheb et al., 2009)
Activated sludge	Artificial additives	DEHP	35000	63	(Stasinakis et al., 2008)

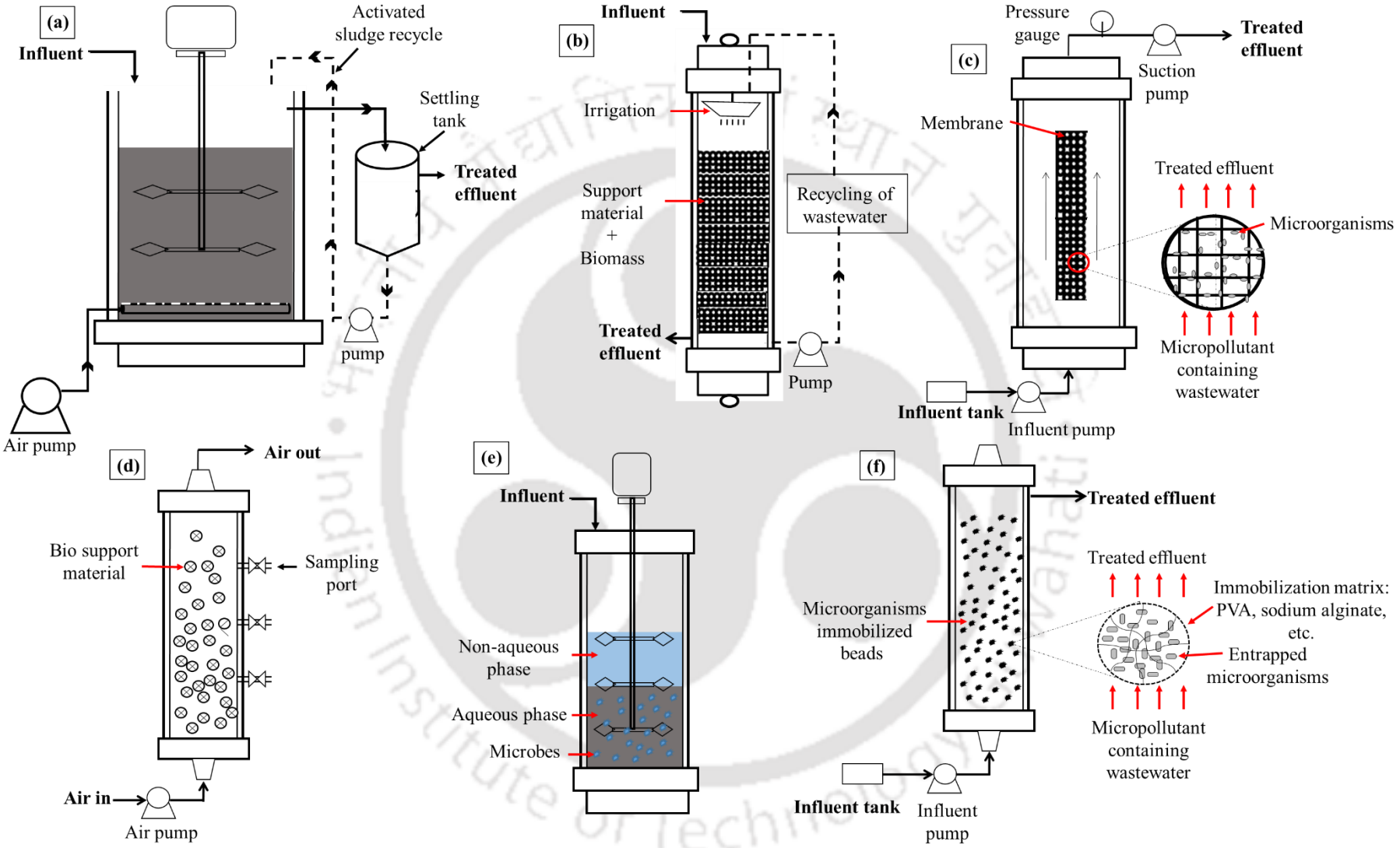


Figure 1.7: Schematic of different bioreactor systems: (a) activated sludge process (b) trickling bed bioreactor, (c) membrane bioreactor, (d) moving bed bioreactor, (e) two-phase partitioning bioreactor (f) expanded bed immobilized cell bioreactor.

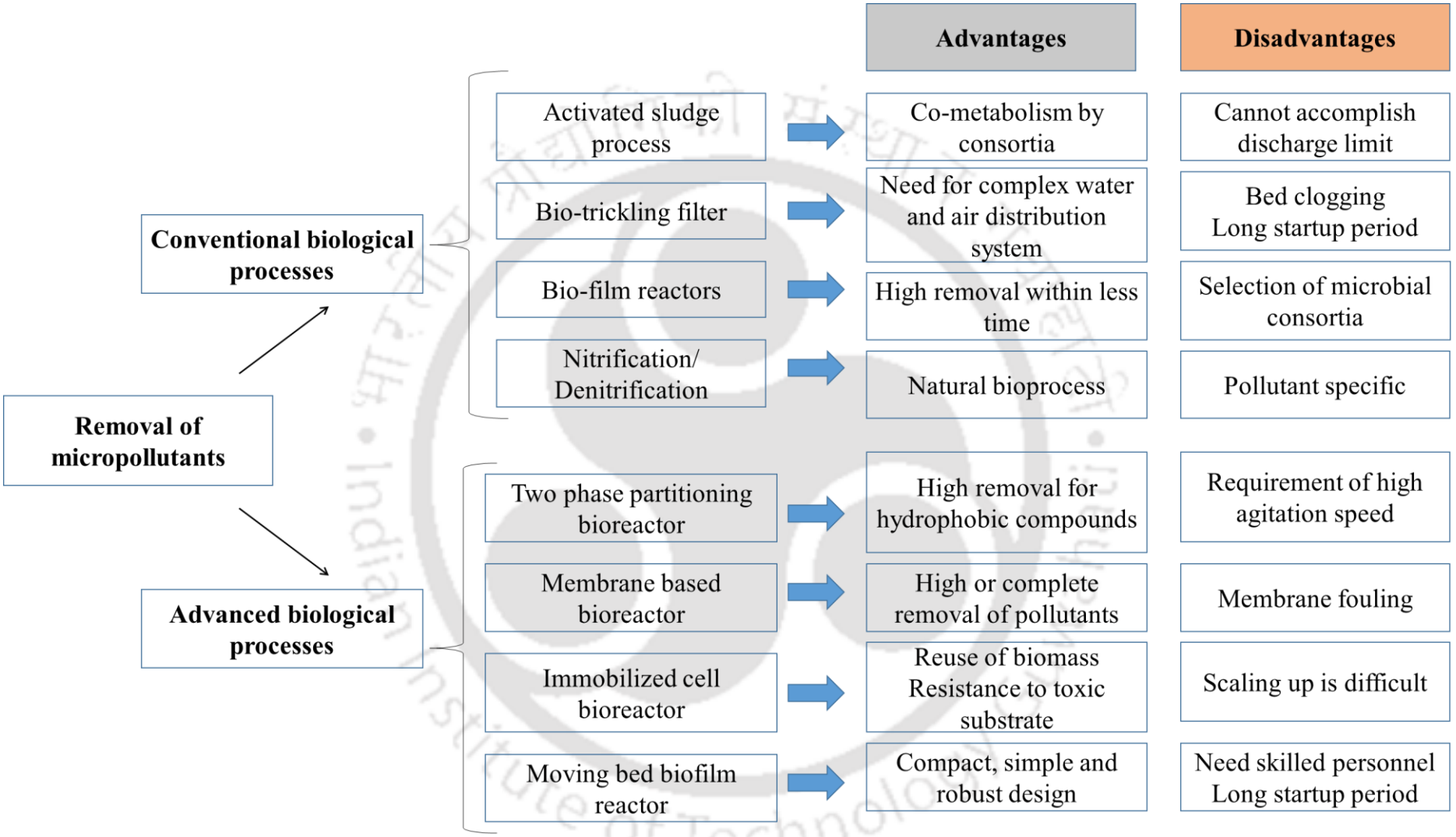


Figure 1.8: Advantage and disadvantages of conventional and advanced biological systems for organic pollutant removal.

1.4. Definition of problem

Complete removal of PAEs employing biological treatment systems is challenging due to their highly toxic nature and recalcitrant to biodegradation. Moreover, treatment efficiencies of the conventional bioprocess systems, e.g., ASP or tricking filters, were also not comparable with the physicochemical processes. In addition, the technical aspects, required for the implementation of engineered bioremediation systems such as MBR, TPPB, immobilized bioreactor, etc., to treat EDPs under natural environmental conditions, are still lacking. The goal of designing such advanced technologies is multi-directional; firstly, improved solubility of PAEs for its enhanced bioavailability, as most of these compounds are less soluble in water at high concentrations. Furthermore, there is a need to understand and elucidate the biodegradation mechanism of PAEs for a better application of such processes. The effect of different bioreactor operation strategies and operation conditions, such as HRT, SRT, pollutant load, F/M ratio, etc., need to be examined in great detail to optimize the treatment process for achieving maximum performance. Hence, there is a need to study EDPs degradation by microorganisms in bioengineered systems that are highly efficient and feasible at a larger scale.

1.5. Aim and objectives

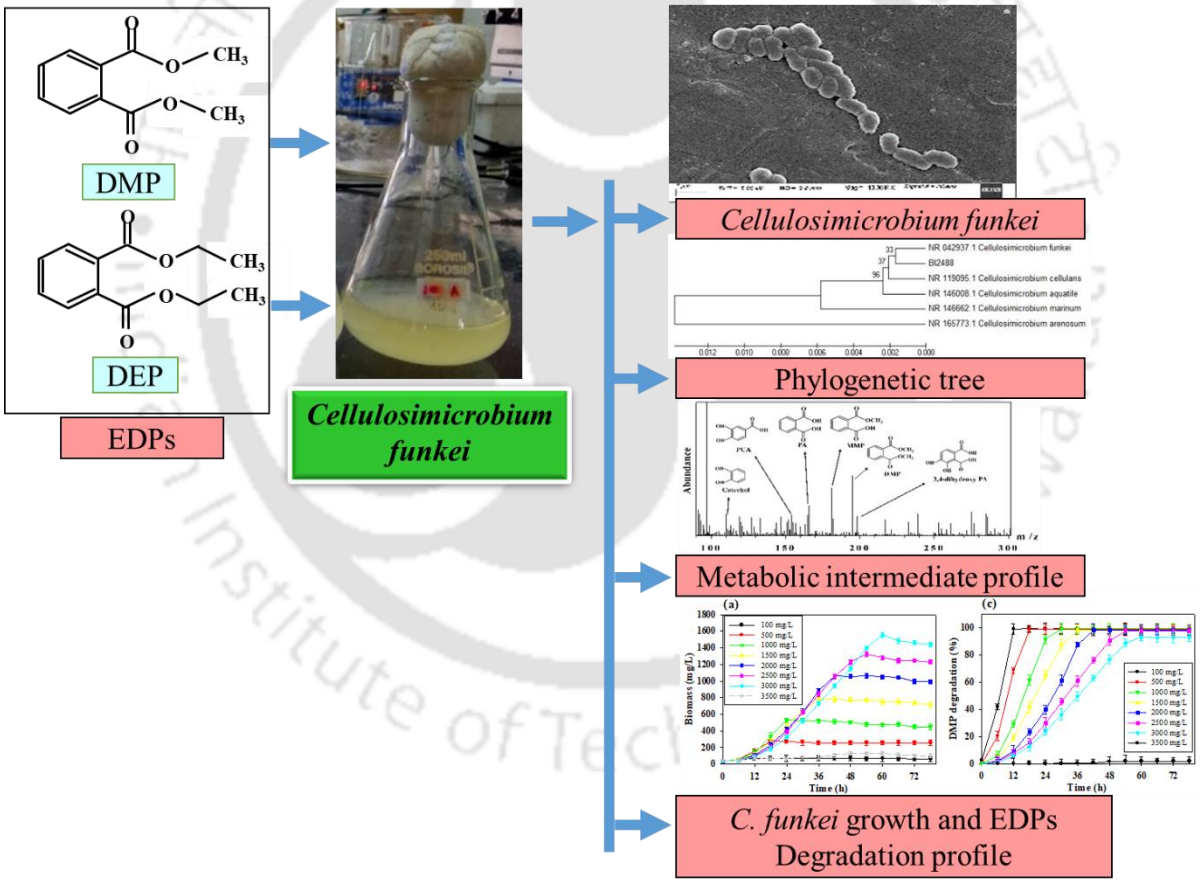
This study aimed at bacterial degradation of EDPs using batch, fed-batch and continuous treatment systems and assessment of their toxicity removal.

To achieve the aforementioned aim, the following investigations were carried out:

1. Screening of potential microorganisms for degradation of EDPs
2. Evaluation of kinetics of biomass growth and EDPs degradation in a simple batch system
3. Elucidation of EDPs biodegradation pathway by the selected microorganism
4. Evaluation of different bioreactor systems, such as slurry phase, two-phase partitioning bioreactor (TPPB), and integrated biodegradation-microfiltration for EDPs biodegradation
5. Ecotoxicity study of EDPs treated wastewater by seed germination and brine shrimp mortality bioassay

Chapter 2

Biodegradation of dimethyl phthalate and diethyl phthalate by *Cellulosimicrobium funkei* in a batch system: kinetics and metabolic intermediate analysis



ABSTRACT

In this study, three different bacterial strains viz. *Cellulosimicrobium sp*., *Ochrobactrum sp.*, and *Rhodococcus opacus* were initially screened for the degradation of dimethyl phthalate (DMP) and diethyl phthalate (DEP) as single and dual substrates at different initial concentrations using shake flasks. In case of single substrate system, complete biodegradation (100%) of DMP and DEP up to 2500 and 1500 mg/L initial concentrations, respectively, was achieved using *C. funkei* in the experiments. Simultaneous degradation of the EDPs was further achieved by adding DMP and DEP in mixture; however, a high concentration of DMP enhanced DEP degradation than at lower concentration. A similar effect of DEP initial concentration on DMP degradation by *C. funkei* was observed. Based on liquid chromatography and mass spectrometry analyses of intermediate metabolites formed during the degradation of EDPs, mono-ethyl phthalate, mono-ethyl mono-methyl phthalate, dimethyl phthalate, mono-methyl phthalate, and phthalic acid were identified as the major metabolites in the DMP and DEP degradation pathway. Results of the batch shake flask experiments on DMP and DEP degradation were fitted to substrate inhibition models reported in the literature. Among the different models, Tessier and Edward models accurately fitted the experimental data with high coefficient of determination (R^2) and least sum of square error (SSE) values. The high values of inhibition constant K_I estimated using the models indicated very good tolerance of *C. funkei* toward DMP and DEP for an efficient degradation of these EDPs in treating contaminated water.

2.1. Introduction

Endocrine disrupting phthalates (EDPs) are exogenous chemicals that adversely affect the hormonal system. Many studies have reported their adverse health effect on human and non-target aquatic organisms (Philips et al., 2017; Ramadan et al., 2020; Wittassek et al., 2011). Among the emerging contaminants, organic compounds impose severe problems during the treatment process. Phthalic acid esters (PAEs) belong to such category of organic compounds and are manufactured for different purposes, mainly for use as plasticizers (Sharma et al., 2021). PAEs include dialkyl or alkyl aryl esters of 1, 2-benzene dicarboxylic acid (phthalic acid). Amongst phthalate esters, diethyl phthalate (DEP) and dimethyl phthalate (DMP) are frequently used for a wide range of commercial applications. PAEs are covalently bound to the plastics polymer and can leach out from the plastics into the environment during use or after disposal and pose severe risks to the ecosystem. Some EDPs also enter into the atmosphere through untreated sewage containing such compounds (Bergman et al., 2012). Owing to its dominant use in a wide range of industrial products, it is prevalent in almost all environmental sections of soil, sediment, water, air and biota.

Humans and wildlife are frequently exposed to these PAEs through food, water, air, medicines, plastics, cosmetics, etc. PAEs and their metabolites are toxic, hepatotoxic, teratogenic, carcinogenic, mutagenic and potential endocrine disrupters (Khadka et al., 2020). DEP is known to cause abnormality in sexual difference, whereas DMP promotes chromosomal damage in human leucocytes, thereby affecting the reproductive and developmental systems of animals and human embryos (Pranaw et al., 2014). DEP and DMP are well known for their endocrine-disrupting and anti-androgenic activities. Moreover, DMP and intermediates formed due to its degradation in animals and humans are reported to cause damage to the liver, nervous and reproductive systems, and interfere in their normal development (Lu et al., 2020). Hence, removal of phthalates from contaminated environment is of utmost importance.

Degradation and removal of PAEs by conventional abiotic methods such as filtration, reverse osmosis, adsorption, chemical hydrolysis, and photodecomposition are often associated with one or more drawbacks, including low removal efficiency, high operation cost, severe reaction condition, etc. (Zhang et al., 2016). On the other hand, conventional wastewater treatment processes involving activated sludge process and trickling filter typically require several days to months for the removal of EDPs from contaminated systems (Kanaujiya et al., 2019). Moreover, biodegradation rate of EDPs is much slower in anaerobic microorganisms than in aerobic microorganisms (Prasad and Suresh, 2015). Although there are several reports available on aerobic biodegradation of PAEs (Pranaw et al., 2014; Tao et al., 2019; Xu et al., 2020; Zhang et al., 2018), their biodegradation kinetics and kinetic modelling are not reported, which are essential for scaling up of the biological treatment systems. Hence, the present study was focused toward biodegradation of phthalate esters as single and dual substrates by a novel isolate in batch shake flasks. The results of EDPs biodegradation kinetics by the novel isolate were further fitted to different substrate inhibition models for a better understanding of the degradation kinetics involved.

2.2. Materials and methods

2.2.1. Chemicals and reagents

DMP and DEP (99.0% Purity) used in this study were purchased from TCI (Tokyo Chemical Industry) Chemicals (India) Pvt. Ltd. (Chennai, India). The solvents methanol and dichloromethane were purchased from Himedia (Mumbai, India). All other chemicals were purchased from Merck and SRL (Mumbai, India). All the chemicals and solvents were of analytical grade.

2.2.2. Screening of microorganisms

Three different bacterial strains were initially screened for degradation of DMP and DEP as single substrate. The bacterial strains *Cellulosimicrobium sp*. (CM) and *Ochrobactrum sp.*

(OB) were procured from Bose Institute, Kolkata, India, and *Rhodococcus opacus* (RO) from Microbial Type Cell Culture (MTCC), Chandigarh, India. The bacterial strains (CM, OB, and RO) were selected for biodegradation of phthalates in wastewater due to their ability to utilize recalcitrant organic compounds for their growth and metabolism (Lu et al., 2009; Kanaujiya et al., 2019). Luria Bertani (LB) broth medium was used to grow and maintain the bacterial cultures. The biodegradation experiments were performed using 250 mL Erlenmeyer flask with 100 mL working volume containing Bushnell Haas - minimal salt medium (MSM) and either 500 mg/L DEP or DMP as the sole carbon source. The flasks were agitated on a temperature-controlled orbital incubator shaker maintained at 28 °C and 150 rpm during the experiment. Bushnell Haas - MSM consisted of (g/L): $MgSO_4 \cdot 7H_2O$ (0.409), $CaCl_2 \cdot 2H_2O$ (0.0265), KH_2PO_4 (1), NH_4NO_3 (1), $Na_2HPO_4 \cdot 12H_2O$ (6), $FeCl_3 \cdot 6H_2O$ (0.0833) and 1 mL trace element solution (g/L) $FeCl_3$ (17), $CaCl_2$ (0.6), $ZnSO_4$ (0.2), $CuSO_4 \cdot 7H_2O$ (0.2), $MnSO_4$ (0.2), $CoCl_2$ (0.8), H_3BO_3 (0.1) and $Na_2MoO_4 \cdot 2H_2O$ (0.3) (Goswami et al., 2017). Initial pH of the medium was adjusted to 7 using NaOH/HCl.

Among the three bacterial strains, *Cellulosimicrobium sp.* was observed to efficiently degrade the EDPs, for 16S ribosomal DNA gene analysis of the organism, its genomic DNA was isolated from the pure culture and primers were designed based on conserved bacterial 16S rDNA sequence regions. 16S rDNA sequence data was then aligned with NCBI-BLAST and analyzed for its homology with that of known bacterial strains. Phylogenetic tree was then constructed employing MEGA X software based UPGMA method to relate the screened bacterial strain with other known species from neighbouring taxa. Morphological analysis through field emission scanning electron microscopy (FE-SEM) was further performed to confirm the identity of bacterium.

2.2.3. Biodegradation of DMP and DEP by *C. funkei*

2.2.3.1. Single substrate system

Biodegradation kinetics of dimethyl phthalate (DMP) and diethyl phthalate (DEP) as single substrates by *C. funkei* was studied using batch shake flasks. The bacterium was initially grown in MSM containing 500 mg/L of DMP and DEP as single substrate for 24 h at 30˚C. Ten millilitres of mid-log phase-grown culture was subsequently used as the inoculum (10%, v/v at OD 1.0) in the experiments. Biodegradation experiments were then carried out using 250 mL Erlenmeyer flask with 100 mL working volume containing MSM and the EDCs as single substrate at an initial concentration ranging from 500 to 3500 mg/L for DMP and 500 to 2500 mg/L for DEP. Controls were maintained without any added inoculum to verify any abiotic loss of EDPs during the experiments. All the flasks were agitated on an orbital rotatory incubator shaker set at 120 rpm and 28˚C for 80 h incubation period. Samples were collected at regular intervals to evaluate the biomass growth and residual DMP/DEP concentration. Each sample analysis was carried out in triplicate and the results presented are arithmetic mean of triplicate sample analysis.

2.2.3.2. Dual substrate system

For biodegradation of DMP and DEP as dual substrates using *C. funkei*, experiments as per a three-level full factorial design were carried out (Table 2.1). All other conditions followed in this mixture study were the same as in the previous single substrate study.

2.2.3.3. Modeling of EDPs biodegradation kinetics

For the analysis of biodegradation kinetics of DMP and DEP as single substrate for *C. funkei*, effect of different initial concentrations, 100, 500, 1000, 1500, 2000, 2500 and 3000 mg/L in case of DMP and 100, 500, 1000, 1500, 2000 and 2500 mg/L of DEP, were chosen. Specific degradation rate (q_s in mg/mg·h) of the compounds was calculated for each initial concentration

as per the following equation (2.1):

$$q_s = -\frac{\mu}{Y_{X/S}} \quad (2.1)$$

Where μ is the specific biomass growth rate (h^{-1}), and $Y_{X/S}$ is the biomass yield.

The following equations were used for calculating μ and $Y_{X/S}$:

$$(\mu) = \frac{ln(X/X_0)}{t} \quad (2.2)$$

$$Y_{X/S} = \frac{X-X_0}{S-S_0} \quad (2.3)$$

Where X and X_0 are the biomass concentrations in mg/L (dry cell weight) at time t and 0, respectively, and S and S_0 are the final and initial substrate concentrations (mg/L). The experimental data was further fitted to different kinetic models (Table 2.2) for estimating the biokinetic parameters involved in degrading the EDPs.

Table 2.1: Initial concentrations of DMP and DEP in the mixture study along with their percentage degradation in each experimental run

Experimental run no.	Initial EDPs concentration (mg/L)		% EDPs degradation	
	DMP	DEP	DMP	DEP
1	1000	1000	98.85	98.02
2	1500	1000	98.16	98.32
3	2000	1000	93.33	94.63
4	1000	1500	98.48	97.53
5	1500	1500	94.75	91.13
6	2000	1500	38.02	29.65
7	1000	2000	71.58	43.90
8	1500	2000	34.32	27.84
9	2000	2000	01.19	00.87

Table 2.2: Various kinetic models applied to describe biodegradation kinetics of DMP and DEP as single substrate using *C. funkei*.

Model	Equation	Reference
Andrew	$q = \frac{q_{max}\, S}{K_s + S + \frac{S^2}{K_I}}$	(Andrews, 1968)
Aiba	$q = \frac{q_{max}\, S}{K_S + S} exp^{\left(\frac{-S}{K_I}\right)}$	(Aiba et al., 1968)
Edward	$q = \frac{q_{max}\, S}{K_s + S + \left(\frac{S^2}{K_I}\right)\left(1 + \frac{S}{K}\right)}$	(Edwards, 1970)
Monod	$q = \frac{q_{max}\, S}{K_S + S}$	(Monod, 1949)
Tiesser	$q = q_{max}\, [exp^{\left(\frac{-S}{K_I}\right)} - exp^{\left(\frac{-S}{K_S}\right)}]$	(Edwards, 1970)
Webb	$q = \frac{q_{max} S \left(1 + \frac{S}{K}\right)}{K_s + S + \frac{S^2}{K_I}}$	(Nemati and Webb, 1997)

q: specific biodegradation rate (mg/mg·h); $\mathbf{q_{max}}$: maximum specific biodegradation rate (mg/mg·h); **S:** substrate concentration (mg/L); $\mathbf{K_S}$: half saturation constant (mg/L); $\mathbf{K_I}$: EDPs inhibition constant (mg/L); **K**: a constant in Edward and Webb model.

2.2.4. Analytical methods

2.2.4.1. Morphological analysis of biomass through FESEM

For FESEM analysis using a field emission scanning electron microscope (Zeiss, Sigma, Germany), one-millilitre of bacterial culture was centrifuged at 10000×g for 10 min, washed with sterile milli "Q" water. The pellet obtained was diluted 10 times with milli "Q" water and vortexed. A single drop of the bacterial culture was placed on FESEM stub and coated with gold through an auto fine coating instrument (JEOL JFC-1300) before final observation.

2.2.4.2. Biomass estimation

C. funkei biomass was determined by measuring the optical density (OD) of the culture at 660 nm wavelength using a UV visible spectrophotometer, and the biomass concentration was determined from a calibration curve between dry cell weight (DCW) of biomass and OD_{660} of the culture (Figure 2.1). For biomass dry cell weight determination by gravimetric analysis, the culture broth was centrifuged at 12,000×g for 15 min at 25 °C, and the cell pellet was washed twice with sterile phosphate buffer and lyophilized before measuring its final weight.

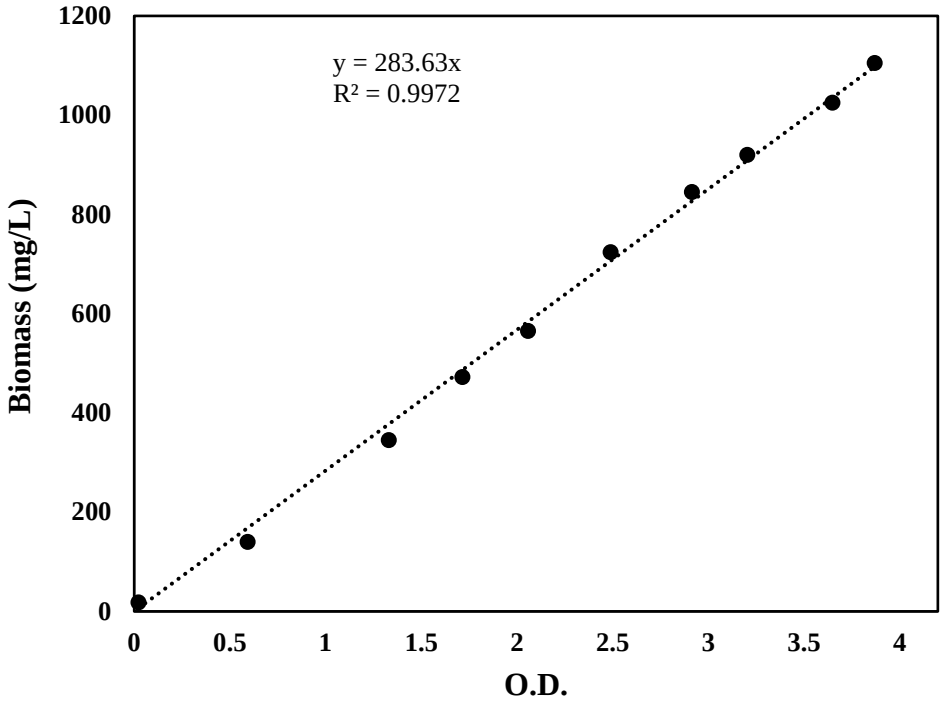


Figure 2.1: Calibration curve to calculate biomass concentration from optical density of *C. funkei* culture.

2.2.4.3. Determination of DMP and DEP

For the analysis of DMP and DEP concentrations, samples collected during the experiments were first extracted with an equal volume of dichloromethane and allowed to air dry at ambient room temperature for evaporating the solvent. Thereafter, an equal amount of methanol was added to dissolve the phthalates and their concentration was determined by using a high-pressure liquid chromatograph (HPLC) (Shimadzu SPD-20A, Japan) fitted with a YMC Triart

C-18 column (YMC, Kyoto-Japan) of dimensions 250mm×4.6mm×5µm and a UV-detector set at 254 nm. Methanol and deionized water in the ratio 8:2 were used as the mobile phase at a flow rate of 1.0 mL/min. Standard solutions of the individual EDPs were prepared in methanol. The experimental sample retention time was identified by comparing it with the standards of the respective EDPs. Standard graphs were plotted between area under the peak and concentration of phthalates for each phthalate to determine the concentration of phthalates in the experimental samples. The following equation was used to calculate percentage degradation of EDPs:

$$\%\ \text{EDPs Degradation} = \frac{C_i - C_f}{C_i} \times 100 \qquad (2.4)$$

Where C_f and C_i are the final and initial concentrations (mg/L) of phthalate, respectively.

2.2.4.4. Identification of metabolic intermediates

Intermediate metabolites formed during EDPs biodegradation by *C.funkei* were analyzed by using a mass spectrometry (MS) system (WATERS, Q-Tof Premier, USA). An isocratic flow was used in the HPLC with an acetonitrile-water solvent (80:20, *v/v*) to elute the metabolites. Metabolites were detected using a TOF/QTOF mass spectrometer under negative electrospray ionization mode (ESI).

2.3. Results and discussion

2.3.1. Screening of microorganisms

Initially, three microbes, *C. funkei* (CF), *Ochrobactrum sp*. (OB), and *R. opacus* (RO), were evaluated to degrade 500 mg/L DMP and DEP as single substrate in batch shake flasks. After three days of a batch run, it is observed that *C. funkei* yielded significant biomass growth on DMP and DEP as the sole carbon source (Figure 2.2a and b). *Ochrobactrum sp*. and *R. opacus* exhibited insignificant growth on these EDPs. The strain *C. funkei* was further identified by 16S rDNA sequence analysis before performing the biodegradation experiments.

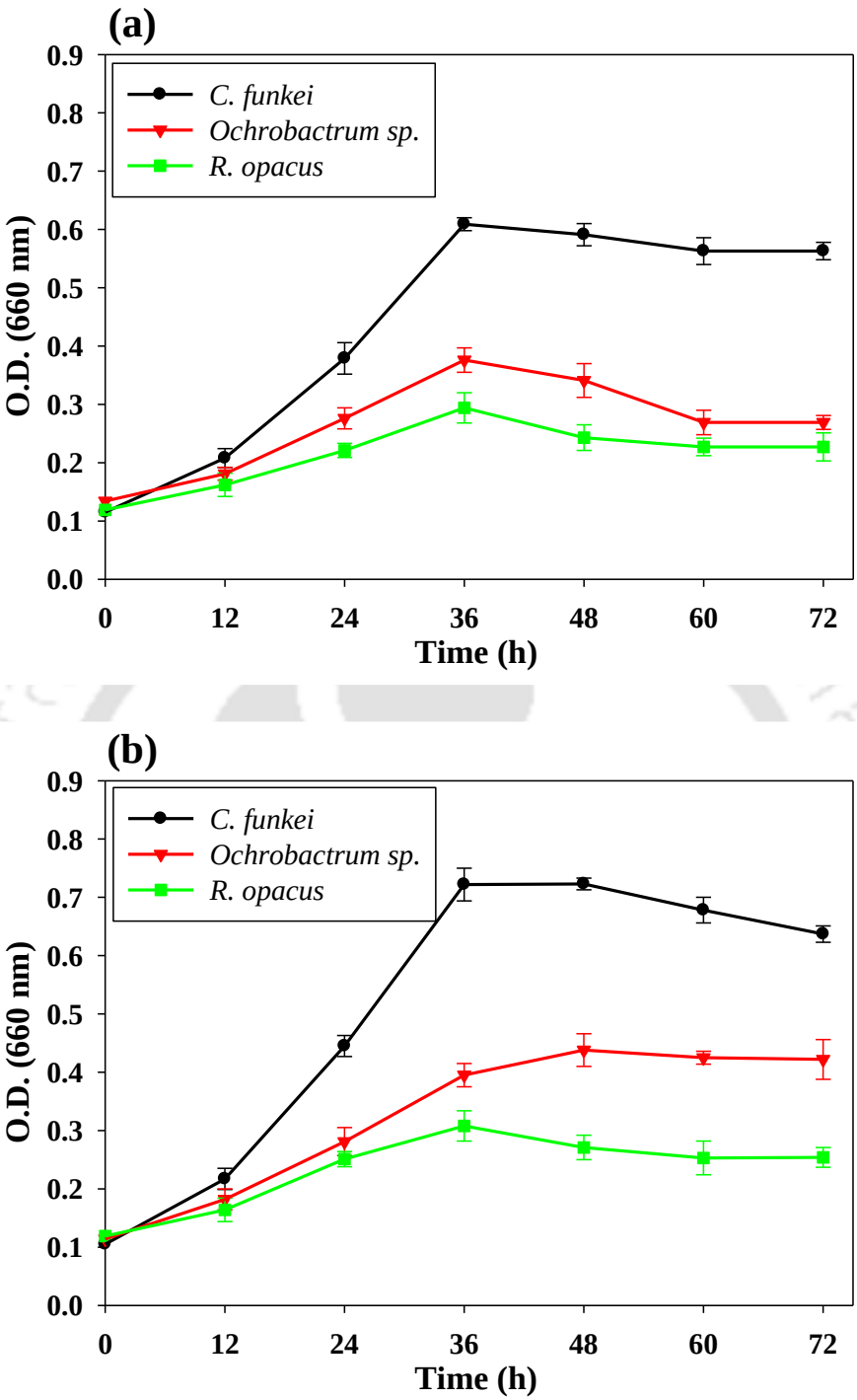


Figure 2.2: Biomass growth profile of the different microbes on (a) DMP and (b) DEP.

2.3.2. 16s rDNA analysis for identification of *C. funkei*

Alignment of the partial 16S rDNA gene sequence of the *C. funkei* revealed 99.67% homology with the already reported *Cellulosimicrobium funkei* (GenBank Accession Number NR_042937.1) followed by 99.54% similarity with *Cellulosimicrobium aquatile*. The phylogenetic relationship of the strain with other related strains based on 16S rDNA sequence

alignment also illustrates that the strain is neighbouring to *Cellulosimicrobium funkei* (Figure 2.3a). Microscopic (FE-SEM) examination of the bacterium revealed that it is rod-shaped and non-flagellar with the dimensions 0.49 – 0.67 µm width and 0.88 – 1.33 µm length (Figure 2.3b). *C. funkei* (strain AR8) has been reported for the remediation of Cr(VI) contaminated industrial effluents (Karthik et al., 2017) and to leach gallium arsenide (GaAs) (Maneesuwannarat et al., 2019). Besides, several *C. funkei* strains have been isolated and reported for its potential diverse environmental applications. However, this is the first study on its ability to degrade EDPs.

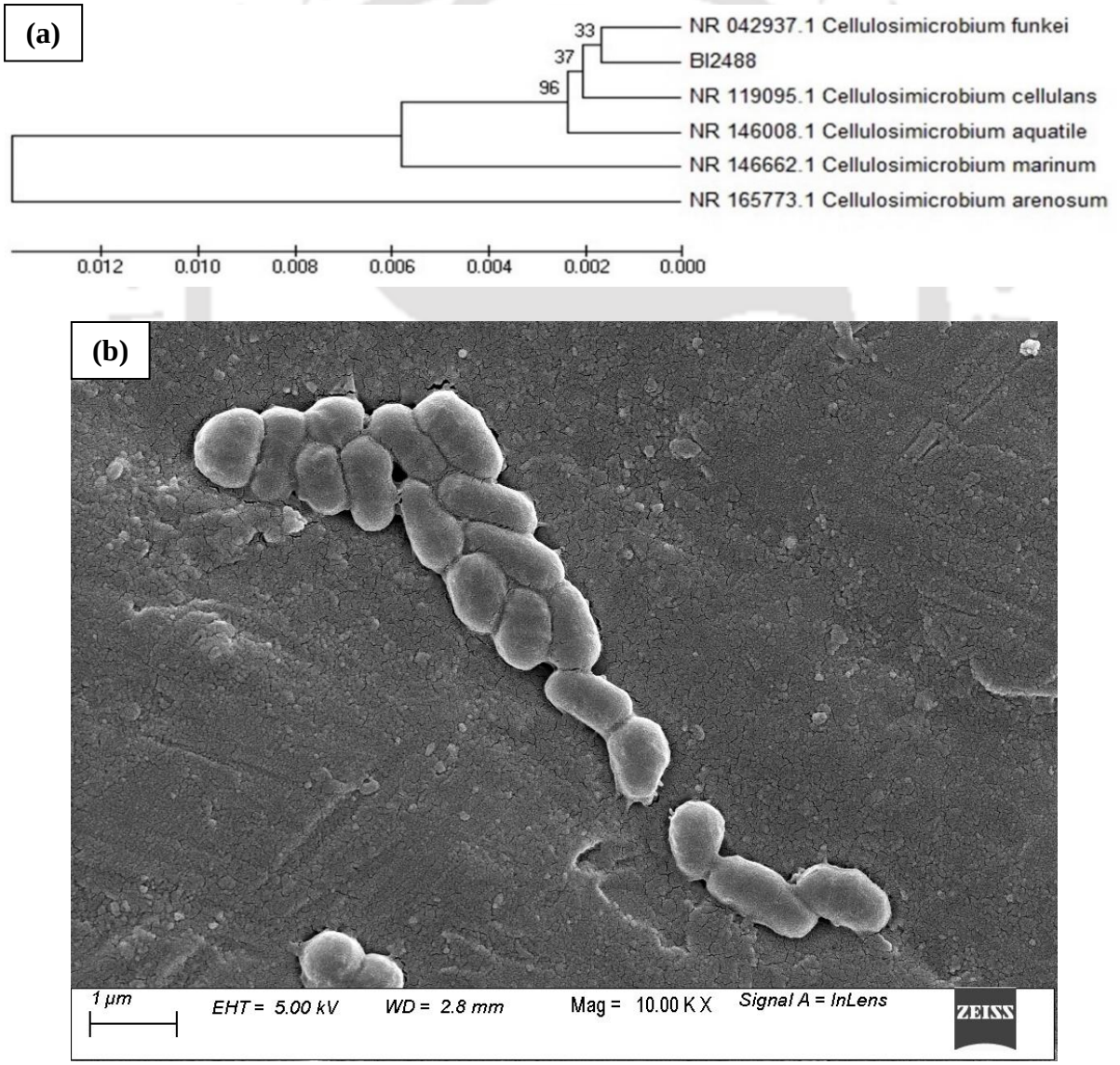


Figure 2.3: (a) *C. funkei* morphology under FESEM and (b) Phylogenetic tree showing its similarity with other strains based on 16S rDNA sequence alignment

2.3.3. Biomass growth and EDPs degradation by *C. funkei*

2.3.3.1. Batch shake flask experiments with single substrate

Figure 2.5 shows the time profile of DMP and DEP degradation as single substrate for *C. funkei* in the batch shake flask experiments, which reveal that the biomass growth increased with an increase in the initial concentration of the EDPs, i.e. up to 3000 mg/L for DMP and 2500 mg/L in case of DEP. The lag phase in biomass growth and EDPs degradation was also prolonged due to an increase in their initial concentration (Figure 2.4 and 2.5). However, the lag phase in biomass growth is short (only 6 h) even at a very high concentration of 3000 mg/L. The value is much less as compared with a literature reported lag phase value of 12 h during biodegradation of 600 mg/L of DMP using *Variovorax sp.* BS1 (Prasad and Suresh, 2015).

Whereas the time taken by the culture for complete degradation of 500 mg/L of DMP/DEP was 18 hours, it was slightly prolonged for degrading 2500 mg/L DMP and 1500 mg/L DEP as single substrate. Moreover, a very high DMP degradation efficiency of 93% was achieved at 3000 mg/L of DMP, but with 3500 mg/L initial concentration the degradation was negligible (Figure 2.5a). In the case of DEP, 67.8% and 29.5% degradation efficiencies were achieved at 2000 and 2500 mg/L initial concentrations, respectively (Figure 2.5b). Maximum specific biodegradation rate of DMP and DEP were achieved at 1000 and 500 mg/L initial concentrations, respectively. Above this concentration, the value gradually reduced and at 3500 mg/L initial concentration biomass growth as well as degradation of the EDPs completely ceased. These results clearly show that *C. funkei* is capable of utilizing the EDPs for its metabolism and growth even up to 3000 and 2500 mg/L initial concentrations of DMP and DEP, respectively, as single substrate. Comparison of biomass growth and DMP biodegradation profiles shown in Figure 2.4a and 2.5a reveals that up to 3000 mg/L initial concentration of DMP, both the biomass growth and DMP degradation values were high. But at higher initial DMP concentration of 3500 mg/L, no significant growth in biomass as well as

very low degradation efficiency are observed. This sharp decline in the biomass growth and DMP degradation at a high initial concentration is attributed to the toxic effect of DMP on *C. funkei* growth, which is confirmed by the inhibition constant (K_I) value of 2841.57 mg/L of DMP, estimated using the substrate inhibition model Tessier model on the growth of *C. funkei* (Table 2.3).

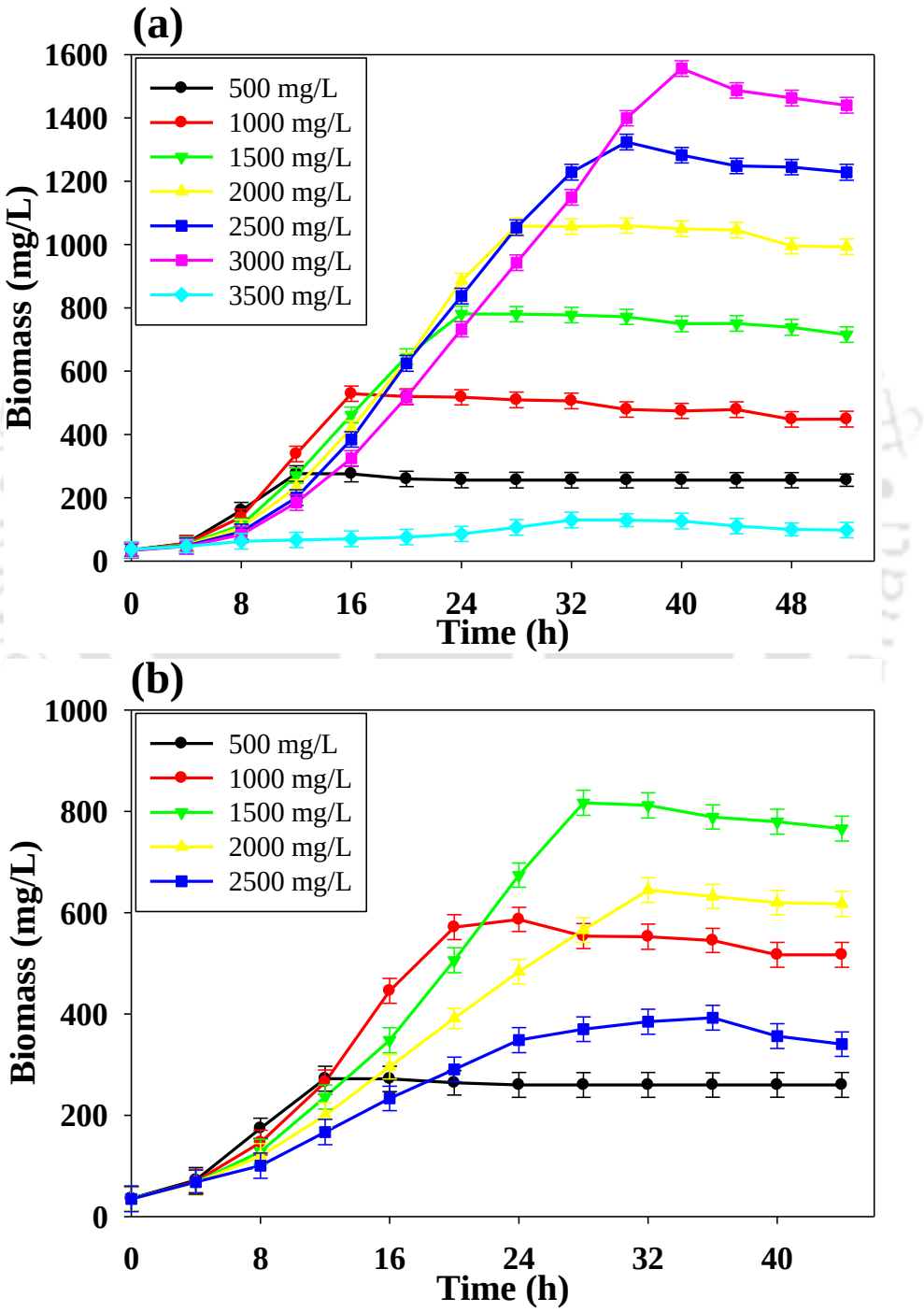


Figure 2.4: Biomass growth of *C. funkei* on (a) DMP and (b) DEP as single substrate.

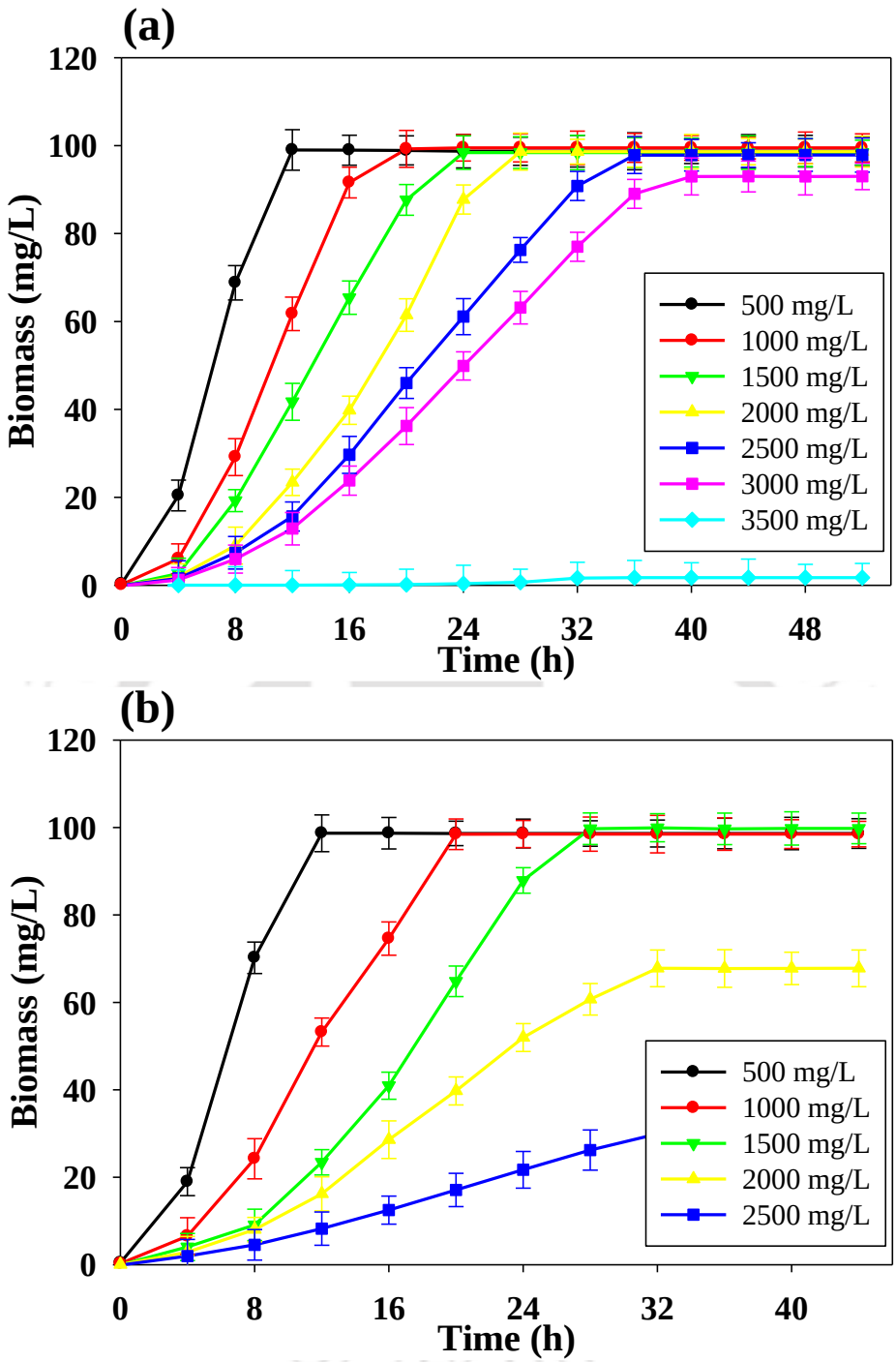


Figure 2.5: Biodegradation efficiency of (a) DMP and (b) DEP at different initial concentrations.

Recently, complete degradation of DMP is reported using *Comamonas testosterone* within 24 h batch treatment time period and at 500 mg/L initial concentration (Li et al., 2017). In another recent study, Xu et al. (2020) reported complete degradation of DMP and DEP at 200 mg/L initial concentration, by using *Paracoccus kondratievae BJQ0001* strain. Compared with these

microorganisms reported for biodegradation of EDPs, *C. funkei* is superior in terms of efficiently degrading DMP and DEP even at high initial concentrations and within a short time.

2.3.3.2. Modeling of biodegradation kinetics of DMP and DEP

From the results of DMP and DEP biodegradation as single substrate using *C. funkei*, maximum specific biodegradation rate values of these compounds were estimated to be 0.26 and 0.24 (mg/mg·h) for 1000 and 500 mg/L initial concentrations, respectively. These values decreased with further increase in their initial concentration (Figure 2.6), which clearly reveals biomass growth inhibition due to these compounds (Pradhan et al., 2012; Sahoo et al., 2014). For a better understanding of the biodegradation kinetics involved, the experimental data was fitted to different kinetic models reported in the literature. The model fitting exercise was carried out using MATLAB (Ver. 9.6, India). Figure 2.6 compares the experimental and model predicted specific biodegradation rate of the EDPs at different initial concentrations.

Values of DMP and DEP biodegradation kinetic parameters estimated from the models are presented in Table 2.3 along with their coefficient of determination (R^2) and sum of squared error (SSE) values between experimental and model-predicted q_s results. From Table 2.3, it is clear that Edward and Tiesser models accurately predicted the experimental data with very low SSE values of 0.089×10^{-3} and 0.008×10^{-3} as well as very high R^2 values of 0.99 and 0.98, respectively, for DEP degradation. These models were also accurate in predicting the experimental q_s values for DMP degradation. The other models (Aiba, Andrew and Webb) although fitted the experimental data well with high R^2 values and low SSE values, the estimated q_{max} values were much higher than the experimentally obtained value. Differences in the accuracy of the different models to predict the experimental data is attributed to the fact that these models were originally derived for describing degradation of substrates other than EDPs by using different microorganisms (Arutchelvan et al., 2006). The estimated K_s values

of the **Edward and Tiesser** models are close to the experimental K_s values. A high value of inhibition constant (K_I) estimated using these two models also reveal a high tolerance and efficiency of *C. funkei* in treating EDPs contaminated systems (Pradhan et al., 2012).

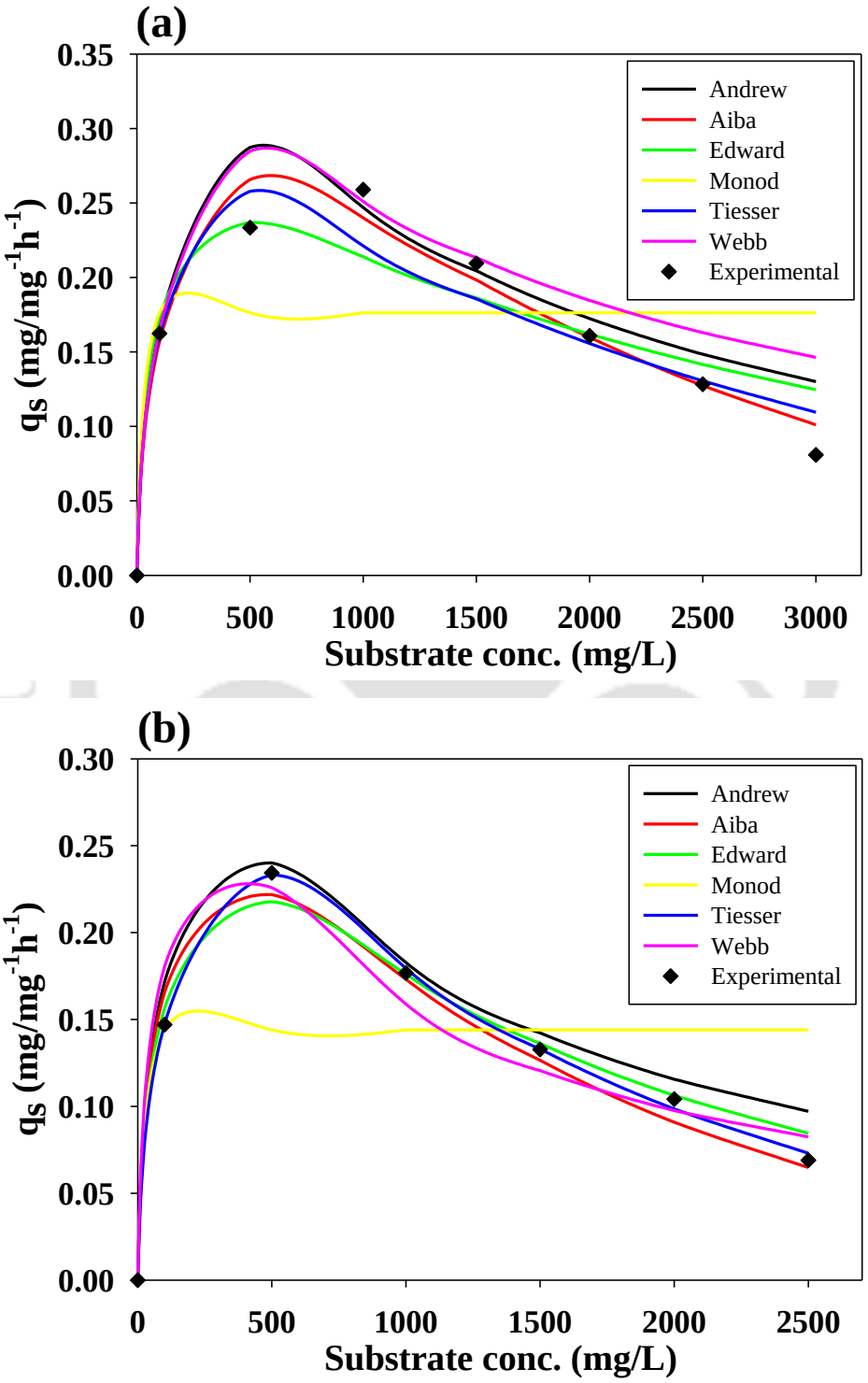


Figure 2.6: Experimental and predicted specific biodegradation rate (q_s) of (a) DMP and (b) DEP at different initial concentrations.

Table 2.3: Estimated biokinetic parameters of DMP and DEP biodegradation as single substrate using *C. funkei*.

		Estimated model parameters					
EDP	**Model**	**q_{max}** (mg/mg·h)	**K_S** (mg/L)	**K_I** (mg/L)	**K**	**SSE**	**R^2**
DMP	**Andrew**	0.593	242.685	861.717	-	0.757×10^{-3}	0.92
	Aiba	0.461	179.423	2056.275	-	0.245×10^{-3}	0.96
	Edward	0.327	84.945	2491.107	9160.47	0.598×10^{-3}	0.91
	Monod	0.176	0	-	-	2.879×10^{-3}	0.54
	Tiesser	0.315	126.480	2841.571	-	0.429×10^{-3}	0.94
	Webb	0.563	232.683	881.705	18385.28	1.095×10^{-3}	90
DEP	**Andrew**	0.578	217.258	513.962	-	0.241×10^{-3}	0.97
	Aiba	0.443	177.217	1399.366	-	0.107×10^{-3}	0.98
	Edward	0.352	118.384	1571	2601.15	0.089×10^{-3}	0.98
	Monod	0.144	0	-	-	2.372×10^{-3}	0.51
	Tiesser	0.328	140.986	1664.164	-	0.008×10^{-3}	0.99
	Webb	0.750	281.013	269.758	17864.59	0.270×10^{-3}	0.94

2.3.3.3. DMP and DEP biodegradation pathway

LC-MS analysis for identifying intermediates formed during the biodegradation of DMP and DEP as single substrate using *C. funkei* revealed the presence of mono-ethyl phthalate, mono-ethyl mono-methyl phthalate, dimethyl phthalate, mono-methyl phthalate, and phthalic acid (Figure 2.7). DEP is reported to be initially degraded by esterase enzyme to mono-ethyl or mono-ethyl mono-methyl phthalate and further into phthalic acid. Moreover, as enzymatic hydrolysis of EDPs mainly involves de-esterification of these compounds (Ahuactzin-Pérez et al., 2016), phthalic acid is identified as the chief intermediate of DEP and DMP biodegradation in this study (Figure 2.8). Amir et al. (2005) reported similar observations on the intermediates

formed during biodegradation of diethyl hexyl phthalate (DEHP), DMP and dibutyl phthalate (DBP). Many other studies (Ahmadi et al., 2017; Navacharoen and Vangnai, 2011; Singh et al., 2017) have also reported that the de-esterification is the main step involved in phthalates biodegradation.

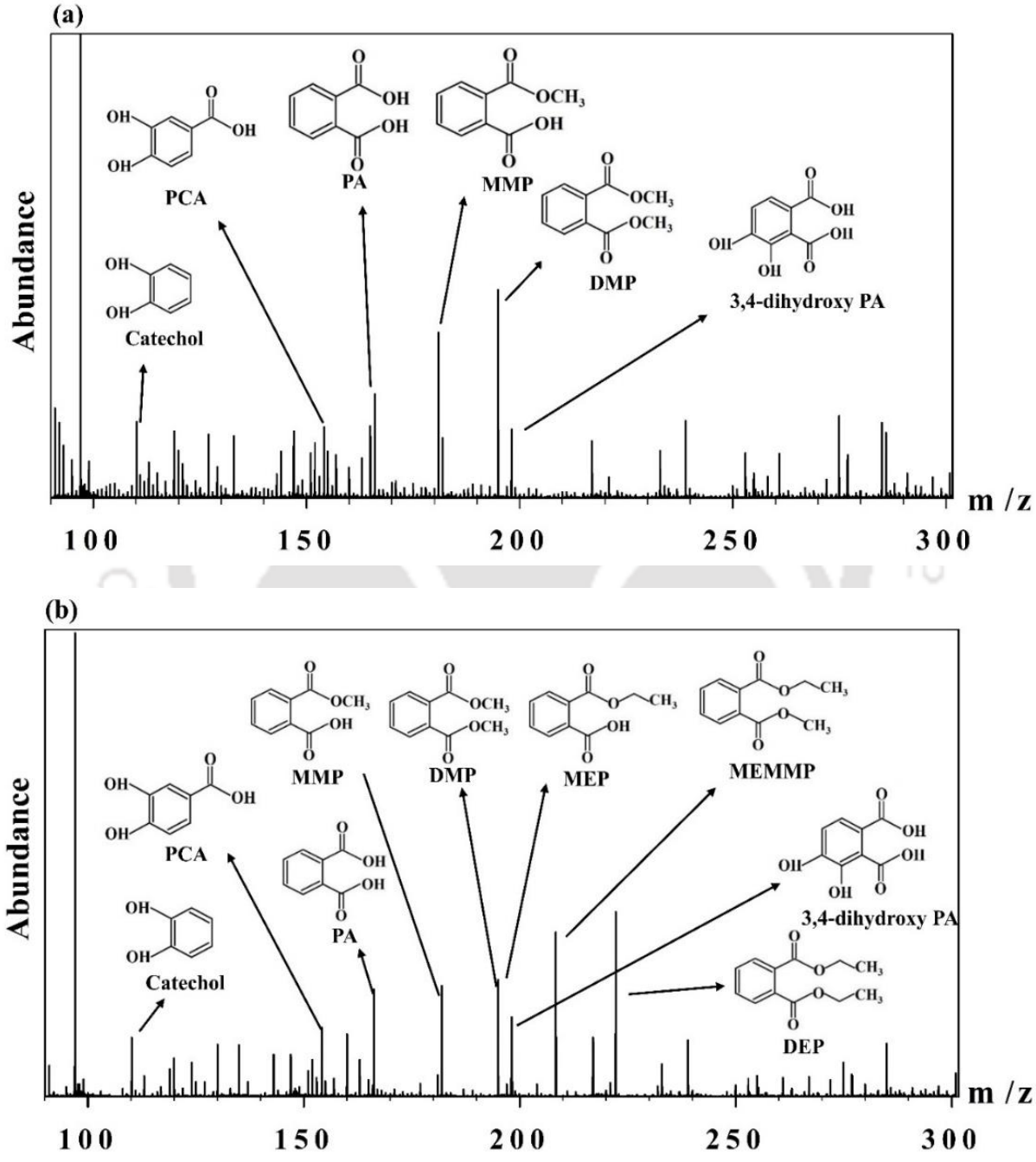


Figure 2.7: LC-MS profile showing metabolites identified during biodegradation of (a) DMP and (b) DEP as single substrate using *C. funkei*.

A similar pathway as that for DEP was observed for DMP biodegradation by *C. funkei*, and mono-methyl phthalate and phthalic acid were identified as the major metabolites. DMP biodegradation as well followed de-esterification as the main reaction. Thus, analysis of DEP and DMP biodegradation intermediates demonstrates that both the compounds share similar de-esterification steps as the main route for their biodegradation by *C. funkei*. Based on these results, proposed pathway involved in biodegradation of DMP and DEP as single substrate using *C. funkei* is presented in Figure 2.8.

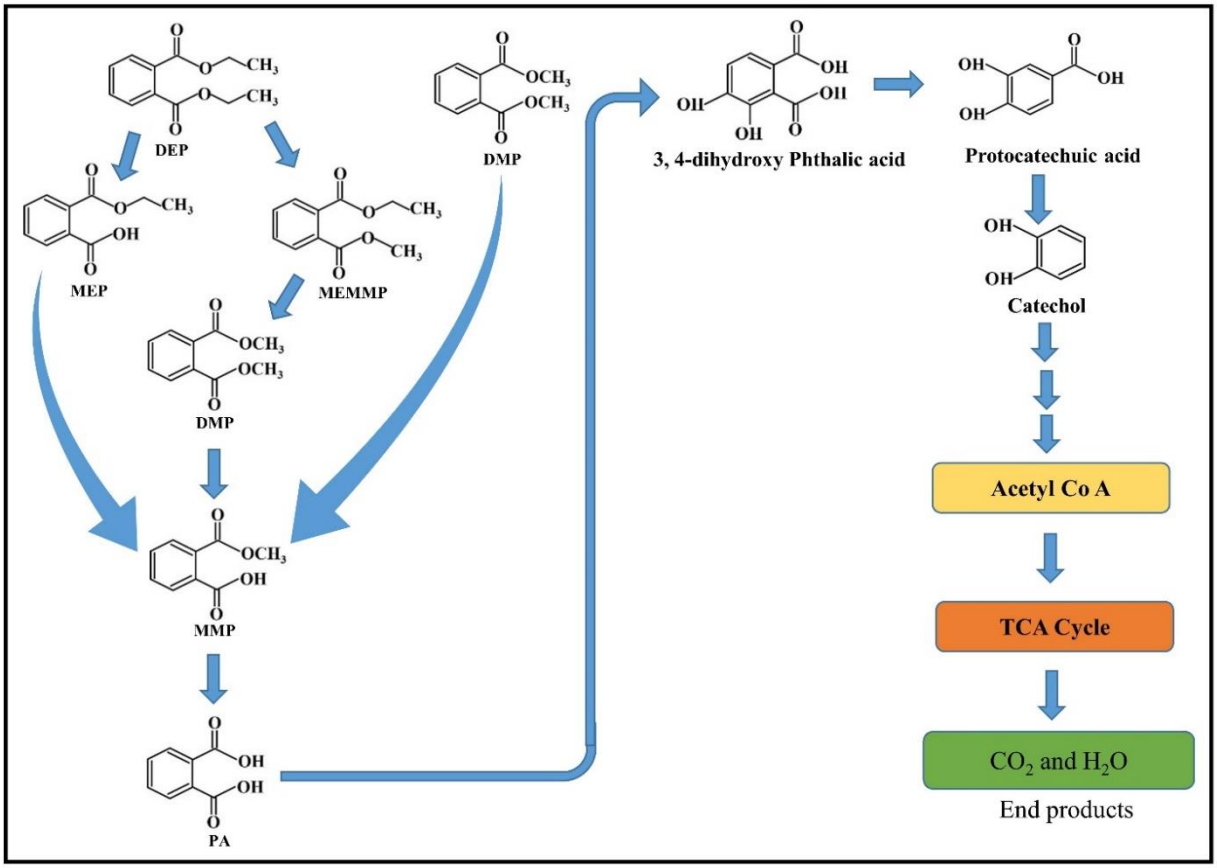


Figure 2.8: Proposed pathway of DMP and DEP biodegradation as single substrate using *C. funkei*.

Biodegradation of phthalic acid further leads to 3,4-dihydroxybenzoic acid (protocatechuic acid), catechol, benzoic acid, 4-hydroxyphthalic acid (4-hydroxyphthalate), 4,5-dihydroxyphthalic acid (4,5-dihydroxyphthalte), 3,4-dihydroxyphthalic acid (3,4-dihydroxyphthalate), etc. as the minor intermediates in the biodegradation of such EDPs

(Zhang et al., 2018). The metabolites produced from phthalic acid involve benzene ring cleavage as the primary degradation mechanism, which subsequently leads to other by-products e.g. 2-hydroxymuconic semi aldehyde. The end products of EDPs biodegradation by such aerobic bacterium are carbon dioxide and water, as reported in the literature (Ahmadi et al., 2017; Navacharoen and Vangnai, 2011; Zhang et al., 2018).

2.3.3.4. Biodegradation of DMP and DEP as dual substrates using *C. funkei*

In order to study the biodegradation of DMP and DEP as dual substrates using *C. funkei*, a 3^2 full factorial design was employed to perform the experiments. The results shown in Figure 2.9 reveal that the bacterial growth was quick at a low concentration combination of the EDPs. At a high concentration combination of the EDPs, the lag phase in biomass growth was prolonged as compared with that in the previous single substrate study.

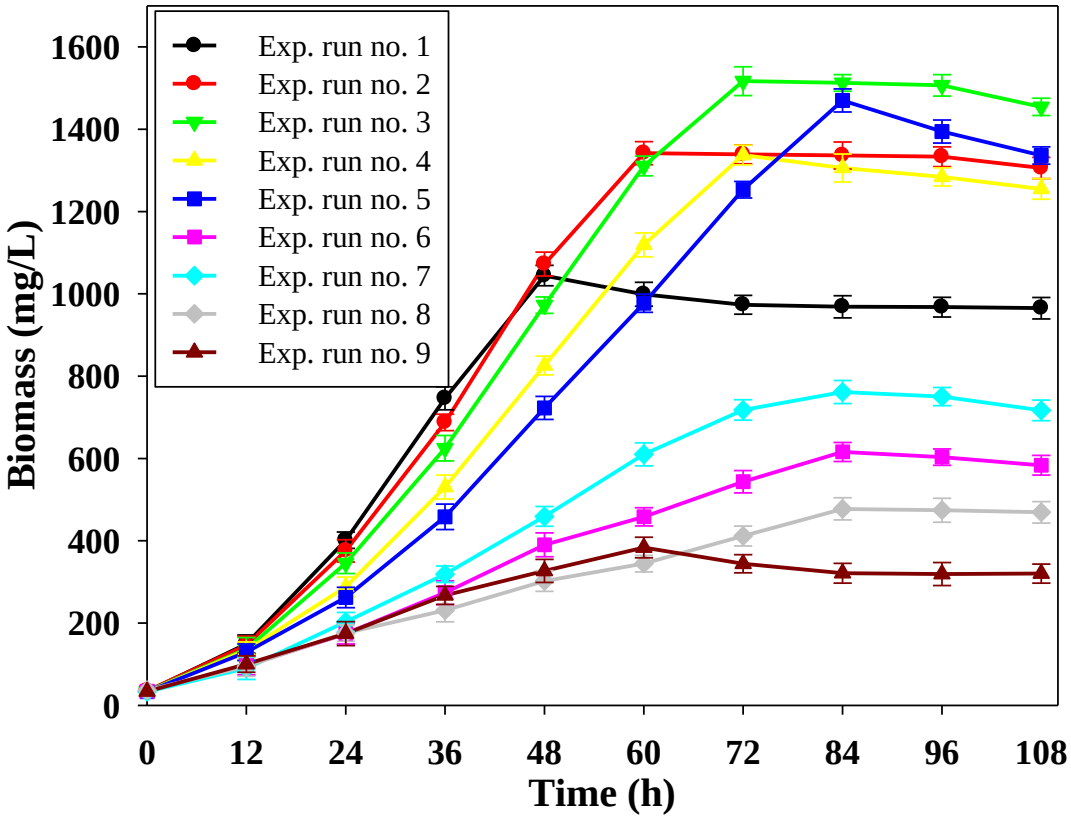


Figure 2.9: Biomass growth of *C. funkei* on DMP and DEP as dual substrates.

Biodegradation efficiency values of the EDPs in the different experimental runs is depicted in Figure 2.10, and the values are presented in Table 2.1 reveal that the degradation of DMP and DEP in the mixture varied in the ranges of 34.32 - 98.85% and 27.84 - 98.02 %, respectively, and depended mainly on their initial concentrations in the mixture. Experimental run nos. 1, 2, and 4, which were carried out with a total initial concentration of the EDPs less than or equal to 2500 mg/L, showed better degradation of the compounds than the other experimental runs. In these experimental runs, the degradation efficiency of both the EDPs is more than 97%. However, in experimental run nos. 3, 5, and 7 (total initial concentration of the EDPs = 3000 mg/L), their degradation efficiency value decreased with an increase in the DEP concentration; on the other hand, an increase in DMP concentration in the mixture favored the EDPs degradation by the bacterium. Owing to its short alkyl side chain and low molecular weight (LMW), DMP induces the degradation of DEP by co-metabolic effect (Ebadi et al., 2017; Xu et al., 2020). DMP is also an important metabolic intermediate of the DEP degradation pathway (Figure 2.8).

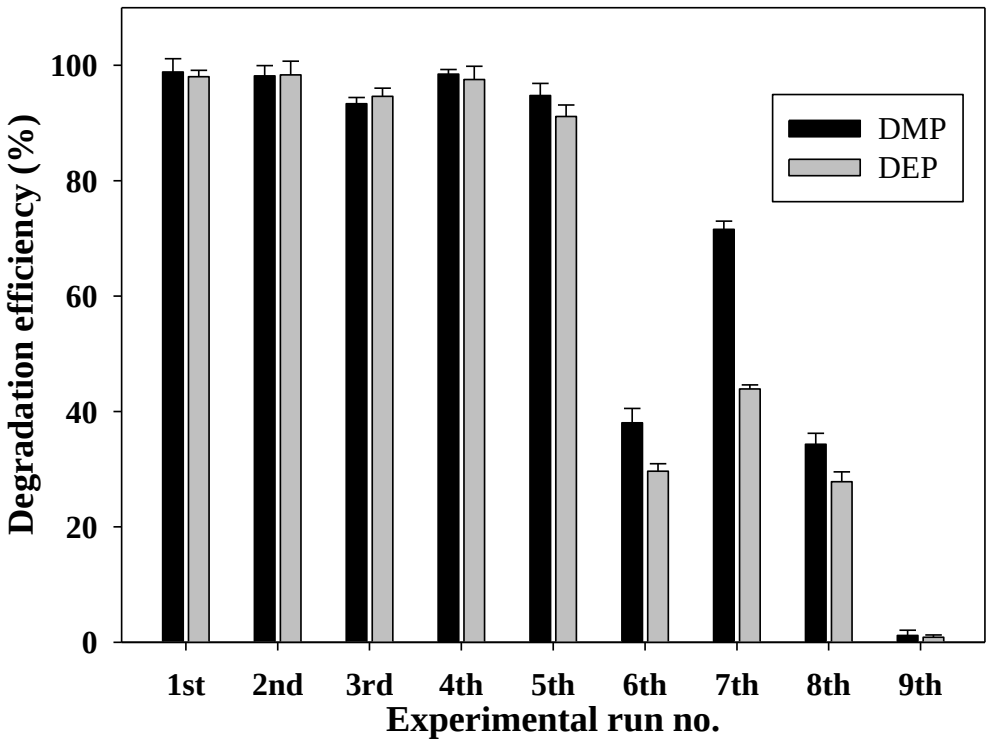


Figure 2.10: Biodegradation efficiency of DMP and DEP in dual substrates system.

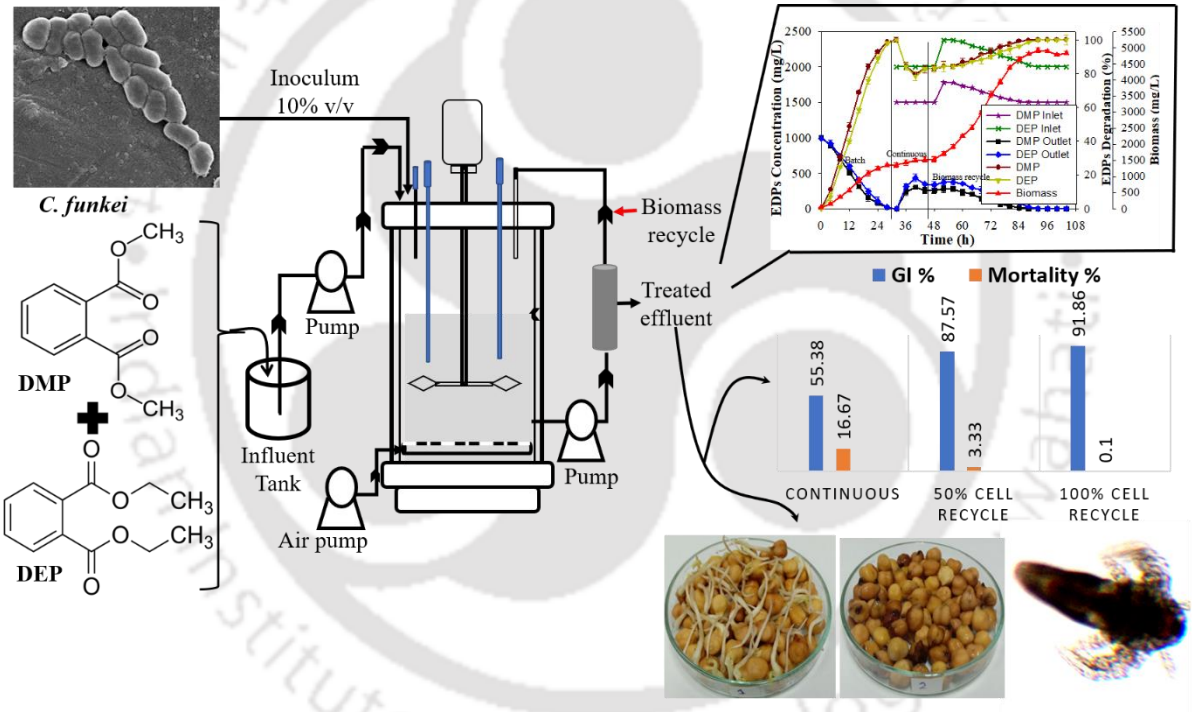
In run nos. 6 and 8 with a total initial concentration of 3500 mg/L, DMP/DEP degradation efficiency reduced to less than 40%, whereas no degradation was observed at 4000 mg/L total initial concentration in the mixture. Xu et al., (2020) reported 100 % degradation of DMP and DEP for a low initial concentration of 200 mg/L in the mixture. Similarly, Prasad and Suresh, (2015) studied degradation of a mixture of DMP, DEP and DBP, with 100 mg/L initial concentration each and obtained 100% degradation by using *Variovorax sp.*. Compared with the literature reports, *C. funkei* was found to efficiently degrade DMP and DEP as dual substrates even up to a very high total initial concentration of 2500 mg/L, which clearly demonstrates its potential to treat EDPs contaminated wastewater.

2.4. Conclusion

Among the three bacteria screened for biodegradation of DMP and DEP, *Cellulosimicrobium funkei* was identified to be the best. Intermediates formed during the biodegradation of DMP and DEP revealed phthalic acid as the central metabolite. In the mixture experiment, due to low molecular weight and short side chain of DMP, its specific degradation rate was more than that of the high molecular weight compound DEP which has a long side chain. Among the different models, Tessier and Edward models accurately fitted the experimental data. The high values of inhibition constant K_I estimated using the models indicated very good tolerance of *C. funkei* toward DMP and DEP for their efficient degradation.

Chapter 3

Biodegradation and toxicity removal of dimethyl phthalate and diethyl phthalate by *Cellulosimicrobium funkei* in a continuous stirred tank bioreactor under different operation modes



Abstract

The present study focused on the biodegradation of DMP and DEP mixture by *Cellulosimicrobium* bacteria in a continuous stirred tank bioreactor (CSTB) under batch, fed-batch, continuous and continuous with biomass recycle modes. Biomass recycling was carried out following microfiltration using an indigenous low-cost tubular ceramic membrane. Under the batch mode of operation, maximum degradation values of 85 and 58 % were observed at 1000 and 2000 mg/L initial concentrations of DMP and DEP, respectively, in mixture, whereas, complete degradation was achieved in the fed-batch system at the same initial concentrations. In continuous operation mode at 24 h hydraulic retention time (HRT), complete degradation was achieved for all inlet DMP and DEP concentrations. The biomass recycle operation mode was found to be the best strategy owing to complete degradation of the phthalates, even at very high inlet concentrations and at a short HRT of 16 h. A high germination index (GI) value of 91.86% and 0% brine shrimp mortality further demonstrated the potential of the CSTB operated under biomass recycle mode for the treatment of phthalate containing wastewater.

3.1. Introduction

Phthalic acid esters (PAEs) are semi-volatile chemicals and used in the making of soft and flexible plastic or as dissolving agents for various types of materials (Huang et al., 2021). Particularly, dimethyl phthalate (DMP) and diethyl phthalate (DEP) are frequently added to commercial plastics and epoxy resins to enhance their properties such as flexibility, durability and adhesion, and hence also known as plasticizers (Tao et al., 2019b). Due to the lack of suitable substitutes, the consumption of PAEs in a wide range of industries seems inevitable in the future (Huang et al., 2021). Their abundant presence in the environment pose a potential threat to the reproductive system of organisms and may affect the hormonal system (Blaauwendraad et al., 2022; Management Association, 2022; Ramadan et al., 2020; Yan et al., 2022). Hence, the ecological risk caused by PAEs in the environment and their removal has been a topic of recent interest among the researchers.

Currently, most of the reports are focused on limited aspects of aerobic biodegradation of PAEs in shake flask with a view to elucidate their biodegradation pathways and mechanisms involved (Song et al., 2022; Tao et al., 2019b; Xu et al., 2022). Very few studies are available on their biodegradation using a bioreactor system or hybrid treatment system aimed toward efficient degradation, economic treatment cost, enhanced safety, least disturbance to the environment etc. (Hu et al., 2021b; Kanaujiya et al., 2019). There is, however, no single report on PAEs degradation using an integrated biodegradation-microfiltration system which has the potential to achieve high treatment efficiency at a fast rate compared to conventional bioreactor systems (Paul et al., 2019a). The bacterium *C. funkei* used in this study has already shown potential to degrade DMP and DEP under batch shake flask and reported in previous Chapter 2. It has ability to utilize phthalates present in wastewater as carbon sources for its biomass growth and metabolic activity.

Hence, the present study focused on biodegradation of DMP and DEP by *Cellulosimicrobium funkei* in a continuously stirred tank reactor operated under different modes. The CSTB was operated under batch, fed-batch continuous and continuous with biomass recycle modes. Toxicity of the treated water was assessed by brine shrimp mortality and seed germination assays.

3.2. Materials and methods

3.2.1. Chemicals

Locally available mineral grade low-cost inorganic precursors, viz. feldspar, quartz, pyrophyllite, ball clay and kaolin, were used for the fabrication of tubular ceramic membrane. Calcium carbonate and carboxymethyl cellulose (CMC) were purchase from Merck (I) Ltd., Mumbai. All other chemicals and reagents used in the study are the same as mentioned earlier in Chapter 2, Section 2.2.1.

3.2.2. Microorganism, culture conditions and media composition

Details of culture conditions followed and media used, for the growth and maintenance of *Cellulosimicrobium sp.*, used in this biodegradation study are the same as mentioned in Chapter 2, Section 2.2.2.

3.2.3. Biodegradation experiments using CSTB under different operation mode

3.2.3.1. Batch operation mode

For biodegradation of DMP and DEP as single and dual substrates for *C. funkei* in a continuous stirred tank bioreactor, a 5L autoclavable lab-scale glass fermenter (BIOSTAT 'A', Sartorius Stedim Biotech, Germany) was used. A schematic of the CSTB is depicted in Figure 3.1a. The fermenter was equipped with necessary sensors for monitoring temperature, pH, dissolved oxygen (DO) and foam level. All online parameters (pH, temperature, DO, and agitation) in the study were monitored using BIOSTAT 'A' software package (BIOSTAT, Germany). The

reactor was operated under controlled conditions of 28 °C temperature, 400 rpm agitation, and 1.0 vvm aeration rate.

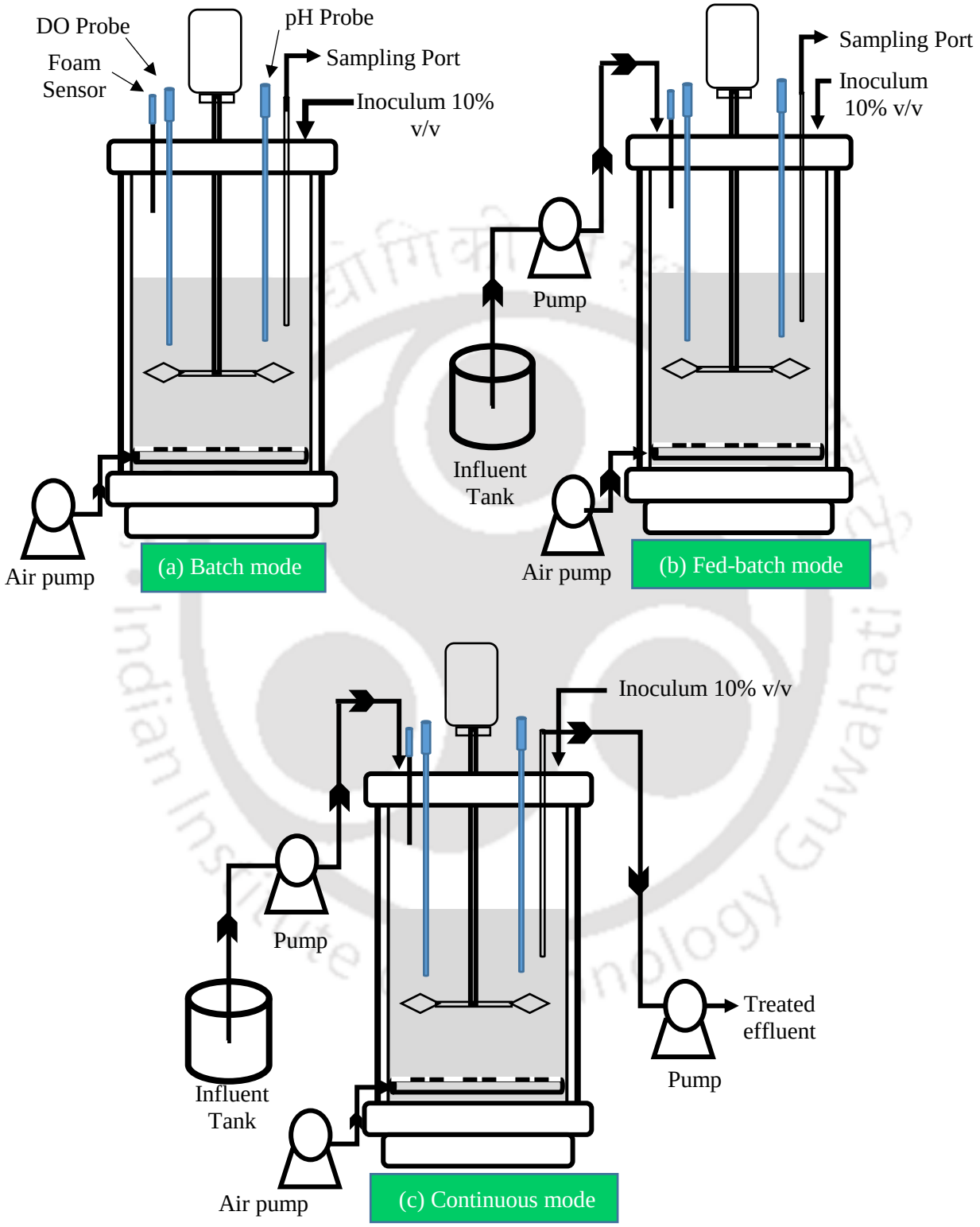


Figure 3.1: Schematic showing different operation modes with the CSTB for EDPs biodegradation in this study: (a) batch, (b) fed-batch and (c) continuous.

The reactor was filled with 2.7 litre MSM, and a calculated amount of DMP and/or DEP, as the only carbon source were aseptically added to the bioreactor prior to the start of the experiments (Table 3.1). The concentrations of DMP and DEP in the experiments were based on the results obtained previously using shake flask. Prior to inoculation with seed culture of the bacterium, the reactor loaded with MSM and phthalates was sterilized at 121 °C for 20 min using an automated autoclave (Equitron Medica Pvt. Ltd., Mumbai, India). After cooling down to room temperature, 300 mL (10%) log phase grown seed culture of the *C. funkei* (OD_{660} = 1) were aseptically added to the reactor. During the experiments, the reactor pH was controlled at 7 by adding HCl or NaOH. Samples (1.5 mL each) were collected in triplicate for evaluating the residual concentrations of DMP and DEP as well as biomass growth of *C. funkei*. Results reported are the arithmetic mean of these triplicate samples.

Table 3.1: DMP and DEP initial concentrations in each experimental run in single and mixture studies along with their percentage degradation values.

Experimental run no.	Initial concentration (mg/L)		% degradation	
	DMP	DEP	DMP	DEP
Single substrate system				
1	3000	2000	97.95	97.74
2	3500	2500	40.27	56.85
Dual substrate system				
1	1000	2000	85.38	58.11
2	2000	1500	62.09	46.44
3	1500	2000	43.02	41.78

Mass balance and stoichiometric analyses

Shuler and Kargi (2002) developed a general reaction mechanism for stoichiometric calculation and mass balance during biological conversion of organic substrate in which no extracellular product is formed except H_2O and CO_2 are as follows:

$$\underset{\text{Substrate}}{CH_mO_n} + aO_2 + bNH_3 \rightarrow \underset{\text{Biomass}}{cC_\alpha H_\beta O_\gamma N_\delta} + dH_2O + eCO_2 \quad (3.1)$$

Based on the above equation and the results obtained, the mass balance on each element and biomass yield from DEP and DMP as the single substrate were calculated by the following equation (3.2):

$$\text{Yield} = \frac{\text{Molecular weight of biomass}}{\text{Molecular weight of substrate}} \quad (3.2)$$

3.2.3.2. Fed-batch operation mode

For evaluation of phthalate biodegradation at a high initial biomass concentration, the CSTB was run in fed-batch operation mode. Figure 3.1b depicts schematic of the fed-batch operation mode of CSTB. For this fed-batch study, the bioreactor was initiated as the batch mode with 1500, 2500 and 2000 mg/L initial concentrations of DEP, DMP and their mixture, respectively, and allowed to run until complete degradation of the phthalates was achieved. The substrates were then fed to the reactor in pulse feeding manner, for which a defined amount of specific concentration of the phthalates was fed into the reactor medium, after establishing the exponential growth phase of the culture and complete degradation of DMP and DEP. The operating parameter of the reactor was kept the same as in the batch experiments. The feed concentrations of DMP, DEP and their mixture in the fed-batch study are presented in Table 3.2. Sample collection was carried out in triplicate at regular interval of time and analyzed for residual DMP and DEP concentrations and biomass growth. The results reported are the arithmetic mean of triplicate sample analyses.

Table 3.2: Feed concentrations of DMP, DEP and their mixture along with their percentage degradation in the fed-batch study.

Stages	Initial concentrations (mg/L)		% degradation	
	DMP	DEP	DMP	DEP
	Single substrate system			
Batch	2500	1500	100	100
1st Feed	3000	2000	100	100
2nd Feed	3500	2500	100	100
3rd Feed	4000	3000	42.14	7.53
	Dual substrate system			
Batch	1000	1000	100	100
1st Feed	1000	2000	100	100
2nd Feed	2000	1500	82.81	73.02
3rd Feed	1500	2000	00	00

3.2.3.3. Continuous operation mode

The CSTB was run in a continuous mode of operation to investigate the biodegradation efficiency of *C. funkei* at different HRT and inlet loading rates of the phthalates. Figure 3.1c shows schematic of continuous operation mode of the CSTB. For the continuous experiments, *C. funkei* biomass was obtained by growing the culture using 1000 mg/L each of DMP and DEP as the sole carbon source under batch mode carried out until their complete degradation. After establishing the exponential growth phase of the culture and complete degradation of DMP and DEP, continuous feeding of MSM containing DMP and DEP mixture into the CSTB was started using a peristaltic pump (Watson Marlow, TR11 4RU, UK). Table 3.3 presents the concentration combinations of DMP and DEP used in this continuous study carried out at different HRTs.

Table 3.3: Concentration combinations of DMP and DEP used in the continuous study carried out under different HRTs.

HRT (h)	Time (h)	Inlet concentrations (mg/L)			% Degradation		
		DMP	DEP	Total	DMP	DEP	Total
Batch	0-32	1000	1000	2000	100	100	100
8	32-56	1000	2000	3000	89.85	91.53	90.69
	56-80	2000	1500	3500	86.86	75.00	80.93
	80-104	1500	2000	3500	68.26	65.49	66.88
Batch	0-32	1000	1000	2000	100	100	100
16	32-56	1000	2000	3000	99.92	99.96	100
	56-80	2000	1500	3500	97.92	85.11	91.51
	80-104	1500	2000	3500	84.18	81.11	82.65
Batch	0-32	1000	1000	2000	100	100	100
24	32-56	1000	2000	3000	99.95	99.99	100
	56-80	2000	1500	3500	99.75	99.12	100
	80-104	1500	2000	3500	99.71	99.97	100

The performance of the continuous bioreactor was evaluated at three different HRTs, viz. 24, 16 and 8 h and three different concentration combinations of DMP and DEP in mixture. At the same HRT, all three-concentration combinations of the DMP and DEP were continuously fed to the reactor consecutively and run for 24 h under each condition. The HRTs and concentrations used in this continuous operation mode were selected on the basis of the results obtained in the previous batch study. The physical parameters (temperature, agitation and aeration) in the reactor were the same as previously mentioned in the batch study. Samples were taken from the effluent of the reactor at 4 h time intervals for analysis.

3.2.4. Tubular ceramic membrane for microfiltration of biomass

3.2.4.1. Fabrication of membrane

For biomass separation, a ceramic membrane fabricated using locally available raw materials, quartz-28 wt.%, ball clay-18 wt.%, calcium carbonate-18 wt.%, pyrophyllite-15 wt.%, kaolin-15 wt.%, and feldspar-6 wt.% was used. All the aforementioned clay powder with specified composition were mixed manually until a homogenous mixture is obtained. A paste was prepared by adding required amount of 3 wt.% sodium salt of carboxymethyl cellulose (Na–CMC) aqueous solution of binder to the mixture of clay powders. For tubular-shaped ceramic membrane preparation, extrusion technique was adopted in this work. The bench top horizontal extruder (M/s VB Ceramic Consultants, Chennai, India) was used for fabrication of the ceramic tubes. The extruded membranes were subjected to three signature steps of controlled thermal treatment in order to avoid cracks in the membrane during drying and sintering process. In the first step, the obtained tubular membranes were air-dried at room temperature for 24 h. In the second step, the membranes were dried at 100 °C and 200 °C in a hot air oven for 12 h in each case. In the final step of the thermal treatment, the membranes were sintered at 950 °C for 6 h at a heating rate of 2 °C/min in a furnace. Furthermore, both the ends of the membrane were rubbed to get the desired length by using abrasive paper. To remove the loosened particles from the membrane, the membranes were immersed in water for ultrasonication using a bath sonicator. Finally, the prepared membranes were dried in a hot air oven at 100 °C prior to their characterization and use in the experiments.

3.2.4.2. Characterization of the prepared membrane

The phase composition of the prepared tubular ceramic membranes (sintered and unsintered) was assessed using a X-ray diffractometer (Model: Micromax-007HF, Make: Rigaku) with Cu Kα radiation source at 40 mA operational current and 40 kV voltage. The analysis was carried out in the 2θ range from 3 to 65^{o} with a scanning rate of 0.05 ^{o}/s. Using a field emission scanning

electron microscope (FESEM) (Model: Sigma 300, Make: Zeiss), the surface (inner and outer) morphology of the ceramic membranes was analyzed. Prior to the analysis, samples were coated with a fine layer of gold to impart conductivity. The thermal stability and appropriate sintering temperature of the membrane were identified by using a thermogravimetric analyzer (TGA) (Model: STA449F3A00, Make: Netzsch), over a temperature range of 25 - 950 °C and a heating rate of 10 °K/min. The chemical stability of the prepared membranes was analyzed by treatment using acid and base. For this analysis, the membranes were suspended in HCl of 1.4 pH and NaOH of 13.5 pH solution individually for seven days at atmospheric conditions. The dry weight of the membranes (W_b) was measured before immersion in acid/base solution. After seven days of immersion, the suspended membranes were washed with Millipore^{TM} water followed by sonication and drying to obtain their final dry weight (W_a). The weight loss percent in acid/base solution was evaluated using the following equation (3.3):

$$\text{Weight loss (\%)} = \frac{W_b - W_a}{W_b} \times 100 \quad (3.3)$$

Where W_b and W_a represents the dry weight of the membrane (g) before and after chemical treatment, respectively.

The porosity of the membranes was calculated by the Archimedes' principle. For this test, the dry weight of the membrane was measured before dipping in water for 24 h. The membranes were then taken out and wiped with tissue paper to remove loosely bound water from the membrane surface and the wet weight of the membrane was measured. The following expression was used to evaluate the membrane porosity (3.4):

$$\text{Porosity (\%)} = \frac{W_w - W_d}{\rho_w \times V_m} \times 100 \quad (3.4)$$

Where W_w and W_d are the wet and dry weight of the membrane, respectively, V_m is total volume of the membrane and ρ_w is the density of water.

Water permeation test was conducted by keeping the membrane in a laboratory-made cross-flow microfiltration setup (Purnima et al., 2020). The effective surface area (A) of the

membrane was 0.001727 m^2, whereas the water flux (J_w) was measured by determining the permeate volume (V) collected at every 2 min time interval (t) under applied pressures in the range 69 – 345 kPa, as given by the following equation (3.5). Equations (3.6) and (3.7) were utilized to estimate pure water permeability (L_h) and pore size of the membrane, respectively.

$$\text{Water flux } (J_w) = \frac{V}{A \times t} \quad (3.5)$$

$$\text{Water flux } (J_w) = L_h \times \Delta P \quad (3.6)$$

$$r = \left(\frac{8\mu\tau l L_h}{\varepsilon}\right)^{\frac{1}{2}} \quad (3.7)$$

Where, ΔP represents applied pressure (kPa), ε is the porosity, τ represents the tortuosity factor (taken as 1), μ is the viscosity of water at room temperature (Pa·s), l represents the pore length (m) and L_h is the pure water permeability (m^3/m^2 s kPa).

3.2.5. Continuous biodegradation under biomass recycle mode

In order to improve the phthalate degradation efficiency by overcoming the drawback of biomass washout condition in the continuous process particularly at high HRTs, a continuous biomass recycle process was developed. For biomass recycle followed by microfiltration, the bioreactor was integrated with the previously described indigenous tubular ceramic membrane (Figure 3.2). For the microfiltration of biomass, the membrane was fitted into a stainless steel pellicon holder, fixed with a diaphragm, and connected with pressure gauges at the inlet and outlet ports. Due to biomass concentration polarization and cake formation, the permeate flux reached a steady state after 100 minutes of operation. Hence, the fouled membrane was subsequently regenerated by dipping it in water for ultrasonication using a bath sonicator. Transmembrane flux was achieved using a peristaltic pump which ensured appropriate amount of driving force.

For continuous biomass recycling, the reactor was initially started under batch mode with 1000 mg/L each of DMP and DEP. At the end of the initial batch operation, the reactor was

continuously fed with aqueous medium containing PAEs at 3.125 mL/min flow rate. After 16 h of continuous run, effluent collected from the bioreactor was directed to the tubular ceramic membrane system for the microfiltration of biomass. The biomass rich retentate was then supplied to the bioreactor as a secondary inlet along with the influent containing the PAEs. The biomass recycle bioreactor was operated at 16 h HRT and 218.75 mg/L·h inlet loading rate of phthalate and its performance compared with that of the previous continuous system without biomass recycle. Schematic of the reactor under continuous operating mode with biomass recycle for phthalate biodegradation is presented in Figure 3.2.

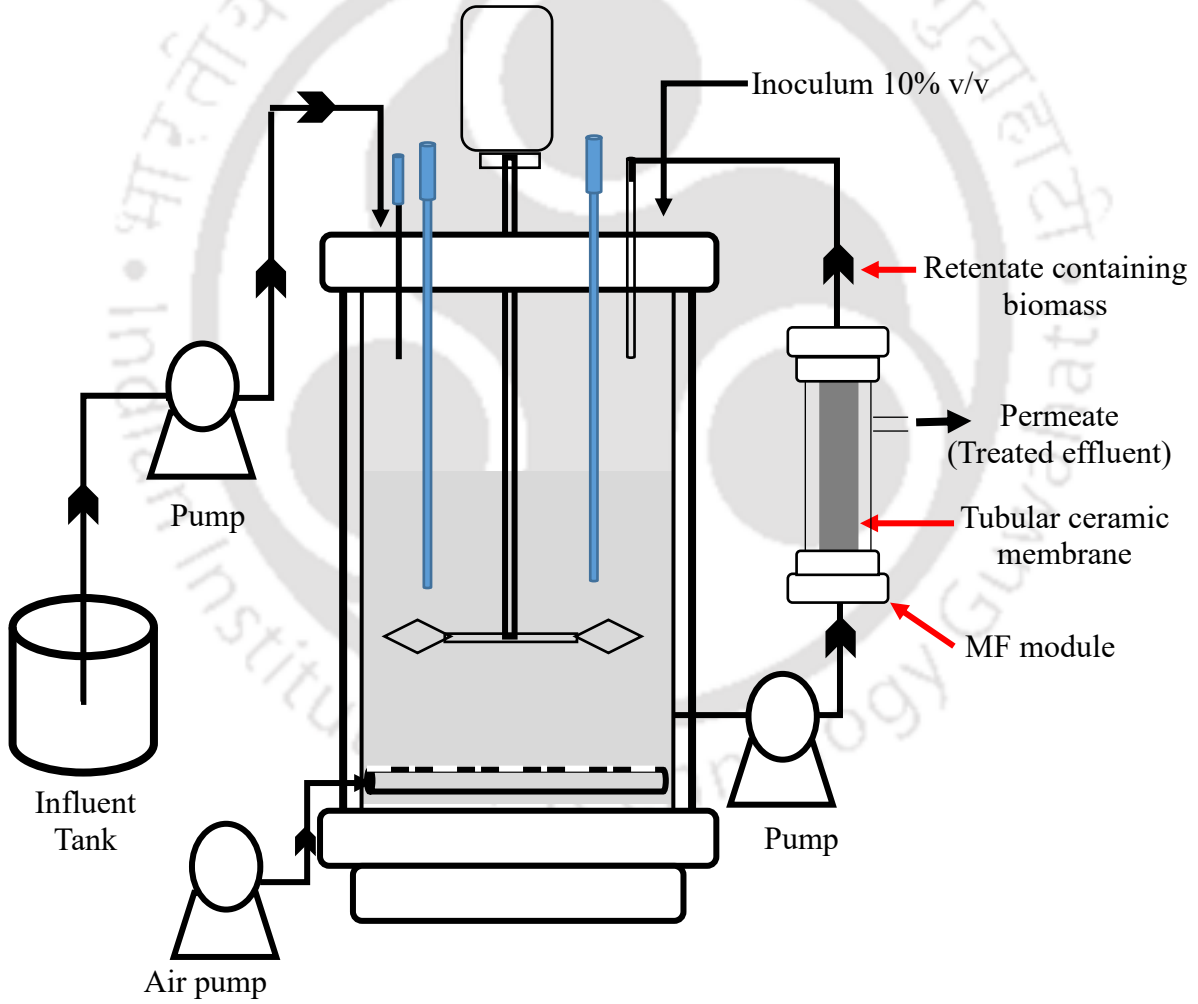


Figure 3.2: Schematic showing integrated biodegradation-microfiltration (MF) set up for continuous mode with biomass recycle bioreactor operation.

In order to evaluate the effect of retentate dilution on phthalate biodegradation, the retentate was diluted 1:1 with distilled water and fed to the reactor along with the inlet. Biomass recycle ratio in the continuous mode with biomass recycle was estimated as per the equation (3.8).

$$X_1 = \frac{Y_{X/S}\,(S_f - S_0)}{(1+\alpha-\alpha C)} \qquad (3.8)$$

where C, X_1, α, S_0 and S_f denote concentration factor in the biomass recycle stream, biomass concentration in reactor effluent, recycle ratio, initial and final phthalate concentrations (mg/L), respectively.

3.2.6. Analytical methods

C. funkei biomass growth and phthalate degradation were determined using the same method as previously described in Chapter 2 under Sections 2.2.4.2 and 2.2.4.3, respectively.

3.2.7. Ecotoxicity of the treated water

3.2.7.1. Phytotoxicity evaluation

In order to assess the toxicity removal of phthalate degraded water due to biodegradation by *Gordonia sp.*, samples taken from the reactor operated under different modes were tested for seed germination. Required quantity of chickpeas (*Cicer arietinum L.*) were placed in 16 petri plates for this phytotoxicity assessment. 60 mL of distilled water, tap water, phthalate-degraded samples and phthalate-containing medium (untreated) were added to each plate separately. The plates were then incubated at 28°C for 24 hours. The seeds were taken out of plates after 24 h, covered in cotton towels moistened with the respective samples and left undisturbed for 48 h. Root length and percentage of the germinated seeds was measured for calculating Germination Index (GI %) as per the equation (3.9). The results were compared with those obtained using distilled water and water containing phthalates.

$$\text{Germination index (GI)}\% = \frac{\text{Seed germination (\%)} \times \text{Root elongation (\%)}}{100} \qquad (3.9)$$

3.2.7.2. Brine shrimp mortality bioassay

Brine shrimp lethality bioassay was carried out to examine the cytotoxicity of phthalate-degraded water due to biodegradation of phthalates mixture by *Gordonia sp*. 1-litre glass beaker containing 600 ml of synthetic seawater (NaCl 30 g/L and pH adjusted to 8.5 using 1N NaOH) was used to hatch brine shrimp eggs (*Artemia salina*) under continuous aeration for 24 h.

Following hatching, ten active nauplii were withdrawn through a glass pipette and placed in separate petri plates with 40 ml of distilled water, tap water, phthalate-degraded samples and phthalate-containing media (untreated) with 30 g/L salt concentration. These dishes were then incubated at room temperature for 24 hours. A magnifying glass was used to examine the number of survived shrimps in each petri plates. The assay was performed in triplicate and the results were expressed as brine shrimp mortality (%), (equation 3.10).

$$\text{Brine shrimp mortality (\%)} = \frac{N_i - N_f}{N_i} \times 100 \qquad (3.10)$$

Where N_i and N_f are the initial and final number of active (survived) nauplii, respectively.

3.3. Results and discussion

3.3.1. Degradation under batch operation mode

3.3.1.1. Single substrate system

The results of the previous batch shake flask study described in Chapter 2 showed that *C. funkei* could efficiently degrade EDPs as single substrate up to 2500 mg/L initial concentration. In order to achieve efficient degradation of the EDPs even at a high initial concentration, a 5 liter bioreactor with provision for monitoring and control of various parameters, including agitation, aeration, dissolved oxygen (DO), pH and temperature, was employed. DMP and DEP degradation as single substrate by *C. funkei* in the CSTB (Figure 3.3) reveal that the lag phase in biomass growth as well as degradation time was reduced compared to that in the batch shake

flask. In the case of DMP, 97.9%, and 40.3% degradation efficiencies were attained within 54 and 66 h at 3000 and 3500 mg/L initial concentrations respectively (Figure 3.4a), whereas in the shake flask, the values were 93% and 0%. In the case of DEP, 97.7% and 56.8% degradation efficiencies were achieved within 40 and 46 h at 2000 and 2500 mg/L concentrations, respectively (Figure 3.4b), whereas these values were 67.8% and 29.5% in the shake flask.

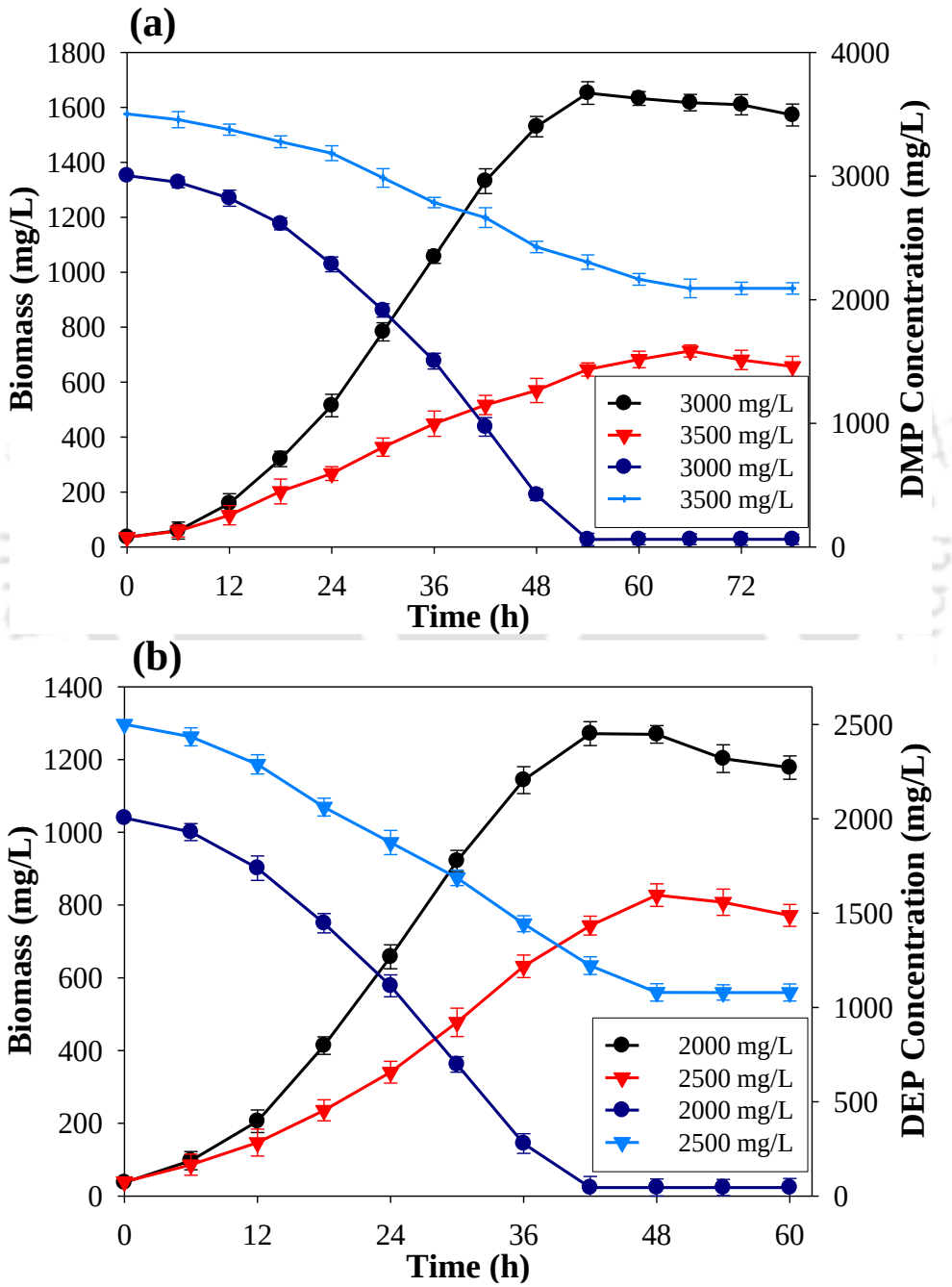


Figure 3.3: Biomass growth of *C. funkei* on (a) DMP and (b) DEP as single substrate along with their biodegradation profile in the CSTB.

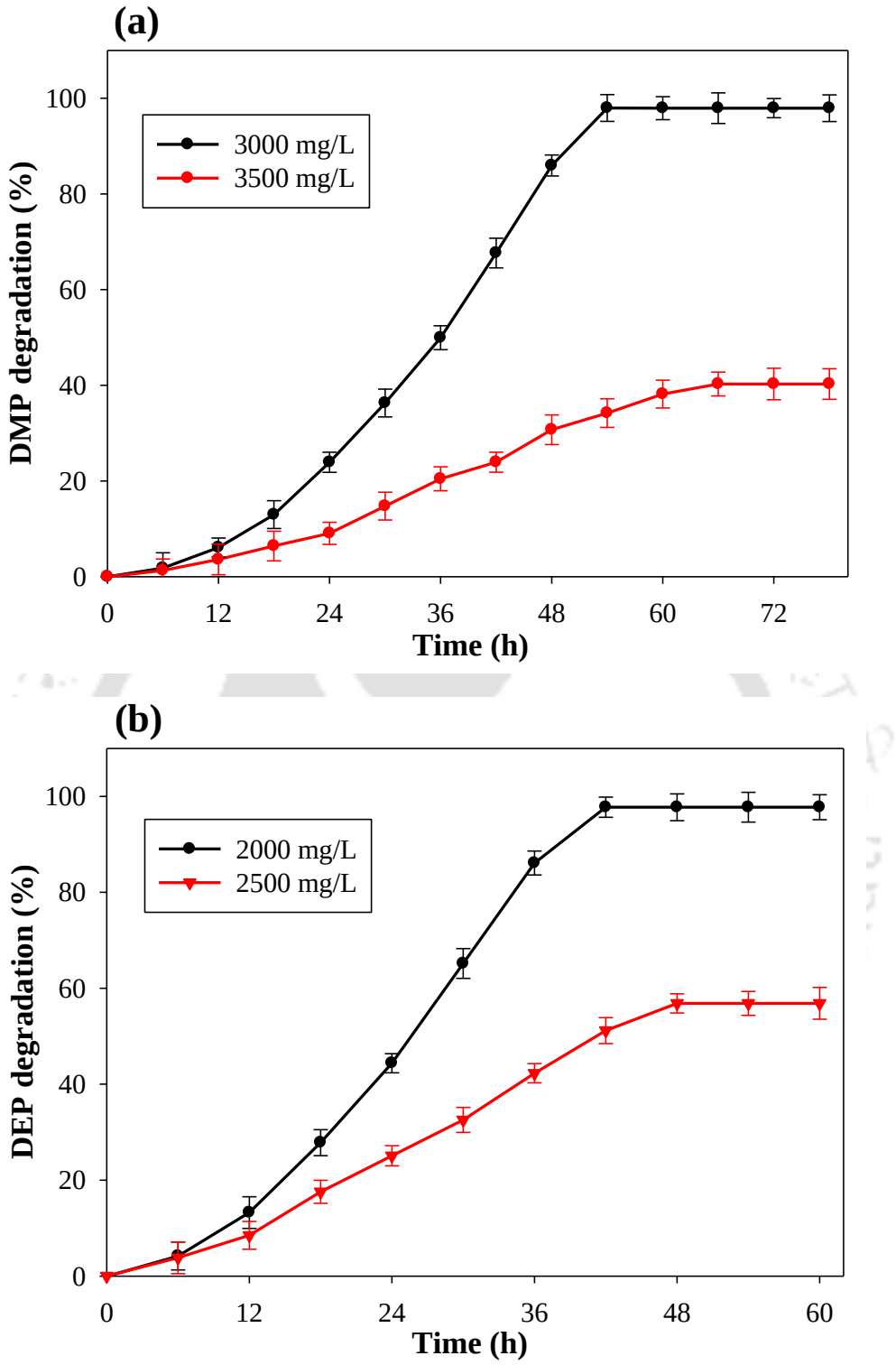


Figure 3.4: Biodegradation efficiency of (a) DMP and (b) DEP in the CSTB.

Very high biodegradation of both DMP and DEP were obtained by *C. funkei* in the CSTB as compared to that in simple shake flask due to efficient transfer of nutrient and oxygen in the CSTB, which are essential for simultaneous biomass growth and degradation of the compounds

by the bacterium. Moreover, the bioreactor was operated under controlled conditions of pH, temperature, dissolved oxygen (DO) and agitation to maintain maximum growth and EDP biodegradation by the bacterium. These parameters were, however, not controlled in the shake flask experiments. Recently, Patil and Jena (2019) used an internal loop airlift bioreactor for biodegradation of DEP by a mixed culture of *Bacillus sp.* and *Micrococcus sp*. and observed 100% degradation efficiency at 1500 mg/L initial concentration in 156 h. In another study on biodegradation of 300 mg/L initial concentration of DEP and diallyl phthalate as single substrate in a moving bed biofilm reactor system, 94.96% and 93.85% removal efficiency values were reported, respectively (Ahmadi et al., 2015). Compared with these literature reports, the continuous stirred tank bioreactor used in this study is found to be superior for biodegradation of EDPs by *C. funkei.* Moreover, this is the first report on biodegradation of DMP and DEP in a stirred tank bioreactor system.

Mass balance and stoichiometric analysis

Mass balance strictly follows the law of conservation of mass that comprises a substrate that enters a system and exits from the system with a known composition. The conversion of substrates from one form to other forms defines the metabolic response of the microbes to treat contaminated wastewater. The basic fundamental of mass balance is given in equation 3.11 below:

$$\mathbf{X_{in} - X_{out} = X_{Accum/rem}} \quad (3.11)$$

Where X_{in} is input concentration, X_{out} is the output concentration, and $X_{Accum/rem}$ is concentration after accumulation or removal.

To treat wastewater containing organic substrate by microbes, the mass balance of input and output concentrations of biomass, substrate, dissolved oxygen, and chemical oxygen demand is an essential step (Kumar and Maitra, 2016). The present mass balance analysis considered concentration of biomass, dissolved oxygen (DO), DEP and DMP in the experiments. Initially,

the culture medium contained an average dissolved oxygen concentration of 7.61 mg/L, which was utilized for simultaneous degradation of the compounds and biomass growth of *C. funkei*. Maximum biomass growth of a culture can be correlated to the uptake of dissolved oxygen in the system (Meng et al., 2019). A decrease in oxygen level during the experiments further confirms that *C. funkei* is aerobic, and degradation of DMP and DEP requires oxygen for their biochemical conversion. It was observed that microbial biomass increased in all the experimental runs as it utilized DMP/DEP as the sole carbon source.

Stoichiometry analysis of DMP and DEP biodegradation and biomass accumulation was carried out based on the dry weight of biomass. The experimental results showed that 3/5 and 2.75/5 of carbon from DEP and DMP, respectively, were converted to biomass. The elemental composition of biomass was assumed as $C_5H_7O_2N$ for this stoichiometric analysis (Patil and Jena, 2019). Based on the above, the stoichiometry equations are as follows:

DMP $C_{10}H_{10}O_4 + \mathbf{a}O_2 + \mathbf{b}NH_3 \rightarrow \mathbf{c}C_5H_7O_2N + \mathbf{d}H_2O + \mathbf{e}CO_2$ (3.12)

Substrate Biomass

DEP $C_{12}H_{14}O_4 + \mathbf{a}O_2 + \mathbf{b}NH_3 \rightarrow \mathbf{c}C_5H_7O_2N + \mathbf{d}H_2O + \mathbf{e}CO_2$ (3.13)

Substrate Biomass

For nitrogen mass balance, NH_3 was considered for stoichiometric calculation as it constituted MSM in the form of NH_4NO_3. From the above stoichiometric equations, the following four different equations were formed to balance C, H, N, and O elements in this study:

DEP		**DMP**	
C balance: 12 = 5c + e	(i)	C balance: 10 = 5c + e	(i)
H balance: 14 + 3b = 7c + 2d	(ii)	H balance: 10 + 3b = 7c + 2d	(ii)
O balance: 4 + 2a = 2c + d + 2e	(iii)	O balance: 4 + 2a = 2c + d + 2e	(iii)
N balance: b = c	(iv)	N balance: b = c	(iv)

After solving the above equations (i) – (iv), the following stoichiometrically balanced equations for DMP and DEP degradation were obtained:

DEP $C_{12}H_{14}O_4 + \mathbf{6.33}O_2 + \mathbf{1.4}NH_3 \rightarrow \mathbf{1.4}C_5H_7O_2N + \mathbf{4.18}H_2O + \mathbf{4.8}CO_2$ (3.14)
Substrate Biomass

DMP $C_{10}H_{10}O_4 + \mathbf{5}O_2 + \mathbf{1.1}NH_3 \rightarrow \mathbf{1.1}C_5H_7O_2N + \mathbf{2.8}H_2O + \mathbf{4.5}CO_2$ (3.15)
Substrate Biomass

From the above equations, biomass yield values were thus obtained as 0.712 and 0.64 for DEP and DMP, respectively. Kumar et al, (2017) performed mass balance analysis of DBP biodegradation in batch shake flask by *Comamonas sp.* and *Pseudomonas sp.*, and obtained biomass yield values of 0.37 and 0.35, respectively. The high biomass yield obtained in the present study indicates the high efficiency of *C. funkei* in utilizing DEP and DMP. These results further demonstrate the importance of CSTB over simple batch shake flasks for DEP and DMP removal from wastewater.

3.3.1.2. Dual substrate system

The CSTB was further evaluated to degrade DMP and DEP as dual substrates at high initial concentrations (≥3000 mg/L). Figure 3.5a reveals that the lag phase in biomass growth in the bioreactor is shorter than the values obtained in shake flask. Biodegradation results presented in Figure 3.5b further reveal efficient but incomplete biodegradation of the EDPs. Figure 3.5b also shows that the degradation efficiency of DMP in all the three experimental runs is high compared with that of DEP, which is due to difference in their chemical structures, in particular the side chains present in these two compounds. EDPs with short side chains are reported to be degraded quickly compared with EDPs containing long side chains (Gao and Wen, 2016; Xu et al., 2020). Maximum biodegradation efficiency of more than 80% and 55% of DMP and DEP, respectively, are achieved at 3000 mg/L total initial concentration in the CSTB, whereas these values were 71.58 % and 43.90 %, respectively, in the shake flask.

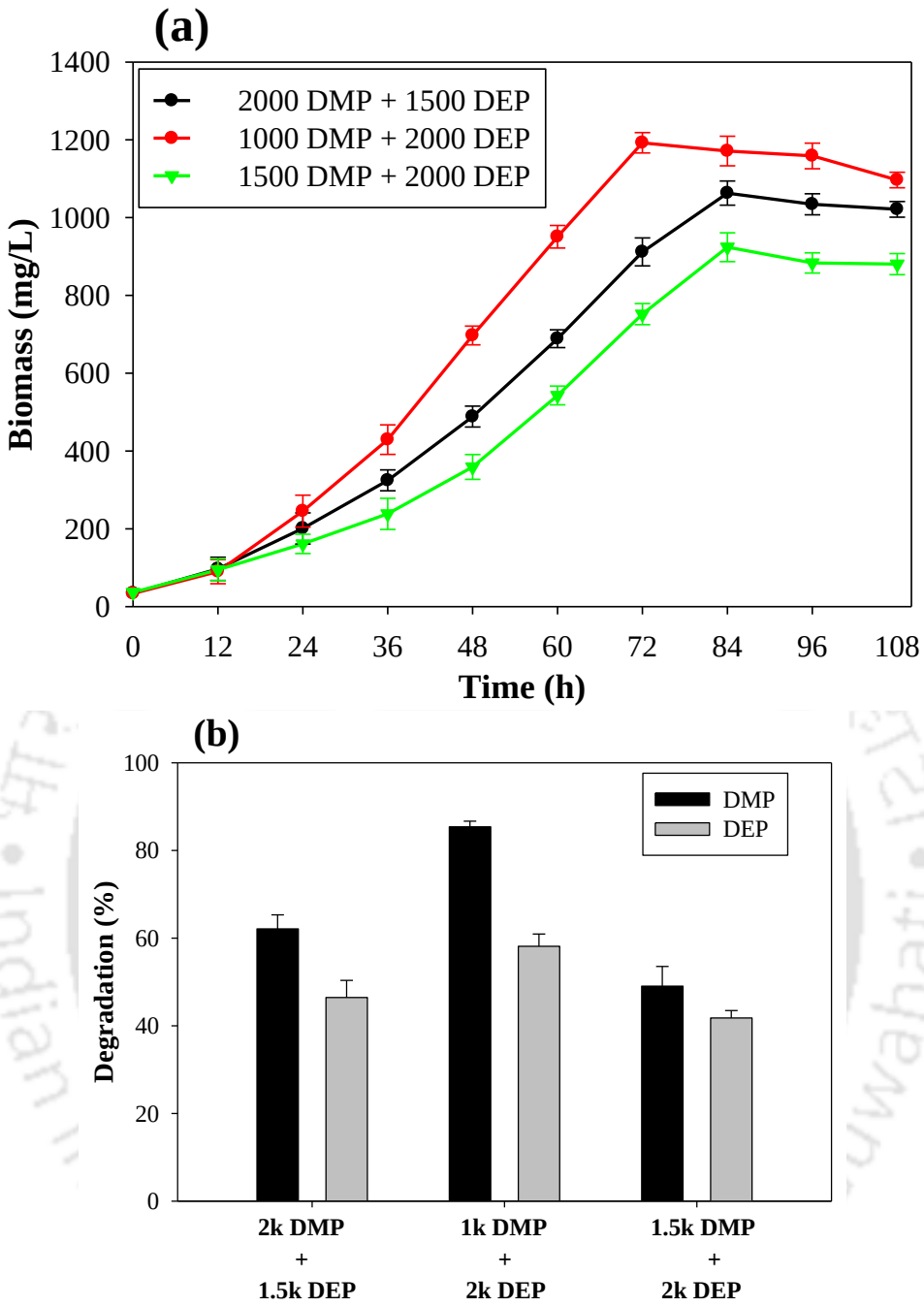


Figure 3.5: (a) *C. funkei* biomass growth and (b) DMP and DEP biodegradation as dual substrates in the CSTB.

3.3.2. Degradation under fed-batch operation mode

Time profile of DMP and DEP biodegradation in single and dual substrate systems along with biomass growth in the fed-batch operated CSTB are depicted in Figure 3.6. It can be seen that complete degradation was achieved under initial batch operation mode with a very short lag

phase of 4 h in biomass growth and degradation. Under fed-batch operation mode, DMP and DEP as the single substrate were completely degraded up to two pulse feeding of DMP and DEP at 3500 and 2500 mg/L concentrations, respectively. Whereas, at the third feeding stage low degradation values of 42.14 and 7.53% were observed for 4000 and 3000 mg/L concentration of DMP and DEP, respectively (Figure 3.6a and b).

When added as dual substrates in the fed-batch operated system, complete degradation of both DMP and DEP were achieved during their first pulse feeding at 1000 and 2000 mg/L concentrations, respectively. Whereas, the degradation values reduced in the 2nd and 3rd feeding stages. The degradation values were 82.81 and 73.02% for DMP and DEP at 2000 and 1500 mg/L concentration, respectively, in the 2nd run, whereas, no degradation was observed during the 3rd feeding stage with 1500 and 2000 mg/L DMP and DEP concentrations, respectively (Figure 3.6c). From the results of the 2nd and 3rd feeding of the two substrates, an increase in the DMP concentration enhanced the biodegradation of DEP, whereas, DEP at a high concentration in the mixture decreased the degradation of DMP. These results are similar to that obtained previously on biodegradation of a mixture of DMP and DEP by *C. funkei* in batch shake flasks. Figure 5.6 reveals that degradation time were lower during the 1st and 2nd pulse feeding stages even at high concentrations than during the 3rd stage. The better treatment efficiency during the 1st and 2nd feeding stages is attributed to the well-acclimatized biomass present in the reactor. A similar effect due to biomass acclimatization was reported by Bianco et al. (2022) for the biodegradation of phenanthrene from a spent sediment washing solution under fed-batch operation. However, low degradation efficiency of the compounds as single or dual substrates at their high concentrations during the 3rd feeding stage reveals the inhibitory effect of the phthalates on the biomass growth of *C. funkei*.

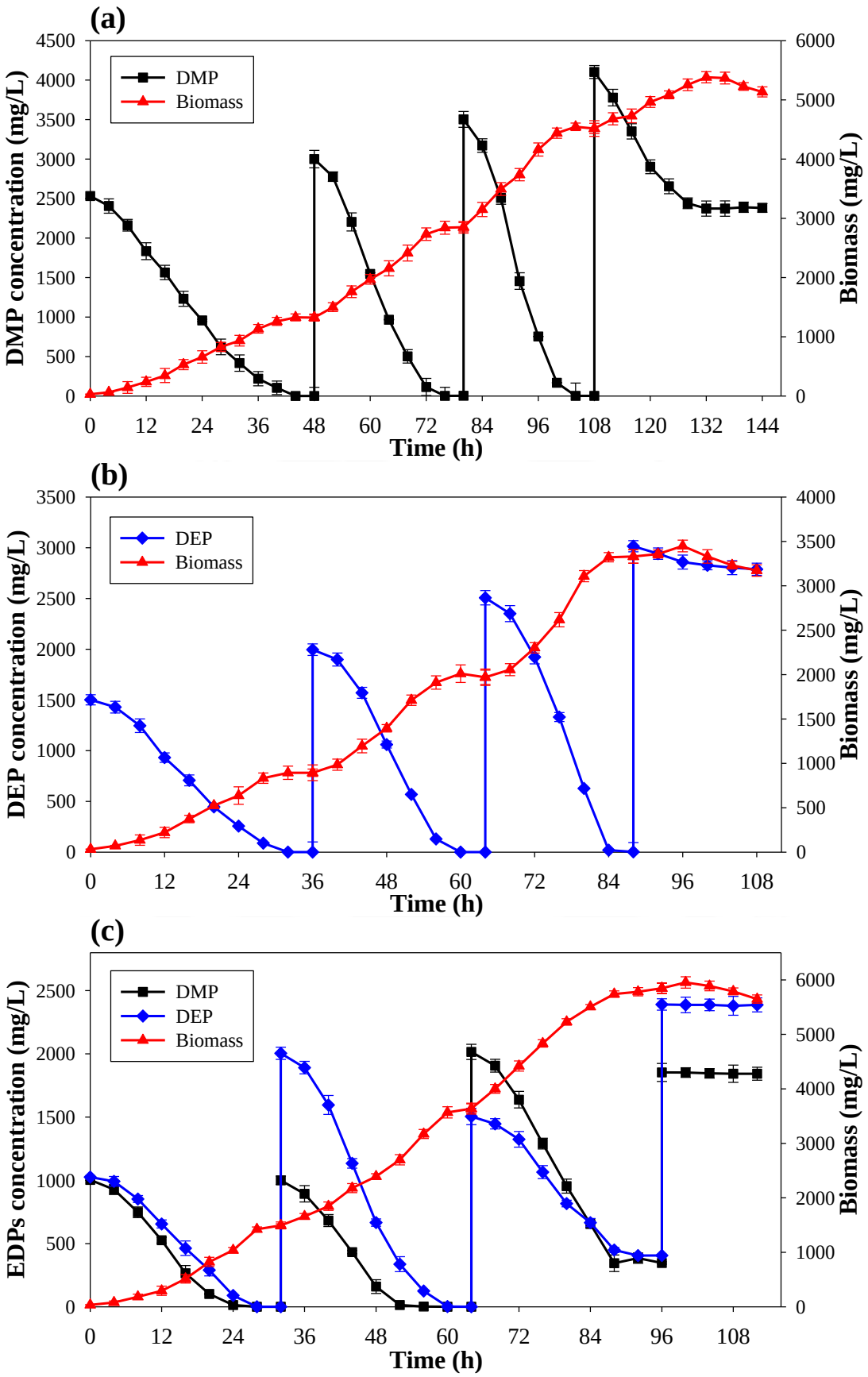


Figure 3.6: Time profile of biomass growth and phthalate degradation by *C. funkei* in the fed-batch operated CSTB: (a) DMP, (b) DEP as single substrates and (c) as dual substrates.

3.3.3. Degradation under continuous operation

To evaluate the continuous degradation of DMP and DEP by *C. funkei*, the CSTB was initiated under batch mode with 1000 mg/L initial concentration each of DMP and DEP. Complete degradation was achieved within 28 h under the batch mode, thereafter the reactor operation mode was switched over to continuous mode. Three different HRTs viz. 8, 16 and 24 h were investigated in this continuous study, which corresponded to 0.12, 0.06 and 0.04 h^{-1} dilution rates, respectively. Figure 3.7a, b and c show the combined time profile of *C. funkei* biomass growth, inlet and outlet concentrations of DMP and DEP and their degradation efficiency at 24, 16 and 8 h HRT, respectively. At a high HRT (24 h), complete degradation of DMP and DEP were obtained at all inlet concentrations (Figure 3.7a). However, at 16 h HRT, 97.92 and 85.11% degradation were obtained at 2000 and 1500 mg/L inlet concentrations of DMP and DEP, whereas, the degradation values were 84.18 and 81.11% at 1500 and 2000 mg/L, respectively. Complete degradation of the compounds was observed at 1000 and 2000 mg/L inlet concentrations of DMP and DEP, respectively, at 16 h HRT (Figure 3.7b). At a short HRT of 8 h, the degradation values decreased to 89.85 and 91.53% for 1000 and 2000 mg/L inlet concentrations of DMP and DEP, respectively, and these values were further low due to an increase in the inlet concentrations (Figure 3.7c). Figure 3.7 reveals very low biomass growth rate at all the three HRTs due to biomass washout in the continuous system.

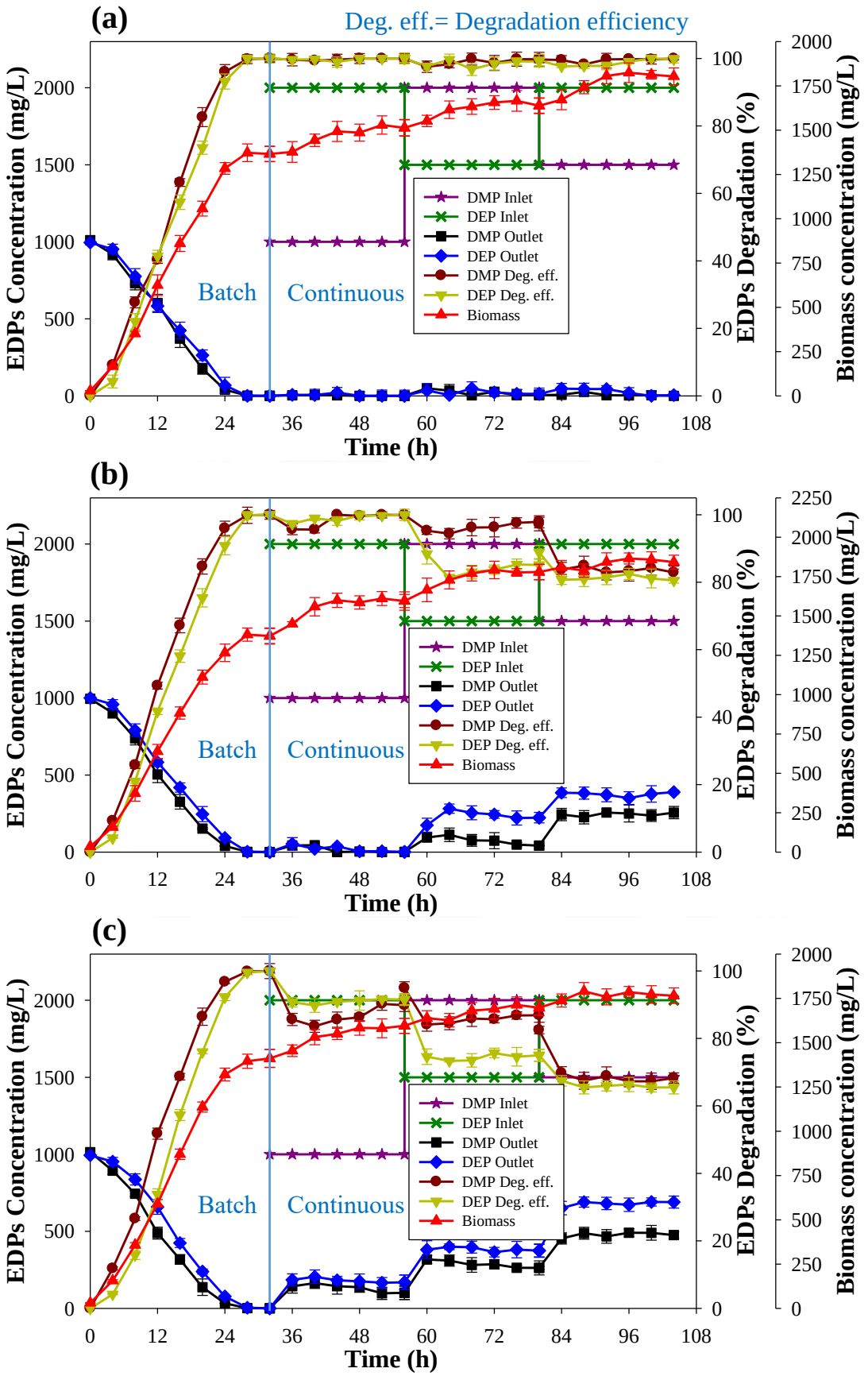


Figure 3.7: Time profile of biomass growth, inlet and outlet concentrations of DMP and DEP and their percentage degradation at (a) 24, (b) 16 and (c) 8 h HRT in the continuous operation.

Figure 3.8 shows the DMP and DEP biodegradation rates in the CSTB under continuous operation mode for different inlet loading rates (ILRs). From the figure, maximum degradation efficiency can be seen at a low ILR, suggesting that the microbial activity is unaffected up to 83.33 mg/L·h ILR of DMP and DEP. Whereas, with an increase in the ILR, degradation rate and degradation efficiency of the compounds decreased, due to inhibition of the microbial activity (Figure 3.8a and b). However, the values of the degradation rates at 125 mg/L·h ILR of DMP and 125 and 250 mg/L·h ILR of DEP, were still high. In general, the diagonal line passing through the figure's origin denotes a stable performance of the system under various input conditions. The degradation rate values which are offset from the diagonal line indicate that ILR values beyond this point are inhibitory to the microbial activity, that hinders its ability to degrade phthalates. Hence, it could be surmised that phthalates loading rate of 125 mg/L·h or more is toxic and inhibitory to microbial activity and, therefore, detrimental to the biodegradation performance of the system. Therefore, to achieve satisfactory biodegradation of phthalates under continuous operation, the bioreactor should be operated at 83.33 mg/L·h ILR or lower.

Phthalate biodegradation using shake flasks, biodegradation pathways and degradation mechanism are well reported in the literature. However, there is a very less understanding on biodegradation of phthalates mixture using bioreactors under continuous operation mode. The results of the phthalate biodegradation obtained in this study revealed that at 24 h HRT phthalate degradation is maximum at all inlet phthalates concentrations, whereas at 16 h HRT the degradation value was maximum only for low inlet phthalate concentrations (Figure 3.8a and b). Maximum degradation of these phthalates was achieved at 24 h HRT for all inlet phthalates concentrations, suggesting that the reactor requires a prolonged HRT to treat high phthalate containing wastewater (Figure 3.8). This is substantiated by the low degradation rates of DMP and DEP at high ILRs. Hence, a novel treatment strategy based on continuous biomass

recycle followed by microfiltration using indigenous ceramic membrane was evaluated to improve the degradation efficiency at lower HRT.

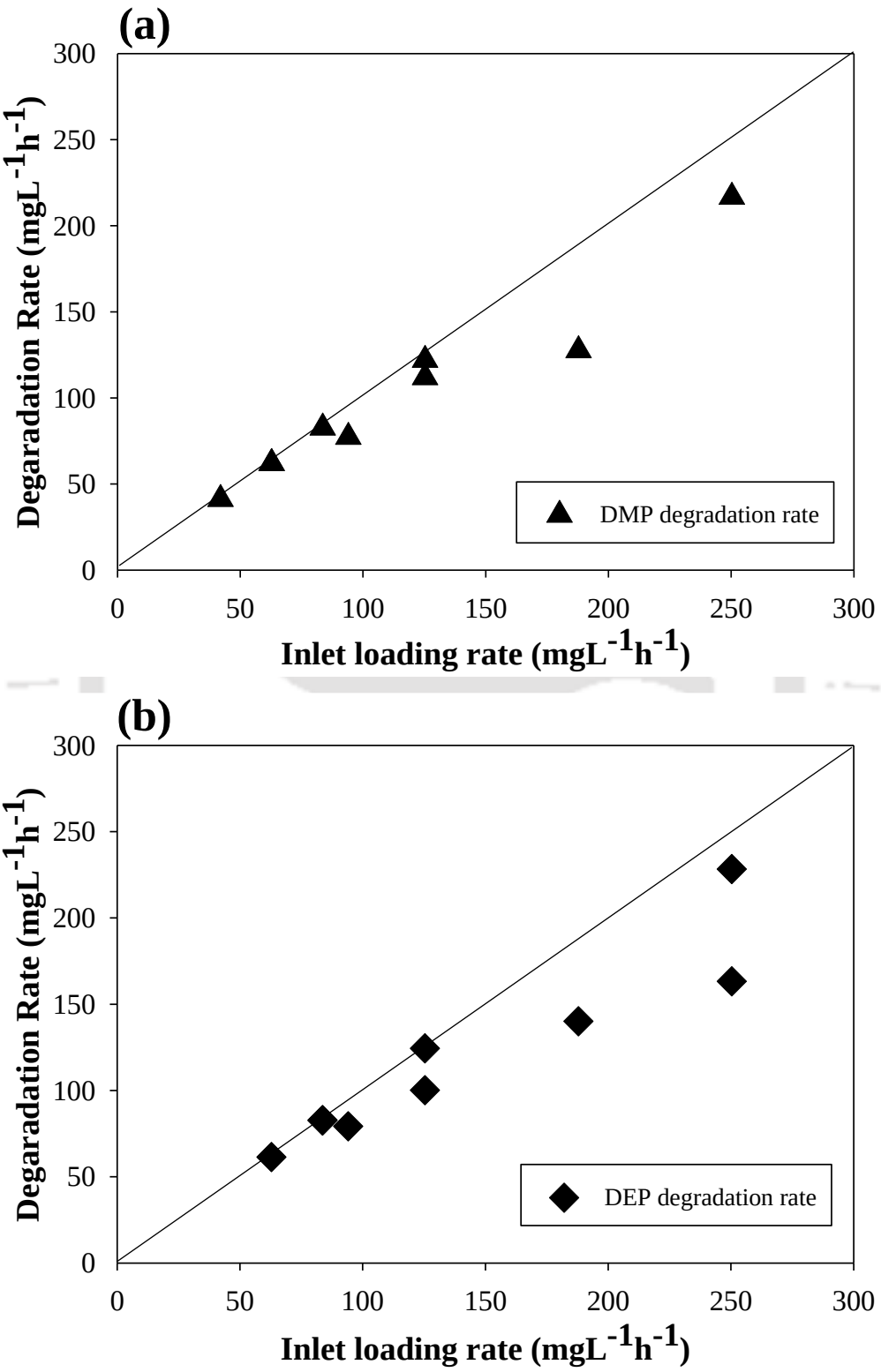


Figure 3.8: (a) DMP and (b) DEP degradation rates with respect to their inlet loading rates.

3.3.4. Characterization of prepared membrane

The XRD analysis results for determining the phase composition of unsintered membranes as well as the membranes sintered at 950 °C are illustrated in Figure 3.9a, which clearly reveals that the major phase of the unsintered membrane is the quartz and this phase remained the same even after the sintering process. During sintering, the calcium carbonate decomposes to generate calcium oxide; as evidenced by the peak disappearance for calcium carbonate at 2θ value of 29.35° in the unsintered membrane and the peak appearance for calcium oxide at 2θ value of 28° in the XRD profile of sintered membrane. The phase change of kaolin to mullite via metal kaolinite was observed at the sintering temperature of 950 °C (Figure 3.9a). Similar patterns were observed by Vasanth et al. (2011) during fabrication of economically viable ceramic membrane for the microfiltration of oil and bacterial solution.

Figure 3.9b represents the TGA and DTG results of the unsintered membrane. The weight loss at 75 – 130 °C is due to the evaporation of physically attached water molecules present in the sample. The weight loss between 200 -300 °C is due to the decomposition of Na-CMC binder. The weight loss from 400-500 °C is attributed to the elimination of structural water/surface hydroxyl group by dehydroxylation reaction and the major weight loss from 610-760 °C represents the formation of calcium oxide and CO_2 due to the decomposition of calcium carbonate (Purnima et al., 2020). The DTG analysis results show four endothermic peaks. The first one below 150 °C is due to the evaporation of physically bound water. The second endothermic peak in the region of 230 to 300 °C represents the thermal degradation of side chains present in the Na-CMC to form CO_2. The elimination of structural water from the membrane occurs by dehydroxylation reaction in the temperature range 420-520 °C and another major peak in the region of 650 to 760 °C represents the thermal degradation of $CaCO_3$ to CaO and CO_2. These results are consistent with the XRD profile, which revealed the appearance of a new peak due to CaO.

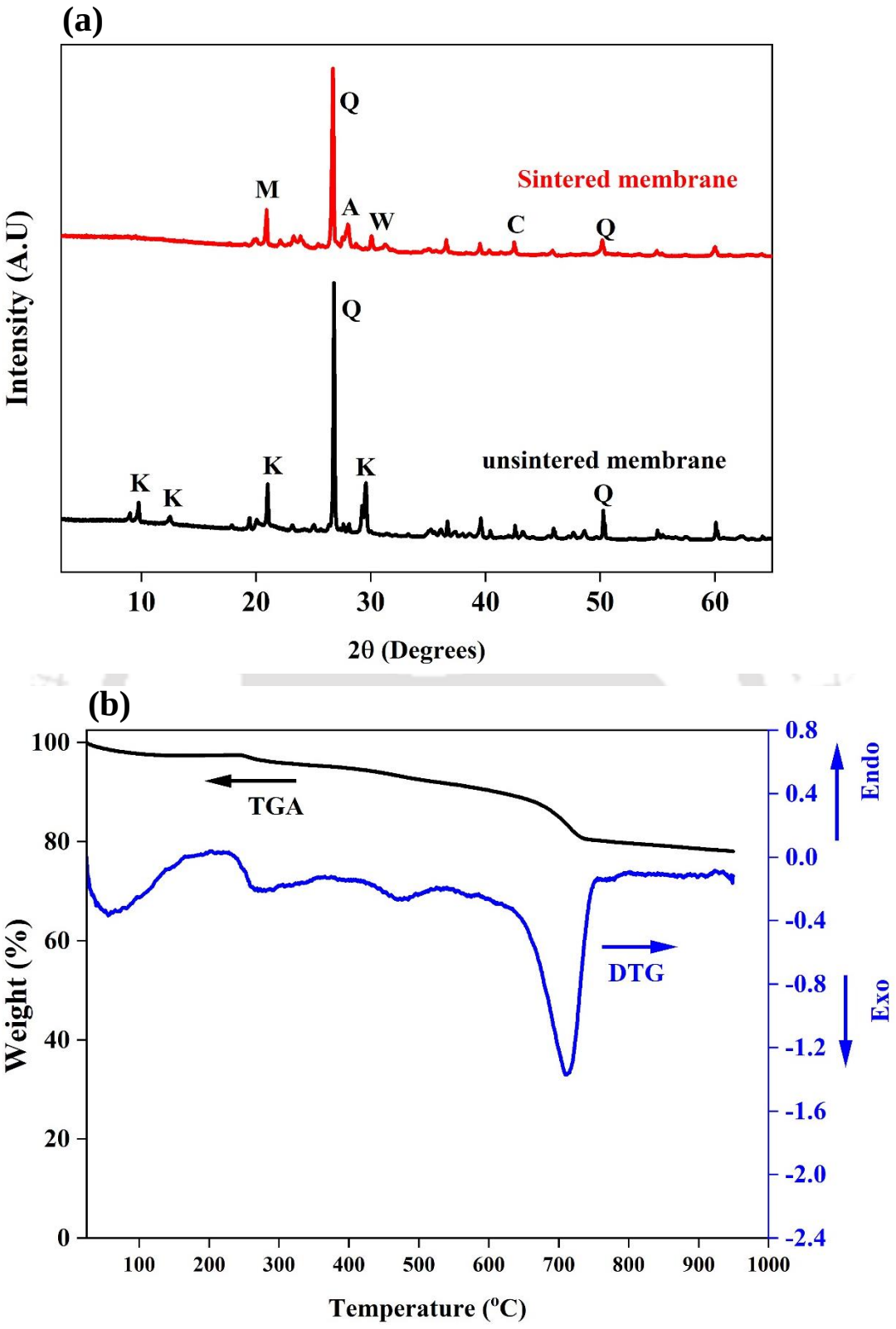


Figure 3.9: (a) XRD analysis of unsintered and sintered membrane (K – kaolin, Q – quartz, C – Calcium oxide, M – Mullite, A – Anorthite W – Wollastonite) and (b) TGA/DTG analysis of unsintered membrane.

In Figure 3.10a and b which show FESEM images of the inner and outer surfaces of the membrane, the darker portions represent the pore and lighter portion depict clay. The membranes contain numerous pores on both inner and outer surfaces. From the images, it is evident that the membrane surface is homogeneous and devoid of any cracks. All these results confirm the exceptional quality of the membrane, mainly owing to the absence of large-sized pores, defects, cracks, etc. in the membrane.

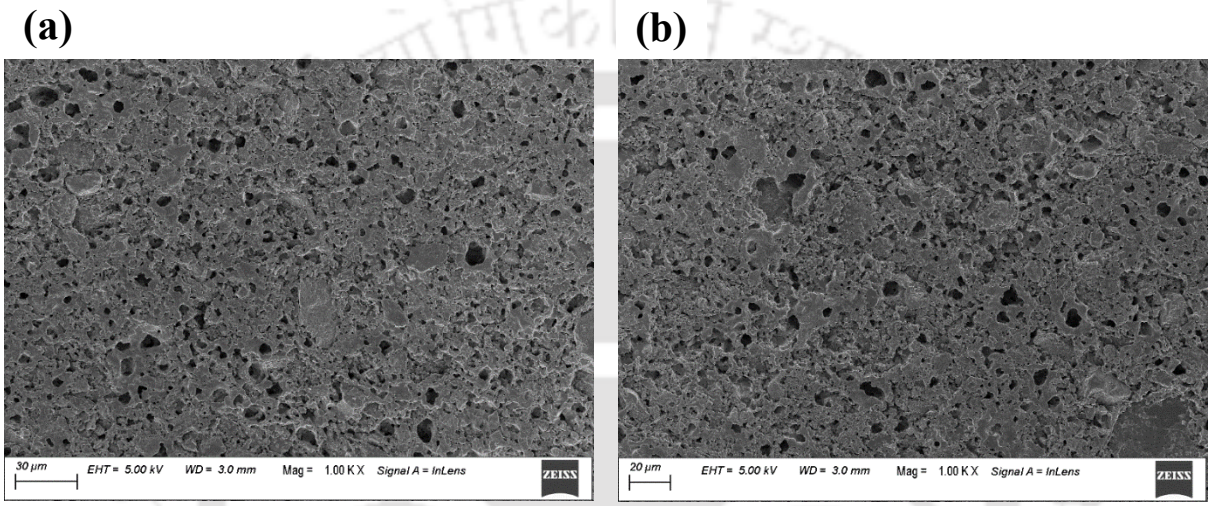


Figure 3.10: FESEM images of the fabricated membrane: (a) inner and (b) outer surface.

The membrane weight loss in alkaline environment was insignificant and was 0.05±0.02%, which makes it suitable for application even in highly alkaline environment. However, the membrane weight loss in acid was quite higher than in the alkaline environment and was 2.43±0.01%. The porosity was measured using equation (3.4) and was found to be 43.4± 0.2%. The pure water flux measurements were carried out in a cross-flow mode by changing the applied pressure from 69 to 345 kPa at a constant cross flow rate of 15 L/h and the results are depicted in Figure 3.11a. The permeate flux increased with an increase in the applied pressure owing to the enhancement of driving force on the membrane surface. Figure 3.11b indicates that the permeate flux increased linearly as per the Darcy's law. The pure water permeability and average pore size of prepared membrane were calculated using equations (3.6), (3.7) and the values were 5.24×10^{-8} $m^3/m^2{\cdot}s$ kPa and 0.11µm respectively (Table 3.4).

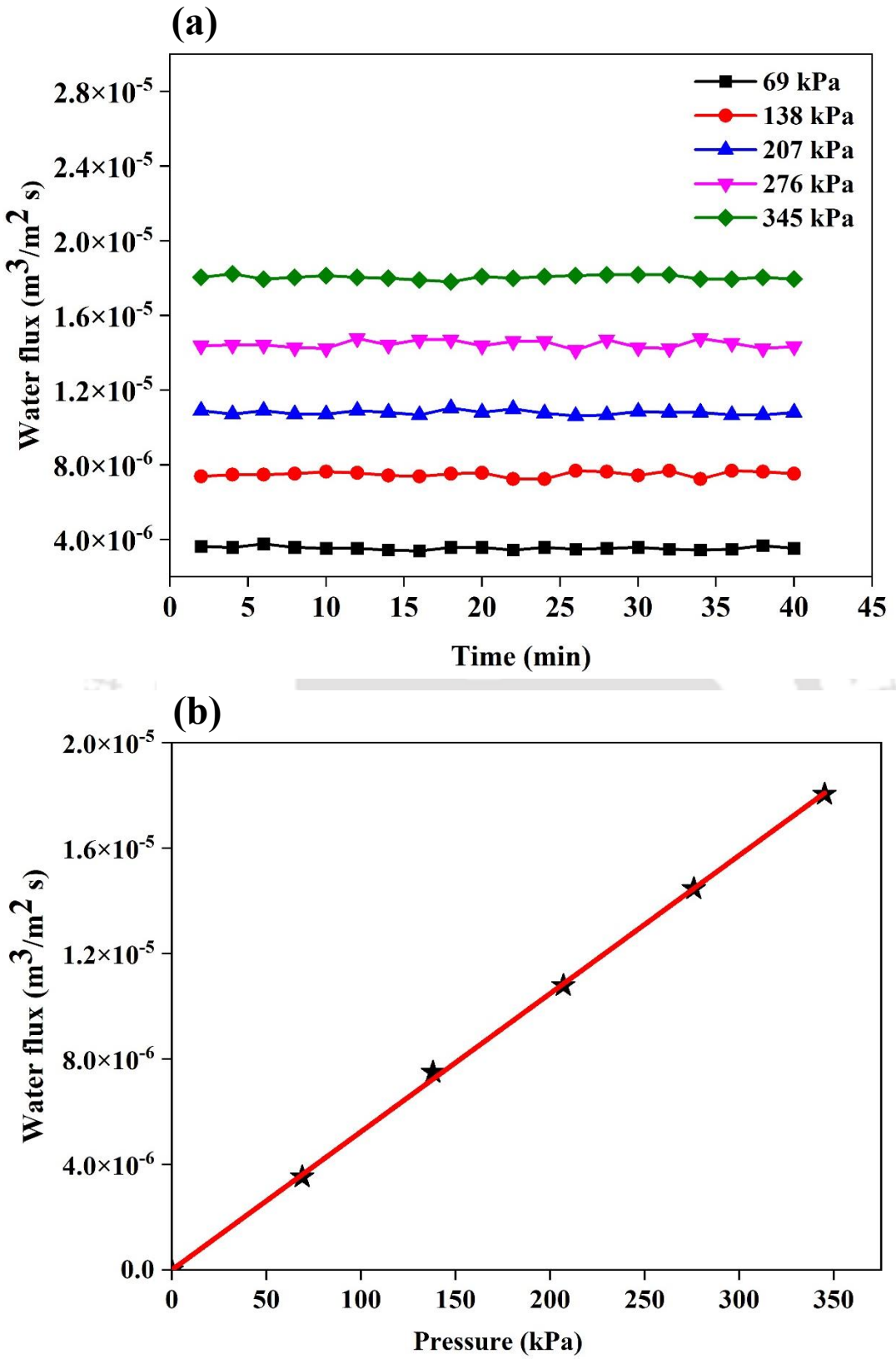


Figure 3.11: (a) Pure water flux vs time at different applied pressure and (b) water flux versus pressure using the tubular ceramic membrane.

Table 3.4: Properties of the fabricated tubular ceramic membrane.

Membrane properties	Values	Conditions
Pure water flux	1.76×10^{-5} $m^3/m^2\cdot s$	At 345 kPa pressure
Porosity	43.4± 0.2%.	
Pure water permeability	5.24×10^{-8} $m^3/m^2\cdot s\cdot kPa$	
Average pore size	0.11µm	
Chemical stability (in terms of weight loss)	(2.43 ± 0.01) %	In acidic medium
	(0.05 ± 0.02) %	In basic medium

The fabricated membrane was subsequently used to separate the *C. funkei* biomass from the reactor effluent by microfiltration operation. The experiment was conducted at room temperature, 345 kPa applied pressure and cross flow velocity of 15 L/h. After 100 min of the operation, the permeate flux almost reached steady value owing to the concentration polarization and cake formation (Figure 3.12). Permeate flux value of the membrane during steady state was 3.17×10^{-6} $m^3/m^2\cdot s$ under a constant pressure of 345 kPa. The fouled membrane was regenerated by immersion in water for ultrasonication using a bath sonicator. After 5 cycles of continuous microfiltration and membrane separation, the concentrated biomass was obtained in 1.5 L of the retentate and the same retentate volume was returned to the bioreactor along with fresh influent for the continuous biodegradation experiments with the CSTB.

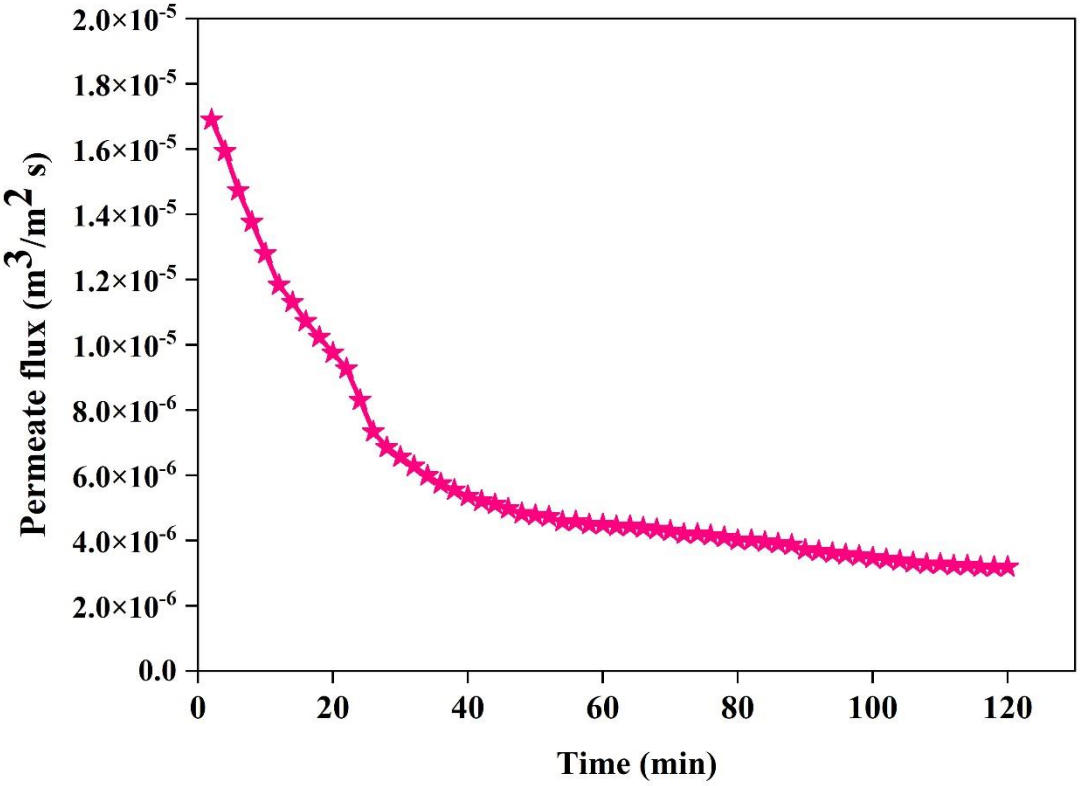


Figure 3.12: Permeate flux of *C. funkei* culture.

3.3.5. Continuous biodegradation under biomass recycle mode

For enhancing the biodegradation efficiency of the continuous bioreactor system, biomass from the bioreactor was recycled following microfiltration using the indigenous tubular ceramic membrane module. The biodegradation experiments were carried out with the membrane flux value of 1.76×10^{-5} $m^3/m^2{\cdot}s$ under a constant pressure of 345 kPa. The biomass recycle experiments were initiated under batch mode and at the end of the exponential growth phase of the biomass, the reactor was switched over to continuous mode and operated further for 16 h. Thereafter, the reactor operation was converted to biomass recycle mode at 16 h HRT. Feed containing 3500 mg/L total inlet concentration of a mixture of DMP and DEP (1500 and 2000 mg/L DMP and DEP, respectively) was added to the system along with retentate containing biomass from the microfiltration system.

In order to assess the effect of different biomass recycle values on EDPs degradation by *C. funkei* in the CSTB, experiments were carried out with 100 and 50% biomass recycle. The results presented in Figure 3.13, depicts the inlet and outlet concentrations (mg/L) of DMP and DEP along with their degradation efficiency (%) and biomass concentration (mg/L) profile with time (h). Complete degradation of DMP and DEP were achieved with 100% biomass recycle at a total inlet loading rate of 218.75 mg/L·h and within 48h of biomass recycle operation, whereas in case of 50% biomass recycle it took 60 h for complete degradation (Figure 3.13a and b). Compared with this result, the degradation value was only 82.65% at the same total ILR using the continuous operated CSTB without biomass recycle. The maximum biomass concentration value of 4738 mg/L was observed at 100% biomass recycle, while the value was 3457 mg/L with 50% biomass recycle. Figure 3.13a and b also reveal that the combined inlet concentration of the phthalates in the reactor was initially high due to residual phthalates already present in the recycle stream. These values clearly reveal that the biomass recycling overcomes the challenges faced due to biomass washout in a continuous system by providing a high concentration of biomass in the reactor, which is necessary to improve the process performance by enhancing the biomass productivity and biodegradability in the system (Gupta et al., 2017; Paul et al., 2019a).

Membrane bioreactor is suitable for wastewater treatment involving low sludge load and high sludge age as it enables prolonged retention of the bacteria inside the bioreactor for degradation and toxicity removal of the organics present in the wastewater. This study distinctly demonstrates that biomass recycle reactor is best suited for the complete degradation of DMP and DEP from wastewater using *C. funkei* even at very high ILR. Similar results on COD removal from refinery wastewater and lipid production by *R. opacus* in a biomass recycle reactor were recently reported by Paul et al. (2019). However, this is the first report on biodegradation of DMP and DEP using *C. funkei*.

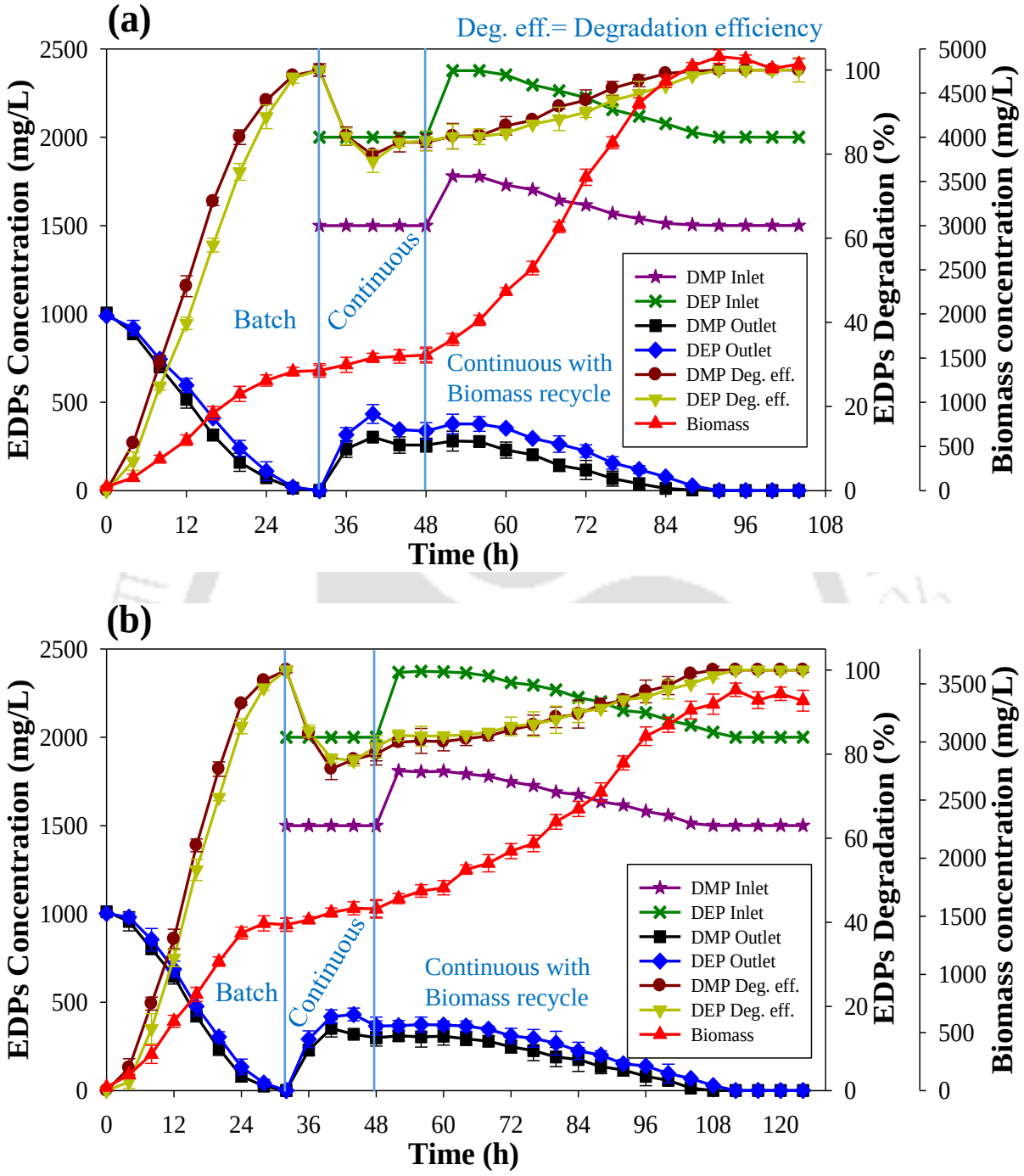


Figure 3.13: Time profile of *C. funkei* biomass growth, inlet – outlet concentration and percentage degradation of DMP and DEP with (a) 100 % and (b) 50 % biomass recycle.

3.3.6. Ecotoxicity assessment

3.3.6.1. Seed germination

To assess the phytotoxicity of DEP and DMP, before and after their degradation by *C. funkei*, GI of the *Cicer arietinum* (Chick pea) seeds were evaluated. Seed germination occurs in the initial stages of a plant growth and in which a quiescent dry seed becomes metabolically active with water uptake. GI of seed depends on the quality of water fed to it, which in turn determines the plant development, growth and productivity.

The chick pea seeds soaked in the different samples, taken before and after DMP and DEP degradation, as well as in distilled water are presented in Figure 3.14. Table 3.5 is the summary of the results obtained. Figure 3.14a shows that the seeds soaked in tap water (control) has a maximum GI value of 98%, whereas, the value was 2.37% for seeds soaked in raw phthalate-containing medium, revealing its very high toxicity (Figure 3.14b). In the case of samples obtained from the batch CSTB treating 3,500 mg/L EDPs mixture, the maximum GI value of 76.63% was observed (Figure 3.14c), Whereas, the value was 81.22% for the water sample from the fed-batch CSTB treating the same concentration (Figure 3.14f). In the case of water sample from the continuous system without biomass recycle, maximum GI was achieved at 24 h HRT for all inlet concentrations (Figure 3.14i-k). However, highest GI in the study was shown by the seeds soaked in sample collected from the biomass recycle system; the values were 91.86 and 87.57% for 100 and 50% biomass recycle, respectively (Figure 3.14r and s). All these results clearly demonstrate the efficiency of CSTB with biomass recycle reactor for toxicity removal of EDPs contaminated wastewater. Paul et al. (2019) recently reported a GI of 73.63% for treated refinery wastewater by *Rhodococcus opacus* using biomass recycle system operated under continuous mode. The value is less as compared to that obtained in this study.

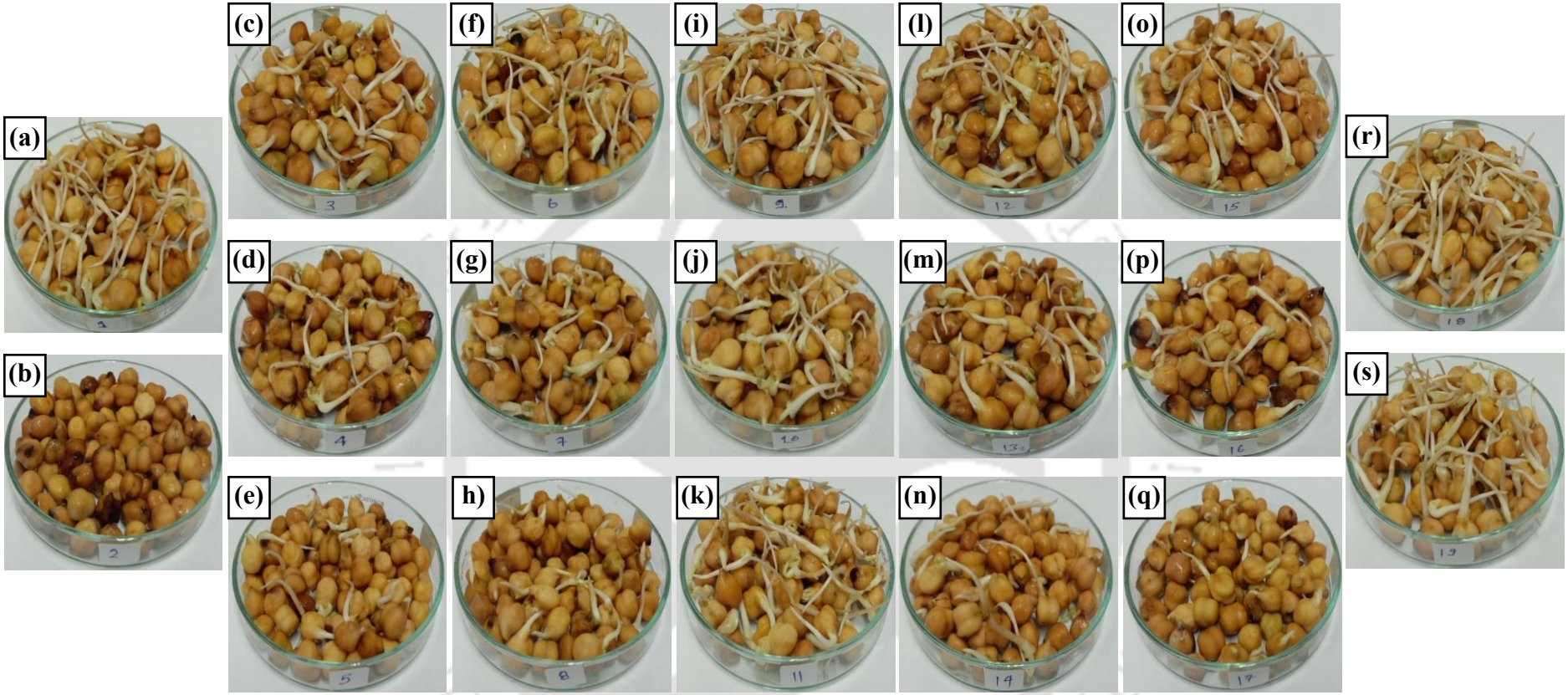


Figure 3.14: Images of germinated chickpea seeds in (a) distilled water, (b) raw phthalate containing medium, and samples from CSTB operated under (c-e) batch (f-h) fed-batch, (i-k) continuous at 24 h HRT, (l-n) continuous at 16 h HRT, (o-q) continuous at 8 h HRT, (r-s) continuous with100% and 50% biomass recycle modes (16 HRT).

Table 3.5: Results of ecotoxicity study with phthalate degraded water

		Total initial phthalate concentration (mg/L)	GI (%)	% Mortality
Distilled water (control)		0	98.00	0.00
Raw/untreated water		2000	02.37	100
Treated water from batch operated CSTB		3000	76.63	10.00
		3500	57.72	16.67
		3500	42.19	40.00
Fed-batch	1st feed	3000	81.22	3.33
	2nd feed	3500	68.43	16.67
	3rd feed	3500	19.48	66.67
Treated water from continuously operated CSTB at different HRTs	24 h HRT	3000	85.44	0.00
		3500	83.67	3.33
		3500	84.71	0.00
	16 h HRT	3000	80.67	3.33
		3500	71.94	10.00
		3500	55.38	16.67
	8 h HRT	3000	68.86	16.67
		3500	44.18	33.33
		3500	32.74	43.33
100% biomass recycle	16 h HRT	3500	91.86	0.00
50% biomass recycle	16 h HRT	3500	87.57	3.33

3.3.6.2. Brine shrimp mortality

Toxicity of phthalate degraded samples which were collected from the CSTB operated under different modes, were further assessed by brine shrimps (*Artemia salina*) mortality assay. A dead brine shrimp nauplii is shown in Figure 3.15, and the results of brine shrimp mortality tests are presented in Table 3.5. From the table, 100% mortality is observed for shrimps incubated with raw phthalate containing medium, which reveals very high toxicity of the

phthalates, whereas, no mortality was observed when distilled water was used. In case of samples collected from batch, fed-batch and continuous CSTB, treating low initial/inlet concentrations of phthalates the mortality % value was low, whereas, the mortality percentage increased with increase in the inlet phthalates concentrations owing to their low degradation values of the EDPs. However, among the different phthalate degraded samples, no mortality was observed with the sample from CSTB with 100 % biomass recycle treating even high inlet concentration (3500 mg/L) of the phthalates. The above findings show that biomass recycle strategy is more suited for complete degradation of phthalate at high inlet loading rates compared with the other treatment strategies. Paul et al. (2022) studied the toxicity of refinery wastewater using brine shrimp and reported a very low mortality value of 39%. In another study, this test was adopted for assessing the toxicity of freshly synthesized triazoles which showed 100% mortality at 300 mg/mL concentration (Ahmed et al., 2016).

In addition to the capability of *C. funkei* for toxicity removal of phthalate-containing wastewater by biodegradation, the biomass recycle system which combines both biodegradation and microfiltration can be used for recycle and reuse of the treated water.

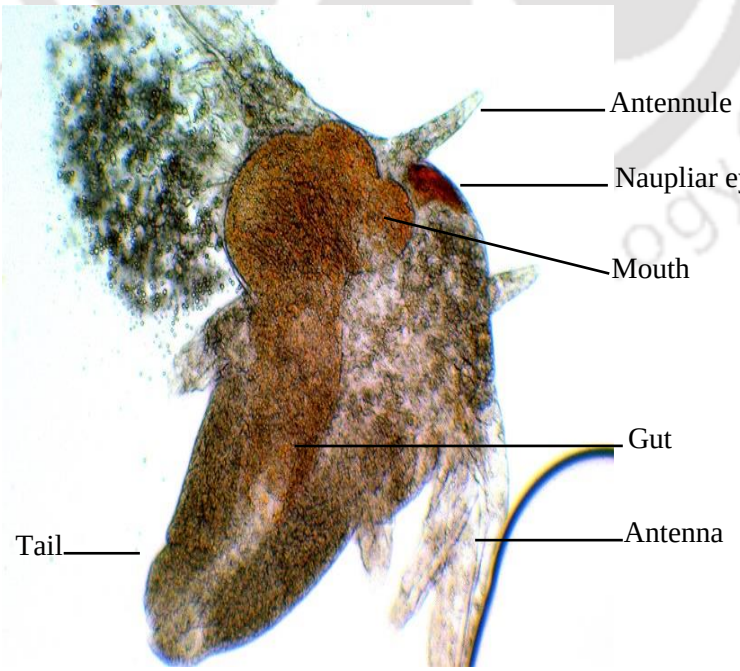


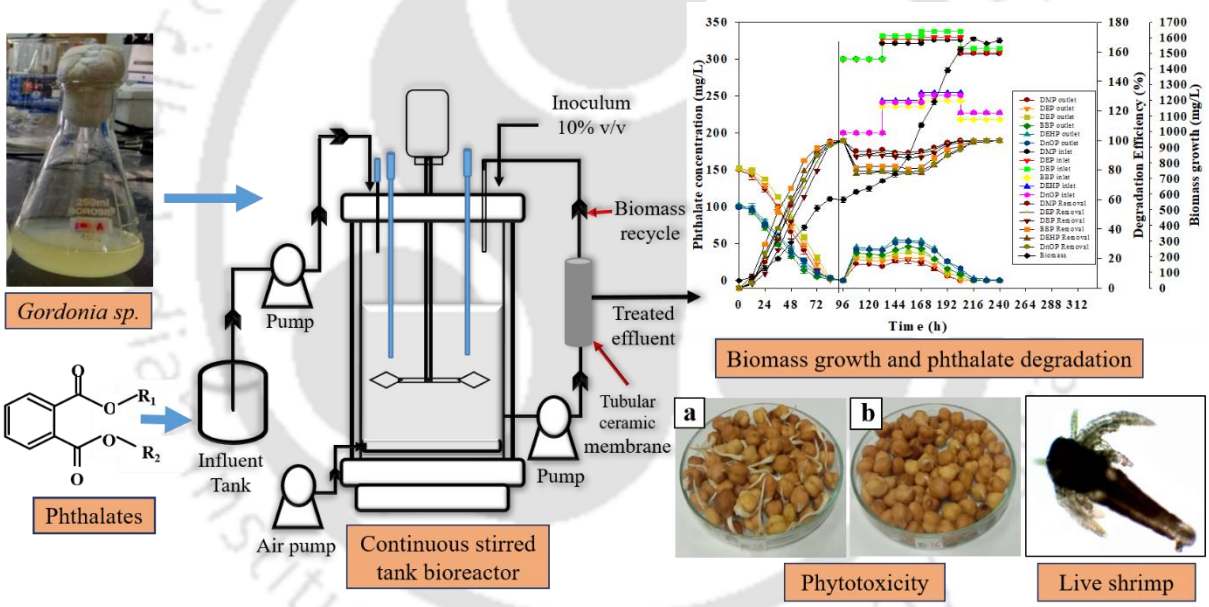
Figure 3.15: Microscopic image of a dead *Artemia salina* (Brine shrimps).

3.4. Conclusion

This is the first study on continuous biodegradation of a mixture of DMP and DEP in biomass recycle system followed by microfiltration using an indigenous low-cost tubular ceramic membrane. As compared with the batch, fed-batch and continuous operated CSTB without biomass recycle, the biomass recycle system proved more efficient in continuous biodegradation as well as toxicity removal of the DMP and DEP mixture. The order of treatment efficiency of the different modes of operation with the CSTB for biodegradation and toxicity removal of the two phthalates was as follows: batch<fed-batch<continuous<continuous with biomass recycle. Toxicity assessment of the phthalate degraded sample using chick peas and brine shrimps demonstrate the reuse potential of the treated water which can be explored further.

Chapter 4

Biodegradation and toxicity removal of a mixture of low and high molecular weight endocrine disrupting phthalates by *Gordonia sp.* in a continuous stirred tank bioreactor under different operation modes



ABSTRACT

This study examined biodegradation of a mixture of dimethyl, diethyl, dibutyl, benzyl butyl, di-2-ethylhexyl, and di- n-octyl phthalates by *Gordonia sp.* in a continuous stirred tank bioreactor (CSTB) under batch, fed-batch, continuous, and continuous with biomass recycle operation modes. For operating the CSTB under biomass recycle mode, microfiltration using an indigenous tubular ceramic membrane was employed. Ecotoxicity assessment of the treated water was carried out to evaluate the toxicity removal efficiency by the integrated bioreactor system. From the batch experiments, the EDPs cumulative degradation values were 90% and 75% at 1250 and 1500 mg/L total initial concentration of the mixture, respectively, whereas complete degradation was achieved at 750 mg/L. In the fed-batch study, 93% degradation was achieved at 1500 mg/L total initial concentration of the mixture. In continuous operation mode, 94 and 85% degradation efficiency values were achieved at 43.72 and 52.08 mg/L·h inlet loading rate of phthalate mixture. However, continuous feeding with 100% biomass recycle revealed complete degradation at 41.67 mg/L·h inlet loading rate within the 84 h operation period. High seed germination index and low mortality percentage of brine shrimps observed with phthalate degraded water from the integrated bioreactor system revealed its excellent potential in the treatment and toxicity removal of phthalates contaminated water environment.

4.1. Introduction

Endocrine-disrupting compounds (EDCs) belong to the class of man-made chemicals which can mimic the natural activity of hormones and enzymes of endocrine system. Phthalate esters (PEs) are typical synthetic organic compounds grouped under EDCs, and they are mainly synthesized from esterification of appropriate alcohol and phthalic acid (Hu et al., 2021a). Manufacturing and processing of polyvinyl chloride (PVC) and other plastic polymers use PAEs as plasticizers to enhance their flexibility and durability by transforming their physical properties (Huang et al., 2019). Globally, an estimate of 7.5 million tons plasticizers is consumed annually (Hu et al., 2021a). The massive consumption and production of plasticizers pose serious risk to the human, animal and aquatic organisms via the exposure to PAEs through dermal contacts, ingestion and inhalation (Kanaujiya et al., 2019). Hence, their degradation and removal from contaminated environment are of utmost importance.

To protect human and animal health and environment from the adverse effect of PAEs, several treatment strategies, such as physical, chemical, advanced oxidation processes and microbial degradation or combinations of these techniques have been suggested (Xiaoyan et al., 2015). Among these treatment strategies, microbial degradation is the most preferred and promising strategy, due to its prominent advantages such as high treatment efficiency, environment friendly, cost effective and greater safety. Numerous PAEs degrading microbial strains have been isolated from various ecosystems, including water, sediment, soil, etc. Organisms belonging to the genera *Rhodococcus*, *Gordonia*, *Arthrobacter*, *Pseudomonas*, and *Fusarium* have been thoroughly examined and demonstrated to degrade PAEs worldwide (Fan et al., 2018b; Iwaki et al., 2012; Nahurira et al., 2017). A number of other microorganisms, such as *Bacillus mojavensis* B1811, *Burkholderia pyrrocinia* B1213, *Pleurotus ostreatus*, *Pseudoxanthomonas sp*., *Agromyces sp.* MT-O, are also reported to degrade a broad range of PAEs (Huang et al., 2019). Isolation of PAEs degrading microorganisms and their metabolic -

-degradation pathway are of heightened interest. However, these studies are limited to PAEs biodegradation as single or dual substrate in simple batch shake flask system.

In the previous two chapters, *C. funkei* was reported to efficiently degrade the low molecular weight phthalates such as DMP and DEP. However, it failed to degrade some of the high molecular weight phthalates, including, BBP, DEHP and DnOP (Appendix 1). Hence, there is a need to explore more robust organism for degrading both high and low molecular weight phthalates. Moreover, mixture of different PAEs, DMP, DEP, DBP, BBP, DEHP and DnOP that are extensively used phthalate esters, is not investigated for their degradation in bioreactor systems. Therefore, the main objective of the present study was to degrade mixture of the aforementioned phthalates by a novel organism, *Gordonia sp.*, in CSTB under batch, fed-batch, continuous modes of operation and evaluate the performance of an integrated CSTB-microfiltration system for their degradation and toxicity removal. Assessment of toxicity removal was performed by phytotoxicity and brine shrimp mortality assays with the treated water.

4.2. Materials and methods

4.2.1. Chemicals and reagents

All six phthalates used in this study viz. di(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), di-n-octyl phthalate (DnOP), di-butyl phthalate (DBP), di-ethyl phthalate (DEP) and di-methyl phthalate (DMP) were purchased from TCI (Tokyo Chemical Industry) Chennai, India. Details of other reagents and chemicals used are the same as previously mentioned in Chapter 2 (Section 2.2.1).

4.2.2. Culture conditions

Gordonia sp. used in the biodegradation experiments was supplied by Bose Institute, Kolkata, India. For repetitive growth (at 150 rpm orbital shaking and 30°C temperature) and

maintenance of *Gordonia sp.* culture, LB (Luria Bertani) broth medium was used. BH-MSM (Bushnell Hass minimal salt medium) was used to support the metabolic activity and biomass growth of *Gordonia sp.* during biodegradation of phthalates. The MSM was prepared as per the protocol and composition given earlier in Chapter 2 under Section 2.2.2.

4.2.3. Biodegradation of mixture of 6 EDPs in CSTB

4.2.3.1. Batch experiments

In order to study the combined effect of phthalates on their biodegradation by *Gordonia sp.* in a CSTB, three different experimental runs were performed at 1500, 1250 and 750 mg/L total initial concentrations of phthalates which corresponded to low, medium and high degradation rates of the compounds, based on the preliminary study conducted using the bacterium in batch shake flask. Details of the bioreactor used in this study are mentioned in Chapter 3 under Section 3.2.3.1.

The reactor was run at a controlled temperature of 28 °C, agitation speed of 400 rpm, and aeration rate of 1.5 vvm, which were found to be optimum based on our previous batch experiment results. Calculated amount of DMP, DEP, DBP, BBP, DEHP and DnOP in MSM were added to the reactor vessel (working volume 3 L) and 10% v/v mid-log phase grown *Gordonia sp.* culture at OD_{660} = 1 was used as the inoculum. Five-millilitre samples were taken at regular time intervals during the experiments, and each sample was analyzed in triplicate. The results were reported as arithmetic mean of the triplicate sample analyses.

4.2.3.2. Fed-batch experiments

In order to investigate the biodegradation of phthalates mixture under fed-batch operation mode using the CSTB, the reactor was initially operated under batch mode with 750 mg/L total initial concentration of phthalates mixture. After reaching stationary growth phase of the culture and exhaustion of phthalates in the reactor, feeding was started in pulse mode and continued up to

1500 mg/L total initial concentration of phthalates mixture. Two feed concentrations of 1250 and 1500 mg/L total concentration of phthalates mixture were followed in this fed-batch experiment; these concentrations were not removed efficiently in the earlier batch experiments. Samples were collected at regular time intervals for the analysis of biomass growth and residual phthalate concentrations.

4.2.3.3. Continuous experiments

For continuous biodegradation of phthalates by *Gordonia sp.*, the reactor was initially operated under batch mode with an initial concentration of phthalate mixture of 750 mg/L in MSM. This continuous experiment was carried out at the same operating conditions of temperature, agitation and aeration followed previously in the batch experiment. 10 % v/v of freshly grown enriched culture of *Gordonia sp.* was used as the inoculum, and after the culture growth was established, the reactor was switched over to the continuous operation mode. For the continuous reactor operation, peristaltic pumps (Watson Marlow, TR11 4RU, UK) were used to supply the feed and withdraw the reactor contents. The continuous degradation of phthalate mixture in the CSTB was evaluated at three different hydraulic retention time (HRT) viz. 24, 36 and 48 h, by maintaining feed flow rates at 2.08, 1.38 and 1.04 mL/min, respectively, with the aid of the peristaltic pump. The reactor effluent flow rate was the same as that of the feed so as to maintain the reactor volume constant. Samples were collected from the outlet at regular time interval and analyzed for biomass growth and residual phthalates concentration. Table 4.1 presents variations in inlet concentration of phthalates, and operation time followed to study continuous biodegradation of phthalates mixture using the CSTB.

Table 4.1: Inlet phthalate concentration and HRT followed for continuous biodegradation of phthalates by *Gordonia sp.* in the CSTB.

HRT (h)	Time (h)	Inlet phthalate concentration (mg/L)						
		DMP	DEP	DBP	BBP	DEHP	DnOP	Total concentration of phthalates mixture
Batch	0-96	150	150	150	100	100	100	750
	96-168	150	150	150	100	100	100	750
24	168-240	150	300	300	200	100	200	1250
	240-312	300	300	300	200	200	200	1500
Batch	0-96	150	150	150	100	100	100	750
	96-168	150	150	150	100	100	100	750
36	168-240	150	300	300	200	100	200	1250
	240-312	300	300	300	200	200	200	1500
Batch	0-96	150	150	150	100	100	100	750
	96-168	150	150	150	100	100	100	750
48	168-240	150	300	300	200	100	200	1250
	240-312	300	300	300	200	200	200	1500

4.2.3.4. Continuous experiments with biomass recycle

For continuous biomass separation and recycle following biodegradation of phthalates by *Gordonia sp.* in the CSTB, a microfiltration setup with indigenous tubular ceramic membrane was connected to the bioreactor as shown in Figure 4.1. The details of fabrication and characterization of the tubular ceramic membrane used in this study are the same as mentioned earlier in Chapter 3 under Section 3.2.4. The bioreactor effluent was treated by the membrane system for microfiltration and recycle of biomass. In this continuous operation mode, the working volume of the bioreactor was maintained constant at 3 L by feeding the biomass rich retentate from the membrane system along with fresh feed containing a known concentration of the phthalates. The reactor was initially operated under batch mode with 750 mg/L total initial concentration of phthalates mixture, and it was switched over to continuous operation mode at the end of the initial batch operation (96 h).

Based on the results of the previous continuous experiments with the CSTB, 36 h HRT and 1500 mg/L total concentration of phthalate mixture were chosen for this continuous with biomass recycle study as the biodegradation efficiency was not satisfactory under continuous operation mode. The bioreactor was initially operated for 36 h under continuous operation mode without biomass recycling, and following which the effluent of the bioreactor was fed to the microfiltration setup operated at a constant pressure of 345 kPa for biomass separation and recycling to the bioreactor in continuous mode. During the entire operation, the microfiltration system was monitored carefully for maintenance of the desired pressure. In order to restore the permeate flux of the membrane, the ceramic membrane was washed with Milli Q water and sonicated. Samples were collected from the bioreactor effluent after every 12 h and analyzed for biomass and residual phthalate concentrations. Analyses of the samples were carried out in triplicates and the results obtained were within ±2% standard deviation.

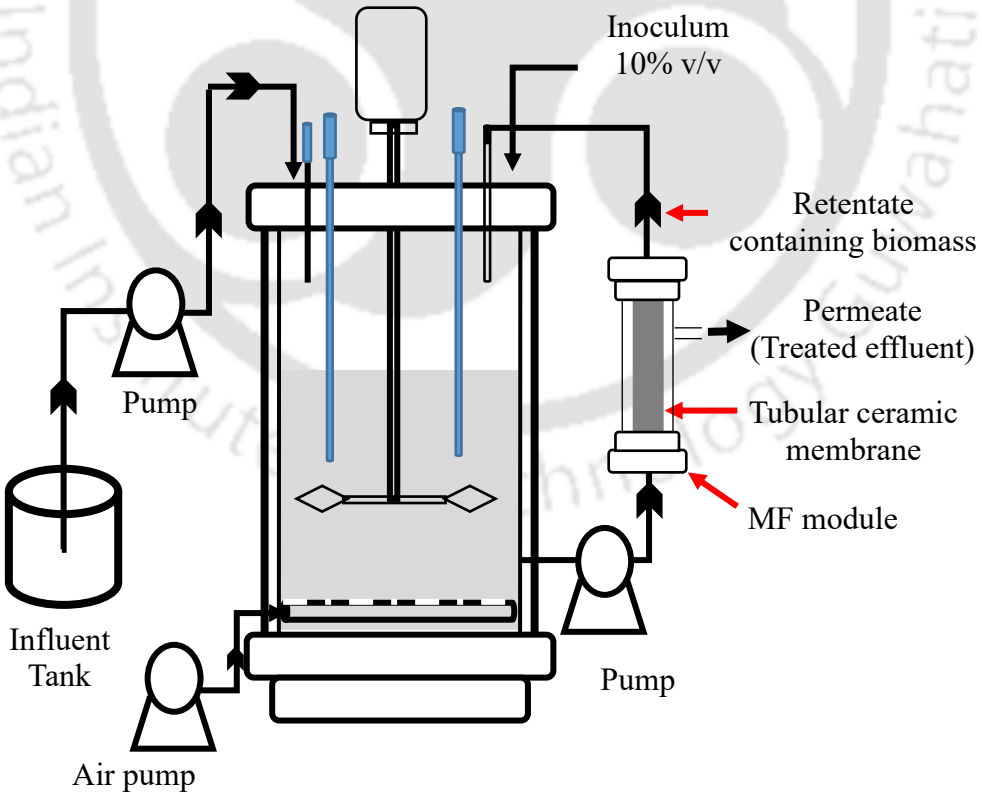


Figure 4.1: Schematic showing integrated biodegradation-microfiltration (MF) setup used in this study

In order to evaluate the effect of biomass amount recycled in the bioreactor on phthalate biodegradation, the retentate containing biomass was diluted 1:1 with distilled water and fed to the reactor along with the fresh substrate inlet. The biomass recycle ratio was calculated by Equation 3.8 (Chapter 3).

4.2.4. Ecotoxicity of the treated water

In order to assess the toxicity removal of phthalate degraded water due to biodegradation by *Gordonia sp.*, samples taken from the reactor operated under different modes were tested for seed germination. The method followed for phytotoxicity assessment was the same as mentioned earlier in Chapter 3 under Section 3.2.7.1.

Brine shrimp lethality bioassay was further carried out to examine the cytotoxicity of phthalate-degraded water due to biodegradation of phthalates mixture by *Gordonia sp*., as detailed earlier in Chapter 3 under Section 3.2.7.2

4.2.5. Analytical methods

For determination of *Gordonia sp.* biomass growth and residual phthalates concentration, methods as previously described in Chapter 2 under Section 2.2.4 were followed.

4.3. Results and discussion

4.3.1. Degradation of EDPs mixture and biomass growth in CSTB

4.3.1.1. Batch operated CSTB

The bacterial strain *Gordonia sp.* used in this study was earlier shown the potential to degrade both low and high molecular weight phthalates in our preliminary study (Appendix 2). Therefore, in the present study, only *Gordonia sp.* was selected to evaluate its performance in degrading phthalate mixture. *Gordonia sp.* is a non-pathogenic bacterium found in variable environments such as soil, sediment, sludge and wastewater, which can tolerate different

environmental pollutants. Thus, it can be considered as a good alternative for the degradation of phthalates from polluted environments.

Figure 4.2 and 4.3 shows biomass growth and phthalates degradation efficiency, respectively, by *Gordonia sp.* in the CSTB, which reveals that at a low total initial concentration (750 mg/L) of the phthalates, the biomass growth was quick with no lag phase along with complete degradation (100%) of the phthalates. Figure 4.2a and 4.3 revealed that at the low total initial concentration of 750 mg/L, complete degradation of the phthalates is achieved in the CSTB within 96 h time period. However, the degradation values were approx. 90% and 60% at the 1250 and 1500 mg/L total initial concentration of the phthalates, respectively, in the CSTB (Figure 4.2b, c and 4.3). Compared to the biodegradation of phthalates mixture in a previously batch shake flask study, these values are much higher in the CSTB.

It is known that a high initial concentration of PAEs leads to substrate inhibition on microbial metabolism and their growth (Nahurira et al., 2017; Zhao et al., 2018). Hence, low initial concentration of the phthalates at 750 mg/L was easily degraded by the bacterium for its metabolism and growth as compared to the high initial concentration 1500 mg/L. The aerobic bacterium *Gordonia sp.* used in this study depends on the DO level of the media for metabolizing the phthalates as its sole carbon and energy source. As agitation speed and aeration rate in a reactor strongly influence the DO level inside, both these physical factors were maintained at optimum values of 400 rpm and 1.0 vvm, respectively, which resulted in maximum degradation of phthalate by *Gordonia sp.* in the continuously stirred tank bioreactor.

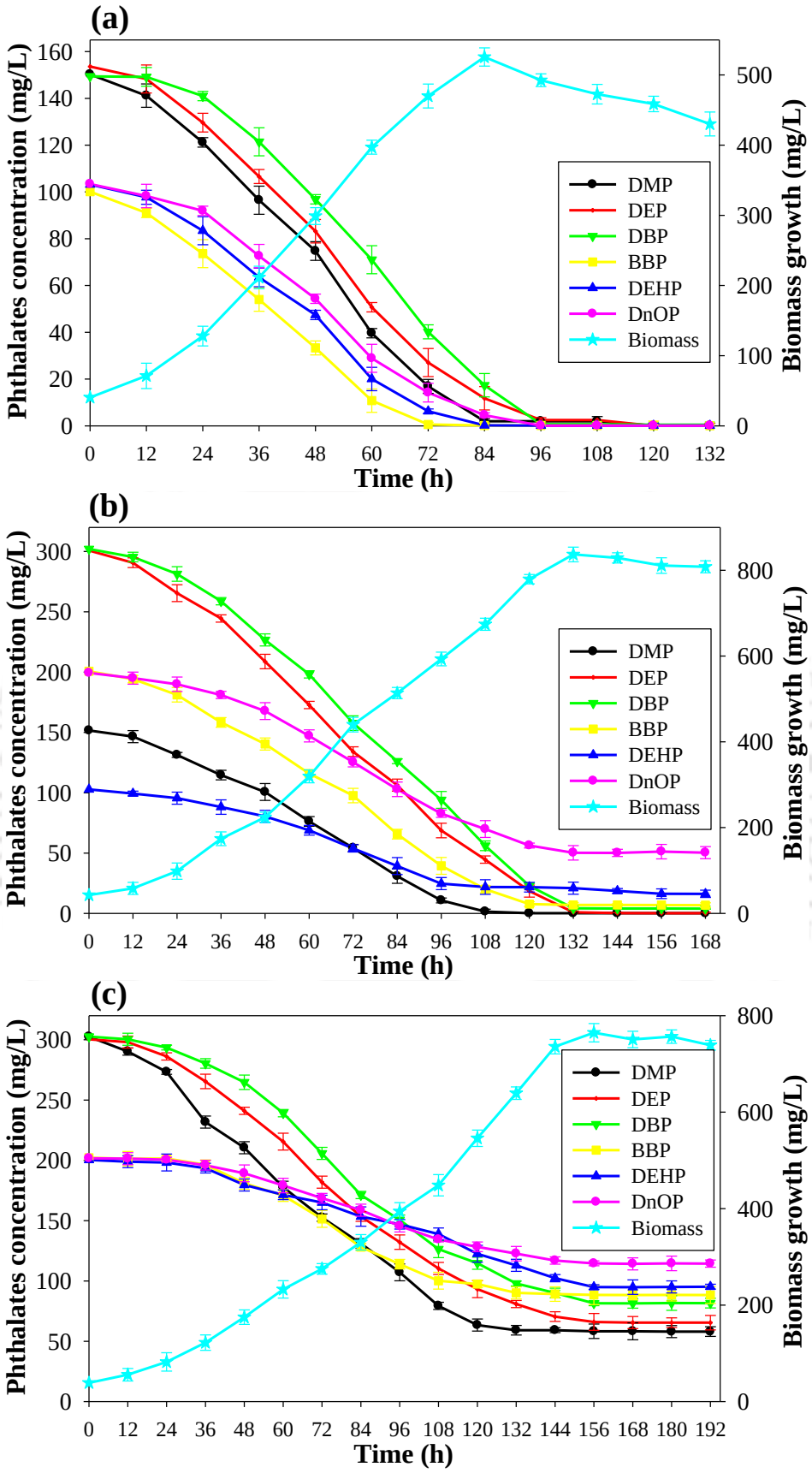


Figure 4.2: Biomass growth and phthalates mixture degradation profile for different total initial concentrations (a) 750, (b) 1250 and (c) 1500 mg/L in the batch operated CSTB.

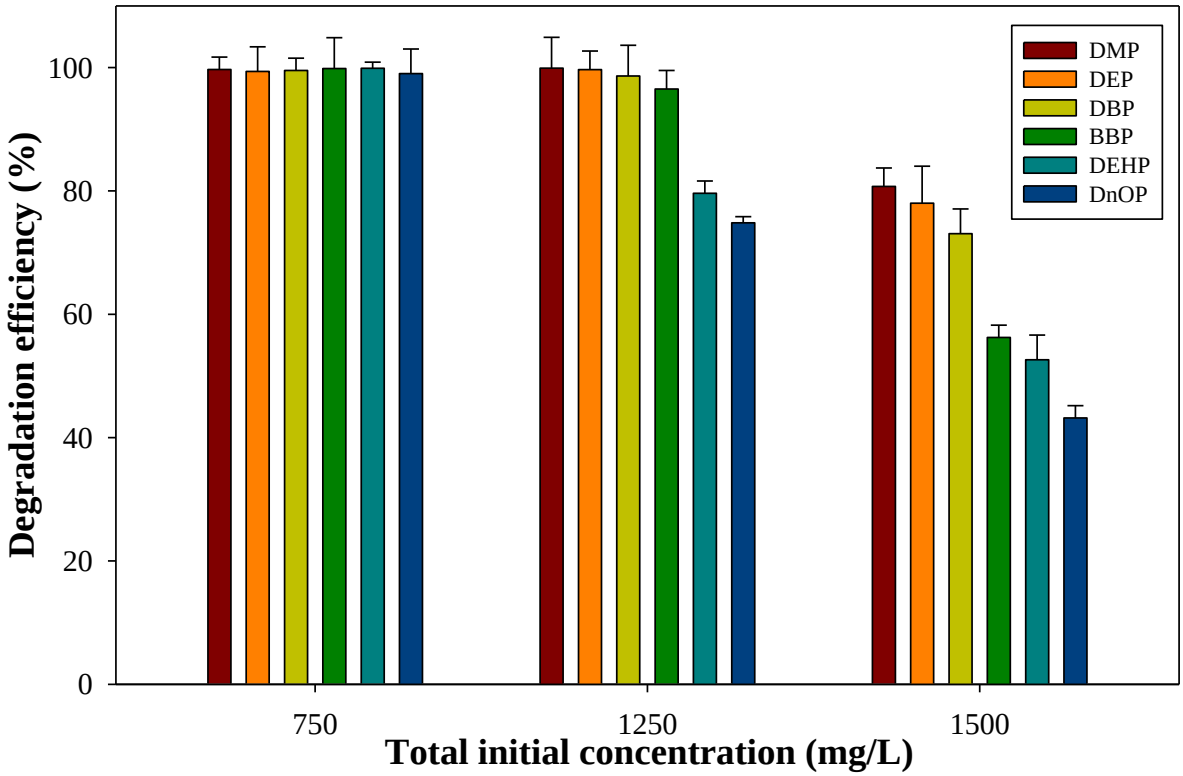


Figure 4.3: Biodegradation efficiency of phthalates mixture at total initial concentration 750, 1250 and 1500 mg/L in the batch operated CSTB.

Several studies have reported degradation of a mixture of two to four phthalates using batch shake flasks (Table 1.4), but this is the first report on biodegradation of a complex mixture of six phthalates by *Gordonia sp.* in CSTB. Patil and Jena (2019) recently reported an internal loop airlift bioreactor for biodegradation of DEP by a mixed culture of *Bacillus sp.* and *Micrococcus sp.*, and observed 100% degradation efficiency at 1500 mg/L initial concentration in 156 hr. In another study on biodegradation of DEP and diallyl phthalate using a moving bed biofilm reactor system, 94.96%, and 93.85% removal efficiency of the compounds were reported at 300 mg/L total initial concentration of the phthalate mixture (Ahmadi et al., 2015). Compared with these literature reports, the continuous stirred tank bioreactor used in this study is found to be superior for biodegrading complex mixture of six phthalates by *Gordonia sp.* even at a high total initial concentration.

4.3.1.2. Fed-batch operated CSTB

Following batch biodegradation of phthalates using the CSTB, the effect of pulse feeding of phthalates mixture on biomass growth and their biodegradation by *Gordonia sp.* was investigated. The previous batch study revealed that the high initial concentration of phthalates mixture inhibited *Gordonia sp*. biomass growth as well as phthalates degradation. Hence, in order to overcome the inhibitory effect of the phthalates at high initial concentration, the bioreactor was started with a low total initial concentration of phthalates mixture, i.e. 750 mg/L under batch operated mode. Figure 4.4, shows complete degradation of the phthalates within 96 h at the low initial concentration combination. The figure further reveals 558.5 mg/L biomass concentration and 5.818 mg/L·h biomass production rate was obtained at the end of the batch operation mode with CSTB.

Following complete degradation of the phthalates at 750 mg/L initial concentration, feed containing 1250 mg/L of total phthalates was added under pulse mode (fed-batch). Owing to the significant amount of active biomass present at the time of the pulse feeding the phthalates, an exponential rise in the biomass concentration and their degradation was obtained without any lag phase. In this fed-batch experiments, phthalates were degraded completely within 108 h, whereas, only 90 % degradation was achieved in the previous batch study conducted with 1250 mg/L total initial concentration. The cumulative degradation rate and biomass growth rate values were 11.574 and 8.416 mg/L·h, which were 30.98 and 32.75 % more than the value observed in the previous batch study. From Figure 4.4, it can be seen that following the second pulse feed of 1500 mg/L, the high molecular weight (HMW) phthalates, i.e., BBP, DHEP and DnOP, degradation efficiency values of 93, 90 and 75 %, respectively, are observed. Whereas, in case of the low molecular weight (LMW) phthalates, i.e., DMP, DEP and DBP, their complete degradation was obtained. In this experiment, the biomass growth rate decreased to 6.27 mg/L·h, whereas, the cumulative degradation rate was 11.14 mg/L·h, which is close to the

value obtained in the previous feeding stage. It is known that HMW phthalates inhibits biomass growth even at low initial concentrations (Kanaujiya et al., 2022). In a study on enzymes involved in phthalate degradation by *T. chlorobenzoica* in a 200 L fermenter, continuous feeding of phthalates resulted in their high degradation due to high cell density and enzymes produced in the system (Ebenau-Jehle et al., 2016). Similarly, very high biodegradation of the six phthalates mixture is obtained in the present study carried out under pulse feed operation mode.

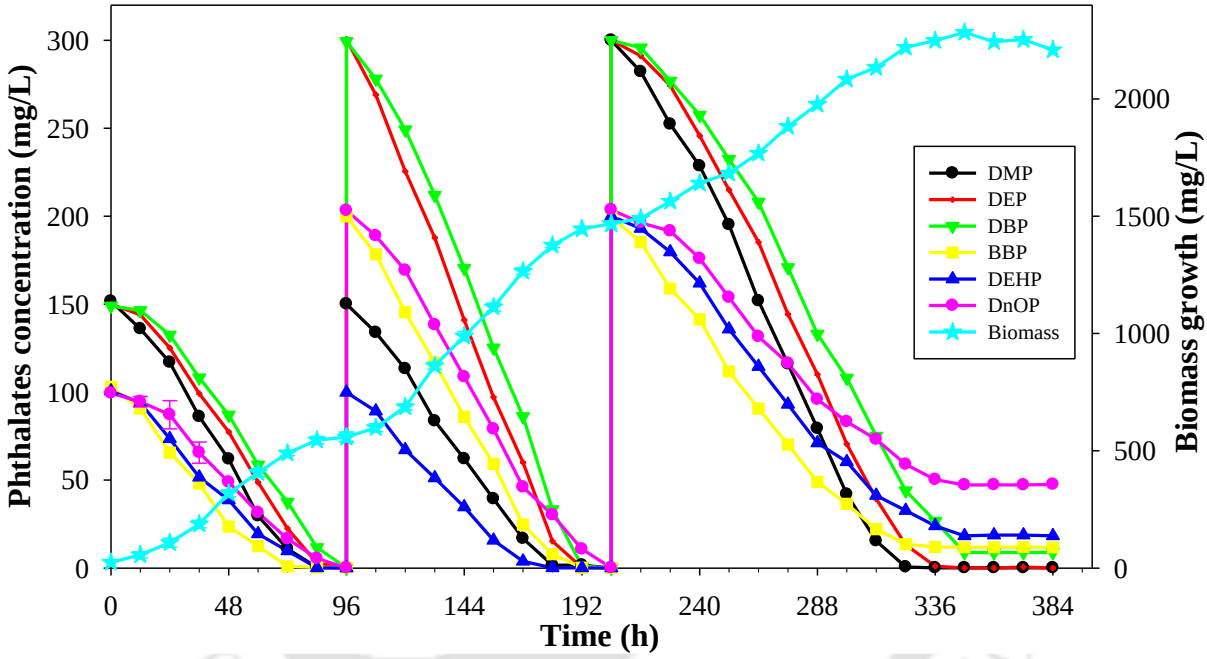


Figure 4.4: Biomass growth of *Gordonia sp.* and phthalates degradation profile in the CSTB operated under pulse feed mode.

4.3.1.3. Continuous operated CSTB

For continuous biodegradation of phthalate mixture by *Gordonia sp.* in the CSTB, the bioreactor was initially operated under batch mode for 96 h with 750 mg/L concentration of phthalate mixture which was found to be non-inhibitory to the microorganism. Figure 4.5 presents the combined profiles of biomass growth, inlet - outlet concentration and percentage removal efficiency of phthalates mixture at different HRT, viz. 48, 36 and 24 h. From Figure

4.5 a-c, it could be seen that lag phase in biomass growth and biodegradation of phthalates is absent due to their low initial concentration as well as enrichment of the culture to grow on phthalates mixture as the sole carbon source. Complete degradation of phthalate mixture was achieved during the initial batch run and within 96 h, which resulted in a high concentration of active and acclimatized biomass. Hence, the same time duration was considered favourable for switching over from batch operation to continuous operation mode of the bioreactor.

Under continuous operation mode, 100% degradation efficiency of phthalate mixture was achieved at 750 mg/L concentrations of phthalate mixture at all the three HRTs. Moreover, at 48 h HRT, complete degradation efficiency was achieved at all three inlet concentrations (750, 1250 and 1500 mg/L) of phthalate mixture. However, the cumulative biodegradation of phthalates was substantially decreased when the bioreactor was operated at 36 and 24 h HRT at the higher inlet concentrations. In case of 36 h HRT, more than 94 and 86% cumulative degradation efficiency were achieved at 1250 and 1500 mg/L inlet concentration of phthalate mixture, respectively. Whereas, only 85 and 75% cumulative degradation efficiency were achieved at 1250 and 1500 mg/L inlet concentration of phthalate mixture, respectively, at 24 h HRT. These results are consistent with the literature reported results on biodegradation of DBP, DEHP, bisphenol-A (BPA) and diclofenac (DCF) by acclimatized sludge under continuous operation; their biodegradation efficiency values substantially declined from 93-75% to 93-50%, respectively (Boonnorat et al., 2016).

From the results of biodegradation of the individual phthalates, it is observed that LMW phthalates as compared to HMW phthalates are easily and quickly degraded by the bacterium. Many studies have reported that PAEs with short ester hydrocarbon side chain are more easily and rapidly degraded than other EDPs with long ester side chains (Abdel daiem et al., 2012; Fan et al., 2018a; Gavala et al., 2003; Zhu et al., 2022).

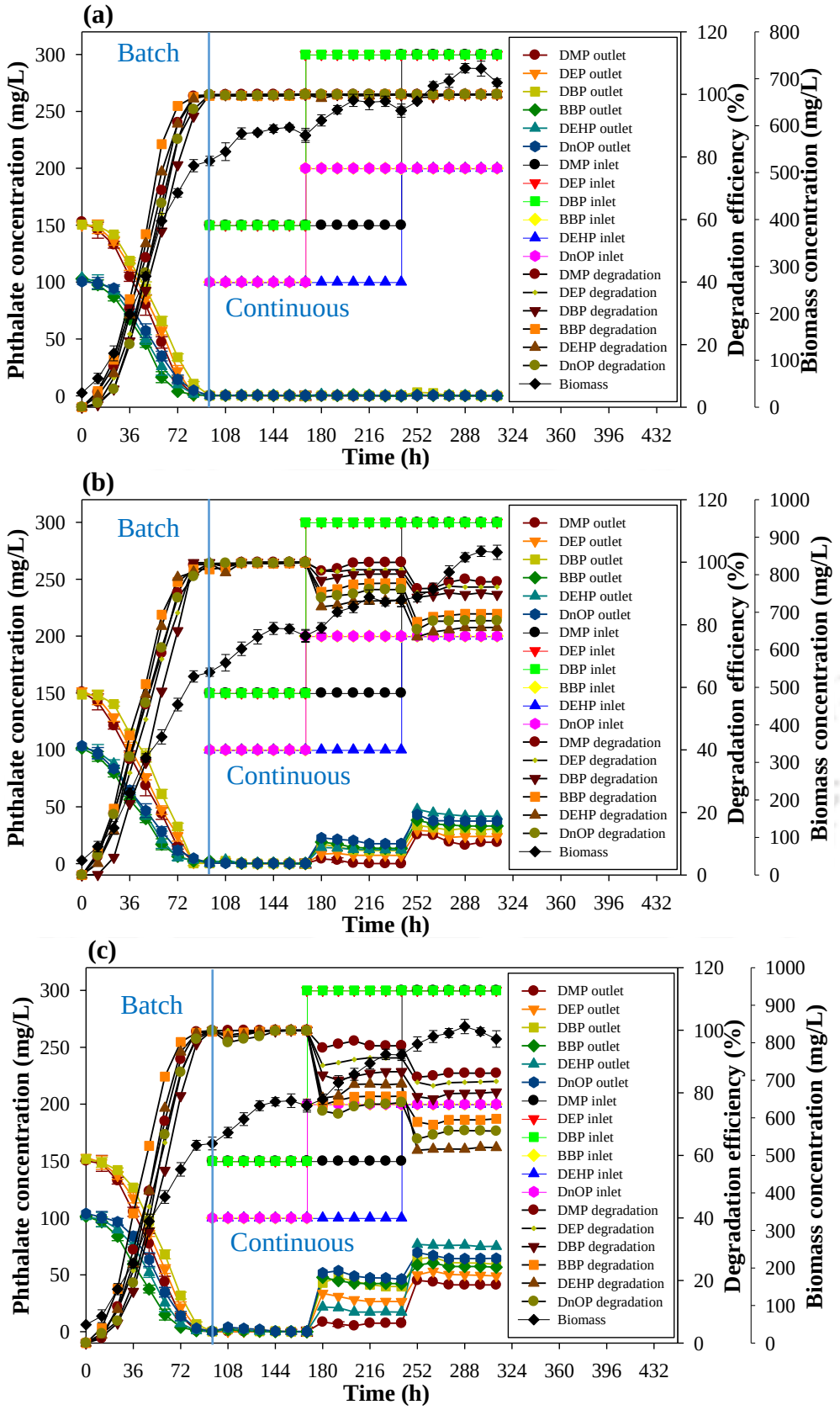


Figure 4.5: Time profile of biomass growth, inlet – outlet concentration of phthalates and % phthalates degradation by *Gordonia sp.* in the continuously operated CSTB for different HRTs: (a) 48 h (b) 36 h and (c) 24 h.

Effect of inlet loading rate

Figure 4.6 reveals the performance of CSTB under continuous operation mode, in terms of degradation rate of phthalates with respect to their inlet loading rate (ILR). In these figures, the straight line through the origin indicates a stable performance of the bioreactor under continuous operation for different ILRs. The degradation rate values that are offset from the line indicate that ILR values beyond this point are inhibitory and detrimental to the bioreactor's performance for phthalates biodegradation. Hence, it could be surmised that maximum degradation efficiency is favored at a low ILR, suggesting that microbial activity is unaffected below 6.25 mg/L·h of DMP, DEP and DBP (LMW) (Figure 4.6 a-c). With an increase in the phthalates ILR, the phthalates degradation rate and efficiency decreased, indicating an inhibitory effect at or above 8.33 mg/L·h of ILR. In the case of HMW phthalates, in particular BBP and DnOP, an ILR value of 4.17 mg/L·h or higher reduced their biodegradation (Figure 4.6d and f). Among the three HMW phthalates, DEHP was identified as the most inhibitory substrate for the microorganism and, therefore, it is suggested to keep its concentration lower than the other phthalates to achieve a better performance with the CSTB (Figure 4.6e). From Figure 4.6g, which shows the cumulative phthalate degradation rate vs their ILR, maximum degradation rate is achieved at 31.25 mg/L·h cumulative ILR value, and above which degradation rate was not consistent with the increase in ILR value. Hence, in order to achieve efficient biodegradation under continuous operation using the CSTB, the bioreactor should be operated at less than 31.25 mg/L·h cumulative ILR of the phthalates.

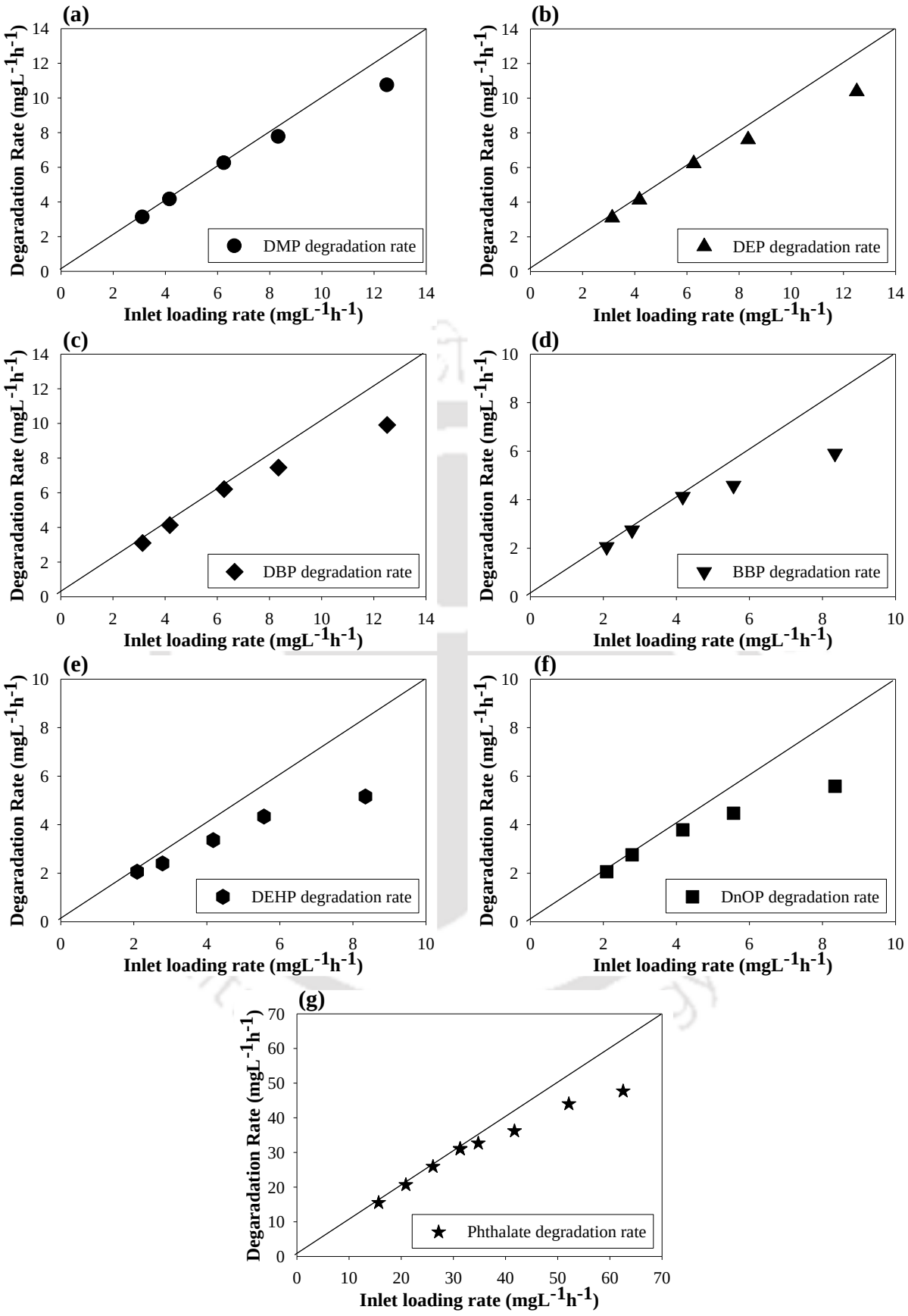


Figure 4.6: Degradation rate as a function of inlet loading rate of different phthalates: (a) DMP, (b) DEP, (c) DBP, (d) BBP, (e) DEHP, (f) DnOP and (g) cumulative degradation rate.

4.3.1.4. Continuous operation with biomass recycle

For enhancing the phthalates biodegradation at a high inlet concentration under continuous operation mode using the CSTB, the bioreactor was integrated with the indigenously made low-cost tubular ceramic membrane for the recycle of biomass followed by microfiltration of the reactor effluent. Experiments were carried out with the membrane flux set at 1.76×10^{-5} $m^3/m^2{\cdot}s$ under a constant pressure of 345 kPa. After 100 min of microfiltration operation at 345 kPa pressure and 15 L/h cross flow velocity, the permeate flux reduced and reached to a steady value of 3.29×10^{-6} $m^3/m^2{\cdot}s$ due to concentration polarization and biomass cake formation. The fouled membrane was regenerated by ultrasonication in distilled water and was reused for microfiltration of biomass.

The results of the bioreactor experiments carried out under continuous mode with biomass recycle are shown in Figure 4.7, which depicts biomass concentration (mg/L), inlet - outlet phthalate concentrations (mg/L), and phthalate biodegradation (%) efficiency profiles with time (h). As depicted in the figure, complete degradation of phthalates was achieved within the initial batch run of 96 h. Thereafter, the bioreactor was run under simple continuous mode and 84.91% cumulative degradation was achieved at 41.67 mg/L·h ILR (Figure 4.7a and b). Under continuous with biomass recycle mode, a short lag phase in phthalates biodegradation is observed due to an increase in the ILR of phthalates (48.41 mg/L·h) as a result of residual phthalates already present in the recycle stream. However, complete degradation of phthalate mixture was achieved quickly at 84 h of the 100 % biomass recycle operations carried out at 41.67 mg/L·h ILR and 36 h HRT (Figure 4.7a). Compared to this result, only 85% cumulative degradation of phthalate mixture was achieved without biomass recycle for the same ILR and HRT values. Moreover, more than 97% cumulative degradation efficiency of the phthalates were achieved when only 50% biomass was recycled during the 156 h operation period.

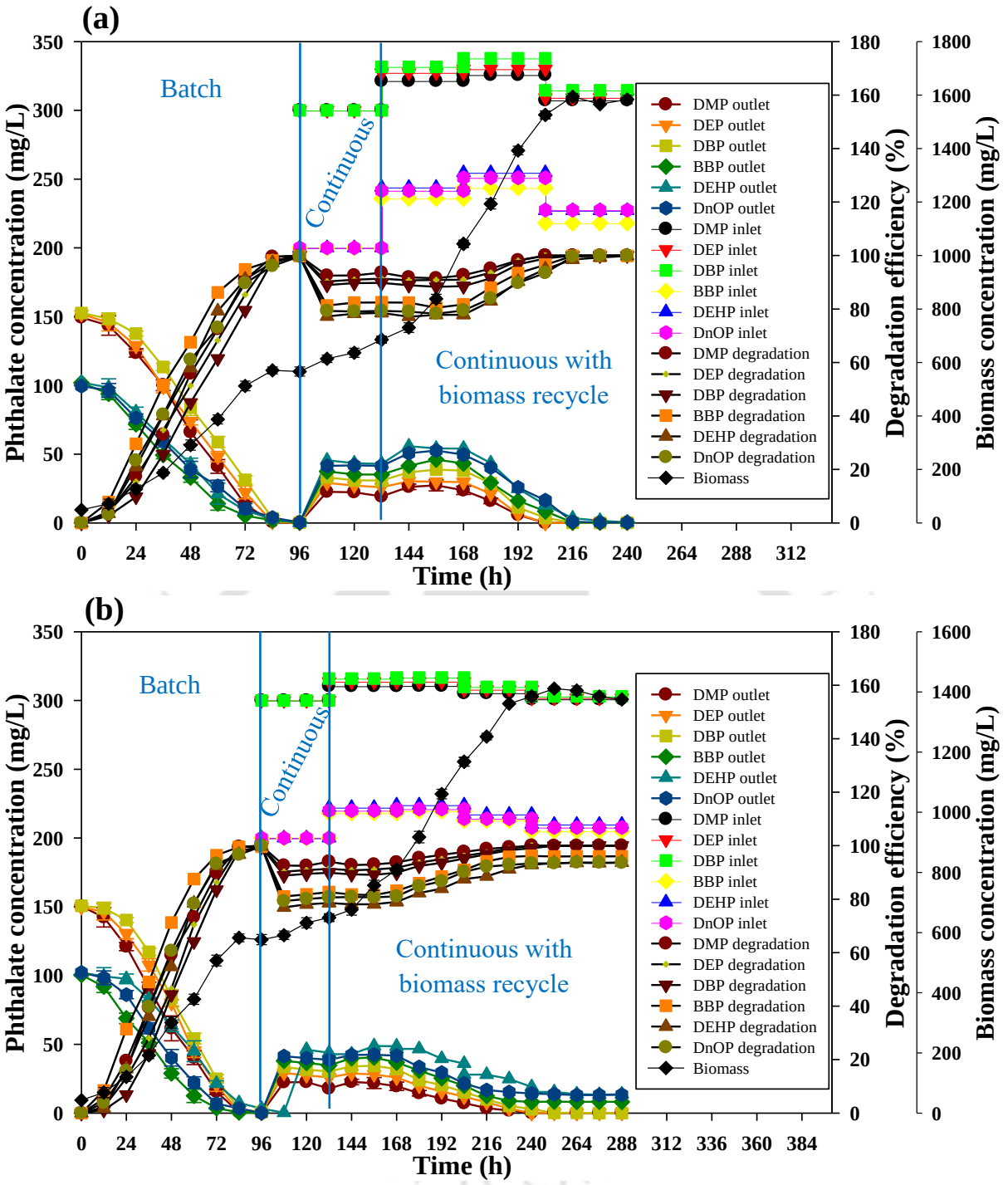


Figure 4.7: Time profile of biomass growth, inlet and outlet concentrations of phthalates and % phthalate degradation by *Gordonia sp.* in the CSTB operated under continuous mode with cell recycle at 36 h HRT: (a) 100% and (b) 50% cell recycle.

Phthalates degradation profiles in case of both 100 and 50% biomass recycle operations reveal that even high ILR of phthalates can be easily treated due to the presence of highly active *Gordonia sp.* in the bioreactor. Hence, it can be concluded that biomass recycling helps in

achieving enhanced biodegradation of phthalates even at high inlet concentrations. Moreover, biomass recycle reduces the biomass washout at high HRT in continuous treatment systems. All these results prove that the CSTB under continuous operation mode with biomass recycle is ideally suited for treating phthalate contaminated wastewater.

4.3.2. Ecotoxicity study of treated water

4.3.2.1. Seed germination bioassay

Following the biodegradation of phthalates mixture by *Gordonia sp.* in the CSTB, the effluent was evaluated for phytotoxicity by measuring the germination index (GI) of *Cicer arietinum L*, which is commonly used for ecotoxicity assessment owing to its sensitive nature towards harmful pollutants and potential for protein digestion after germination (Paul et al., 2019b). The GI index of *C. arietinum* seeds is an excellent parameter for ecotoxicity assessment that can serve as a suitable tool to determine phytotoxicity of pollutants in water. In this study, the GI was calculated using the seeds soaked in different samples: distilled water (negative control), liquid medium before and after degradation of phthalate, and the results are presented in Table 4.2. Figure 4.8 shows the image of germinated seeds obtained using the different samples for GI calculation. The GI of <1% in case of seeds soaked in medium containing untreated phthalate indicates acute toxicity. On the other hand, seeds soaked in distilled water display a very high GI of 98%.

The GI values of the treated effluent containing degraded phthalates from the different experiments followed the order: continuous treatment at 24 h HRT< batch treatment< continuously treatment at 36 h HRT< fed-batch treatment< continuous treatment at 48 h HRT< continuous treatment with 50% biomass recycle < continuous treatment with 100% biomass recycle. The GI values obtained in the case of 1500 mg/L degraded phthalates were lower than the values obtained with 750 and 1250 mg/L degraded phthalates due to its low biodegradation efficiency value obtained at a high concentration. A very high GI value of 92.94 % for seeds

soaked in treated effluent from the CSTB operated under continuous mode with 100% cell recycle clearly reveals that the treated water is safe and free of lethal phytotoxic agents for reuse purpose.

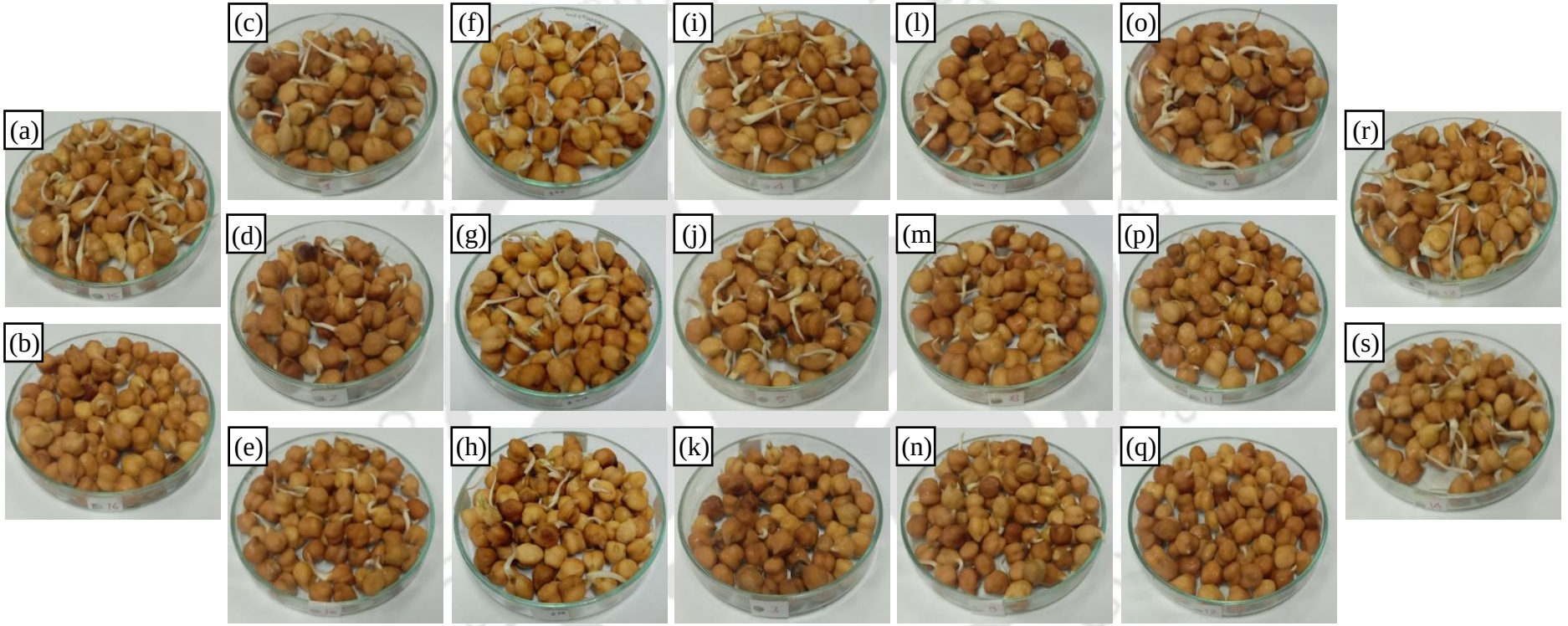


Figure 4.8: Germinated *Cicer arietinum L.* seeds soaked with: (a) distilled water (negative control), (b) phthalate containing medium, (c-e) 750, 1250 and 1500 mg/L samples from batch experiment, (f-h) 750, 1250 and 1500 mg/L samples from fed-batch experiment (i-k) 750, 1250 and 1500 mg/L samples from continuous experiment at 48 h HRT, (l-n) 750, 1250 and 500 mg/L samples at 36 h HRT, (o-q) 750, 1250 and 1500 mg/L samples at 24 h HRT, (r) samples from continuous mode with 100% biomass recycle and (s) with 50% biomass recycle.

Table 4.2: Results of ecotoxicity assessment of degraded phthalates.

		Total Initial phthalate concentration (mg/L)	GI (%)	% Mortality
Distilled water		0	98.00	0.00
Raw/untreated effluent		750	0.85	93.33
Treated effluent from batch operated CSTB		750	74.98	03.33
		1250	35.98	33.33
		1500	13.00	66.67
Treated effluent from fed-batch	1st feed	300	84.55	03.33
	2nd feed	3500	78.24	03.33
	3rd feed	3500	63.72	16.67
Treated effluent from continuously operated CSTB at different HRTs	48 h HRT	750	88.95	0.00
		1250	81.17	13.33
		1500	79.06	13.33
	36 h HRT	750	85.82	0.00
		1250	53.60	16.67
		1500	20.14	43.33
	24 h HRT	750	81.67	0.00
		1250	23.65	40.00
		1500	9.73	66.67
100% cell recycle	36 h HRT	1500	92.94	0.00
50% cell recycle	36 h HRT	1500	83.43	3.33

4.3.2.2. Brine shrimp lethality bioassay

The brine shrimp lethality assay is a fast, inexpensive and simple bioassay, and it has been developed for the evaluation of ecotoxicity of various natural and synthetic hazardous compounds (Eom et al., 2020; Paul et al., 2022b). Brine shrimps have been used for toxicity assessment of various plant extracts (Madjos et al., 2019). In this report, the active brine shrimp nauplii were used to determine the toxicity removal owing to biodegradation of phthalates by *Gordonia sp.* Nauplii incubated with distilled water was used as the negative control. An image showing the brine shrimp used in this study is shown in Figure 4.9 and the mortality percentage values are presented in Table 4.2, which reveal very high mortality of nauplii (93.33%) due to raw/untreated wastewater containing phthalates. Zero percent mortality is observed in the case of nauplii incubated in distilled water. Relatively high mortality of 66.67% is observed even in the case of degraded phthalates from the batch operated CSTB. Whereas insignificantly low mortality of nauplii is observed with phthalate degraded effluent from the CSTB operated under continuous mode with 100% cell recycle. These results of brine shrimp lethality assay correlate well with the results of toxicity assessment by seed germination assay and further confirm toxicity removal of phthalates by continuous treatment with 100% biomass recycle.

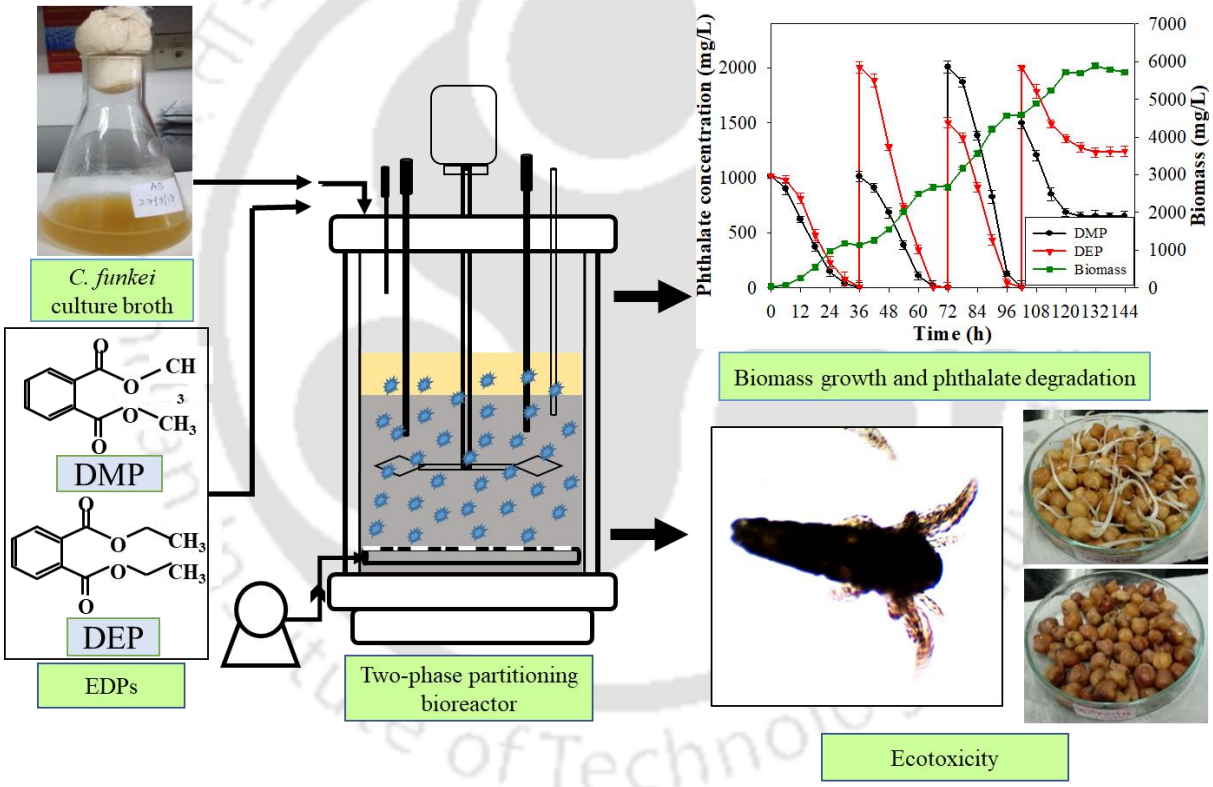
Figure 4.9: Microscopic image of brine shrimp nauplii used in the bioassay.

4.4. Conclusion

This study demonstrated the efficiency of a bioengineered system containing *Gordonia sp.* in an integrated bioreactor - microfiltration (MF) system for biodegradation and toxicity removal of phthalates mixture. Complete degradation of phthalates, even at very high inlet loading rates, was achieved in continuous with biomass recycle mode using the integrated CSTB - MF system with tubular ceramic membrane. The bacterium *Gordonia sp.* efficiently degraded both LMW and HMW phthalates, particularly at a low inlet concentration combination in the bioreactor. In addition, toxicity analysis of the degraded phthalates using the bioengineered system revealed very high GI and low mortality of brine shrimps suggesting that it could be scaled up for industrial treatment of phthalate containing wastewater.

Chapter 5

Biodegradation and toxicity removal of dimethyl phthalate and diethyl phthalate by *Cellulosimicrobium funkei* in two-phase partitioning bioreactor system



ABSTRACT

In the present study, biodegradation of DEP as the single substrate and a mixture of DMP and DEP as dual substrate were examined in a two-phase partitioning bioreactor (TPPB) using *Cellulosimicrobium funkei*. The TPPB system was run under batch and fed-batch modes of operation at different initial concentrations of DEP and mixture of DMP and DEP. Under the batch mode of operation, 93% degradation was achieved by *C. funkei* within 60 h with 38.75 mg/L·h degradation rate. In the fed-batch system, complete degradation was observed up to 3500 mg/L total initial concentration of the phthalates (1500 mg/L DEP and 2000 mg/L DMP) within 24h. Phytotoxicity assessment of the treated water by seed germination and brine shrimp lethality assays revealed a germination index value of 86.17 and 13.33% brine shrimp mortality. Overall, this study revealed a very good potential of the TPPB system for detoxification of endocrine disrupting chemicals in the environment.

5.1. Introduction

Phthalic acid esters (PAEs) are anthropogenic compounds, composed of alkyl or dialkyl esters of phthalic acid, and globally used to manufacture plastic and polyvinyl chloride by providing flexibility and mechanical strength (Lu et al., 2020). A critical environmental concern due to PAEs is their persistence in the ecosystem for extended period of time due to their high hydrophobicity and low volatility (Sharma et al., 2021). Therefore, different life forms, including human, animal and aquatic organisms, are exposed to PAEs through contaminated environment. Frequent exposure to phthalates is associated with toxic effects on humans and wildlife (Ahmad et al., 2017; Kambia et al., 2015). Phthalates interfere with the normal functioning of various receptors present in estrogen, progesterone, peroxisome and glucocorticoid (Josh et al., 2014; Lee et al., 2017; Sheikh et al., 2016). Hence, the removal of PAEs from polluted environments is of raising interest among the researchers.

In the previous Chapters, continuous stirred tank bioreactor was reported to efficiently degrade the phthalic acid esters in slurry phase system but for their complete degradation it is still a challenge, particularly at high concentration. Moreover, due to hydrophobicity and low aqueous solubility of PAEs, their bioavailability to microbes is seen as a hindrance to their biodegradation in natural as well as engineered treatment systems (Josh et al., 2014). In order to overcome this limitation, a two-phase partitioning bioreactor (TPPB) system can be considered for biodegradation of such pollutants (Baskaran et al., 2020; Daugulis, 2001c; Mahanty et al., 2010; Praveen and Loh, 2015). A TPPB system incorporates two immiscible aqueous phases: a biocompatible organic solvent in which PAEs are dissolved and an aqueous phase that contains microbes and other nutrients required for biodegradation. The organic phase of a TPPB system aids in the treatment process by transfer of the target pollutant to the aqueous phase based on real-time demand of the microorganisms and equilibrium considerations (Dafny, 2017; Han et al., 2018).

Several recent reports have successfully highlighted the effectiveness of TPPB system for microbial degradation of hydrophobic contaminants (Arriaga and Aizpuru, 2019; Cheng et al., 2016; Han et al., 2018; Hernández et al., 2011; Poleo and Daugulis, 2013). However, there is no report available yet on the biodegradation of PAEs using TPPB system. Hence, this study focused on biodegradation of a mixture of DMP and DEP in a TPPB system by *Cellulosimicrobium funkei*, under batch and fed-batch modes of operation. The effect of non-aqueous phase liquid (organic phase) on *C. funkei* growth and oxygen transfer were evaluated in this study with an aim to produce non-toxic water from phthalate contaminated aqueous medium.

5.2. Materials and methods

5.2.1. Chemicals and solvents

Silicone oil (SO), isopropyl myristate (IPM) and n-hexadecane (nH) were obtained from Himedia, Mumbai, India. Rest of the chemicals used in this study are the same as previously mentioned in Chapter 2 under Section 2.2.1.

5.2.2. Bacterial culture conditions

Cellulosimicrobium funkei used in this biodegradation study was examined previously to degrade DMP and DEP in a TPPB under batch and fed batch operation modes. Detailed characteristics of the bacterium and culture conditions are mentioned in Chapter 2 under Section 2.2.2.

5.2.3. Two phase partitioning bioreactor (TPPB) experiments

5.2.3.1. Selection of non-aqueous phase liquid

In this study, various organic solvents viz. isopropyl myristate, silicone oil and n-hexadecane were considered to screen the suitable and biocompatible non-aqueous phase liquid (NAPL) for this study. For the screening study, six 125 mL Erlenmeyer flasks each containing 40 mL

of Bushnell Haas-mineral salt medium (BH-MSM medium) and 5 mL of solvents were taken indivisualy. DEP was added at 1000 mg/L in 3 flasks as the carbon source and 3 flasks were kept without DEP. One extra flask having all the components except NAPL was taken as the positive control. Five mL of 24 h grown *C. funkei* culture (OD_{660} =1) was used as the inoculum and added to each flask. All of the flasks were cultured in an orbital shaker incubator for 48 hours at 28°C and 150 rpm; biomass was collected at the end of the experiments followed by washing with PBS (to remove solvents) and lyophilization. The effect of different organic solvents on relative metabolic activity of the bacteria was quantified by dividing the value of *C. funkei* biomass in each case by the value obtained without any added NAPL which served as the positive control in the experiments (Mahanty et al., 2010).

5.2.3.2. Effect of volume fraction of silicone oil on DEP biodegradation.

In order to determine the optimal volume fraction of silicone oil, which was found suitable as the NAPL for this study for efficient delivery of DEP into the medium, batch experiments were conducted with four different volume fractions of silicone oil, i.e. 5%, 10%, 15% and 20%. DEP (2000 mg/L) was homogenized in different fractions of the organic phase by ultra-sonication, and then added to BH-MSM taken in 125 mL Erlenmeyer flasks. These flasks were inoculated with 5 mL of *C. funkei* culture (OD_{660} = 1) and incubated in an orbital shaker incubator at 150 rpm and 28°C for 112 h. A control flask without any added NAPL and containing all other components were considered to observe the sole effect of NAPL on biomass growth and DEP biodegradation. Samples were taken after every 8 h time interval for the analysis of residual DEP and biomass concentration. All the experiments were carried out in triplicates.

5.2.3.3. Effect of NAPL on oxygen transfer in stirred tank reactor

Volumetric oxygen mass transfer coefficient (k_La) is a very crucial parameter for aerobic system, which might be affected by the addition of NAPL in the bioreactor system. Hence,

prior to studying biodegradation of PAEs using the TPPB system, the effect of NAPL addition on k_La in the system was evaluated at different combinations of agitation and aeration rates. For this hydrodynamic study, all the experiments were performed using a 5 L laboratory scale bioreactor as mentioned earlier in Chapter 3 (Section 3.2.3.1) and operated under different agitation and aeration rates in the ranges 400 – 800 rpm and 0.5 – 1.5 vvm, respectively. Oxygen mass transfer coefficients, with and without NAPL, in the system were calculated by the dynamic gassing out method (Shuler and Kargi, 2002).

5.2.3.4. Biodegradation of DEP and DMP in TPPB

5.2.3.4.1. Batch experiments

All experiments in this study were carried out using the pure culture of *C. funkei* in a closed environment (bioreactor) and using sterile MSM containing phthalates as sole carbon source. The physical dimensions and other features of the CSTB used in this study are mentioned in Chapter 3 under section 3.2.3.1. Figure 5.1 shows schematic of the TPPB system used in this study. The working volume of the reactor was 3 liter and operating conditions were maintained optimum at 1 vvm aeration rate, 600 rpm agitation, 7 pH and 28°C temperature. At all the above operating conditions, the initial DO of the BH-MSM medium was at 100% saturation (7.82 mg/L); however, during the growth phase of the bacterium, it varied between 90 and 95% (7.04 - 7.43 mg/L). The batch experiment was run at 2500 mg/L initial concentration of DEP. A pre-determined amount of the DEP was added in 300 mL, i.e. 10% of total working volume of silicone oil as the NAPL and incubated in an ultrasonic water bath for 40 min to make a homogeneous mixture of DEP with silicone oil before finally adding the mixture to the bioreactor. The TPPB containing both the DEP loaded silicone oil and 2.4 L of BH-MSM was inoculated with 10% v/v of freshly grown *C. funkei* culture (OD_{660} = 1). For adjusting the medium pH to 7, NaOH/HCl was used in the experiments. Samples in triplicate were

withdrawn at every 6 h for measuring the residual DEP concentration and biomass growth. The samples were analysed in triplicate and the findings were averaged.

DEP biodegradation efficiency by the *C. funkei* in concomitant presence of DMP was further evaluated by using the TPPB system. For simultaneous biodegradation of DEP and DMP, the organic phase was prepared by dissolving DEP and DMP at initial concentrations of 2000 and 1000 mg/L, respectively.

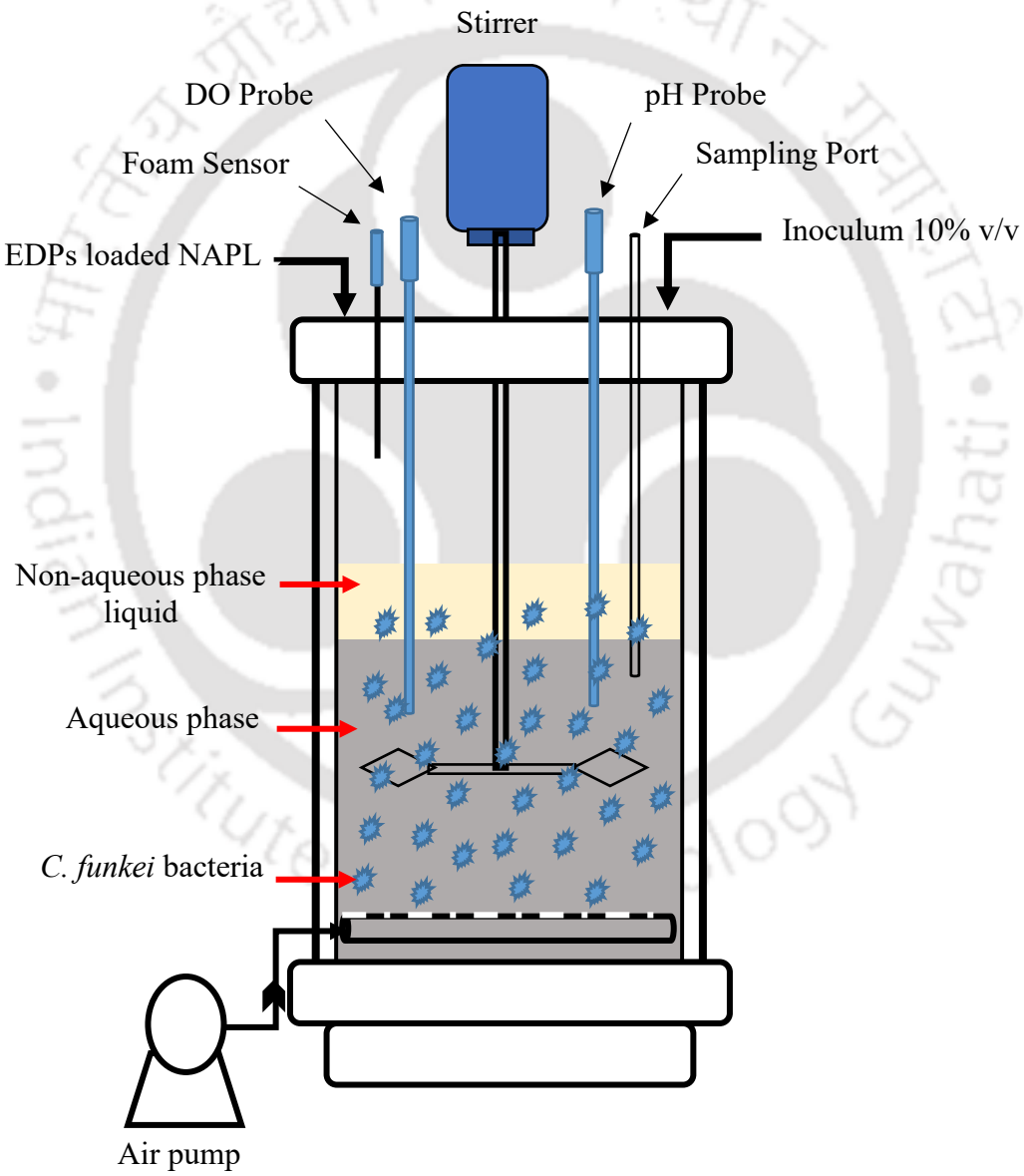


Figure 5.1: Schematic representation of the two-phase partitioning bioreactor system used in this study.

5.2.3.4.2. Fed-batch experiments

For phthalate biodegradation using the TPPB system under fed-batch mode of operation, 10% v/v NAPL was used and the bioreactor was initially operated under batch mode with 2000 mg/L DEP as the single substrate. At the end of the batch run, i.e. after complete degradation of DEP by *C. funkei*, DEP was added at 2500 mg/L initial concentration in the TPPB and the concentration in the subsequent pulse feed was increased by 500 mg/L than the previous one and up to 3500 mg/L.

Similarly, for degrading a mixture of DMP and DEP, the TPPB was started under batch mode with 2000 mg/L total initial concentration of the mixture (1000 mg/L each). After the initial batch operation, phthalate mixture was added in pulse-feed mode for up to three times of different concentrations as mentioned in Table 5.1. The concentration combinations of DEP and DMP for the fed-batch study were chosen based on the outcomes of our earlier study described in Chapter 2 which revealed high, moderate and low biodegradation efficiency of the two EDPs. Triplicate samples were withdrawn at 6 h interval for measuring the biomass growth and residual DEP and DMP concentrations.

Table 5.1: Different phthalate concentration used in the fed batch experiments with the TPPB and their biodegradation percentage values.

Experimental run	Initial concentration (mg/L)		% Degradation	
	DMP	DEP	DMP	DEP
Initial concentration in batch	1000	1000	99.64	98.96
1st feed	1000	2000	99.96	99.89
2nd feed	2000	1500	99.83	99.77
3rd feed	1500	2000	56.38	37.88

5.2.4. Analytical methods

Determination of *C. funkei* biomass growth and EDPs in the aqueous phase were carried out as per the same protocol mentioned in Chapter 2 under Sections 2.2.4.2. and 2.2.3.4., respectively. Silicone oil samples were pre-treated with anhydrous sodium sulphate to eliminate trace amount of water and extracted with equal volume of methanol by vortexing for 5 min followed by centrifugation for 10 min at 10,000×g for phase separation (Mahanty et al., 2010). The residual concentration of DMP and DEP in the extracted methanol sample were determined by the same protocol as followed for the aqueous phase determination of the EDPs.

5.2.5. Ecotoxicity of the degraded phthalates

5.2.5.1. Phytotoxicity evaluation

For evaluation of the toxicity removal of DEP and DMP owing to their degradation by *C. funkei,* sample taken from the TPPB system was examined for seed germination, and the obtained result was compared with that of distilled water, tap water and phthalate containing water (untreated). Details of the phytotoxicity assays are mentioned in Chapter 3 under Section 3.2.7.1.

5.2.5.2. Brine shrimp lethality bioassay

To examine the cytotoxicity removal of phthalates due to their biodegradation by *C. funkei* in the TPPB system, brine shrimp lethality bioassay was carried out. For brine shrimp lethality bioassay, the same method as mentioned in Chapter 3 under Section 3.2.7.2 was followed.

5.3. Results and discussion

5.3.1. Selection of organic solvent as NAPL in the TPPB system

Different solvents were screened based on their biocompatibility with *C. funkei* and their efficiency to deliver phthalates to the degrading microorganism in the aqueous phase as well as their recalcitrance to biodegradation. Figure 5.2 shows the results of screening experiments

in terms of the relative metabolic activity of *C. funkei* in the presence of various solvents, which clearly reveals that very high relative metabolic activity is observed even when IPM was used without any added DEP in the medium. Hence, IPM was not chosen as NAPL for TPPB system which otherwise can affect the biodegradation of DEP by *C. funkei*. Whereas, in the case of silicone oil and n-hexane as the NAPL, very low metabolic activity of *C. funkei* is observed in the absence of DEP as the sole carbon source. Therefore, these solvents are considered as non-bioavailable to the organism. However, compared with silicone oil as the NAPL n-hexane inhibited the metabolic activity of *C. funkei* in the presence of DEP as the carbon source, due to its toxic effect, and therefore, it was considered as non-biocompatible solvent for use in the TPPB system (Baskaran et al., 2020; Darracq et al., 2012). It is apparent from Figure 5.2 that relative metabolic activity of the bacterium in the presence of silicone oil and DEP was the same as in the control which contained only DEP as the sole carbon source. Hence, silicone oil was considered as biocompatible, non-bioavailable and suitable for DEP delivery to the microbes. Moreover, it is known that the NAPL (silicon oil) is immiscible with water and can be separated easily from aqueous medium by simple settling, heating, distillation, centrifugation, etc. Thus, based on these results, silicone oil was used as the NAPL for further experiments.

Proper selection of a non-aqueous phase liquid (NAPL) is very crucial for TPPB system in order to avoid bacterial growth inhibition or unnecessary utilization of NAPL for growth as well as to enhance the delivery of target pollutant to degrading bacterium. Bioavailable NAPL are usually avoided for use in the TPPB systems, as it reduces the biodegradation efficiency of microorganisms. Silicone oil is well reported to be highly stable, hydrophobic and chemically resistant to oxidative attack, and it has been used in several studies dealing with two-liquid phase systems. (Baskaran et al., 2020; Mahanty et al., 2010; Praveen and Loh, 2015).

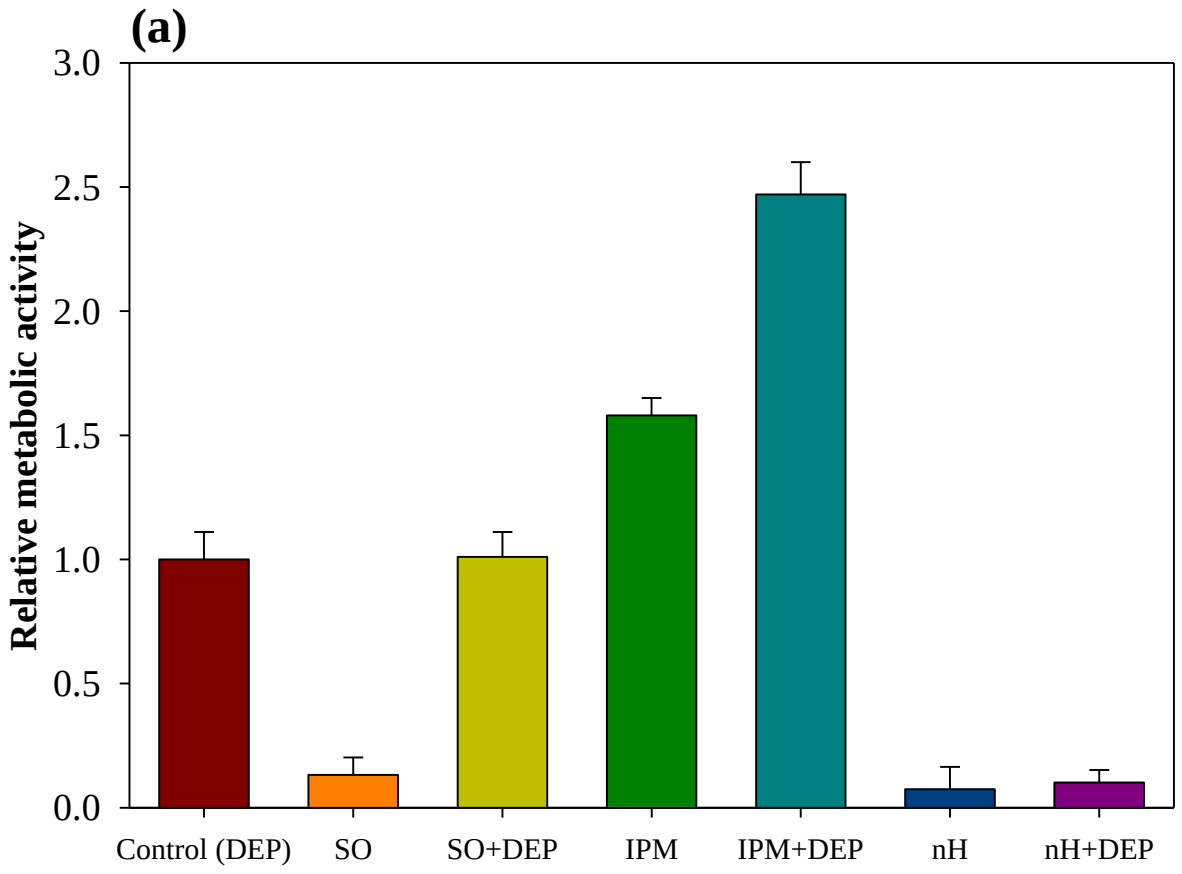


Figure 5.2: Relative metabolic activity of *C. funkei* in the presence of different NAPL.

5.3.2. Effect of NAPL volume fraction on biodegradation

For determining the optimal NAPL volume required in the TPPB system, experiments were conducted using 5 different volume fractions, viz. 0, 5, 10, 15 and 20 % of NAPL along with 2000 mg/L DEP as the sole carbon source. DEP biodegradation by *C. funkei* and their biomass growth were determined for different volume fractions of NAPL (Figure 5.3). Figure 5.3a reveals a very short lag phase in the biomass growth at 10% NAPL, whereas at a high NAPL concentration the lag in biomass growth is prominent. Maximum biomass concentration of 1214.22 mg/L is observed within 72 h for 10% NAPL, whereas, at 15% NAPL concentration the culture took a prolonged time of 112 hours for achieving the maximum biomass concentration value. These results clearly reveal slow release of the DEP at higher volume fraction of NAPL which yielded high biomass concentration in the experiments.

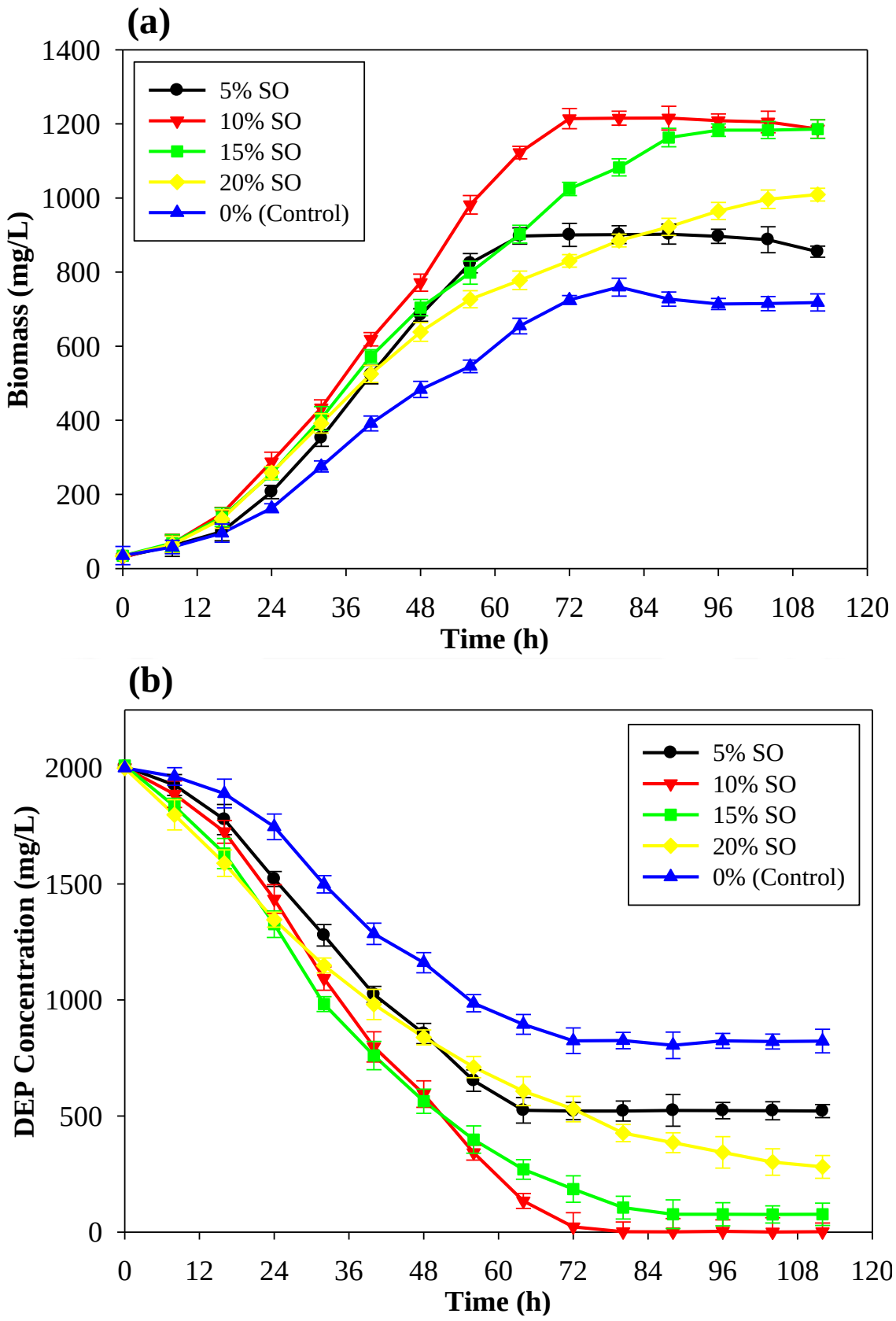


Figure 5.3: (a) Biomass growth of *C. funkei* and (b) DEP degradation at different volume fractions of silicone oil.

From Figure 5.3b, only 75% biodegradation of DEP is observed at 5% NAPL. Moreover, quick release of DEP into the aqueous phase from NAPL was observed, which resulted in inhibition of the biomass growth. On the other hand, a higher volume fraction of silicone oil (15 and 20%) revealed poor delivery of DEP to the aqueous media which resulted in a low degradation rate of DEP. Whereas, without any added NAPL very low degradation of DEP was achieved due to its strong inhibitory effect on biomass growth by the organism. At 10% v/v of silicone oil in the TPPB system, complete DEP degradation within a short time is achieved due to its efficient delivery as needed by the organism for its metabolism. In this TPPB system, DEP loaded silicone oil droplets served as the delivery vehicle in ensuring simultaneous *C. funkei* growth and degradation of the compound. Hence, based on the biodegradation results and cost consideration, 10% (v/v) of silicon oil was chosen for further experiments.

5.3.3. Determination of volumetric oxygen mass transfer coefficient in the TPPB

For an efficient aerobic biodegradation of the phthalates by *C. funkei*, a high oxygen mass transfer rate from gas bubble to NAPL or the aqueous phase is essential, and it is mainly governed by the size of the interfacial area between the different phases. The interfacial area is in turn a function of surface average droplet diameter and dispersed phase volume fraction (phase ratio) (Shuler and Kargi, 2002). The optimal phase ratio that generates the maximum interfacial area is influenced by certain parameters, including agitation, aeration and medium viscosity of the TPPB system. For instance, an increase in agitation and aeration rates results in an increase in the number of bubbles and droplets in the liquid medium. However, the distribution of size and mean diameter of the droplets are due to their simultaneous breakage and coalescence: a high mixing rate produces small sized NAPL drops with a high tendency toward coalescence (Abufalgha et al., 2021; Hassan and Robinson, 1977). Therefore, choosing the best set of all these operating conditions define the success of a TPPB system.

The measured values of k_La in single and two phases under different agitation and aeration rates in the reactor are displayed in Figure 5.4. It is observed from the figure that when aeration was increased from 0.5 to 1 vvm aeration rate in the TPPB system, it resulted in about two-fold increase in the oxygen mass transfer coefficient value, but when the aeration was further increased to 1.5 vvm, the k_La value remained constant. At low aeration rates, the effect of agitation on k_La was the same in both the single and two liquid phase systems. However, at an agitation rate of 400 rpm, large sized air bubbles were observed in the two-phase reactor system and the liquid phases remained distinct and not well-mixed. Whereas higher agitation led to small sized air bubbles which raised the k_La value owing to an enhancement in breakage and coalescence of NAPL drops in the aqueous medium. On the other hand, when the agitation rate was further increased from 600 to 800 rpm, no further increase in the k_La value was observed. Thus, 1.0 vvm aeration and 600 rpm agitation were fixed throughout the TPPB study owing to efficient oxygen mass transfer rate, shearing and dispersing of air bubbles, necessary to achieve high DEP biodegradation efficiency.

The addition of silicone oil resulted in approximately two-fold increase in oxygen volumetric mass transfer coefficient compared to the value observed in single aqueous phase system. Few studies also support the finding that addition of silicone oil as NAPL in TPPB system enhances the oxygen volumetric mass transfer coefficient (Bordel et al., 2010; Dumont et al., 2005).

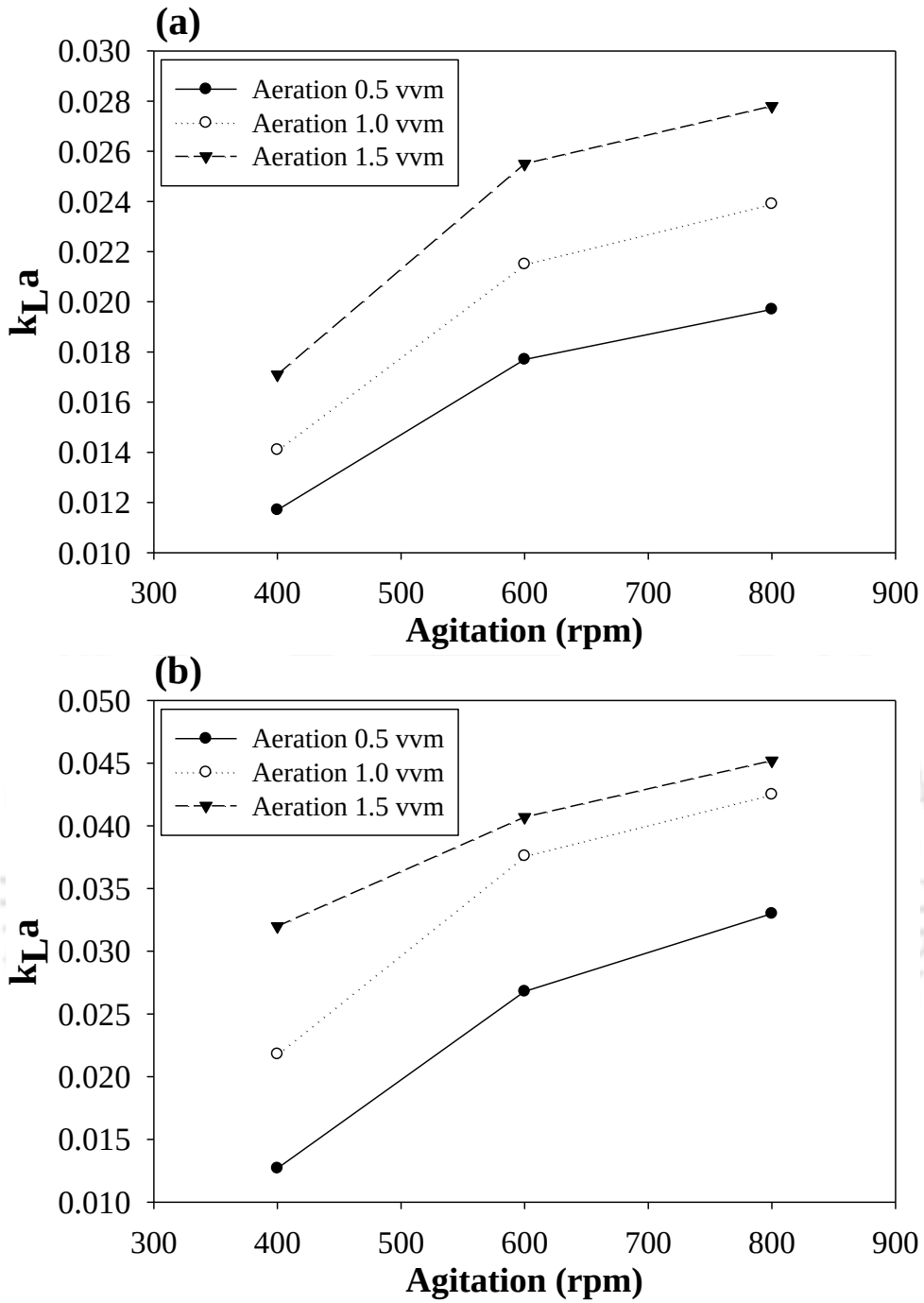


Figure 5.4: Observed volumetric oxygen mass transfer coefficient values in (a) single and (b) two-phase systems.

5.3.4. Biomass growth and EDPs degradation by *C. funkei* in TPPB system

5.3.4.1. Batch operation mode

Using the CSTB operated under batch mode, *C. funkei* could effectively degrade DEP only up to 1500 mg/L initial concentration in single phase system. Above 2000 mg/L of initial DEP

concentration, *C. funkei* growth was inhibited and the biodegradation efficiency reduced. To overcome the inhibitory effect of the high concentration of DEP, the two-phase partitioning bioreactor developed in this study was initially operated under batch mode, and the results of biomass growth and DEP biodegradation by the bacterium are shown in Figure 5.5a. From the figure, 93% degradation of DEP is achieved within 60 h for an initial concentration of 2500 mg/L, whereas the value was only 58% in the single-phase system (Chapter 3). Moreover, no lag phase in biomass growth and DEP degradation were observed in the present TPPB system as compared with that in the single-phase system.

In order to assess the performance of the batch TPPB system to degrade a mixture of DEP and DMP by *C. funkei*, a total combined concentration of 3000 mg/L (2000 DEP and 1000 DMP) was chosen due to their low rate and efficiency of biodegradation in the single-phase system at the same initial concentration (Chapter 3). The mean concentrations of PAEs, including DMP, DEP and DEHP in domestic wastewater for discharge and aqueous environments are reported to be in the range from 1 µg to 400 mg/L (Gani et al., 2017; Salaudeen et al., 2018; Tuan Tran et al., 2022). However, raw wastewater from industries, such as plastics, paint, ink, fertilizers, personal care products etc. contains substantially high concentrations of these compounds (Anne and Paulauskiene, 2021; Ishchenko et al., 2018; Magnusson et al., 2016). Hence, the concentration range chosen for the present biodegradation study matches with the literature reported value.

Biomass growth of *C. funkei* and EDPs biodegradation profile presented in Figure 5.5b reveal absence of lag phase and complete degradation of DMP within 48 h; DEP was 83.7% degraded within 60 h. The degradation values in the single-phase system were 85.37% and 58.12% for DMP and DEP, respectively (Chapter 3). The pathway involved in DMP and DEP degradation by *C. funkei* and the degradation intermediates are reported in Chapter 2. These results clearly reveal very high biodegradation efficiency of the compounds by *C. funkei* in the TPPB system

than in the single-phase system. Limited number of reports are available on biodegradation of phthalates using conventional bioreactor system (Fang et al., 2010; Xu et al., 2021b; Yousefzadeh et al., 2017), but this is the first report on DEP and DMP biodegradation in a two-phase partitioning bioreactor system.

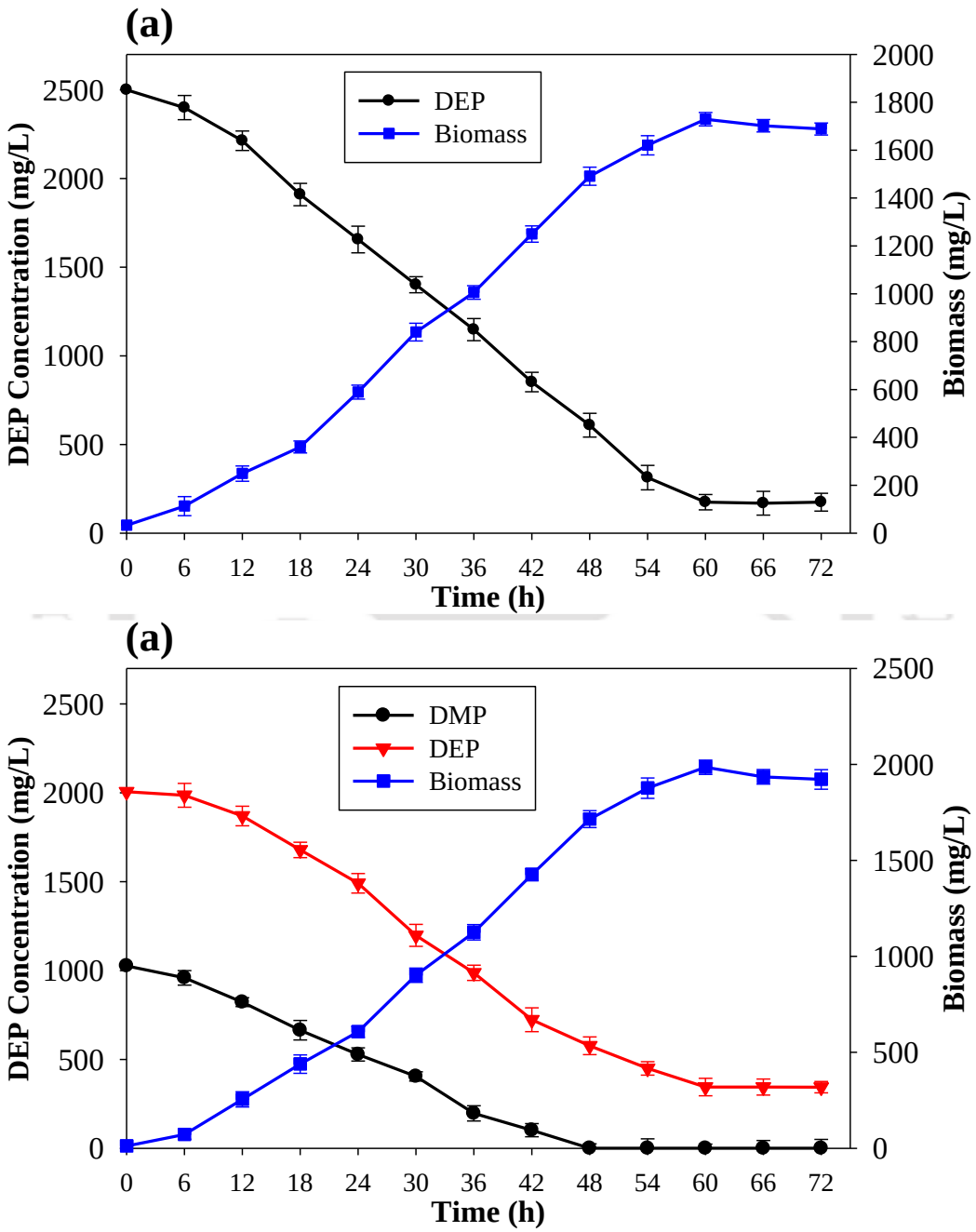


Figure 5.5: Biomass growth of *Cellulosimicrobium funkei* and EDPs biodegradation profile in the TPPB system operated under batch mode: (a) single substrate (DEP) and (b) mixed substrate (DMP and DEP).

5.3.4.2. Fed-batch operation mode

For assessing the TPPB system under fed-batch operation mode to degrade EDPs, the reactor was run initially for 48 h under batch operation mode and later switched over to fed-batch mode of operation. Figure 5.6a shows the time profile of DEP biodegradation and biomass growth of *C. funkei*. Initially, the TPPB system was started with a low DEP concentration of 2000 mg/L, which resulted in quick degradation of the compounds within 36 h. Thereafter, the substrate concentration was increased in a step-wise manner from 2000 to 2500, 3000, and finally up to 3500 mg/L. Figure 5.6a reveals complete degradation of DEP up to 2500 mg/L within 78 h. At 3000 mg/L, 95% degradation is achieved, whereas very low degradation is observed at 3500 mg/L. Thus, compared with the batch study, fed-batch study using the TPPB system resulted in a high DEP degradation efficiency even at high initial concentrations and within a short time period.

Performance of the TPPB system operated under fed batch mode was further examined to degrade a mixture of DEP and DMP at different concentration combination by feeding under pulse mode. Biodegradation profiles of DEP and DMP in the mixed substrate system, along with biomass growth of *C. funkei*, are presented in Figure 5.6b. From the figure, it is observed that in the first experimental run with DMP and DEP at initial concentrations of 1000 mg/L each EDPs were degraded completely within 36 h. In the second run, a mixture of 2000 and 1000 mg/L of DEP and DMP, respectively, was supplied and their complete degradation were again achieved within 36 h. In the third run, the DEP concentration was kept low at 1500 mg/L and the DMP concentration was increased to 2000 mg/L to make the total initial concentration of the mixture equal to 3500 mg/L. Complete degradation of EDPs were achieved even at this high concentration and within 24 h. More strikingly, in all the experimental runs, no lag phase was observed in the EDPs degradation profiles (Figure 6b). Moreover, the time required for EDPs biodegradation was reduced due to sufficiently high amount of well-acclimatized

biomass present in the TPPB system. However, in the fourth run, the reactor was operated with very high concentrations of 2000 mg/L DEP and 1500 mg/L DMP, which resulted in only 37.88 and 56.36% degradation of DEP and DMP, respectively, probably due to inhibitory effect of DEP on the biomass activity at a very high concentration relative to DMP in the mixture.

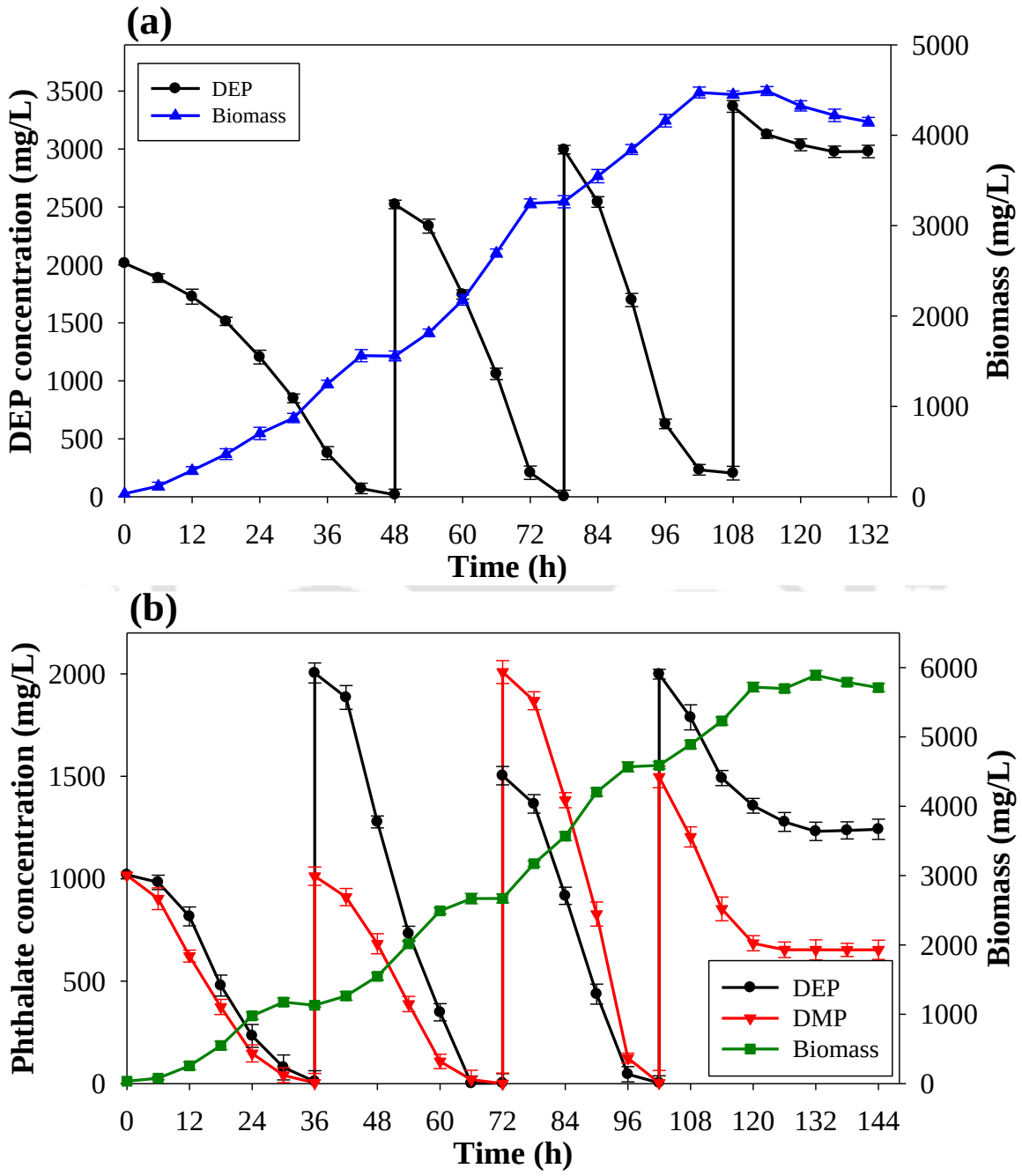


Figure 5.6: Biomass growth of *Cellulosimicrobium funkei* and EDPs biodegradation profile in the TPPB system operated under fed-batch mode: (a) single substrate (DEP) and (b) mixed substrate (DMP and DEP).

In the literature, an anaerobic fixed film baffled bioreactor and an up-flow anaerobic fixed bed bioreactor operated in continuous mode for biodegradation of DEP in wastewater were reported to achieve 91.11 and 88.72% degradation efficiency, respectively, at 700 mg/L influent concentration (Yousefzadeh et al., 2017). In a recent study by Xu et al. (2021), biodegradation of diethyl phthalate using a sponge–based immobilized bioreactor was run under continuous mode and 67.87% degradation efficiency was observed at 1.71 mg/L·h loading rate. Compared to these reports, the TPPB system followed in this study demonstrated complete degradation of the EDPs even at a relatively high concentration and within a short time period, more so when the system was operated under fed-batch mode.

Different physical removal methods have been reported for the complete removal of phthalates from contaminated system. However, all these methods are known to generate huge amount of hazardous sludge as secondary waste requires further treatment for dispose. Moreover, membrane techniques, including nanofilters, reverse osmosis and ultra-filtration, are expensive due to high membrane cost and high operating pressure. On the other hand, biological methods are advantageous in terms of low-cost of the process, high removal rate, non-toxic degradation product, and eco-friendly approach, which are the main elements required for sustainable remediation (Ahmadi et al., 2017; Patil and Jena, 2019; Singh et al., 2017; Yousefzadeh et al., 2017). Furthermore, the NAPL used in this study can be reused in the biodegradation process.

5.3.5. Ecotoxicity assessment of the degraded phthalates

5.3.5.1. Phytotoxicity

Following biodegradation of DMP and DEP by *C. funkei* in the TPPB system, phytotoxicity of the treated liquid medium was assessed based on their ability to promote/supress germination of chickpea seed. Chickpeas are often used for such phytotoxicity assessment due to its high sensitivity to hazardous contaminants (Paul et al., 2019b). The seeds GI is a highly sensitive

toxicity assessment parameter that is used to monitor changes in the phytotoxicity of contaminants in water.

The GI values of seeds soaked in phthalate degraded sample from TPPB, phthalate containing medium, distilled water and tap water were calculated using Equation 3.9. A negative control in the experiment comprised of seeds soaked in distilled water. Images of the germinated *Cicer arietinum L* seeds used for the GI calculation are shown in Figure 5.7. Seeds soaked in untreated phthalate-containing sample had a very low GI value of 1.53%, demonstrating highly toxic effect of the phthalates. Seeds soaked in phthalate degraded sample from the TPPB had a high GI value of 86.17%, proving that it is free from phytotoxic chemicals. However, distilled water and tap water showed very high GI values of 97 and 93.63%, respectively. The GI value (86.17%) obtained in this study is much higher as compared to the GI value of 68.43% obtained after biodegradation of a mixture of DMP and DEP using a continuous stirred tank bioreactor (Chapter 3), which reveals that the TPPB system is more efficient in toxicity removal than the simple CSTB.

Figure 5.7: Germinated chickpea seeds soaked in (a) phthalate containing medium, (b) phthalate degraded sample from TPPB, (c) distilled water and (d) tap water.

5.3.5.2. Brine shrimp assay

The brine shrimp lethality assay is a quick, low-cost, and simple bioassay; it has been developed for the evaluation of ecotoxicity of various natural and synthetic hazardous compounds. Brine shrimps are also used for the toxicity assessment of various plant extracts

(Madjos et al., 2019; Naidu et al., 2014). In the present study, the active brine shrimp nauplii were used to determine the toxicity removal due to biodegradation of phthalate by *C. funkei* in the TPPB system. The brine shrimp mortality in tap water, distilled water, untreated and treated phthalate samples were evaluated using Equation 3.10; nauplii incubated in distilled water served as negative control in the study.

Table 5.2 presents brine shrimps mortality percentage value with different samples. A very high mortality of 86.67% is observed for the nauplii incubated in phthalate containing medium. Whereas, 13.33% mortality is observed for the nauplii incubated in phthalate degraded sample from TPPB. A very low mortality of 3.33% and 6.67% are observed for the nauplii incubated in distilled water and tap water, respectively. The high GI value and low brine shrimp mortality achieved in this study can be attributed to the formation of non-toxic intermediate compounds or the end products of CO_2 and H_2O during DMP and DEP biodegradation by *C. funkei*, as known previously from the intermediate analysis discussed in Chapter 2.

Table 5.2: Brine shrimp mortality in different water samples.

	No. of nauplii at 0 h	**No. of live nauplii at 24 h (Average of three test)**	**Mortality %**
Phthalate containing medium	10	1.33±1.53	86.67
Phthalate degraded sample from TPPB	10	8.67±0.57	13.33
Distilled water	10	9.67±0.57	3.33
Tap water	10	9.33±0.57	6.67

5.4. Conclusion

The present study established the importance of TPPB system over single-phase system for achieving high biodegradation efficiency of phthalates even at very high initial concentration and within a short time. The mechanism of phthalate degradation in the TPPB system involved slow release from the non-aqueous phase (silicone oil) to the aqueous phase as per real time demand of *C. funkei*, which aided in overcoming the substrate inhibition effect of the EDPs. Besides, very high volumetric oxygen mass transfer coefficient due to the added silicone oil in the TPPB was observed for enhancing the EDPs biodegradation. A high GI value of 86.17% of chickpea seeds soaked in phthalate degraded sample taken from the TPPB system along with a low mortality value of 13.33% of brine shrimps demonstrate the utility of TPPB system in toxicity removal of phthalates from contaminated systems.

Chapter 6

Summary and Conclusions

Phthalic acid esters are also known as the endocrine disrupting phthalates owing to their adverse effect on various organs of the endocrine system. PAEs the emerging environmental pollutants that are released into the environment from a wide variety of sources during production, use and disposal. Water contamination due to PAEs is therefore a serious concern. Hence, this study focused on the biodegradation of different PAEs at various concentration combination using different bioengineered system, viz. continuous stirred tank bioreactor (CSTB), two-phase partitioning bioreactor (TPPB) and continuous with biomass recycle system followed by microfiltration under aerobic condition.

In a preliminary experiment using three bacteria *(Rhodococcus opacus, Cellulosimicrobium funkei and Ochrobactrum sp.*), *C. funkei* was identified to be the best for the biodegradation of DMP and DEP as a single substrate. However, using batch shake flask, low degradation efficiency was obtained at high initial concentration of the phthalates. Hence, a continuous stirred tank bioreactor (CSTB) was examined for enhancing the biodegradation of DMP and DEP by *C. funkei*, and complete degradation was achieved even up to 3000 and 2000 mg/L initial concentrations of DMP and DEP, respectively. High degradation efficiency using the CSTB was attributed to the controlled conditions of aeration, agitation and pH in the bioreactor. Stoichiometric and mass balance analyses carried out in this batch bioreactor study clearly established the effectiveness of the biodegradation process using the CSTB. Degradation of a mixture of DMP and DEP was also found to be enhanced using the CSTB.

As compared to batch operation, fed-batch operation further revealed the high efficiency of degradation of the DMP and DEP mixture, even at their high initial concentrations. Under the continuous mode of operation using the CSTB, a high degradation rate (178.37 mg/L·h) was achieved even at high total inlet loading rate (ILR) of 218.75 mg/L·h. The degradation rate under the continuous mode of operation was further enhanced (218.68 mg/L·h) by recycling

the biomass into the bioreactor by microfiltration using an indigenous tubular ceramic membrane. High GI and low brine shrimp mortality values were observed from the phthalate degraded samples, demonstrating the potential of the CSTB integrated with microfiltration for treating PAEs containing wastewater. Thus, *C. funkei* demonstrated the efficient degradation of DMP and DEP as single and dual substrates using the CSTB under different operation modes, in particular continuous with 100% biomass recycle.

In addition to *C. funkei*, another novel bacterium, *Gordonia sp.*, showed complete degradation of a mixture of six phthalates including BBP, DEHP and DnOP as high molecular weight phthalate in the CSTB system. Complete degradation (100%) of phthalates, even at very high total ILR (61.67 mg/L·h) was achieved using CSTB under continuous with biomass recycle mode suggesting that integrated biodegradation-microfiltration approach was best suitable for efficient degradation of phthalates for treating PAEs containing wastewater. In addition, toxicity analysis of the degraded phthalates revealed very high GI and low mortality of brine shrimps, further confirming the potential of the bioengineered system for treating such wastewater. Hence, the bioengineered system consisting of CSTB with the degrading bacterium demonstrated successful biodegradation of different phthalates in wastewater under different operation modes: batch, fed-batch, continuous and continuous with biomass recycle.

The major mechanism of PAEs biodegradation by *Gordonia sp.* and *C. funkei* was shown to involve the hydrolysis and/or esterification of alkyl side chain of phthalates. Intermediates formed during the biodegradation of PAEs further confirmed phthalic acid as the central metabolite. The mixture study reveals that an increase in the concentration of low molecular weight (LMW) phthalates (DMP, DEP and DBP) resulted in a high degradation of high molecular weight (HMW) phthalates (BBP, DEHP and DnOP), whereas LMW phthalate degradation efficiency values reduced with an increase in HMW phthalate concentrations.

Due to the low molecular weight and a short side chain of LMW phthalate, its specific degradation rate was more than that of the HMW phthalates.

A novel bioengineered system, a two-phase partitioning bioreactor (TPPB) system showed very high biodegradation efficiency of phthalates by *C. funkei* even at very high initial concentration of DMP and DEP, and within a short duration. In this TPPB system, the non-aqueous phase liquid (NAPL) silicone oil used was found to be biocompatible, non-bioavailable and suitable for DEP delivery to the degrading bacterium. The mechanism of phthalate degradation in the TPPB system involved the slow release of the EDPs from the non-aqueous phase (silicone oil) to the aqueous phase as per real-time demand of *C. funkei*, which also aided in overcoming the substrate inhibition effect of the EDPs. Besides, a very high volumetric oxygen mass transfer coefficient due to the added silicone oil in the TPPB was observed for enhancing the EDPs biodegradation in this bioengineered system. Very high biodegradation efficiency values of the compounds by *C. funkei* were reported using the TPPB system. A high GI value of 86.17% of chickpea seeds soaked in phthalate degraded sample taken from the TPPB system along with a low mortality value of 13.33% of brine shrimps, demonstrated the utility of the TPPB-based bioengineered system in toxicity removal and treatment of phthalates from wastewater.

Scope for future work

Following are some scope for future research work based on the findings of this thesis:

1) Biodegradation of a mixture of low and high molecular weight PAEs by *Gordonia sp.* in the TPPB system.
2) PAEs biodegradation using co-culture of *C. funkei* and *Gordonia sp.*: process intensification and optimization
3) Cost analysis of different treatment systems used for biodegradation of PAEs.
4) Genetic engineering strategies to overcome substrate inhibition at high PAEs concentration.

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Appendix

Appendix 1:

Screening of microorganisms for phthalate degradation

In the screening, *C. funkei* (CF), *Ochrobactrum sp*. (OB), and *R. opacus* (RO), were evaluated to degrade 500 mg/L DBP, BBP, DEHP and DnOP as single substrate in batch shake flasks. After three days of a batch run, it is observed that none of the bacterium yielded significant biomass growth on any of the phthalate used as the sole carbon source (Figure A1a - d). Hence, these bacteria were not used in the biodegradation experiments of the mixture of 6 phthalates (DMP, DEP, DBP, BBP, DEHP and DnOP).

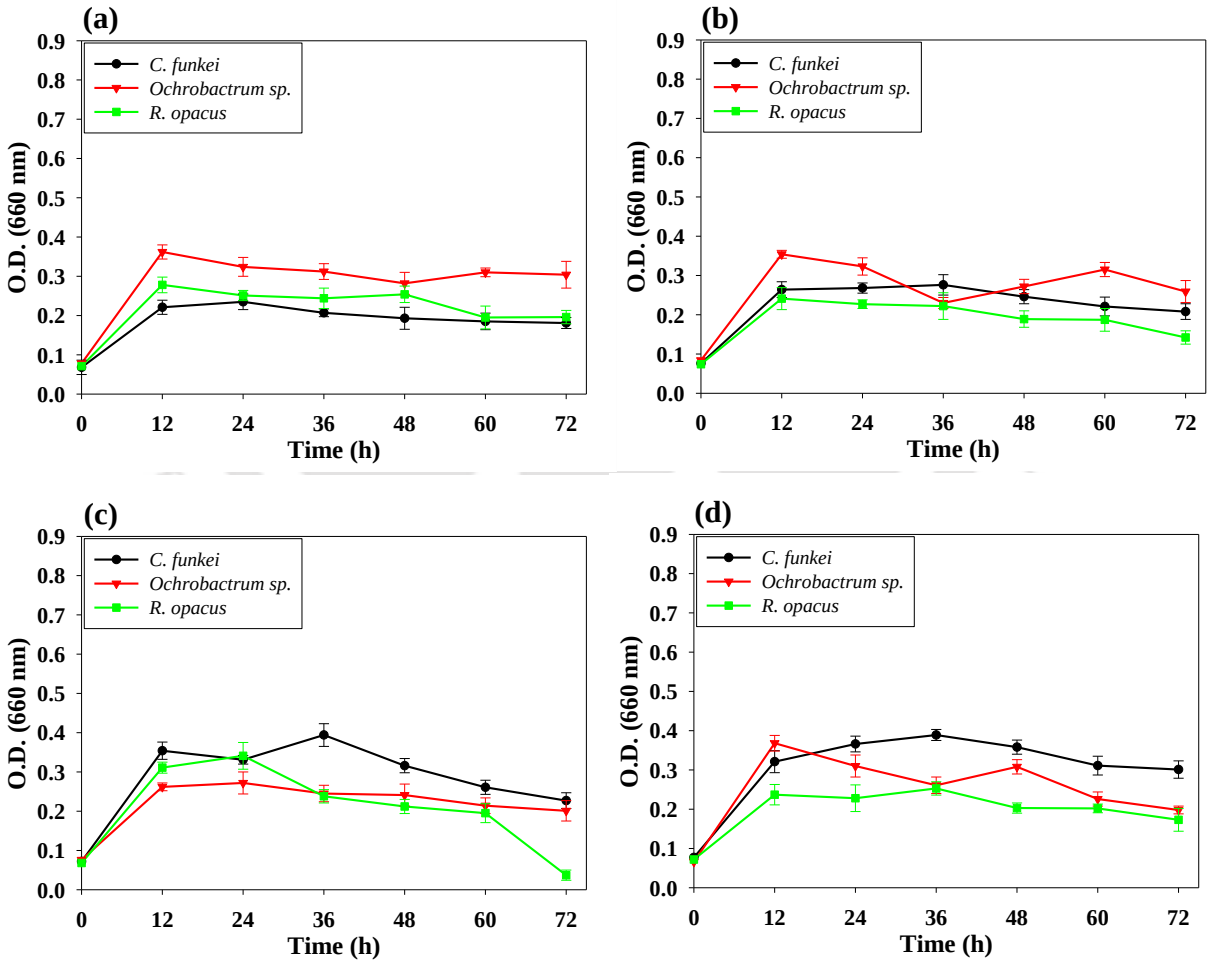

Figure A1: Biomass growth profile of the different microbes on (a) DBP, (b) BBP, (c) DEHP and (d) DnOP.

Appendix 2:

***Gordonia sp.* biomass growth and phthalate degradation in batch shake flasks**

The bacterial strain *Gordonia sp.* used in this study was earlier shown to degrade both low and high molecular weight phthalates but in single component system (Chatterjee et al., 2005; Huang et al., 2019; Jin et al., 2012; Nahurira et al., 2017; Sarkar et al., 2013). Therefore, in the present study only *Gordonia sp.* was selected to evaluate its performance in degrading phthalate mixture. Biomass growth profile of *Gordonia sp.* in batch shake flasks (Figure A2) reveals that the lag phase in biomass growth increased with an increase in the concentration of phthalates in the mixture. Maximum biomass growth was achieved in experimental run 4 which was carried out with a total initial phthalate concentration of 1150 mg/L. Phthalates degradation in the various experimental runs is depicted in Figure A3. All these results clearly reveal that the biomass growth and phthalate biodegradation by *Gordonia sp.* depend on the total initial concentration of phthalates and their respective molecular weight in the different experimental runs. For instance, in run nos. 1, 4, and 7, complete degradation of the phthalates was achieved, which is due to the low initial concentration of the HMW phthalates (BBP, DEHP, and DnOP) in the mixture.

In addition, phthalates with a low molecular weight (LMW), viz. DMP, DEP, and DBP enhanced the biodegradation of the other phthalates with a high molecular weight (HMW) in particular at their high initial concentrations in the mixture due to co-metabolism. Thus, biodegradation efficiency of BBP, DEHP, and DnOP (HMW phthalates) are higher in run nos. 6, 12, and 13 than in run nos. 5, 10, 11, 15 and 16 due to a relatively high initial concentration of DMP, DEP and DBP (LMW phthalates) in the mixture. On the other hand, HMW phthalates such as BBP, DEHP, and DnOP at a high initial concentration in the mixture resulted in a low biodegradation efficiency of LMW phthalates.

Hence, both initial concentration of phthalates and their molecular structure played an important role in their biodegradation in mixture. For instance, DMP and DEP with low molecular weight and simple structure were easily degraded even at high initial concentrations as compared with the high molecular weight phthalates DEHP and DnOP (Figure A3). Moreover, degradation rate of high molecular weight phthalates were lower (even at low initial concentration) than that of the low molecular weight compounds due to their long ester side chains which caused steric hindrance effect towards hydrolytic enzymes present in the bacterium (He et al., 2013; Zhao et al., 2018). These results confirm that the preferred substrates for *Gordonia sp.* were the LMW compounds (DMP, DEP, and DBP), which were degraded quickly even at their high initial concentration in the mixture.

The degradation rates of BBP, DEHP, and DnOP were also lower than for DMP, DEP, and DBP, owing to the differences in substrate preference by the bacterium. He et al. (2013) described that differences in phthalate biodegradability are likely due to steric effect of side chain present in the compounds, which hinders binding of hydrolytic enzymes with phthalates for their degradation. This study also showed that phthalates with short alkyl side chains were degraded quickly by the bacterium as compared to phthalates with long and branched chains. Recent studies further support the low degradation efficiency of phthalates with long and complex alkyl side chains at their high initial concentration in mixture (Ebadi et al., 2017; Ren et al., 2016; Wang et al., 2012; Y. Xu et al., 2020). However, the degradation efficiency values obtained in this study are higher than the literature reports on biodegradation of phthalates mixture (Table 1).

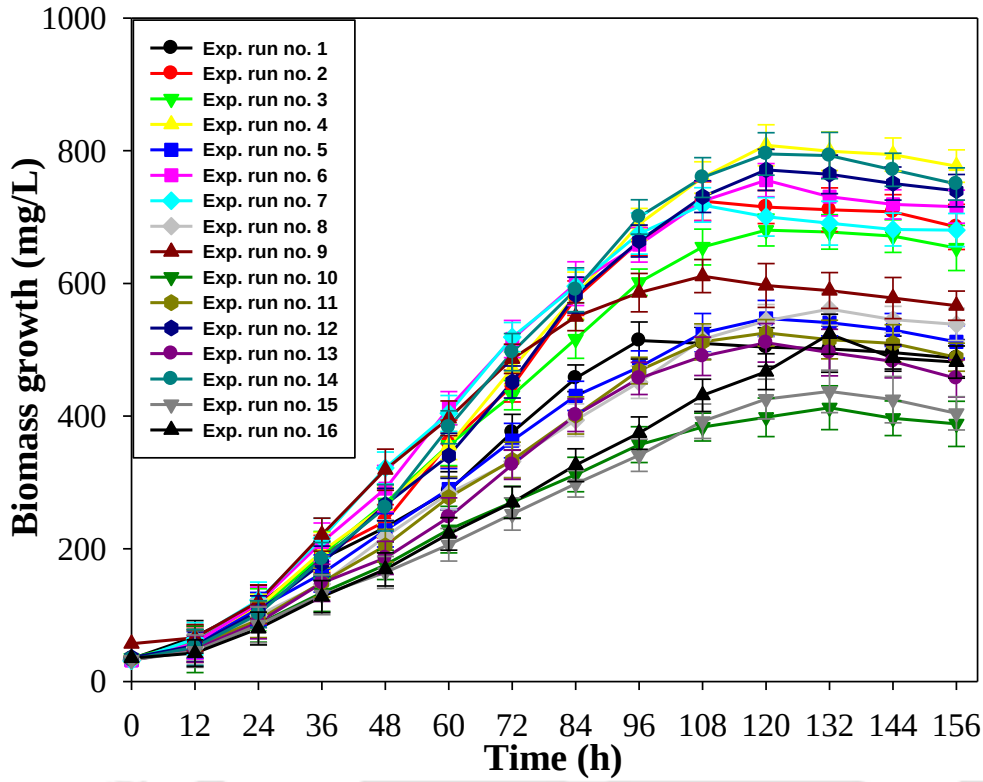

Figure A2: Biomass growth of *Gordonia sp.* in the batch shake flask study.

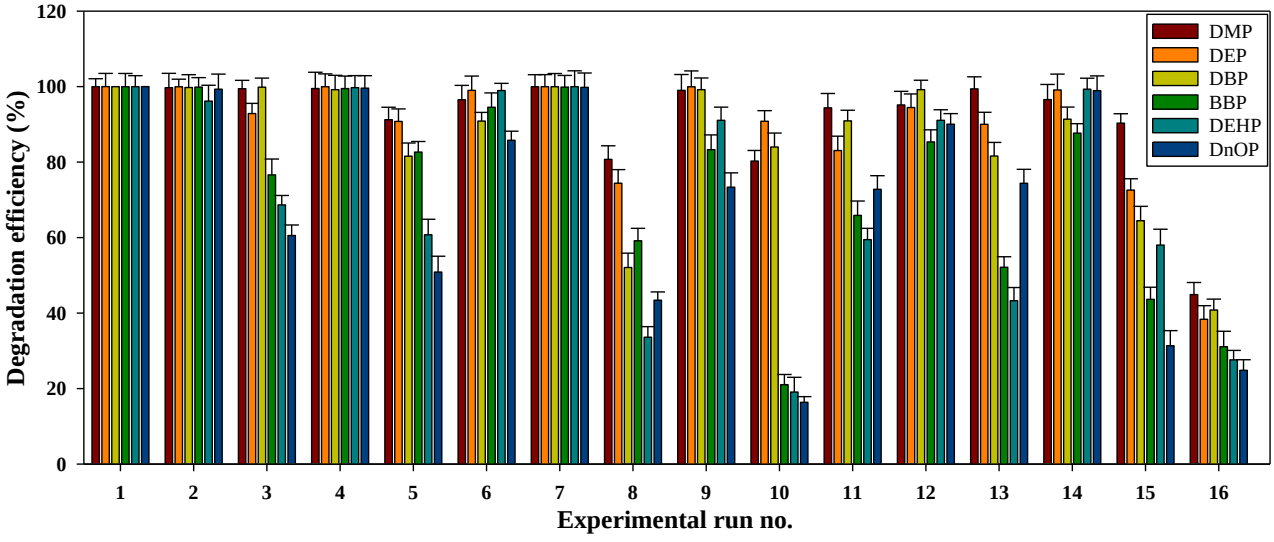

Figure A3: Biodegradation efficiency of phthalates mixture in the batch shake flask study.

List of publications

Manuscripts published in peer-reviewed International Journals

1. **Kanaujiya, D. K.**, Paul, T., Sinharoy, A., & Pakshirajan, K. (2019). Biological treatment processes for the removal of organic micropollutants from wastewater: a review. *Current pollution reports*, *5*(3), 112-128. **IF 8.097**

2. **Kanaujiya, D. K.**, & Pakshirajan, K. (2021). Mass balance and kinetics of biodegradation of endocrine disrupting phthalates by *C. funkei* in a continuous stirred tank reactor system. *Bioresource Technology*, 344, 126172. **IF 11.889**

3. **Kanaujiya, D. K.**, Sivashanmugam, S., & Pakshirajan, K. (2022). Biodegradation and toxicity removal of phthalate mixture by *Gordonia sp.* in a continuous stirred tank bioreactor system. *Environmental Technology & Innovation*, *26*, 102324. **IF 7.758**

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5. **Kanaujiya, D. K.**, Chhantyal A.K., Pugazhenthi G. and Pakshirajan, K. (2023). A novel hybrid system for continuous biodegradation and toxicity removal of low molecular weight phthalates. *Journal of Environmental Chemical Engineering*, 11(3), 109983. **IF 7.968**

6. **Kanaujiya, D. K.,** Purnima M., Pugazhenthi G., Dutta T.K. and Pakshirajan, K. (2023). An indigenous tubular ceramic membrane integrated bioreactor system for biodegradation of phthalates mixture from contaminated wastewater. *Biodegradation.* (Under Review)

Presentations in international and national conference

1. **Kanaujiya, D. K.**, Purnima, M., Pugazhenthi, G. and Pakshirajan, K. Biodegradation of endocrine-disrupting phthalates by *Gordonia* sp. in a continuous stirred tank reactor integrated with indigenous tubular ceramic membrane for microfiltration and biomass recycle. *International Conference on Bioprocess for Sustainable Environment and Energy*. June 20-24, 20222.
2. **Kanaujiya, D. K.**, & Pakshirajan, K. Two liquid phase partitioning bioreactor: A novel method for detoxification of endocrine disrupting phthalates in the environment. *North-East Research conclave India.* May 20-22, 20222
3. **Kanaujiya, D. K.**, & Pakshirajan, K. Biodegradation of Endocrine Disrupting Chemical (EDC) from synthetic wastewater by *Arthrobacter sp.* in batch mode operation. *Bioprocessing India Conference*. December 16 – 18, 2018

Manuscripts from collaborative work published in International Journals

1. **Kanaujiya, D. K.**, Atharva, M., Chhantyal, A.K., Karn, R. and Pakshirajan, K. (2023). Biodegradation of low, medium and high molecular weight phthalate by *Gordonia* sp. in a batch system: Kinetics and phytotoxicity analyses. *Bioengineered*. **(Under Review)**
2. Nandi, M., Paul, T., **Kanaujiya, D. K.**, Baskaran, D., Pakshirajan, K., & Pugazhenthi, G. (2021). Biodegradation of benzyl butyl phthalate and dibutyl phthalate by Arthrobacter sp. via micellar solubilization in a surfactant-aided system. *Water Supply*, *21*(5), 2084-2098. **IF 1.768**
3. Ekka, J. P., Bala, K., Muthukumar, P., & **Kanaujiya, D. K.** (2020). Performance analysis of a forced convection mixed mode horizontal solar cabinet dryer for drying of black ginger (*Kaempferia parviflora*) using two successive air mass flow rates. *Renewable Energy*, *152*, 55-66. **IF 8.634**
4. Ekka, J. P., Muthukumar, P., Bala, K., **Kanaujiya, D. K.**, & Pakshirajan, K. (2021). Performance studies on mixed-mode forced convection solar cabinet dryer under different air mass flow rates for drying of cluster fig. *Solar Energy*. **IF 7.188**