Phenol degradation by free and immobilized bacterial consortia isolated from crude oil contaminated sites of Assam, India

A Thesis

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DOCTOR OF PHILISOPHY

by

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CERTIFICATE

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It is certified that the work described in this thesis entitled ***"Phenol degradation by free and immobilized bacterial consortia isolated from crude oil contaminated sites of Assam, India"*** by **Sounak Bera** 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 Centre for Energy, Indian Institute of Technology Guwahati, India. The contents of this thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

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ABSTRACT

Rapid industrialization and development of nations around the world have increased the demand for exploring energy resources. Majority of the world's energy demand still heavily rely on fossil fuels, primarily petroleum and petroleum products. Phenolic compounds are amongst the most noxious derivatives of crude oil and their removal from wastewaters is imperative before releasing into the environment. The thesis focuses on isolation of bacterial cultures which are capable of tolerating and degrading high concentration of phenol in wastewaters. Firstly, an efficient phenol degrading mixed bacterial culture was isolated from sludge sample collected from one of the petroleum refinery located in Assam, India. On further investigation it was found that the mixed bacterial consortia consisted mainly of three culturable bacterial strains. These were identified as *Stenotrophomonas acidaminiphila* strain DBK (GenBank Accession no. KC992293), *Brevibacterium sp*. strain DBK1 (GenBank Accession no. KP231222) and *Brucella sp*. strain DBK2 (GenBank Accession no. KP231223). Further, batch biodegradation experiments were conducted for a wide range of initial phenol concentrations at optimized pH and temperature conditions. It was found that the mixed culture was able to degrade a maximum phenol concentration of up to 1000 mg L^{-1} within 96 h while the maximum specific growth rate (μ_{max}) was observed at 100 mg L^{-1}. The pH and temperature required for optimal phenol degradation was 6.5 and 37 °C respectively. The mixed culture degrades phenol via the *ortho*-cleavage pathway by formation of an intermediate (*cis, cis*-muconate) which was detected spectrophotometrically at 260 nm. The experimental data were validated by fitting the growth and substrate utilization curves with their corresponding simulated dynamic profiles obtained by solving Haldane's equation via MATLAB.

In the next part of the study, an attempt was made to immobilize the mixed culture on two unreported (for phenol biodegradation) lignocellulosic matrices. The matrices used in the study were dried areca nut husks and dried mature luffa sponge fibres. These matrices are available in most Asian countries very abundantly. The phenol-acclimatized mixed bacterial consortia was immobilized on the matrices via natural adsorption in *via* a solid-state approach. Phenol degradation studies were performed in batches to optimize the physicochemical parameters. Optimum pH and temperature for phenol degradation was found to be 8.0 and 37 °C. At an optimum pH and temperature, the areca nut husk and luffa sponge systems immobilized with the mixed culture could degrade 1000 mg L^{-1} phenol in 28 h and 30 h

respectively. The highest experimental degradation rates in areca nut husk and luffa sponge systems were 0.37 h^{-1} and 0.21 h^{-1} respectively at 200 mg L^{-1} phenol. Degradation kinetic studies were carried out using several inhibition models. Further studies revealed that both matrices with immobilized microbes could be reused for several successive batch degradation experiments and stored at 4 °C for several weeks without any noticeable loss in degradation efficiency. Further, lab-scale packed bed reactors were fabricated and degradation studies were carried out with synthetic phenol feed at varying feed flow rates.

Keywords: Mixed bacterial culture; Phenol degradation; Substrate inhibition; Maximum specific growth rate; *Ortho*-cleavage pathway; Immobilization; Areca nut husk; Luffa sponge fibre; Kinetic parameters

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THESIS OUTLINE

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A brief outline of each chapter is furnished below.

Chapter I: Introduction

This chapter starts with an introduction to energy and energy resources. It then highlights the fact that majority of the world's energy demand is met by fossil fuel and these crude resources needs to be refined in order to be used as fuel and other purposes. It highlights the fact that the ecosystem around the points of pollution are exposed to highly toxic amounts of hydrocarbons and that these need to be treated. Thereafter, the different chemicals present in the effluents of these refining industries have been mentioned along with their toxic effects. It then gives a brief idea about treatment methods and emphasizes on biological treatments. Biological systems of treatment have been discussed in brief along with their advantages.

Chapter II: Review of Literature and objectives

This chapter starts with an introduction to phenol. Phenol was recognized as the most common and toxic pollutant in petroleum refinery wastewater and further literature review have been carried out. In the later part of this chapter, literature review on phenol was extensively carried out. Different types of phenols and derivatives and their toxic effects have

been discussed. The chapter then starts to look into the various technologies for phenol removal from the environment with an emphasis on microbial remediation. The chapter then discusses about pathways and kinetics of phenol degradation. Thereafter, different reactors employed for wastewater treatment and phenol degradation have been discussed. Based on the discussion in the chapter five objectives were decided and planned accordingly.

Chapter III: Materials and Experimental Methods

For the isolation of phenol and phenolic compounds degrading bacteria, samples were collected from four different hydrocarbon contaminated environments in Assam, India. Total 15 samples were collected from these sites. The samples, mainly of three types, i.e., a) oily sludge, b) hydrocarbon contaminated wastewater and c) crude oil were collected in aseptic plastic bags, sample bottles and were stored at 4 °C until use. The pH, electrical conductivity, temperature, ORP, dissolved oxygen was recorded. These samples were enriched in liquid mineral salt medium (MSM) containing phenol as a sole source of carbon. Native bacteria were isolated by subsequent serial dilution and plating them on MSM agar plates (1.8% agar). Bacterial isolates were identified by 16S rDNA sequencing.

Batch studies were conducted using a phenol tolerant mixed microbial culture for optimizing the ideal environmental conditions for growth and degradation of phenol. Growth studies were carried out by measuring optical density (OD) of the cultures at 600 nm (Thermo Fischer Scientific USA). Optimization studies were carried out in an incubator shaker (ZHICHENG Instruments ZHWY-2112B) at 120 rpm and different desired temperatures. Phenol was used as the sole source of carbon throughout the optimization studies at varying concentrations (75-1000 mg L^{-1}). The factors that were optimized included inoculum dosage, temperature, pH and initial concentration of phenol.

After batch studies using suspended bacterial cultures, immobilization studies were conducted. Two different lignocellulosic biomass were considered as immobilization matrices of choice for this study. These were dry areca nut husks and dry mature luffa fibers. Lignocellulosic biomass was obtained from local market. These were then cleaned, washed and dried in a hot air oven at 85 °C to remove moisture completely. The biomasses were then chopped into pieces, washed, drained, autoclaved and dried again for long term storage. Bacterial culture was immobilized on the matrices by a solid state culture method. Physical parameters (pH, temperature and initial phenol concentration) for maximum degradation of the pollutant were optimized for these immobilized microbes. Residual phenol concentrations were

measured via High Performance Liquid Chromatography (Model: Agilent Technologies 1220 Infinity LC) fitted with a Variable Wavelength Detector (VWD) and a reverse phase column (Agilent ZORBAX Eclipse XDB-C18, particle size 5 µm, dimensions 3.0 x 150 mm). Growth kinetic studies were carried out by fitting experimental data to various established substrate inhibition models. Storage and reusability studies were also carried out.

Two upflow packed-bed reactors were set-up, which consisted of a tubular acrylic glass column with an internal diameter of 2.5 cm and a length of 50 cm and a total volume of 245 mL. The reactor consisted of an inlet at the bottom for feeding fresh medium and an outlet at the top of the column for the exit of effluent. A perforated stainless steel plate was set up at 1 cm above the bottom of the column to support the matrices. The reactors were packed with the lignocellulosic matrices with previously immobilized organisms. About 34 g equivalent of dry areca nut husk and 14 g equivalent of dry luffa sponge fibers were packed. Peristaltic pumps were used to feed a fixed volume of 1 L media through the bottom of the reactor. The reactor was operated at a fixed flow rate of 7.2 mL min^{-1} with a phenol concentration of 1000 mg L^{-1} till it was stable. Initial feed pH was maintained at 8.0. Temperature of 37 °C was maintained in the reactor using a water jacket. Different feed flow rates were tested for degradation of 1000 mg L^{-1} of phenol. Outlet effluent was collected at regular intervals for analysis of residual phenol.

Chapter IV: Biodegradation of phenol by a native mixed bacterial culture isolated from crude oil contaminated site

A total of 15 samples were collected from 4 different sampling sites from different parts of Assam which has a long history of crude oil drilling and refining. 23 bacterial isolates were obtained from all the samples. A mixed bacterial culture capable of tolerating high phenol load was obtained from a sample designated as DB2. This mixed culture had 3 bacterial strains namely, *Stenotrophomonas acidaminiphila*, *Brevibacterium sp*. and *Brucella sp*. respectively. The strains were designated as DBK (GenBank Accession no. KC992293), DBK1 (GenBank Accession no. KP231222) and DBK2 (GenBank Accession no. KP231223) respectively. Further studies were carried out using this mixed culture. The pH and temperature required for optimal phenol degradation was 6.5 and 37 °C respectively. It was found that the mixed culture was able to degrade a maximum phenol concentration up to 1000 mg L^{-1} within 96 h while the maximum specific growth rate (μ_{max}) was observed at 100 mg L^{-1}. The experimental data were validated by fitting the growth and substrate utilization curves with their corresponding

simulated dynamic profiles obtained by solving Haldane's equation via MATLAB R2015a with μ_{max} = 0.155 h^{-1} and K_I = 400 mg L^{-1}. Phenol was degraded via *ortho*-cleavage pathway by formation of an intermediate (*cis*, *cis*-muconate) which was detected spectrophotometrically at 260 nm.

Chapter V: Areca nut (Areca catechu) husks and Luffa (Luffa cylindrica) sponge as microbial immobilization matrices for efficient phenol degradation and their use in packed bed reactor with varying flow rates

Immobilization studies were carried out with the dry areca nut husks and mature luffa fibers. Scanning electron microscopy (FESEM) was performed to visualize the lignocellulosic matrices with and without immobilization of the mixed bacterial culture. Phenol degradation studies were performed in batch to optimize the physicochemical parameters. Optimum pH and temperature for phenol degradation was found to be 8.0 and 37 °C. At an optimum pH and temperature, the areca nut husk and luffa sponge systems immobilized with the mixed culture could degrade 1000 mg L^{-1} phenol in 28 h and 30 h respectively. The highest experimental degradation rates in areca nut husk and luffa sponge systems were 0.37 h^{-1} and 0.21 h^{-1} respectively at 200 mg L^{-1} phenol. Degradation kinetic studies were carried out using several inhibition models.

Studies with the packed bed reactor was carried out with 1000 mg L^{-1} of phenol as a sole source of carbon. Studies were carried out at different feed flow rates (3.6, 7.2, 10.8 & 14.4 mL min^{-1}). With the increase in flow rate, phenol removal efficiency deteriorated. Both biomass could remove phenol to a point where it was undetectable at a feed flow rate of 3.6 mL min^{-1} in 48 h. At a flow rate of 7.2 mL min^{-1} both systems could achieve more than 90% phenol removal. However, with further increase in feed flow rate there was noticeable deterioration in phenol removal efficiency. This was mainly due to insufficient residence time.

Chapter VI: Conclusions and Future Scope

In this chapter inferences drawn from the present work and future scopes have been discussed. The study concluded that the mixed microbial culture consisting of three bacterial strains *Stenotrophomonas acidaminiphila* DBK, *Brevibacterium sp*. DBK1 and *Brucella sp*. DBK2 which were isolated from petroleum contaminated sludge could degrade 1000 mg L^{-1} of phenol in 96 h. Potential of two previously unreported lignocellulosic immobilization matrices namely, areca nut husk and mature luffa vegetable fibers, for phenol degradation was studied. The optimum pH and temperature for efficient phenol degradation were determined to

be 8.0 and 37 °C respectively. The enhanced tolerance of the immobilized systems towards adverse conditions have also been demonstrated in this study. Storage and reusability studies showed promising results. The matrices were also used in lab scale packed bed reactors for the degradation of phenol (fixed feed volume with recirculation from the outlet). A feed of 1 L containing 1000 mg L^{-1} phenol could be treated within 48 hours with no detectable phenol and COD in the effluent. Potential of these matrices in continuous reactors and stirred tank reactors need to be studied. The future work can also include study in laboratory microcosms and field studies in mesocosms. Further research can explore the molecular biology of the enzyme responsible for phenol degradation. Upon sequencing the gene for the enzyme, suitable primers can be designed and expression levels can be checked by quantitative PCR.

CHAPTER 1

INTRODUCTION

This chapter introduces us to energy and energy resources and gives a brief insight to the world's energy demand and supply statistics. It gives an idea about the toxic effects of hydrocarbons on the ecosystem around the points of pollution and emphasizes on the importance of treatment of petroleum wastes. The chapter then gives a brief idea about treatment methods while emphasizing on biological treatment methods.

Chapter 1

INTRODUCTION

1.1 Introduction

Energy is an indispensable resource for the development of human race. It is the key for developing a sustainable civilization and plays a vital role in reshaping human lives. The evolution of human society and civilization is solely dependent on man's ability to discover and master new energy resources. Hence, one of the primary commitments of human life has always been the production, conversion and consumption of energy.

The world's current energy requirement is met through fossil fuels such as coal, petroleum and natural gas, nuclear energy, hydrothermal energy, wind, solar power etc. Although strenuous efforts are being made to substitute fossil fuels with renewable sources of energy, it still holds a major share among the raw materials used globally for energy generation. Crude oil, amidst these, holds a significant share followed by coal and natural gas (Figure 1.1. a). As compared to the global scenario, a significant supply of energy demand is met through biofuels and wastes (21.2% as compared to 9.5% globally) in India. However, in India, the total primary energy supply is met primarily by coal (44.3%) followed by crude oil (25.3%) (Figure 1.1. b) (according to latest statistical data from IEA or International Energy Agency). India is the fourth largest consumer of oil in the world and second largest in the Asia Pacific region with a reported consumption of about a staggering 5.16 million barrels per day (bpd) in 2018 alone. India is also the fourth largest crude oil importer of the world, importing about 4.57 million bpd. India holds about 4.5 billion barrels of proved oil reserves as of December 2018, and is the second largest in the Asia-Pacific region after China. In 2018, India recorded a production of 869 thousand bpd of total oil (BP Statistical Review of World Energy, 2019, 68th Edition, 2019).

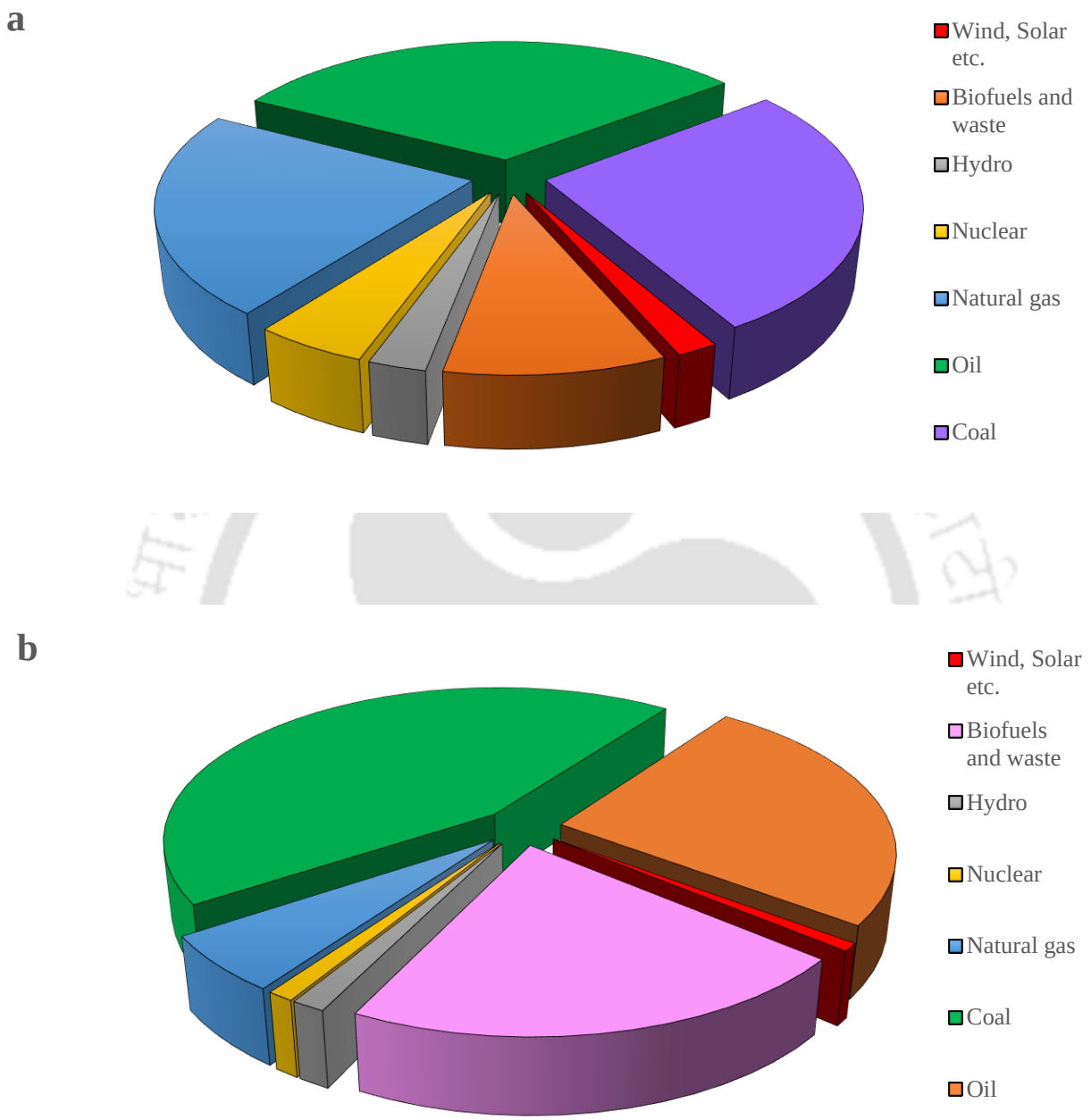

Figure 1.1. Total primary energy supply by source (a) World; (b) India (latest data for the year 2017) (Source: International Energy Agency, https://www.iea.org/statistics/)

For usage of petroleum as fuel it needs to be purified from the unprocessed crude oil which is a naturally occurring yellow to black liquid occurring under the Earth's surface. Today a large number of oil refineries are operational for the processing of crude oil to obtain various consumer products like petrol, diesel, kerosene, asphalt, and several other chemicals which are of great importance to numerous industries. Oil pollution has been an ecological problem for most part of this century. Pollution of aquatic environments and agricultural fields can occur by crude oil spills and also oil refinery effluents. Oil refinery effluents contain many different chemicals such as ammonia, sulphides, hydrocarbons and phenol and its derivatives at varying concentrations (Veeresh et al., 2005). Phenols are the most vicious xenobiotic compounds in petroleum industry wastewater and effluents. Phenols can be manufactured as well as it can be a natural product. Pure phenol is a flammable, colourless to white solid and has an obnoxiously sweet and tarry odour. The compound can be tasted and smelt at concentrations which are far lower than those that can cause deleterious effects (National Center for Biotechnology Information, 2020). The toxicity of phenols is well known and severe toxic effects have been observed in and close to the area of the oil refinery fallouts (Wake et al., 2005). Oily sludge and petroleum refinery effluents have been recognized as extremely hazardous in many countries and can pose a serious threat to the environment and human health (J. Liu et al., 2009; Rossiter et al., 2010; Diya'Uddeen et al., 2011; Hu et al., 2013). Therefore, these effluents need to be treated before their release in the environment. It is also important to treat the refinery fallouts as harmful chemical leeches into the groundwater.

1.2 Background and importance of bioremediation of phenol

Bioremediation is a process by which the earth is naturally detoxified. Microorganisms like bacteria, fungi or algae can be used for the purpose of removing pollutants from contaminated earth or water. These microbes act just like white blood cells of our body to destroy and eliminate pollutants and xenobiotic compounds. Bioremediation is a process which occurs naturally and has a high rate of success even on its own. So, the question that arises is, "why do humans need to interfere with a process which can occur and succeed on its own?". The answer to this is; with human interference the positive effects of bioremediation can be multiplied several folds. This process is accelerated several folds when provided with optimum temperature, pH, nutrients and several other physicochemical parameters without which this natural process could take years. This is a sustainable and inexpensive process and can be combined with other technologies to achieve even greater degrees of positive results. Bioremediation can ensure complete breakdown of pollutants into harmless compounds. Some

microbes may also exhibit PGPR (plant growth promoting rhizobacteria) properties after bioremediation.

Contamination of soil and ground water is a pressing issue and is ever increasing due to continued development of industries and agriculture which causes the release of hazardous wastes in form of different chemicals, crude oil, pesticides etc. The demand and consumption of crude oil has seen a steady rise due to global developmental activities (Chaudhary and Kim, 2019) and this has led to increased drilling and refining activities which releases various toxic chemicals in the environment. Among all the chemicals present in the oil refinery effluents, phenol and its derivatives are highly soluble in water and persist in the environment for over a long time. Long range transportation, bioaccumulation in animal tissue and biomagnification in the ecological food chain are characteristic features of these aromatic organic compounds (Nair et al., 2008). Phenol and its derivatives have been listed amongst the priority pollutants by US Environmental Protection Agency (Keith and Tellliard, 1979). This enlistment in the priority pollutants list by the USEPA is due to the fact that phenol is responsible for severe adverse effects on animals, plants and humans due to short and long term exposure. It is well known for its toxic effects on plants, animals and humans. Studies have revealed lethal and teratogenic effects of phenol on *Bufo arenarum* embryos (Paisio et al., 2009). Therefore, considering their adverse effects on the environment, treatment of wastewaters containing phenol and its derivatives is of utmost importance.

1.3 Bacteria as agents for biodegradation of phenol

Phenolic effluents from industries can be treated via a number of conventional and advanced techniques. Some of these techniques are distillation, adsorption, photocatalytic oxidation, membrane separation and chemical oxidation (Alshabib and Onaizi, 2019). These methods of treatment of phenolic wastewaters, although very effective, have certain drawbacks in the form of energy inefficiency and production of certain toxic secondary by-products. On the contrary, biological methods of wastewater treatment using microorganisms has the potential to breakdown pollutants such as phenol completely.

A large number of bacterial species have been reported to actively degrade phenol and utilize the compound as the sole source of carbon and energy. These include species such as *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp., etc. The genus *Bacillus* and *Pseudomonas* are the most widely studied, reported, and applied for the biodegradation of phenolic compounds and other petroleum hydrocarbons. At very high concentrations of phenol, these

organisms however, are affected by the phenomena of substrate inhibition and their growth maybe subdued (Prieto et al., 2002). There are certain external physicochemical parameters which affects the process of phenol metabolism. The most important of these factors are substrate concentration, pH, temperature and availability of oxygen. Since, phenol is highly toxic and exhibits severe substrate inhibition properties, the optimization of initial substrate concentration is extremely crucial for developing an efficient bacterial phenol degradation system (Nair et al., 2008). The degradation process can be further enhanced by immobilizing the bacterial cells (Annadurai et al., 2000). Immobilizing the cells can also negate the effect of substrate inhibition to a certain extent.

Microorganisms such as bacteria can break open the aromatic rings of phenolic compounds and harness the energy and carbon from the same using certain intracellular enzymes (Chiong et al., 2014). Aerobically, this aromatic ring cleavage is achieved via either of two pathways namely *ortho* (intradiol) or *meta* (extradiol) pathways (Yang and Humphrey, 1975). The two key enzymes for the *ortho* and *meta* pathways are identified as catechol 1,2-dioxygenase and catechol 2,3-dioxygenase respectively. Via these pathways, bacteria can break down phenolic compounds to end-products which may be as simple as carbon dioxide, water and certain essential non-toxic compounds like succinate, Acetyl-CoA or pyruvate which are further utilized in the citric acid cycle of the organism.

1.4 Strategies and reactors for treatment of phenols

As mentioned earlier phenol and phenolic compounds are highly toxic to all life forms and microbial growth can be severely impeded at higher concentrations of the pollutant. Therefore, certain strategies should be devised in order to achieve optimal and efficient degradation of the pollutant. Among several approaches, the most common is isolation of microbial strains which are tolerant to highly noxious concentrations of the target pollutant. There are ample number of reports of efficient phenol degrading microbes in literature. However, as human civilization continues to progress, the environment is constantly exposed to even higher concentrations of pollutants such as phenols and other newer emerging pollutants. Hence, seeking out new and more efficient strains of organisms for the degradation of xenobiotic compounds is always desirable. Researchers also explore the possibility of isolating newer and less reported genus of bacteria for efficient degradation of xenobiotic compounds. Most of the reported studies have been carried out using pure strains of bacteria. However, mixed bacterial cultures may prove to be more effective in metabolizing compounds like phenol and its derivatives. Studies involving defined mixed cultures have proven to be

more effective in the degradation of phenol compared to the pure individual strains (Y. J. Liu et al., 2009). Indigenous and undefined mixed bacterial culture after acclimatization can also degrade phenol and its derivatives. These mixed cultures have been shown to degrade either sole phenol or a mixture of multiple phenolic substrates efficiently (Saravanan et al., 2008a, 2008b) by acting in synergy (Monteiro et al., 2000). Research also showed that bacterial cultures, when immobilized in a suitable matrix are much more efficient than the freely suspended counterpart. Immobilization of microbial cells in a matrix like sodium alginate, polyvinyl alcohol etc. is a promising strategy. Apart from increasing the degradation efficiency of the organisms, immobilization also protects them from the harsh environmental conditions like suboptimal pH, temperature and high pollutant concentrations. Immobilization also helps the biomass to be reused for repeated biodegradation experiments (El-Naas et al., 2009), hence making the process of bioremediation more cost-effective. Degradation processes are much more advantageous if carried out in continuous mode rather than in batches (Banerjee and Ghoshal, 2016). In the industries, batch processes simply might not be feasible when conducting operations in large scales. Continuous mode of degradation can be achieved with the help of bioreactors which might be a basic stirred tank reactor or a more sophisticated process like membrane reactors. Packed bed reactors are the best example where immobilized microbial cells have been widely used for the treatment of xenobiotic compounds. Cells immobilized in a wide variety of matrices have been applied in these studies. Immobilized cells, when used in a continuous treatment process, can resist the washout of cells even in unfavorable conditions (Aksu and Bülbül, 1998). Some of the other reactors which have been used for treatment of phenolic wastewaters are fluidized bed reactor, sequencing batch reactor, trickle bed reactor, rotating biological contactor, fuel cells, membrane reactors etc.

The present research reports the isolation of phenol utilizing bacterial strains from sites which have a diverse and long history of crude oil contamination. A suitable mixed bacterial culture was isolated. Various physicochemical parameters for phenol degradation were optimized and growth kinetics were studied. Phenol degradation pathway was also determined. Further, immobilization studies were carried out by two previously unreported lignocellulosic biomasses as immobilization matrices. Phenol degradation was also studied in lab scale packed bed bioreactors using these matrices as packing materials.

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CHAPTER 2

REVIEW OF LITERATURE AND OBJECTIVES

In this chapter background, sources and toxic effects of phenol have been discussed in details along with the concentration of phenolic compounds in the wastewaters of various industries. Various phenol removal methods have also been discussed with an emphasis on biological treatment methods. Biological degradation pathway of phenol has been depicted. This chapter also discusses the effects of various physicochemical and environmental parameters on microbial phenol degradation efficiency. The chapter also discusses about the various reactors employed for biodegradation of phenol. Based on the literature survey, the major objectives of the research were formulated and presented at the end of this chapter.

Chapter 2

REVIEW OF LITERATURE AND OBJECTIVES

2.1 Background on phenol

Phenol is an aromatic organic compound which can be derived from the simplest aromatic hydrocarbon, benzene. It is also known as hydroxybenzene and consists of a single hydroxy substituent which is added to a carbon of the benzene ring by replacing a hydrogen. It is a crystalline colourless chemical with a characteristic odor and is soluble in water and organic solvents. Phenol was first isolated (in its impure form) from coal tar in 1834 by the German chemist Friedlieb Ferdinand Runge (Busca et al., 2008). On an industrial scale, phenol used to be synthesized by extraction from coal tar. The traditional separation process of phenolic compounds involves a neutralization reaction which uses strong aqueous alkaline solutions such as NaOH or Na_2CO_3. Phenolic salts (such as phenolate) formed in the reaction are transferred from the coal tar to water. In the next step phenolate salts are acidified by mineral acids followed by a solvent extraction process to recover the phenols (Meng et al., 2014). Although the process is very efficient, it is not very cost-effective and environmental friendly due to usage of strong acids and alkali. Moreover, the process produces effluents and wastewater rich in phenolic compounds (Jiao et al., 2015) which are very difficult to treat. Cumene peroxidation is an efficient process for manufacturing phenol and the world's major demand for phenol is met through this process. The process involves liquid phase air oxidation of cumene to cumene peroxide. This in turn is acidified to synthesize phenol, and acetone is generated as a co-product. Some other methods by which phenol can be manufactured are toluene two-stage oxidation, Raschig phenol process, chlorobenzene caustic hydrolysis and benzene sulfonate process.

Phenols find extensive usage in household products and also as raw materials in many industries. It is used in very low concentrations in household cleaners, mouthwashes, cough drops etc. It was the first surgical antiseptic used by the British surgeon Joseph Lister (Lister, 1967). Phenol is used as raw material in chemical industries such as - production of alkylphenols, cresols, xylenols, phenolic resins, aniline etc. It is also used in other industries like manufacturing of plastics, explosives, pesticides and insecticides, dyes, textiles, pharmaceuticals etc. (Michałowicz and Duda, 2007). If released in low quantities, the half-life of phenol in air is less than a day and it is also relatively short-lived in soil (2-5 days for complete removal). However, in water it tends to persist for more than a week. On the contrary,

release of large amounts of phenol at once or a constant release of small amounts over time helps phenol persist in the environment for long, thus contributing to the xenobiotic nature of the compound (ATSDR, 2008).

2.2 Sources of phenol in the environment

Sources of phenol in the environment can be both anthropogenic and natural. Phenol pollution is widespread in the soil and water ecosystems in and around the crude oil drilling sites, refineries and refinery fallouts (Bera et al., 2017). High concentrations of phenol has been reported in effluents of industries such as phenol manufacturing plants, phenol-formaldehyde resin, coal conversion, coking plant, leather, textiles, pulp mills, pharmaceutical etc. (Kumaran and Paruchuri, 1997; Pinto et al., 2003; Huang et al., 2014; Wang et al., 2014). The occurrence of phenol and its derivatives in the environment might also be attributed to the usage and degradation of phenolic pesticides and phenoxyherbicides like 2, 4 - dichlorophenoxyacetic acid or 2, 4, 5 - trichlorophenoxyacetic acid (Michałowicz and Duda, 2005). Industrial wastewaters might contain the compound in concentrations of above 10,000 mg L^{-1} whereas, domestic wastewaters may contain lesser concentrations (0.1 - 1.0 mg L^{-1}) (Schie and Young, 2000). It was also estimated that 0.3 - 0.4 mg of phenol is released per cigarette when it burns (Michałowicz and Duda, 2007).

In the nature, phenols can be synthesized during natural processes like biosynthesis of chlorinated phenols by fungi and plants and decomposition of lignocellulosic and organic matter. It can also be formed as a result of chemical reactions occurring in the atmosphere in condensed water vapors that form clouds (Michałowicz and Duda, 2007). Polyphenolic compounds are produced by plants and microorganisms and are the most widely distributed class of plant secondary metabolites. In plants and other organisms, these phenolic compounds play important roles, such as, UV protection agents, defense against pathogens and herbivores, contribute to the color of the plant etc. (Hättenschwiler and Vitousek, 2000). Other natural sources of phenolic compounds may include forest fire, natural runoff from urban areas where asphalt is used as binding material, production by marine algae, plants and invertebrates etc. Phenols can also be produced in ruminant animals during anaerobic degradation of ligninaceous plant materials and excreted in urine and faeces. In non-ruminant animals, including humans, phenolic compounds are produced by the microorganisms that reside in the intestines from aromatic amino acid precursors such as tyrosine (Schie and Young, 2000; Nair et al., 2008). The chemical structures of various phenolic compounds usually present in

various industrial effluents are depicted in Figure 2.1.

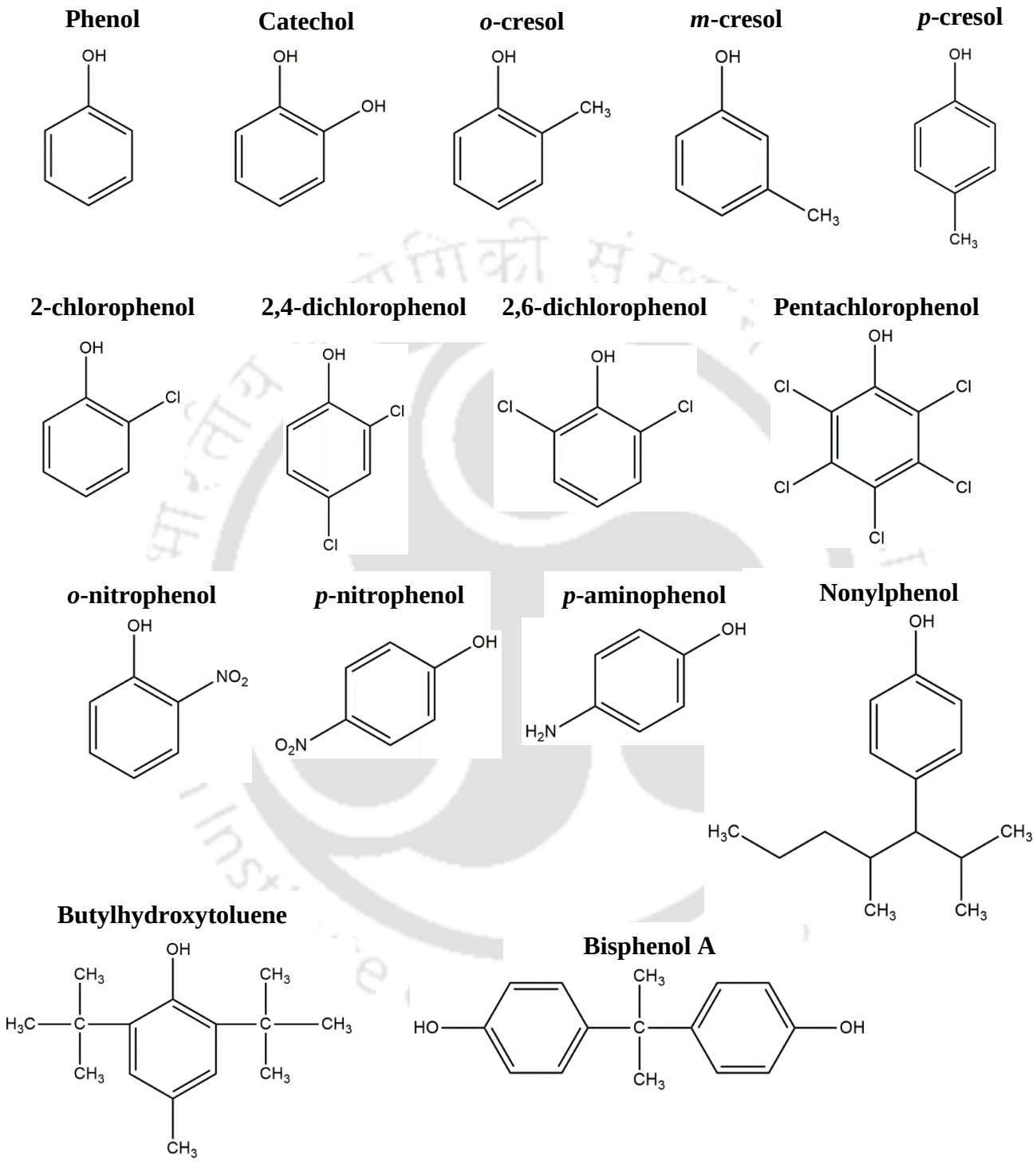

Figure 2.1. Chemical structure of phenol and its derivatives found in industrial effluents (Michałowicz and Duda, 2007; Raza et al., 2019)

2.3 Toxic effects of phenol and its occurrence in industrial discharges

Phenol has been categorized in the list of priority pollutants by the United States environmental protection agency (USEPA) (Keith and Telliard, 1979). Phenol has a tarry odor and is corrosive in nature. Phenolic compounds have been shown to be toxic to humans, animals and plants (Lin and Wu, 2011). Its vapors are corrosive to skin eyes and respiratory tract. Severe or prolonged exposure to phenol have been reported to cause disorders of the central nervous system, hypothermia, dermatitis, muscular convulsions and weakness, myocardial depression, and even death (Nair et al., 2008). It has also been reported to cause renal damage, eye conjunctional swelling leading to blindness, hepatic damage and anorexia. Exposure to solid or concentrated solutions of phenol causes burns, whitening and erosion of skin and hence use of proper protective gear and clothing is recommended while handling phenol. Phenol can impair various biochemical functions in human beings (Nuhoglu and Yalcin, 2005). Although it is a suspected carcinogen, studies by IARC (International Agency for Research on Cancer) established that it is not classifiable under carcinogen category. No existing data was found which could relate toxic effects of phenol leading to reproductive and developmental defects (Health Protection Agency, 2007). 1 g of phenol is reported to be lethal for human beings if ingested (Nuhoglu and Yalcin, 2005) and concentrations of even 1 mg L^{-1} affects aquatic life (Chang et al., 1995). Phenol has been reported to be either toxic (reduces enzyme activity) or lethal to fish at concentrations of 5 - 25 mg L^{-1} (Tsai and Juang, 2006). Even at very low concentrations phenols impart obnoxious taste and odor to drinking water. Chlorinated phenolic wastewater (chlorinated for disinfection) can result in the formation of polychlorinated phenols which are even more toxic (Chung et al., 2003). Hence, the USEPA recommends a phenol concentration of 0.5 ppb or less in potable and mineral waters. However, the agency imposes a limit of 0.5 ppm and 1 ppm (ppm = parts per million) for surface waters and sewage waters respectively (Aksu and Bulbul, 1999; El-Ashtoukhy et al., 2013). The central pollution control board (CPCB) of India has set a limit of 5 ppm for phenolic compounds in wastewaters from small scale industries (Bajaj et al., 2008).

Coal tar and wastewater from industries such as resins, plastics, iron and steel, leather, synthetic fuels manufacturing, rubber etc. are some of the most common anthropogenic sources of phenols in natural water sources (Figure 2.2.). It might also be released from publicly owned treatment works and sewage overflows (ATSDR, 2008). Various industrial effluents (Table 2.1) may contain phenolic compounds in copious amounts (up to 17500 ppm). These wastewaters undeniably harm the environment and hence, need to be treated before their

release. Phenolic wastewaters with high levels of toxicity poses threat to the microbial population in treatment plants. Unless the microbial population is acclimatized to higher levels of the contaminant, there are high chances that the biological treatment plant might fail.

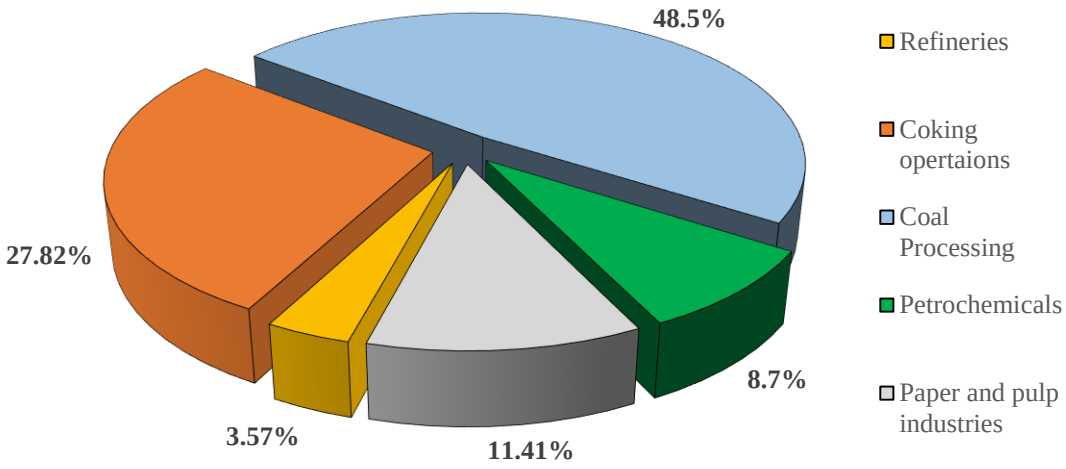

Figure 2.2. Concentration of phenols (%) in wastewaters and effluents of various industries (Busca et al., 2008; Raza et al., 2019)

Table 2.1. Concentration of Phenolic compounds in Industrial wastewaters

Industrial Sources	Concentration of Phenolic compounds (mg L^{-1})	Reference
Oil Refineries waste water	500 - 1500 (phenol)	(El-Ashtoukhy et al., 2013)
Coking Plants	200 - 1200 (phenol)	
Weak Ammonia liquor	900 - 5000 (phenol)	(Kostenbader and Flecksteiner, 1969)
Coke oven effluent	283.5 (phenol) 16.2 (catechol) 95.0 (cresols) 14.8 (resorcinol) 21.9 (hydroquinone)	(Kumaran and Paruchuri, 1997)
Gas Plant effluent	131.25 (phenol) 31.9 (catechol) 114.5 (cresols) 11.25 (resorcinol) 1.55 (hydroquinone)	
Low Temperature Carbonization effluent	3395 (phenol) 1955 (catechol) 1425 (cresols) 770 (resorcinol) 61.25 (hydroquinone)	
Coal gasification (10% dilution)	207 (phenol) 57 (o-cresol) 139 (m- and p-cresol) 60 (Dimethylphenols)	(Veeresh et al., 2005)
H-coal liquefaction	4900 (phenol) 586 (o-cresol) 1230 (m-cresol) 420 (p-cresol) 320 (Dimethylphenols)	
Lignite gasification	5500 - 7260 (total phenols)	

Lignite liquefaction	3290 - 11700 (total phenols)	
Low temperature carbonization	9250 - 17500 (total phenols)	
Aircraft maintenance	200 - 400 (total phenols)	
Plastic factories	600 - 2000 (total phenols)	
Fiberboard manufacturing	150 (total phenols)	
Phenolic resin	1600 (total phenols)	
Orlon manufacturing	100 - 150 (phenol)	
Fiberglass manufacturing	40 - 400 (phenol)	
Wood Carbonizing	500 (phenol)	
Strong liquor before dephenolization (during hot flaking of phenolic resin)	15000 (phenol)	(Weber et al., 1992)
Strong liquor after dephenolization	3000 (phenol)	
Olive oil mill	1000-1500 (phenol)	(Pinto et al., 2003)

2.4 Development of bacterial resistance to phenol

The noxious effect of any xenobiotic compound relies on numerous factors. The bactericidal effect of phenol is largely due to the capability of the compound to disrupt the bacterial lipid membrane and the reactivity of the compound with the cell's biomolecules (Michałowicz and Duda, 2007). Different enzymes in cells can catalyze one-electron reactions, leading to the synthesis of phenoxy radicals, reactive oxygen species, and intermediate metabolites (like hydrogen peroxide), which can interact with cellular biomolecules. The interaction of these compounds with specific cellular structures depend on reactivity of phenols. Highly reactive phenols can incite quick peroxidation of lipids of the cell membrane, while lesser reactive phenols can penetrate the cell causing damage to the cell organelles and their components (Hayashi et al., 1999). With the disruption of the lipid membrane which is the sole protector of the bacterial cytoplasm from the harsh outer world, its functionality is impaired and K^+ ions, ATP and nucleotides leak out (Heipieper et al., 1991), causing cell death (van Schie and Young, 2000). Toxic effect of xenobiotic compounds also depends on parameters like pK_a (K_a is the compound dissociation constant) and log P (P is the octanol-water partition coefficient of the un-dissociated acids). Enhanced membrane penetration by the xenobiotics is directly proportional to the increase in hydrophobicity, the value of log P and a decrease in the value of pK_a (Michałowicz and Duda, 2007). Inhibition of bacterial growth in phenol may occur at a phenol concentration of as low as 50 ppm and the bactericidal effects of phenol kicks in at 2000 ppm (Bajaj et al., 2008).

However, microorganisms have been reported to develop mechanisms to survive in phenol concentrations which are otherwise fatal. These mechanisms negate the effect of reactive phenols which amplifies the fluidity of the lipid membrane of the cell. There are reports, where a *Pseudomonas putida* P8, which can degrade phenol, isomerize *cis*-unsaturated fatty acids to their *trans*-configuration (Heipieper et al., 1992). The *trans*-fatty acids tend to align together closer and more tightly forming a more rigid lipid membrane as compared to the *cis*-unsaturated ones. It was found that the phenol adapted *Pseudomonas putida* P8 cells could readily utilize up to 750 ppm phenol as the carbon source, while, the non-adapted ones could barely survive 250 ppm concentrations. Another similar resistance mechanism was reported in *Escherichia coli* K-12, which involves the conversion of unsaturated fatty acids to saturated ones for thriving in a phenol concentration of 1000 ppm. Saturated fatty acid chains, alike the *trans*-fatty acids, can align closely in the lipid membrane. This helps the organism nullify the amplified fluidity of the lipid membrane caused by the effect of reactive phenols (Keweloh et

al., 1991). Researchers often aim at isolating bacteria which thrive in high phenol concentrations, as these microorganisms can be especially useful for the treatment of wastewaters, sludges etc. where phenol exerts noxious inhibitory effects (Schie and Young, 2000).

2.5 Removal of phenol from the environment

2.5.1 Physicochemical methods

Physicochemical processes such as distillation, ion-exchange, chemical oxidation, liquid-liquid extraction, adsorption etc. have been studied by various research groups for phenol removal. Physicochemical processes require secondary treatment due to substantial amounts of organic matters present in the effluents (Chang et al., 1995). Higher concentrations of phenol (higher than 15,000 ppm) require to be treated by thermal decomposition processes, but, the high energy requirement for such processes limits it to be used in small scale (Portela et al., 2001). Adsorption using activated carbon and other sorbents for the treatment of diluted phenolic wastewaters is an effective process. Novel techniques are being developed for treatment of phenolic wastewaters. These include chemically modified activated carbon, coupling with nanoparticles and, switching with low cost biosorbents (Tran et al., 2015). However, as a whole the process is uneconomical considering the high cost involved (Halhouli et al., 1997). Solvent extraction can be used for treating a wide range of phenol concentration and is also a non-destructive process. However, phase dispersion occurring in the process is a major disadvantage because it requires high energy input and also results in emulsification and subsequent problems in the separation process (Praveen and Loh, 2013a). An alternative destructive treatment of aqueous phenol is electrochemical oxidation. The main advantage of the process is that it has no requirement for any reagents. However, the major cost involved here lies in the equipment and energy required (Villegas et al., 2016).

2.5.2 Biological wastewater treatment and removal of phenol

Various governmental organizations across the world have imposed strict regulations on pollution discharge in an effort to combat the ever-increasing burden on our aquatic systems. The industry-developed waste treatment systems are typically thought to yield negative economic returns. However, complying with the strict governmental regulations might also serve as an alternate source of income, if the industries take advantage of the Clean Development Mechanism (CDM) under the Kyoto Protocol 1997 (Chan et al., 2009).

Biological wastewater treatment might be capable of generating revenue from Certified Emission Reduction (CER) credits or carbon credits under CDM. Biological treatment procedures can be used to treat wastewaters with a BOD/COD (BOD = Biochemical oxygen demand; COD = Chemical oxygen demand) ratio of 0.5 or more with ease (Metcalf et al., 1972). When compared to the physicochemical methods biological treatments offers a more practical and rather inexpensive approach for treatment of phenol contaminated wastewaters and sludge with generation of little or no secondary pollutants (Sponza and Uluköy, 2005). Aqueous phenols are usually treated by biological means. Biological methods of phenol treatment, or, as a matter of fact, remediation of any xenobiotic compounds, are usually considered as a baseline or reference method against which other physicochemical methods of treatment are often compared (Villegas et al., 2016). Biological treatment methods are simple to design, easy to maintain, and, phenol can be degraded to simple harmless end products under aerobic or anaerobic conditions by microorganisms (Chan et al., 2009; Villegas et al., 2016). Aerobic microorganisms (aerobes) can utilize free or dissolved oxygen to metabolize organic wastes to biomass and carbon dioxide. On the contrary, anaerobes can degrade complex organic wastes into methane, carbon dioxide and water in the absence of oxygen. This anaerobic conversion pathway comprises of three basic steps of hydrolysis, acidogenesis including acetogenesis and methanogenesis. Conventionally, organic wastewaters have been treated via aerobic treatment processes with high degrees of efficiencies. On the other hand, anaerobic waste treatment methods, while still realizing pollution control, also aims at resource recovery and utilization (Seghezzo et al., 1998). Aerobic processes are used to treat wastewaters with relatively lower COD concentrations (1000 mg L^{-1} or less) as compared to anaerobic systems (can treat COD concentrations of 4000 mg L^{-1} or more) (Chan et al., 2009). Anaerobic treatment methods are more advantageous in terms of energy requirement and has potential of bioenergy production and nutrient recovery. However, aerobic processes can attain improved removal efficiencies of soluble biodegradable organic matter. The produced biomass usually flocculates well, which in turn reduces the concentration of suspended solids in the effluents, finally resulting in the production of higher quality effluents (Grady Jr et al., 2011).

Physicochemical treatment of phenolic wastewaters yields toxic secondary pollutants in the effluents. Halogenation for the treatment of phenolic wastewaters, generate even more toxic halogenated phenols (Marrot et al., 2006). Therefore, biological methods are most widely used for phenol treatment as it degrades phenol completely to non-toxic compounds. Biological phenol remediation has been widely reported with diverse species of *Pseudomonas* (Monteiro

et al., 2000; Tsai and Juang, 2006; Kurzbaum et al., 2010). Bioremediation of phenol has also been reported using *Sulfolobus* (Christen et al., 2012), *Candida* (Wang et al., 2012), *Halomonas sp.* (Haddadi and Shavandi, 2013), *Bacillus* (Banerjee and Ghoshal, 2010a, 2010b) and so on. Mixed cultures also offer a promising alternative for phenol bioremediation as they might actually be able to withstand even higher phenol concentrations compared to pure cultures. Efficient phenol degradation by mixed cultures have also been reported by a number of researchers. A list of different organisms including fungi, algae, bacteria and mixed cultures which have been reported by researchers to degrade phenol and its derivatives, are listed in Table 2.2. For the treatment of phenolic wastewaters, both anaerobic and aerobic treatment methods are used (discussed in later sections).

Table 2.2. A list of phenol degrading microorganisms

Sl no.	Types of Phenol	Concentration (mg L^{-1}) (unless mentioned otherwise)	Temperature (°C)	Microorganisms	Reference
1	Phenol	100 - 2400	21, 32	Activated sludge containing *Bacillus, Pseudomonas, Citrobacter, Proteus* and *E. Coli*.	(Holladay et al., 1978)
2	Phenol	2000	25	*Pseudomonas sp.*	(Bettmann and Rehm, 1984)
3	Phenol	3200	25	Co-immobilized *Pseudomonas putida* P8 *and Cryptococcus elinovii* H1	(Zache and Rehm, 1989)
4	Phenol	500	-	Anaerobic sludge with a dominance of *Methanothrix sp.*	(Chang et al., 1995)
5	Phenol	420	20.5 - 27	Mixed culture with dominance of *Pseudomonas stutzeri* and *Pseudomonas putida*	(Banerjee, 1997)
6	Phenol	1000	25 - 35	*Pseudomonas putida*	(Aksu and Bülbül, 1998)
7	Phenol	100 - 1000	30	*Pseudomonas putida* MTCC1194	(Bandyopadhyay et al., 1998)
8	Phenol	100	25 - 30	*Pseudomonas putida* ATCC11172	(Mordocco et al., 1999)
9	Phenol	25 - 800	30	*Pseudomonas putida* ATCC 49451	(Wang and Loh, 1999)
10	Phenol	200 - 600	30	*Pseudomonas pictorum* NICM-2077	(Annadurai et al., 2000)
11	Phenol	500	19 - 30	*Pseudomonas putida* DSM 548	(Sá and Boaventura, 2001)
12	Phenol	1000	30	*Pseudomonas putida* ATCC 17484	(González et al., 2001b, 2001a)
13	Phenol	500	30	*Pseudomonas putida* ATCC 31800	(Annadurai et al., 2002)
14	Phenol	1000 - 5000	30	*Candida tropicalis*	(Chen et al., 2002)

15	Mixture of Phenol, 4-chlorophenol, 2,4,6-trichlorophenol	20 mg L^{-1} each	30	Defined mixed culture of *Pseudomonas testosteroni* CPW301 *and Pseudomonas solanacearum* TCP114	(Kim et al., 2002)
16	Phenol	1000	23 ± 3 (continuous)	*Rhodococcus erythropolis* UPV-1	(Prieto et al., 2002)
17	Phenol	1000	30	*Pseudomonas putida* CCRC14365	(Chung et al., 2003)
18	Phenol	660	65	*Bacillus thermoleovorans*	(Feitkenhauer et al., 2003)
19	Low molecular weight Phenols	-	-	*Ankistrodesmus braunii* and *Scenedesmus quadricauda* (Algal strains)	(Pinto et al., 2003)
20	Phenol	300	28	*Ralstonia taiwanensis*	(Chen et al., 2004)
21	Phenol and 2,4-dichlorophenol	239.5 - 400.1 (phenol) 15.6 – 22.5 (2,4-DCP)	25	*Achromobacter sp.*	(Quan et al., 2004)
22	o-cresol	30 - 600	25	Waste activated sludge	(Maeda et al., 2005)
23	Phenol	100 - 2800	30	*Pseudomonas putida* CCRC14365	(Chung et al., 2005)
24	Phenol	1250	28	*Pseudomonas putida*	(Pazarlioğlu and Telefoncu, 2005)
25	Phenol and Cresols	3000 (phenol)	-	Methanogenic sludge showing presence of *Methanothrix sp.*	(Veeresh et al., 2005)
26	Phenol	1000	29.9 ± 0.3	*Pseudomonas putida* MTCC1194	(Kumar et al., 2005)
27	Phenol	100 - 2700	26	Mixed culture with predominance of *Alcaligenes* and *Acinetobacter*	(Tziotzios et al., 2005)
28	Phenol	1450	25 ± 1	Mixed culture	(Nuhoglu and Yalcin, 2005)
29	Phenol	1254	30	*Pseudomonas sp.* SP -1	(Vinod and Reddy, 2005)
30	Phenol	500 - 3000	Ambient	Mixed culture	(Marrot et al., 2006)

31	Phenol contaminated air	22160 mg/h.m3 (phenol loading rate)	-	*Pseudomonas putida*	(Nikakhtari and Hill, 2006)
32	Phenol	600	25	*Pseudomonas stutzeri* OX1	(Viggiani et al., 2006)
33	Phenol	100 - 500	30	*Pseudomonas putida* BCRC 14365	(Juang and Wu, 2007)
34	Phenol	1500	30	*Acinetobacter sp.*	(Adav et al., 2007)
35	Phenol	100 - 1100	30	*Acinetobacter sp.* PD12	(Wang et al., 2007a)
36	Phenol	190 - 5170	25 ± 5	Mixed culture	(Bajaj et al., 2008)
37	Phenol	100 - 500	30	Binary mixed culture of *Pseudomonas aeruginosa* and *Pseudomonas fluorescence*	(S E Agarry et al., 2008; S. E. Agarry et al., 2008)
38	Phenol and m-cresol	100 - 800 (phenol) 400 (m-cresol)	26 ± 1	Mixed culture with predominant presence of *Pseudomonas sp.*	(P Saravanan et al., 2008; Pichiah Saravanan et al., 2008a, 2008b)
39	Phenol	1000	28	*Rhizobium sp.* CCNWTB 701	(Wei et al., 2008)Wei et al., 2008
40	Phenol	800	30	*Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03	(Liu et al., 2009)
41	Phenol	100 - 900	30	*Pantoea sp.* NII-153	(Dastager et al., 2009)
42	Phenol and m-cresol	2600 (phenol) 300 (m-cresol)	30	*Candida tropicalis*	(Jiang et al., 2010)
43	Phenol	100 - 2000	37	*Bacillus cereus* MTCC9817 & MTCC 9818	(Banerjee and Ghoshal, 2010a, 2010b, 2011)
44	Phenol	51 - 745	80	*Sulfolobus solfataricus* 98/2	(Christen et al., 2012)
45	Phenol	250	30	*Exiguobacterium aurantiacum*	(Jeswani and Mukherji, 2012)
46	Phenol and 4-chlorophenol	150 and 900 (phenol) 150 (4-chlorophenol)	-	*Candida tropicalis* W1	(Wang et al., 2012)
47	Phenol	120 - 1200	37	Mixed culture	(Rosenkranz et al., 2013)

48	Phenol	100 - 1100	30	*Halomonas sp.* PH2-2	(Haddadi and Shavandi, 2013)
49	Phenol	100 - 4000	30	*Pseudomonas putida* ATCC11172	(Praveen and Loh, 2013a, 2013b)
50	Phenol	497.2 - 1183	30	Mixed culture	(Kılıç and Dönmez, 2013)

2.5.2.1 Anaerobic phenol degradation

Degradation of organic matter by microbial activity in limited access of oxygen develops anaerobic conditions (Schink et al., 2000). Several pathways might be possible for anaerobic degradation of phenol. Anaerobic phenol-degrading bacteria accomplishes phenol degradation by an intricate and slow cooperation with acetogens and methanogens (Li et al., 2019). Phenol is first metabolized into fatty acids and alcohols by bacteria. Following this, the acetogens and methanogens together, produce methane via a syntrophic metabolism process by making use of reaction intermediates for example, hydrogen or formate, which are associated with interspecies hydrogen transfer (IHT) (Gieg et al., 2014). Figure 2.3 depicts phenol degradation pathway under denitrifying conditions where, phenyl-phosphate is formed by phosphorylation of phenol, and later is converted to 4-hydroxybenzoate. However, phenol-degrading bacteria gets inhibited at higher concentrations of phenol which in turn affects the IHT by impeding the activity of hydrogen-exploiting methanogens, the outcome of which is the obstruction of methanogenesis (Li et al., 2018; Oshiki et al., 2018). The anaerobic treatment processes can withstand higher organic loading rates (OLR) and generates reduced quantity of sludge. Additionally, it also produces energy (Rosenkranz et al., 2013). Nevertheless, the biodegradability of the xenobiotic compound and competence of the microbial communities rules the productivity of anaerobic digestion process (Franchi et al., 2018). Bacterial inhibition due to high phenol concentrations can result in a lower degradation rates in anaerobic processes. Therefore, microbial inoculum should be chosen wisely, as a robust and well adapted inoculum can improve biodegradability and help mitigate wastewater toxicity (Xu et al., 2015).

A variety of microorganisms, both pure and mixed, can degrade phenol anaerobically. Nitrate and sulfate reducing bacterial pure cultures are reported to degrade phenol and phenolic compounds. Pure cultures of denitrifying pseudomonads isolated from anaerobic sewage sludge or sediment samples have been reported to metabolize phenol and *p*-cresol (Tschech and Fuchs, 1987). A sulfate reducing *Desulfobacterium phenolicum* Ph01 isolated from marine sediments was able to degrade phenol and its derivatives (Bak and Widdel, 1986). Many other researchers also reported mixed cultures for treatment of phenols (Wang et al., 1986; Collins et al., 2005; Fang et al., 2006).

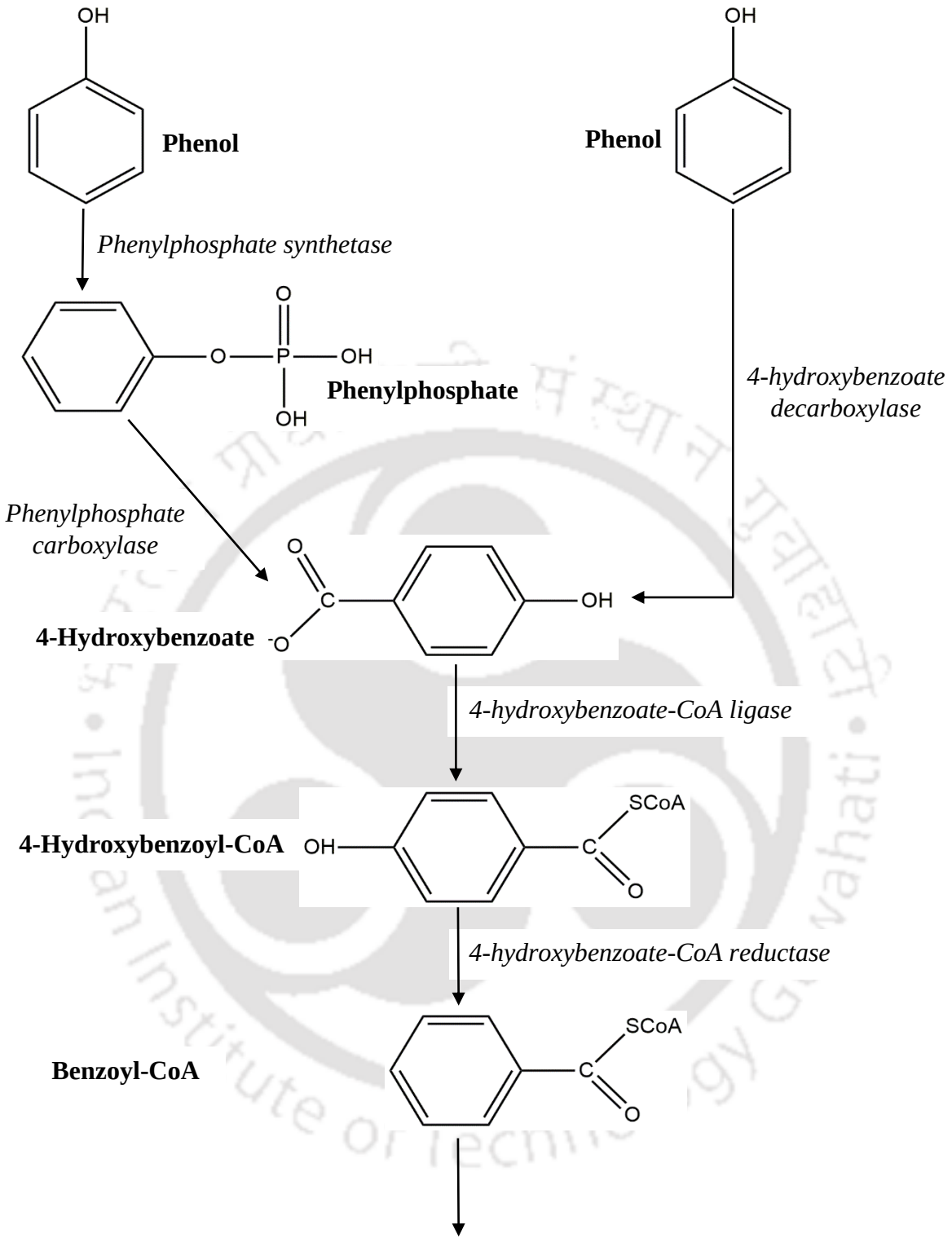

Figure 2.3. Anaerobic phenol degradation pathway (Gao et al., 2009)

2.5.2.2 Aerobic phenol degradation

Aerobic degradation processes are favoured for phenol removal from wastewaters, largely, because of their ability to completely mineralize the compound. Phenol is the most appropriate model compound for studying the degradation kinetics of xenobiotic aromatic compounds (Christen et al., 2012). The substrate inhibition property of phenolic compounds can be subdued by adopting various approaches. One such strategy of acclimatizing the microorganisms to noxious concentrations of phenol is by gradually increasing the pollutant concentration (Banerjee, 1997). Other strategies might include cell immobilization (biofilm formation), gene manipulations and targeted metabolic engineering (Zídková et al., 2013; Pradeep et al., 2015).

When phenol diffuses into the bacterial cells, an enzyme called phenol hydroxylase adds a second hydroxyl group in the *ortho*-position of the benzene ring. A reduced pyridine nucleotide ($NADH_2$) and molecular oxygen is required in the reaction. The resultant intermediate of the reaction is catechol or 1,2-dihydroxybenzene which can then be degraded by *ortho*-cleavage (*β*-ketoadipate) or *meta*-cleavage pathway (Figure 2.4). In the *ortho*-cleavage or *β*-ketoadipate pathway, the enzyme catechol 1.2-dioxygenase cleaves the benzene ring of the catechol between the two hydroxyl groups via intradiol fusion (Harwood and Parales, 1996). The intermediate formed thereafter is *cis, cis*- muconate, which is further broken down to *β*-ketoadipate. The *β*-ketoadipate is metabolized to succinate and acetyl-Coenzyme A which then enters the TCA cycle (tricarboxylic acid cycle).

In the *meta*-pathway, however, the aromatic ring of the catechol is cleaved adjacent to the two hydroxyl groups via an extradiol fission (Yang and Humphrey, 1975). This reaction forms an intermediate called 2-hydroxymuconic semialdehyde and the enzyme that catalyzes this reaction is catechol 2,3-dioxygenase. Eventually, 2-hydroxymuconic semialdehyde is broken down to produce acetaldehyde and pyruvate (a few more of the intermediates are shown in Figure 2.4.) which are utilized in the Krebs cycle. Substituted phenolic compounds are typically metabolized via the *meta*-pathway. However, catechol and its precursors are catabolized more specifically by the *β*-ketoadipate pathway (Feist and Hegeman, 1969).

Aerobic breakdown of phenol can be achieved by a wide array of organisms. The most common bacterial cultures reported to degrade phenols are *Pseudomonas* sp. (Reardon et al., 2000; El-Naas et al., 2009; Mollaei et al., 2010; Surkatti and El-Naas, 2014) and *Bacillus* sp. (Bandyopadhyay et al., 1998; Shourian et al., 2009; Banerjee and Ghoshal, 2010a). Mixed

bacterial cultures have also been used by several research groups for efficient phenol degradation (Pichiah Saravanan et al., 2008a; Bajaj et al., 2009; Pradhan et al., 2011; Ma et al., 2015). There are also reports of degradation of phenols by algal species like *Ankistrodesmus braunii* and *Scenedesmus quadricauda* (Pinto et al., 2003) and fungal strains like *Candida tropicalis* (Wang et al., 2012).

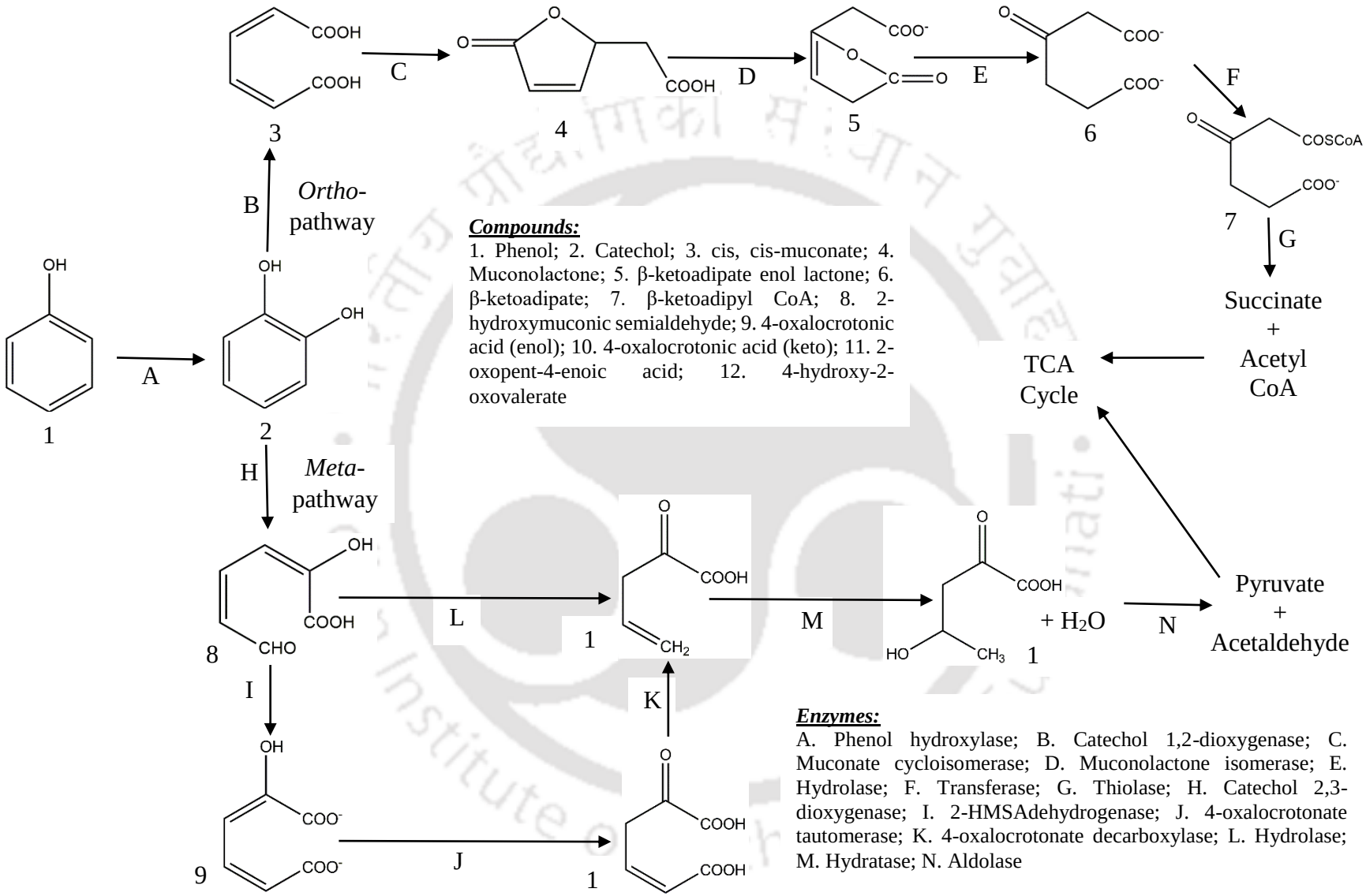

Figure 2.4. Aerobic Phenol degradation pathway (Hughes and Bayly, 1983; Aghapour et al., 2013; Nešvera et al., 2015)

2.6 Factors influencing the biodegradation of phenol

Several physicochemical parameters must be optimized for every microorganism to degrade any xenobiotic compound efficiently under laboratory conditions. Initial substrate concentration, pH of the culture media and temperature of incubation are the most important of the several factors which effects the biodegradation process of phenol or as a matter of fact any xenobiotic compound. A few other parameters that influences phenol degradation are presence of auxiliary nutrient source, bioavailability of phenol, oxygen content, salinity (in case of certain organisms) (Halhouli et al., 1997; Haddadi and Shavandi, 2013).

2.6.1 Effect of initial substrate concentration

Phenol, being toxic in nature is well-known as a microbial growth inhibitor particularly at higher concentrations. This is known as 'substrate inhibition'. A *Pseudomonas putida* strain could be acclimatized in high phenol concentrations of up to 1000 mg L^{-1} (Kumar et al., 2005). However, the lag periods were considerably longer and 1000 mg L^{-1} phenol was degraded in 162 h. Wang et al, (2007) isolated a strain of *Acinetobacter* sp. (PD12), which took 9 h to degrade 500 mg L^{-1} of phenol by 99.6%. PD12 could also metabolize phenol at concentrations of up to 1100 mg L^{-1}, which took much longer and displayed much lesser value of degradation rate constant. In another study, *Ralstonia taiwanensis* strain R186, isolated from *Mimosa pudica* root nodules, showed phenol tolerance and degradation abilities up to a concentration of about 900 mg L^{-1} (Chen et al., 2007). The research involved studying diauxic growth, where the authors found that glycerol acted as a bio-stimulant and could be utilized by the *Ralstonia* cells as a source or energy and simultaneously the toxicity of phenol was "antagonized". However, when the culture medium was augmented with other readily utilizable nutrients like acetate or yeast extract, the bacterial cells preferred to consume these nutrients and the noxiousness of phenol was not attenuated. A mixed microbial consortium was able to degrade phenol up to concentrations of 800 mg L^{-1}. Within a phenol concentration range of 100 - 500 mg L^{-1}, the culture system did not exhibit much inhibitory effect and the contaminant was degraded within 30 h. However, beyond 500 mg L^{-1}, the inhibitory effect was obvious and it took 69 h for the mixed microbial consortium to degrade 800 mg L^{-1} phenol (Pichiah Saravanan et al., 2008a). Banerjee and Ghoshal, (2010b) studied the effect of phenol concentrations of up to 2000 mg L^{-1} with two strains of *Bacillus cereus*, MTCC9817 strain AKG1 and MTCC9818 strain AKG2 isolated from petroleum refinery and oil exploration sites. The calculated growth rate for MTCC9817 strain AKG1 was found to max out at 600 mg L^{-1} whereas, for the strain MTCC9818 AKG2 it was observed to be at 1000 mg L^{-1} phenol. The microorganisms exhibited

insignificant growth at and beyond 2000 mg L^{-1} of phenol, which indicated substrate inhibition and their inefficiency to thrive at such higher concentrations.

2.6.2 Effect of incubation temperature

The degradation rate of phenol by microorganisms is significantly influenced by temperature changes. All phenol degrading organisms have different optimum temperatures for the efficient utilization of phenol. Nonetheless, most researchers report an optimum temperature in the range of 25 - 37 °C for efficient phenol degradation by microorganisms (Table 1.2.). Higher temperatures appear to be more unfavorable for phenol degradation when compared to lower temperatures (Mordocco et al., 1999). However, there are reports of phenol degradation by extremophiles like *Bacillus thermoleovorans* and *Sulfolobus solfataricus* and these strains work at higher temperatures of 65 °C and 80 °C respectively (Feitkenhauer et al., 2003; Christen et al., 2012). When it comes to degradation rate, even a difference of about 5°C could affect the degradation rate of phenol by 50% (Marrot et al., 2006). Sá and Boaventura, (2001) found that, although phenol removal efficiencies were high at 30 °C, the TOC (Total organic carbon) removal efficiencies were affected in their trickling bed reactor setup. This could be possibly due to the heightened metabolite production at that temperature due to phenol degradation. Chung et al., (2003) studied the effect of immobilization with *Pseudomonas putida* systems and found that the optimum temperature for phenol degradation in both suspended and immobilized systems was 30 °C. However, the degradation rate in the free cell suspension system was about 1.45 times higher when compared to the immobilized systems. Immobilization, however, increased the thermal stability of the cells and the immobilized cells could degrade phenol and tolerate a temperature of up to 35 °C. Introduction of microorganisms to suboptimal temperatures may be unfavorable for the enzymes responsible for the benzene ring cleavage, which is the key step in the biological degradation process. Exposure of microbes to lower temperatures is expected to slow down the metabolic activity (El-Naas et al., 2009).

2.6.3 Effect of pH

pH is another key factor that must be optimized to achieve enhanced levels of phenol biodegradation. The pH acts as an indicator of phenol degradation wherein, a drop in the medium pH can be observed as phenol is biodegraded (Marrot et al., 2006). Various studies have shown microorganisms to grow and degrade phenol at varying pH ranges. A *Pseudomonas putida* strain MTCC1194 was reported to have its highest phenol degrading capacity at neutral pH (Bandyopadhyay et al., 1998). Another study reported that,

Pseudomonas putida couldn't resist pH change and as the pH deviated from neutral condition the biochemical reactions required for phenol degradation was significantly affected (Annadurai et al., 2002). Yet another study involving two strains of *Bacillus cereus* reported efficient growth and phenol degradation at almost neutral pH (Banerjee and Ghoshal, 2010a). The surface charge of microbial cells present in activated sludge is said to be influenced by pH changes. Within the pH range of 3 - 10, biomass mostly exhibit negative surface charge, while, phenol converts to a negatively charged phenoxide ion above pH 9.0. Due to the isoelectric point of activated sludge, below a pH value of 3.0, the overall surface charge of the cells become positive. Hence, there will be insignificant electrostatic attraction between the activated sludge biomass and phenol (Aksu and Gönen, 2004). Most studies indicate that, a near-neutral pH (6.5 - 7.5) is best suited for phenol degradation. However, there are microorganisms that may deviate from this and degrade phenol in an acidic or alkaline pH environment. These microorganisms can be categorized into acidophilic and alkaliphilic microorganisms. A strain of *Bacillus brevis* degraded sole phenol optimally at a pH of 8.0 with an inoculum load of 5% (v/v) (Arutchelvan et al., 2006). Another study reported phenol degradation by *Halomonas campisalis* (a haloalkaliphilic bacterium) over a wide alkaline pH range of 8.0 to 11.0 in the presence of 100 g L^{-1} of NaCl (Alva and Peyton, 2003). *Paecilomyces variotii* JH6, a filamentous fungal strain could degrade phenol optimally at an acidic pH of 5.0 with the addition of 100 mg L^{-1} of glucose as a supplementary source of carbon. The study also reported complete phenol degradation in the presence of other toxic chemicals such as m-cresol and quinoline (Wang et al., 2010).

2.6.4 Other factors

2.6.4.1 Auxiliary Carbon and Nitrogen sources

While degrading toxic xenobiotic compounds, auxiliary carbon and nitrogen sources might play an important role. Supplementary nutrient sources such as yeast extract, mannitol, glucose, urea etc. can act as growth inducers and helps attain higher biomass concentration in a short time span (Banerjee and Ghoshal, 2010a). Shourian et al, (2009) showed the effect of various auxiliary carbon and nitrogen sources on phenol degradation by a *Pseudomonas* sp. SA01. The supplementary energy sources used in the study were sucrose, glucose, glycerol, maltose, galactose and mannitol. Co-nitrogen feeding was done using ammonium sulfate, potassium nitrate, casein, yeast extract, tryptone, arginine and urea. Mannitol, casein, tryptone and yeast extract were the best add on auxiliary carbon and nitrogen sources for the degradation of phenol (700 mg L^{-1}). Complete degradation was achieved in 20 hours. The research also

reported that other compounds like arginine, urea, potassium nitrate and ammonium sulfate prolonged the duration of phenol degradation. When combined with urea and arginine, it took as long as 60 h for the organism to completely degrade phenol (Shourian et al., 2009). Another study involving *Pseudomonas putida* used glucose along with phenol for acclimating the strain. This was done by gradually decreasing the glucose concentration while increasing the phenol proportionately (Pazarlioğlu and Telefoncu, 2005).

2.6.4.2 Immobilization

Another strategy which may prove to be effective for biodegradation of phenol is immobilization of bacterial cells. The bacterial biomass can be immobilized using various solid supports like alginate, poly-acrylamide, polyvinyl alcohol, chitosan etc. Immobilization could drastically increase degradation efficiency. Liu et al, (2009) observed elevated degradation rate in case of immobilized cells (1.20 h^{-1}) as compared to free cells (0.95 h^{-1}) at optimum temperature. Studies revealed that immobilization of microbial cells could help retain the phenol degradation efficiency even after a storage of 30 days (Banerjee and Ghoshal, 2011). Immobilized bacterial cells gains improved stability and durability. Immobilized cells can be used for repeated phenol degradation experiments. Banerjee and Ghoshal, (2011) used immobilized *Bacillus cereus* for at least three consecutive batch phenol degradation experiments without any significant drop in the degradation efficiency. They also demonstrated that immobilization of bacterial cells results in improved tolerance to the toxic effects of phenol. At a phenol concentration of 2000 mg L^{-1} they achieved 54.0 % and 52.5 % degradation by two bacterial strains of *Bacillus cereus* in their study, which were higher than the respective suspension cultures of the strains. Yet another study by Wang et al., (2007b) revealed that immobilized bacterial cells (in polyvinyl alcohol) could degrade 99% of phenol even after storage for 50 days at 4 °C. Immobilized cells have been reported to handle pH and temperature changes better than free cells. At 45° C and pH 6.0, when the activity of the free cells almost ceased, the immobilized cells still maintained an acceptable degradation rate of 0.72 h^{-1} and 0.51 h^{-1} respectively (Liu et al., 2009). Juang and Wu, (2007) achieved 46.0 % phenol removal at pH 5.0 by microbial cells immobilized on microporous hollow fibers. Alginate is the one of the most widely used gel matrices for cell entrapment. They offer high biomass loading and good substrate diffusion within the matrix (Chen and Lin, 2007).

2.7 Kinetics of phenol degradation

Kinetics of biodegradation of phenol and phenolic compounds have been extensively studied over the years for pure microbial cultures (Wang and Loh, 1999; Monteiro et al., 2000; Chung et al., 2003; Singh et al., 2008; Banerjee and Ghoshal, 2010b). Researchers have also studied growth and degradation kinetics of phenolic compounds for mixed microbial cultures (Kumaran and Paruchuri, 1997; Nuhoglu and Yalcin, 2005; Saravanan et al., 2009). To assess the biodegradation potential of microorganisms and for operation of reactors and treatment units, we need to be acquainted with the growth and degradation kinetics (Monteiro et al., 2000; Kumar et al., 2005). Bacterial growth kinetics in a batch reactor may be represented by the following equation:

$$\frac{dX}{dt} = \mu X - k_d X = \mu_{net} X \quad (1)$$

where, X = concentration of biomass (mg L^{-1}); t = time (h); μ = specific growth rate (h^{-1}); k_d = decay coefficient (h^{-1}); $\mu_{net} = \mu - k_d$, net specific growth rate (h^{-1}) (Kumar et al., 2005).

Substrate consumption rate (mg L^{-1} h^{-1}) can be represented by:

$$\frac{dS}{dt} = -\frac{1}{Y}\left(\frac{dX}{dt}\right) = -\frac{\mu X}{Y} \quad (2)$$

where, μ is a function of S; Y = cell mass yield (Kumar et al., 2005).

Kinetics of bacterial growth on phenols have been extensively studied and represented by various kinetic models. Phenol, being a well-known toxic compound, severely inhibits microbial growth. This phenomenon is known as *substrate inhibition*. Kinetics of bacterial growth can be represented by two approaches. The first approach is defined by the Monod's model. The Monod's model does not take into consideration the inhibitory effects of the carbon or energy source. Hence, this approach is mostly not considered while describing the bacterial growth kinetics in phenol or any other pollutant which can inhibit microbial growth. The Monod's model kinetic equation can be expressed as follows:

$$\mu = \frac{\mu_{max} S}{K_S + S} \quad (3)$$

where, μ_{max} = maximum specific growth rate (h^{-1}); Ks = half saturation coefficient (mg L^{-1}) (Kumar et al., 2005).

The other approach takes into consideration the phenomenon of substrate inhibition. Several kinetic models have been studied by researchers for representing the microbial

degradation of phenol and other toxic pollutants. Owing to its mathematical simplicity, the Haldane's model is most extensively studied (Monteiro et al., 2000; Tsai and Juang, 2006; Agarry and Solomon, 2008; El-Naas et al., 2009). The Haldane's model (Haldane, 1965) can be expressed as:

$$\mu = \frac{\mu_{max} S}{K_S + S + \frac{S^2}{K_I}} \tag{4}$$

where, μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (mg L^{-1}), K_S is the half saturation coefficient (mg L^{-1}) and K_I is the inhibition coefficient (mg L^{-1}).

At very high substrate concentrations, $S >> K_s$, the above-mentioned Haldane's equation reduces to:

$$\mu = \frac{\mu_{max} S}{S + \frac{S^2}{K_I}} \tag{5}$$

or,

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{S}{K_I \mu_{max}} \tag{6}$$

This is the linearized Haldane's equation.

Apart from the Haldane's model various other models have also been proposed and used to describe the dynamics of microbial growth on phenol. These models are as follows:

Aiba et al., 1968: $\mu = \frac{\mu_{max} S}{K_S + S} exp\left(\frac{-S}{K_I}\right)$ (7)

Edwards, 1970: $\mu = \mu_{max} S \left[exp\left(\frac{-S}{K_I}\right) - exp\left(\frac{-S}{K_S}\right)\right]$ (8)

Yano et al., 1966: $\mu = \frac{\mu_{max} S}{K_S + S + (S^2/K_I)[1 + S/K]}$ (9)

Webb, 1963: $\mu = \frac{\mu_{max} S[1 + S/K]}{S + K_S + (S^2/K_I)}$ (10)

where, μ = Specific growth rate (h^{-1}); μ_{max} = Maximum specific growth rate (h^{-1}); S = Substrate concentration (mg L^{-1}); K_S = Substrate affinity constant (mg L^{-1}); K_I = Substrate inhibition constant (mg L^{-1}); K = Constant in Yano and Webb model (mgL^{-1}).

Kumar et al, (2005) reported in their study that bacterial growth kinetics in phenol simply could not be represented by Monod's model or linearized Haldane's model. However, Haldane's growth kinetic model (Equation 4) fitted their experimental data quite well. Yet another study by El-Naas et al, (2009) involving a strain of *Pseudomonas putida* also confirmed that Haldane's model gave a better fit of their experimental data when compared to Monod's model. Another study reported that the Haldane's kinetic model gave a good fit in spite of significantly higher concentrations of phenol (0.5-3.0 g L^{-1}) in their experiments (Marrot et al., 2006). Kumaran and Paruchuri, (1997) in their comparative study between Monod's and Haldane's model reported that Monod's model could be used for estimation of the kinetic constant μ and Haldane's model can be used for estimation of μ_{max} and K_I. Haldane's model has also been used to study degradation of other phenolic compounds (o-cresol by Maeda et al., 2005). All these studies point out to the fact that Haldane's model is widely accepted and gives a good fit for a wide range of inhibitory substrate concentrations. However, Wang and Loh, (1999) and Nuhoglu and Yalcin, (2005) reported the inadequacy of the Haldane's kinetic model at higher initial substrate concentrations. In contrast, another study by Marrot et al, (2006) confirmed the inadequacy of the Haldane's model even at lower substrate concentrations (100 mg L^{-1}).

2.8 Bioreactors employed in the degradation of phenol

Biological treatment of industrial effluents and noxious xenobiotic pollutants has been gaining popularity over the years. Biological methods are being preferred over other physicochemical treatment methods, as they are cost efficient, environment friendly and even ensures complete breakdown of xenobiotic compounds into harmless end products. Further, xenobiotic compounds can be degraded more efficiently in bioreactor systems. Several configurations of bioreactors have been used in industries and by researchers all over the world for the treatment of xenobiotic compounds like phenol and its derivatives. Bioreactor configurations may be as simple and traditional as the activated sludge process or other state-of-the-art technologies. Some reactor configurations are stirred tank reactors, fluidized bed reactors, packed bed reactors, membrane based reactors, biofilm based reactors, bubble column reactors, internal loop airlift reactors etc. These reactors may be operated in batch, semi-batch or continuous modes. Wastewater treatment facilities managing municipal and industrial wastes make use of continuous reactors for treating wastewaters aerobically. However, for lab-scale studies, anaerobic treatments or pharmaceutical industries researchers often employ batch and semi-batch reactor configurations (Sundstrom, D.W. and Klei, 1979). Another fact that is

worth mentioning is the effect of immobilization of the microbial biomass on solid supports. When compared to their suspended counterparts, immobilized microbial biomass improves reactor operation (Aksu and Bülbül, 1998).

2.8.1 Stirred tank reactors

Stirred tank batch reactors, also referred to as discontinuous stirred reactor, corresponds to a closed thermodynamic system and are considered as ideal and simplest type of reactor (Caccavale et al., 2011). It consists of a reactor vessel and an agitator or mixer. The schematic diagram of a stirred batch reactor is shown below:

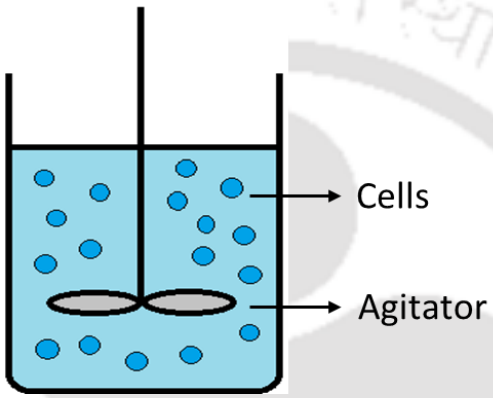

Figure 2.5. Schematic diagram of a typical stirred tank reactor

These reactors can be used for the treatment of solutions with high viscosity, such as soil slurry and, under circumstances where immobilized enzymes and bacterial cells are used. The major disadvantage of this reactor system is physical agitation which could impart mechanical stress on the immobilized biomass. The reactor can also be used as continuous stirred tank reactor (CSTR) which is considered to be an open system. Batch reactors are typically well suited for small volume production or treatment for a short period of time in contrast to the continuous reactors which are used for longer durations for continuous production. Holladay et al., (1978) used CSTR for the treatment of phenolic waste liquor and tested a phenol input of 2400 mg L^{-1}. However, they were unable to achieve an effluent phenol concentration below 100 mg L^{-1}. For inlet phenol concentrations of 1400 mg L^{-1} effluent phenol levels of 1 mg L^{-1} or less were achieved. González et al., (2001b) operated stirred tank reactor in both batch and continuous mode with a phenol concentration of 1000 mg L^{-1}. Complete phenol degradation was attained after 10 days. Continuous experiments with complete phenol removal were carried on with a hydraulic retention time (HRT) of 4 days and

a phenol loading rate of 0.25 g L^{-1} day^{-1}. Chung et al., (2003) used a stirred batch reactor for their phenol degradation experiments with *Pseudomonas putida* immobilized onto Ca-alginate beads. In another study, suspended cells of *Bacillus thermoleovorans sp.* was able to degrade phenol up to a concentration of 7 g L^{-1} day^{-1} in a continuously operated stirred tank reactor at 65 °C (Feitkenhauer et al., 2003). Sponza and Uluköy, (2005) used an upflow anaerobic sludge blanket reactor (UASB) and an aerobic CSTR system in sequence for treatment of 2,4 dichlorophenol. Kuscu and Sponza, (2007) used a CSTR in sequence with an anaerobic migrating blanket reactor (AMBR) under increasing organic loading conditions for treatment of p-nitrophenol with a removal efficiency of 91%. Kinetics of phenol degradation and efficient removal in CSTRs have been widely studied and reported by various research groups (Wei et al., 2010; Christen et al., 2012).

2.8.2 Fluidized bed reactors

Fluidized bed reactors (FBRs) have also drawn attention of researchers for biological wastewater treatment due to certain advantages it offers. It has excellent heat and mass transfer characteristics. In this type of reactor, a fluid (liquid or gas), which acts as substrate, is passed in an upward direction inside the reactor tube/vessel, through a solid material onto which enzymes or biomass are immobilized. The substrate is passed at high enough velocities so as to suspend the solid particles as if it were a fluid. The process is known as fluidization. Design of such reactor systems with adequate fluid velocities is important because high velocities could result in loss of the biomass or enzymes and the fluidized bed organization could be destroyed. The major advantages of fluidized bed reactors are, uniform particle mixing due to the fact that the solid material behaves essentially like a fluid inside the reactor, uniform temperature gradients, no bed clogging, small external mass transfer resistance and large mass transfer surface area, high organic loading rates (OLR), low HRT, and ability to operate the reactor in a continuous state. The disadvantages of FBR are increased reactor vessel size, erosion of the internal components of the reactor vessel as well as the solid particles and excessive growth of biomass on the solid support particles, control of bed expansion and biofilm thickness (Lazarova and Manem, 1994) and high-energy consumption due to the very high liquid recirculation ratio. Three basic processes occurring in the FBR during biodegradation of phenol has been reported (Vinod and Reddy, 2005):

a) Oxygen transfer from the gas phase into the bulk liquid.

b) Phenol, oxygen and nutrient transfer from the liquid phase to the surface of biofilm on the supporting matrix.

c) Simultaneous diffusion and reaction of phenol, oxygen and other nutrients within the biofilm.

Holladay et al., (1978) achieved a phenol degradation rate of 8 g L^{-1} day^{-1} in a 3 L FBR (tapered column). The fluidizing packing consisted of anthracite coal particles. A complete mixing type three phase fluidized bed bioreactor setup containing biofilm and suspended sludge was used by Hirata et al., (1998) for the treatment of synthetic phenolic wastewater. The solid supports used in the study for biofilm immobilization were poly vinyl alcohol particles coated with activated carbon. González et al., (2001b) used a setup of FBR for the biodegradation of phenol using microorganisms immobilized in calcium-alginate beads. In their study, they achieved complete degradation of 1000 mg L^{-1} phenol after 12 days of operation in batch mode. The reactor was also operated in continuous mode with a HRT of 4 days and no phenol was detected in the effluent. In another study using immobilized *Pseudomonas putida*, >90% removal efficiency was observed for phenol concentrations of up to 1000 mg L^{-1} in FBR operated in batch mode (González et al., 2001a). They also operated the FBR in continuous mode with an HRT of 4 days and a phenol loading rate of 62.5 mg L^{-1} day^{-1} and achieved complete phenol removal. Gallego et al., (2003) carried out their experiments for the degradation of phenolic compounds and achieved 99.8% removal in a fluidized bed reactor under environmental conditions (without sterilization). Vinod and Reddy, (2005) investigated the biodegradation of phenolic wastewater in FBR using *Pseudomonas sp*. immobilized on plastic beads. Effluent concentration of 50 mg L^{-1} was observed for a feed inlet of 1254 mg L^{-1}, whereas detectable phenol concentrations were absent in the effluent of feed concentrations of up to 1034 mg L^{-1}. The authors in their study have used steady state biofilm model to describe the biodegradation of phenol using immobilized bacteria and have obtained satisfactory results.

2.8.3 Packed bed reactors

These are tubular reactors and are filled with solid matrix or packing material on which microbial cells are immobilized. Cells may be immobilized inside the solid support or could be grown on the surface of the packing material. The main advantages of a packed bed reactor are that, the active bacterial cells are immobilized on a solid stationary surface, the reactor can quickly recover form pollutant shocks and it has a multi stage mass transfer mechanism. The

major disadvantage of the packed bed reactor lies in the fact that it develops excessive biomass which can easily result in clogging of the packed bed and makes the continuous operation of the reactor difficult (Holladay et al., 1978). Two packed bed reactors (PBRs) of different inner diameters were used by Holladay et al., (1978) in their study. The PBR with greater inner diameter delivered lower conversion rates than that of the smaller one. Aksu and Bülbül, (1998) used PBR for phenol removal using calcium alginate immobilized with *Pseudomonas putida*. Kim et al., (2002) employed PBR for treatment of phenol and its derivatives (chlorophenols) using diatomaceous earth (Celite R-635) as a carrier material. Prieto et al., (2002) used *Rhodococcus erythropolis* immobilized on ceramic beads (Biolite®) for continuous phenol degradation experiments in PBRs. Under optimum conditions they achieved complete phenol degradation in a defined mineral media with a maximum phenol degradation rate of 18 kg m^{-3} day^{-1}. A pilot scale packed bed reactor was used by Tziotzios et al., (2005) to investigate phenol removal by microorganisms isolated and enriched from olive pulp. Pazarlioğlu and Telefoncu, (2005) used pumice particles as carrier materials in recycled and continuous mode packed bed reactors. In yet another study *Alcaligenes sp.* was capable of removing 99% phenol and 92% COD (Chemical oxygen demand) in continuous mode of operation using packed bed reactor with a residence time of 8 hours (Nair et al., 2007). 'Liapor' clay beads were used as carrier material by Bajaj et al., (2008) for treatment of phenol containing synthetic wastewater in an aerobic fixed bed reactor.

2.8.4 A few other reactors

2.8.4.1 Air lift and Bubble column reactors

These reactors are essentially cylindrical vessels with a gas distributor at the bottom. The only difference between an airlift and a bubble column reactor is that airlift reactor contains a draft tube based on which the reactor can be of two types: internal loop airlift reactor and external loop airlift reactor. When compared to a bubble column reactor, recirculation in an airlift reactor is more vigorous and the oxygen transfer rates are low. Bettmann and Rehm, (1984) employed a special airlift fermenter for cultivating phenol acclimatized *Pseudomonas sp*. immobilized in alginate or polyacrylamide-hydrazide. They compared the degradation activity of the immobilized and free microbial cells. Continuous degradation of phenol from 100 mg L^{-1} to concentrations as low as 2.5 mg L^{-1} was achieved using immobilized *Pseudomonas putida* in a bubble column reactor (Mordocco et al., 1999). A degradation efficiency of over 95% was achieved in continuous operation of an airlift fermenter containing polyacrylamide gel entrapped *Candida tropicalis* for inlet phenol concentrations of 4000 and

5000 mg L^{-1} in less than 5 and 10 days respectively (Chen et al., 2002). Effects of phenol loading rate on the biodegradation of 2,4- dichlorophenol in an internal-loop airlift reactor using *Achromobacter sp*. immobilized on honeycomb-like ceramic carrier was investigated by Quan et al., (2004). Studies have also reported bioremediation of phenol polluted air by *Pseudomonas putida* in an external-loop airlift reactor with a small amount of stainless steel mesh packing (Nikakhtari and Hill, 2006). *Pseudomonas stutzeri* OX1 was capable of degrading over 95% of phenol in an internal loop airlift three-phase bioreactor reactor (Viggiani et al., 2006). Saravanan et al., (2008) employed an internal loop airlift bioreactor for degradation of phenol and m-cresol as single and mixed substrates using an indigenous mixed microbial culture. El-Naas et al., (2009) used a bubble column bioreactor for studying phenol degradation by *Pseudomonas putida* immobilized in polyvinyl alcohol gel pellets. Jemaat et al., (2014) studied continuous degradation of o-cresol in an airlift bioreactor.

2.8.4.2 Rotating biological contactor (RBC)

The RBC consists of packs of rotating polythene, PVC (Polyvinyl chloride) or expanded polystyrene discs (known as media) in a tank. About 40% of the disc are is immersed in wastewater. The biological growth occurs on the surface of the discs to form a biofilm. The rotation helps in aeration and also efficient mass transfer of nutrients and products to and from the biofilm. To restrict the film thickness to 1-2 mm the reactor is rotated on a very slow rpm. Tokuz, (1989) used a pilot scale RBC comprised of discs made of high density polyethylene, rotating at a speed of 4 rpm for treatment of synthetic wastewater containing 2- chlorophenol and 2- nitrophenol and achieved a COD removal efficiency of above 85% and 90% respectively. Banerjee, (1997) studied impacts of several operating variables like input phenol concentration, input phenol loading, input hydraulic loading, temperature of wastewater, rotational speed of discs on the rate of phenol degradation using RBC. 99.9% removal efficiency was achieved by Alemzadeh et al., (2002) in a RBC at a phenol loading rate of 4 x 10^{-3} m^3 m^{-2} day^{-1} and COD concentration of 800 mg dm^{-3}. Jeswani and Mukherji, (2012) achieved complete removal of pyridine, quinoline and benzene and 85–96% removal of phenol, naphthalene, phenanthrene, fluoranthene and pyrene using a mixed consortium of a pyrene degrader, *Exiguobacterium aurantiacum* and activated sludge on a three stage RBC with an HRT of 24 h and an OLR of 6.6 g m^{-2} day^{-1}.

2.8.4.3 Trickle bed reactors

In trickle bed reactors a liquid and a gas phase flow downward concurrently through a fixed bed of catalyst particles while reaction takes place (Satterfield, 1975). Trickle bed reactors can be operated without recycling, but recycling allows higher loading and gives better flow distribution. Trickling filters are widely used for removal of organic matter from wastewater by aerobic bacterial action. The packing can be done with stone or other materials. Versteeg and Visser, (1997) used structured packing known as KATAPAK™ in their reactor. Phenol degradation experiments by *Pseudomonas putida* DSM548 was carried on by Sá and Boaventura, (2001) using a siliceous granular material (PORAVER) for biomass support.

2.8.4.4 Upflow anaerobic sludge blanket (UASB)

This is an anaerobic digester used widely for wastewater treatment and produces biogas with high methane concentration as a by-product. Chang et al., (1995) used a UASB for methanogenic fermentation of phenolic wastewater with phenol and COD removal efficiencies of 90% and 80% respectively. In another study UASB was used for co-degradation of phenol and m-cresol at 37 °C where phenol and m-cresol were degraded up to 98% and 20% respectively without a carbohydrate co-substrate, for wastewaters containing up to 900 mg L^{-1} of phenol and 320 mg L^{-1} m-cresol (Zhou and Fang, 1997).

2.8.4.5 Hollow fibre membrane bioreactor (HFMBR)

In these reactors a membrane separates wastewater from the cells which are immobilized in the membrane and the biodegradation takes place under controlled conditions (Juang and Wu, 2007). A microporous polypropylene hollow fibre module was employed by Chung et al., (2005) for phenol biodegradation (>1000 mg L^{-1}) using *Pseudomonas putida* CCRC14365. Various operational conditions using hollow fibre membrane contactors was investigated by Shen et al., (2012). A hollow fibre supported liquid membrane bioreactor (HFSLMB) was developed by Praveen and Loh, (2013b) for two phase biodegradation of phenol by *Pseudomonas putida* ATCC 11172, which could degrade up to 4000 mg L^{-1} of phenol within 76 hours without experiencing any severe substrate inhibition. In another study *Pseudomonas putida* ATCC 11172 could also degrade 1000 and 2000 mg L^{-1} of phenol completely within 26 and 36 hours respectively in a two phase partitioning bioreactor where sixty bundles of extractant-impregnated hollow fibre membranes (EIHFM) prepared using 400 g L^{-1} of trioctylphosphine oxide (TOPO) were added (Praveen and Loh, 2013a).

2.9 Objectives of the work

From the detailed literature review it can be observed that biodegradation of phenol and other phenolic hydrocarbons have been well studied. Researchers have used variety of microorganisms and mixed microbial consortia for efficient phenol degradation. Immobilization studies using various matrices have also been carried out. However, many of these immobilization matrices, although proved excellent for degradation studies, are very expensive. These add to the cost of the treatment process and might make it economically unfeasible. In our present study we have aimed to experiment with different lignocellulosic biomass and use them as immobilization matrices for phenol degrading microbes. The lignocellulosic biomasses used in the present study were dried Areca nut husk and mature Luffa sponge fibres. These two lignocellulosic matrices have not been reported before for phenol degradation studies. Moreover, lignocellulosic biomasses are biodegradable and do not pose a threat to the environment when disposed as some synthetic matrices might do. Phenol was chosen as our target pollutant owing to its significant toxicity and widespread presence in many types of wastewater.

It is also important to isolate new microbial strains from contaminated sites as these microbes have been exposed to unforgiving conditions for many years. Bacterial communities surviving especially at places like refinery fallouts and wastewater of older petroleum refineries and effluent treatment plants are perfect candidates for studies related to petroleum hydrocarbons degradation.

Keeping the above discussion in mind, the following objectives were determined and accomplished in the present work:

- Isolation, characterization, screening and identification of organisms.
- Batch studies for optimization of parameters for growth and degradation: Effect of substrate concentration, pH and temperature.
- Elucidation of the degradation pathway.
- Immobilization studies.
- Phenol degradation study in bioreactors.

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CHAPTER 3

MATERIALS AND EXPERIMENTAL METHODS

This chapter contains the experimental conditions and methods in details. It also describes all the analytical methods used in the experiments. The chapter introduces us to the microbial culture media, sampling sites and procedures used to isolate the phenol tolerant bacterial culture. The chapter also discusses in details about the experimental procedures used for optimization of several parameters for efficient degradation of phenol via suspension cultures as well as immobilized cells. Degradation and growth kinetic studies have also been discussed. Performance of the immobilized cells in packed bed reactors at different feed flow rates have also been covered in this study.

Chapter 3

MATERIALS AND EXPERIMENTAL METHODS

3.1 Chemicals and culture media

Mineral salt medium (MSM) was the culture medium of choice throughout the study. Liquid MSM contained sodium nitrate ($NaNO_3$) (4.0 g L^{-1}), disodium hydrogen phosphate (Na_2HPO_4) (3.61 g L^{-1}), potassium dihydrogen phosphate (KH_2PO_4) (1.75 g L^{-1}), magnesium sulfate ($MgSO_4.7H_2O$) (0.2 g L^{-1}), calcium chloride ($CaCL_2.2H_2O$) (0.05 g L^{-1}), ferrous sulfate ($FeSO_4.5H_2O$) (0.001 g L^{-1}), copper sulfate ($CuSO_4.5H_2O$) (50 µg L^{-1}), sodium molybdate (Na_2MoO_4) (10 µg L^{-1}) and manganese sulfate ($MnSO_4$) (10 µg L^{-1}). For solid media, 1.8% agar was used as a gelling agent. Yeast extract (3.0 g L^{-1}) and phenol (varying concentrations) were used as carbon sources for different experiments. Culture media was autoclaved at 121 °C for 15 min prior to use. pH of the culture media after autoclaving was found to be 7.0±0.2. Phenol stock solution was prepared separately under sterile conditions and stored at 4 °C. Phenol was added to culture medium after autoclaving to avoid thermal decomposition. All the chemical components of mineral medium and pure phenol crystals were obtained from MERCK India and HiMedia® (analytical grade, unless mentioned otherwise).

3.2 Sampling from hydrocarbon contaminated sites and enrichment of the samples in high phenol concentrations

Samples were collected from four hydrocarbon contaminated environments of Assam, India. A total number of 15 samples were collected from these sites. The samples were mainly of three types, a) oily sludge, b) hydrocarbon contaminated wastewater and c) crude oil (Table 3.1). The samples were collected in aseptic plastic bags and sample bottles and were stored at 4 °C until further use. The pH, electrical conductivity, temperature, ORP (Oxidation reduction potential) and dissolved oxygen content of these samples were recorded.

For enrichment of the samples,1 g each of solid samples and 1 mL each of liquid samples were added to 100 mL sterile liquid mineral salt medium (MSM) in 250 mL Erlenmeyer flasks. The composition of MSM has been described in the previous section. 100 mg L^{-1} of phenol was added as the sole source of carbon and incubated at 37 °C and 120 rpm for 48 h in an incubator shaker (ZHICHENG Instruments ZHWY-2112B). The culture flasks were regularly monitored for visual bacterial growth. Gradual increment in phenol concentration of up to 1000 mg L^{-1} was done for the samples. The initial pH of the medium was maintained at 7.0 and the

working volume was maintained at 100 mL. The enrichment process was carried out until a high phenol (1000 mg L^{-1}) tolerant mixed culture was obtained.

3.3 Isolation and identification of the bacterial strains

Isolation of the culturable bacterial strains from the enrichment cultures was carried out by serial dilution and repeated subculturing on solid MSM containing yeast extract (3 g L^{-1}) as carbon source and 1.8% agar as gelling agent. Identification of the bacterial isolates was done by PCR amplification of the 16S rDNA conserved region. Genomic DNA was isolated using a column based DNA isolation kit. The isolated genomic DNA was subjected to 16S rDNA PCR amplification using 16S universal forward primer fD1 (5′ - AGAGTTTGATCCTGGCTCAG-3′) and 16S universal reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The conditions for PCR reactions involved an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 seconds, an extension/elongation step at 72 °C for 90 seconds and a final elongation step at 72 °C for 10 min. The PCR thermal cycler used for the reactions was obtained from Applied Biosystems (Model: 2720 Thermalcycler). PCR Master Mix, DNase and RNase free water and DNA isolation kit was obtained from MERCK Bangalore GeNei™. Isolated DNA fragments were sequenced and the sequences were analyzed using BLAST (http://www.ncbi.nih.gov/BLAST) and submitted to NCBI Nucleotide database using the BankIt sequence submission tool (www.ncbi.nlm.nih.gov/BankIt). The sequences were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw) of the European Bioinformatics Institute (EMBL-EBI) and BioEdit Sequence Alignment Editor Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The construction of phylogenetic tree was done using MEGA6.

Table 3.1: Description of samples and sampling sites

Sampling Site		Nature of the sample	pH	Conductivity (μS cm^{-1})	Temperature (°C)	Dissolved O_2 (DO) (mgL^{-1})	ORP (mV)
Location 1	DB1	Before ETP inlet	8.07	0.0	37.2	0.23	− 66.3
	DB2	Refinery sludge	6.84	0.0	35.6	0.01	182.0
	DB3	After bio-treatment, via aeration tank, superphosphate, urea added(Activated sludge)	Tested by SGS India Pvt Ltd. (Kolkata) On 15 December, 2011				-
			Cr	**Hg**	**Ni**	**Pb**	**V**	**Zn**	**Co**	**As**	
						(mg/Kg)					
			42	2.0	45.9	37.3	27.42	704.63	7.45	2.93	
	DB4	Oil contaminate d soil (70-80 years old)	7.21	28.6	38.4	-	93.8
	DJ1	CBUS (Central Bowser unloading station) (OIL)	6.68	0.0	40.3	2.7	− 45
Location 2	DJ2	Disposed water Central Tank Farm inside plant	7.5	0.66	52.0	2.18	− 230
	DJ3	Central Tank Farm Sludge from Tank 9	-	-	-	-	-
	DJ4	-	-	-	-	-	-
Location 3	J1	Well number 44 Oil collecting station (OCS)	10.3	0.7	34.3	0.8	− 61.3
	J2	Well number 5 Crude oil Oil 95%	7.8	0.0	25.6	0.5	− 7
	J3	Soil near well 5 Soil with spilled crude oil	7.76	0.0	29.2	3.0	− 77
Location 4	GR1	Sludge from new bioremediation pit (Mixed sludge Tank bottom +EJP)	-	-	-	-	-
	GR2	Storage pit undergoing process of bioremediation from TERI	-	-	-	-	-
	GR3	Lagoon 1 sludge from all steps	6.0	0.21	44.9	0.19	− 30
	GR4	Effluent to ATP	4.96	0.80	38.7	2.8	60.4 to 80.0

3.4 Biochemical characterization and field emission scanning electron microscopy

For all the isolated bacterial strains several biochemical characteristics were analyzed according to Bergy's manual. The biochemical tests included Gram's staining, Gelatin hydrolysis, Starch hydrolysis, Catalase test, E_{24} Assay and oil spread assay.

Scanning electron microscopy was done for observing the mixed culture which was able to degrade phenol up to a concentration of 1000 mg L^{-1}. 24 h old phenol grown cells were collected and sample preparation was done by glutaraldehyde treatment and acetone gradation. Sample was then taken on a slide and dried overnight in a vacuum desiccator. Subsequently, the sample was coated with a gold film and community morphology was observed and recorded in Zeiss Field Emission Scanning Electron microscope (model: Sigma) at a magnification of 32.77 KX.

3.5 Batch studies for optimization of growth parameters: Inoculum dosage, substrate concentration, pH and temperature (for suspension cultures)

3.5.1 Optimization of inoculum dosage

Inoculum dosage optimization studies were carried out in 250 mL Erlenmeyer flasks containing 100 mL MSM supplemented with 1000 mg L^{-1} phenol as the sole source of carbon. Using the hyper phenol tolerant mixed bacterial culture, experiments were conducted for inoculum volumes of 0.5%, 1.0%, 1.5%, 2.0% and 2.5% v/v respectively. Optical density (at 600 nm) of the inoculum (3 days old) was recorded to determine the amount of bacterial cells being used for the experiments. Cell growth and residual phenol concentrations were measured at regular intervals. Experiments were also conducted to obtain a calibration curve to convert optical density values into corresponding dry biomass or DCW (dry cell weight): one OD_{600} = 0.866 g dry cells L^{-1} (R^2 = 0.97).

3.5.2 Growth under different initial phenol concentrations

Experiments were carried out with an optimal inoculum dosage at different increasing concentrations of phenol (75-1000 mg L^{-1}) to find out the optimum substrate concentration for growth and to observe the growth and substrate utilization patterns. At first, batch experiments were conducted for three different initial phenol concentrations (75, 300 and 900 mg L^{-1}) and the data obtained was used for model development. In the next step, the model was validated by cultivating the mixed culture under the remaining phenol concentrations. All the experiments were conducted in triplicates. A uniform temperature of 37 °C and pH 7.0 was maintained throughout all the experiments.

3.5.3 Effect of initial pH on degradation of phenol

The effect of initial pH of the culture media on the phenol degradation performance was determined by conducting experiments with the phenol enriched mixed bacterial culture at various initial pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5). The experiments were carried out at 37 °C and a predetermined optimum phenol concentration of 100 mg L^{-1}. Samples were collected at regular intervals for monitoring bacterial growth and phenol utilization.

3.5.4 Effect of incubation temperature on phenol degradation

The effect of incubation temperature on phenol degradation by the mixed bacterial culture was also studied with incubation temperatures of 25 °C, 30 °C, 37 °C and 45 °C at an optimized substrate concentration and pH of 100 mg L^{-1} and 6.5 respectively. Microbial growth and phenol degradation were measured at regular time intervals.

Optimum substrate concentration, pH and temperature were determined on the basis of the value of the specific growth rate, μ (h^{-1}).

3.6 Measurement of bacterial growth

Bacterial growth measurement was carried out at regular intervals using a double beam UV visible-spectrophotometer (Thermo Scientific Evolution 201) at 600 nm. Quartz cuvettes of volume 1000 µL and dimensions 45×12.5×12.5 mm with a path length of 10 mm used for the same.

The specific growth rate, μ (h^{-1}), is an essential parameter for calculating the optimum growth conditions of any microorganism. The microbial growth was presumed to follow first order kinetics:

$$\frac{dX}{dt} = \mu X \tag{1}$$

or, $$\mu = \frac{1}{X}\frac{dX}{dt} \tag{2}$$

Integrating Eq. 2 with the condition t = 0 to t, result in Eq. 3,

$$\ln X_t - \ln X_o = \mu t - \mu t_o \tag{3}$$

$$\text{or,} \quad \mu = \frac{\ln X_t - \ln X_o}{t - t_o} \tag{4}$$

where, X_t = Biomass at a given time t

X_o = Initial biomass at time $t = 0$

Eq. 4 was used to calculate the specific growth rate values for each of the experiments. It has been presumed that microbial growth was inhibited primarily by toxicity of phenol and that the aeration provided was sufficient for growth. Hence, oxygen was not considered to be a limiting factor for growth (Basak et al., 2014b).

3.7 Phenol removal assay and kinetic studies for suspension cultures

To measure the phenol concentrations, cell free supernatant was obtained by centrifugation at 7000 rpm for 10 min. Residual phenol concentrations were measured by the 4-aminoantipyrene spectrophotometric method as described in standard methods (American Public Health Association (APHA), 1998). This method involves the use of 4-aminoantipyrine which reacts with phenol at an alkaline pH in presence of potassium ferricyanide to form a red colored antipyrine dye which can be measured spectrophotometrically against a suitable blank at 500 nm. The residual phenol concentrations were calculated by plotting the values against a suitable standard curve.

Phenol is a well-known inhibitory compound and is toxic to a host range of microorganisms. There are many proposed substrate inhibition models that represents the growth of microorganisms in a batch reactor in the presence of an inhibitory substrate. The most widely used amidst them is the Haldane's model, which can be expressed as,

$$\mu = \frac{\mu_{max} S}{K_S + S + (S^2 / K_I)} \tag{5}$$

where, μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (mg L^{-1}), K_S is the half saturation coefficient (mg L^{-1}) and K_I is the inhibition coefficient (mg L^{-1}). Sensitivity of the microorganisms to the inhibitory substrate is indicated by a lower value of K_I (Banerjee and Ghoshal, 2010a). In the present

study Haldane's model was used for validation of the experimental data. The model was solved using ODE solver *ode23s* of MATLAB (Mathworks, Natick, MA, USA) for simulation of growth and substrate utilization profiles. The values of bio-kinetic parameters like μ_{max}, K_S and K_I were determined by fitting simulated dynamic profiles of biomass and phenol utilization with corresponding experimental data using constrained based linear optimization algorithm '*fmincon*' available in MATLAB (Mathworks, Natick, MA, USA) (Palabhanvi et al., 2014). To find the degree of correlation between the simulated and experimental data, regression coefficient values were determined for each of the experiments using OriginPro 9.0.0 (OriginLab Corporation, Northampton, MA 01060, USA).

3.8 Identification of the phenol metabolic pathway

Identification of the phenol metabolic pathway was carried out by spectrophotometric measurement of formation of intermediate products for detection of the key enzymes of the pathway. For this study, phenol grown cells were harvested by centrifugation at 7000 rpm and 4 °C for 10 min. The cell pellet obtained were washed two to three times with potassium phosphate buffer solution (KH_2PO_4:K_2HPO_4, 50 mM, pH 7.0) and resuspended in the same buffer. The cells were then sonically disrupted using a probe type sonicator (Vibracell, Model: VCX500, 20 kHz, 500 W) under ice-cold conditions to obtain a crude extract of enzyme. Cell debris was separated by centrifugation at 10,000 rpm and 4 °C for 30 min. A reaction mixture of catechol (1 µM) was prepared in phosphate buffer (KH_2PO_4:K_2HPO_4, 50 mM, pH 7.0) and equilibrated at 37 °C (Banerjee and Ghoshal, 2010b). The crude cell extracts were then added to it and the key enzymes, catechol-2,3-dioxygenase and catechol-1,2-dioxygenase, were detected spectrophotometrically (Thermo Scientific Evolution 201 UV-visible spectrophotometer) by measuring the formation of the intermediate products, 2-HMSA (Hydroxymuconic Semialdehyde) and *cis, cis*-muconate at 375 nm and 260 nm respectively (Neumann et al., 2004).

3.9 Preparation of the lignocellulosic matrices and immobilization of the mixed culture

Areca nut husks and luffa sponges were obtained from the local market in Guwahati, Assam, India. Areca nut husks were washed with water and dehydrated in a hot air oven at 85 °C to remove any moisture, as these are prone to fungal contamination. The outside skin or the exocarp of the areca nut husks were removed and only the fibrous part or mesocarp was retained. The luffa sponges were also washed with water and dried at 85 °C to remove moisture.

The dried areca nut husks and mature luffa sponges were then chopped into pieces of 5 mm to 6 mm approximately. The chopped biomasses were washed several times with deionized water to remove pigments imparted by the particles. These were dried again at 85 °C. The untreated biomasses were autoclaved, dried and stored. These were later used as matrices for microbial immobilization. Immobilization of microbial cells onto the dried biomass was performed *in situ* in conical flasks by a semi-solid state approach, where cells were immobilized by natural adsorption (Yu et al., 2007; Basak et al., 2014a).

20 g of sterile and dried areca nut husk was mixed with 200 mL of fresh bacterial culture. The bacterial culture was maintained in 1000 mg L^{-1} phenol for achieving higher bacterial biomass densities. The concentration of the bacterial culture was recorded as 1.09 (OD_{600}) which was equivalent to 0.944 g L^{-1} dry cell weight (DCW) (1.0 OD equivalent to 0.866 g L^{-1} of dry cell biomass, R^2 = 0.97). This was allowed to stand in a static incubator at 37 °C. After 24 h, the flasks were augmented with 50 mL of fresh media substituted with 200 mg L^{-1} phenol and allowed to stand overnight. Thereafter, the entire media was replaced with 200 mL of fresh mineral media containing another 200 mg L^{-1} phenol. This was allowed to stand for another 24 h in a static incubator at 37 °C. This method of immobilization was adopted from Basak et al. (2014) with slight modifications.

Similarly, immobilization of bacterial culture was also carried out for luffa sponges. Since the density of luffa sponge is rather low when compared to areca nut husks, only 6 g of dry luffa sponges were used for immobilization of bacterial culture. The entire immobilization process was achieved in 500 mL conical flasks containing 200 mL of bacterial culture.

3.10 Electron microscopic studies of the immobilized biomass

The lignocellulosic matrices with and without the immobilized mixed bacterial consortium was observed and studied via FESEM (Field emission scanning electron microscopy). 5 mm pieces of areca nut husk and luffa sponges were collected from the immobilization flasks and fixed by treatment with 3.5% glutaraldehyde for 6 h (Yu et al., 2007). These were further washed with sterile crystal-free neutral phosphate buffer and dehydrated by treatment with increasing ethanol gradation (50%, 70%, 90%, 95% and 100%). The samples were finally dried in a vacuum desiccator overnight. The dried samples were directly placed on a double-sided carbon tape on a FESEM stub and coated with gold film. The morphology of the bacterial community immobilized on the lignocellulosic matrices were witnessed and photographed at a magnification of 0.5 – 10 KX via Zeiss FESEM (model: Sigma). The oven-

dried lignocellulosic matrices without the immobilized bacterial culture were directly viewed under FESEM without any kind of pre-treatment.

3.11 Optimization of physical parameters responsible for growth and substrate utilization (for immobilized cells)

3.11.1 Effect of initial pH of the culture media

Batch experiments were performed to ascertain the impact of initial pH of the growth medium on the bioremediation of phenol. A wide range of pH levels were considered for the experiment (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). The initial pollutant concentration in the culture medium was maintained at 200 mg L^{-1}. Experiments were performed in 250 mL conical flasks containing 100 mL of culture media. 15 g of wet areca nut husk and 6 g of wet luffa sponge fibers (with immobilized microorganisms) were used as inoculum for the experiments. All the experiments were carried out at 37 °C in an incubator shaker at 120 rpm. Phenol degradation was monitored at regular intervals. Degradation data was used to calculate the degradation rate of phenol, which helped determine the optimal pH required for substrate utilization.

3.11.2 Effect of incubation temperature

Experiments were also performed to investigate the impact of temperature of incubation on the bioremediation of phenol. A predetermined optimum pH, calculated from the previous set of experiments, was used for the study. 200 mg L^{-1} of phenol was used as a solitary source of carbon for the experiments. Studies were performed at incubation temperatures of 25 °C, 30 °C, 37 °C and 45 °C in 250 mL conical flasks containing 100 mL of culture media in a shaker incubator at 120 rpm. The inoculum volume was maintained at 15 g for wet areca nut husks and 6 g for wet luffa sponge fibers. Consumption of phenol by the immobilized microbes was monitored at regular intervals for calculation of phenol degradation rate and successive ascertainment of the optimum incubation temperature.

3.11.3 Effect of initial phenol concentrations

At an optimum pH and temperature determined from the previous set of experiments conducted in *Section 3.11.1* and *3.11.2* respectively, studies were also conducted to ascertain the optimum phenol concentration for rapid substrate utilization. Various increasing concentrations of phenol (200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1}, 800 mg L^{-1} and 1000 mg L^{-1}) were used as the sole source of carbon and energy for this study. Inoculum load was maintained at 15 g for wet areca nut husk and 6 g for wet luffa sponge fibers. Experiments were performed in 250 mL conical flasks containing 100 mL of mineral media in a shaker incubator at 120 rpm.

Residual concentrations of phenol were estimated by withdrawing samples at fixed intervals. Phenol degradation data was used for calculation of degradation rate, q (h^{-1}) and analysis of degradation kinetics. Degradation kinetic study is discussed in a later section.

The optimum value of environmental parameters were determined based on the value of degradation rate, q (h^{-1}). All experiments discussed above were conducted in triplicates.

3.12 Analysis of residual phenol for immobilization experiments

Nylon membrane filter papers (0.22 µm pore size, Pall Corporation) were used to filter samples. Residual phenol concentration in the filtrates were determined via High Performance Liquid Chromatography (Model: Agilent Technologies 1220 Infinity LC) fitted with a Variable Wavelength Detector (VWD) and a reverse phase column (Agilent ZORBAX Eclipse XDB-C18, particle size 5 µm, dimensions 3.0 x 150 mm) after appropriate dilutions. A mobile phase of water (60%): acetonitrile (40%) was used and a flow rate of 1.0 mL min^{-1} was maintained at room temperature. Residual phenol was detected by injecting aliquots of 20 µL at a wavelength of 280 nm.

3.13 Degradation kinetic studies and comparison of inhibition kinetic models

Experimental degradation data (obtained from batch studies described in *Section 3.11.3*) was fitted to several available kinetic models to elucidate the kinetics of phenol degradation by the immobilized microorganisms. Various established substrate inhibition kinetic models viz., Haldane model (Haldane, 1965), Yano model (Yano et al., 1966), Aiba model (Aiba et al., 1968), Edward model (Edwards, 1970) and Webb model (Webb, 1963) were considered in this study. Degradation rate of phenol, q (h^{-1}), was ascertained from the gradient of plot of the negative logarithm of S/S_o vs. time (t) (Banerjee and Ghoshal, 2011) for various initial substrate concentrations (S_o). The plot of experimental degradation rates (q) versus values of various corresponding initial phenol concentrations (S_o) were fitted to the different substrate inhibition models. Different bio-kinetic parameters such as, q_{max} (maximum degradation rate, h^{-1}), K_I (inhibition constant, mg L^{-1}), and K_S (affinity constant, mg L^{-1}) were calculated for the inhibition models using nonlinear regression analysis in MATLAB R2017b.

3.14 Storage and reusability studies of the mixed culture immobilized on the lignocellulosic biomass

The effect of storage on the stability and biodegradation potential of the microbes immobilized on the lignocellulosic biomass was studied for a period 0-6 weeks. After the

immobilization of the mixed bacterial culture on the lignocellulosic biomass (as described in *Section 2.2*), a biodegradation experiment was conducted for a phenol concentration of 200 mg L^{-1}. Residual phenol concentrations were measured at regular intervals and time taken for complete phenol degradation was noted. In order to maintain similar inoculum quality for the entire course of storage stability experiments, abundant immobilized material was prepared for carrying out biodegradation studies in triplicates for eight batches. The immobilized cells were then stored at 4 °C for conducting the storage stability experiments. Biodegradation batch experiments were conducted in triplicates, every week (for up to 6 weeks), to check for the decline (if any) in biodegradation potential of the immobilized mixed microorganisms. Before commencing the batch experiments, the lignocellulosic biomass was allowed to reach the room temperature.

Reusability of the immobilized microorganisms was also studied by conducting several successive batch degradation experiments. For these studies, the lignocellulosic biomass immobilized with the mixed microbial culture was retrieved from the first batch of experiments. These were then washed with sterile distilled water. Subsequently, the retrieved biomass was subjected to repeated batch biodegradation experiments for up to 15 batches. After each batch, the biomass was retrieved and washed with sterile distilled water. Residual phenol concentration was monitored at regular intervals and time taken for complete degradation was noted for all the batches.

All the studies were conducted in triplicates with a phenol concentration of 200 mg L^{-1} in 250 mL conical flasks. A uniform pH and temperature of 8.0 and 37 °C respectively was maintained in the shaker incubator at 120 rpm for all storage and reusability experiments.

3.15 Composition analysis studies of the lignocellulosic biomass

Compositional analysis studies were carried out for the lignocellulosic biomass without immobilized microorganisms and with immobilized microorganisms after 15 consecutive batch experiments to determine the degradation of the structural components (if any) of the lignocellulosic biomass. Hemicellulose was determined by a method described by Goering and Van Soest (Goering and Soest, 1970) which involved estimating neutral detergent fiber (NDF) and acid detergent fiber (ADF) separately. Hemicellulose content can be determined by calculating the difference between NDF and ADF. Holocellulose was measured by treatment with sodium chlorite (Wise, 1946). Lignin was estimated by TAPPI standard protocol (Technical association of the pulp and paper industry).

3.16 Packed bed reactor setup for the degradation of synthetic phenol feed

Two upflow packed-bed reactors were set-up, which consisted of tubular acrylic glass column with an internal diameter of 2.5 cm and a length of 50 cm and a total volume of 245 mL. The reactor consisted of an inlet at the bottom for feeding fresh medium and an outlet at the top of the column for the exit of effluent. The reactor schematics and photograph are presented in Figure 3.1 and Figure 3.2. A perforated stainless steel plate was set up at 1 cm above the bottom of the column to support the matrices. The reactors were packed with the lignocellulosic matrices with previously immobilized organisms. About 34 g equivalent of dry areca nut husk and 14 g equivalent of dry luffa sponge fibers were packed. Peristaltic pumps were used to feed a fixed volume of 1 L media through the bottom of the reactor. The reactor was operated at a fixed flow rate of 7 ml min^{-1} with a phenol concentration of 700 mg L^{-1} till it was stable. Further Studies were conducted with 1000 mg L^{-1} phenol at different feed flow rates (3.6, 7.2, 10.8 & 14.4 mL min^{-1}).

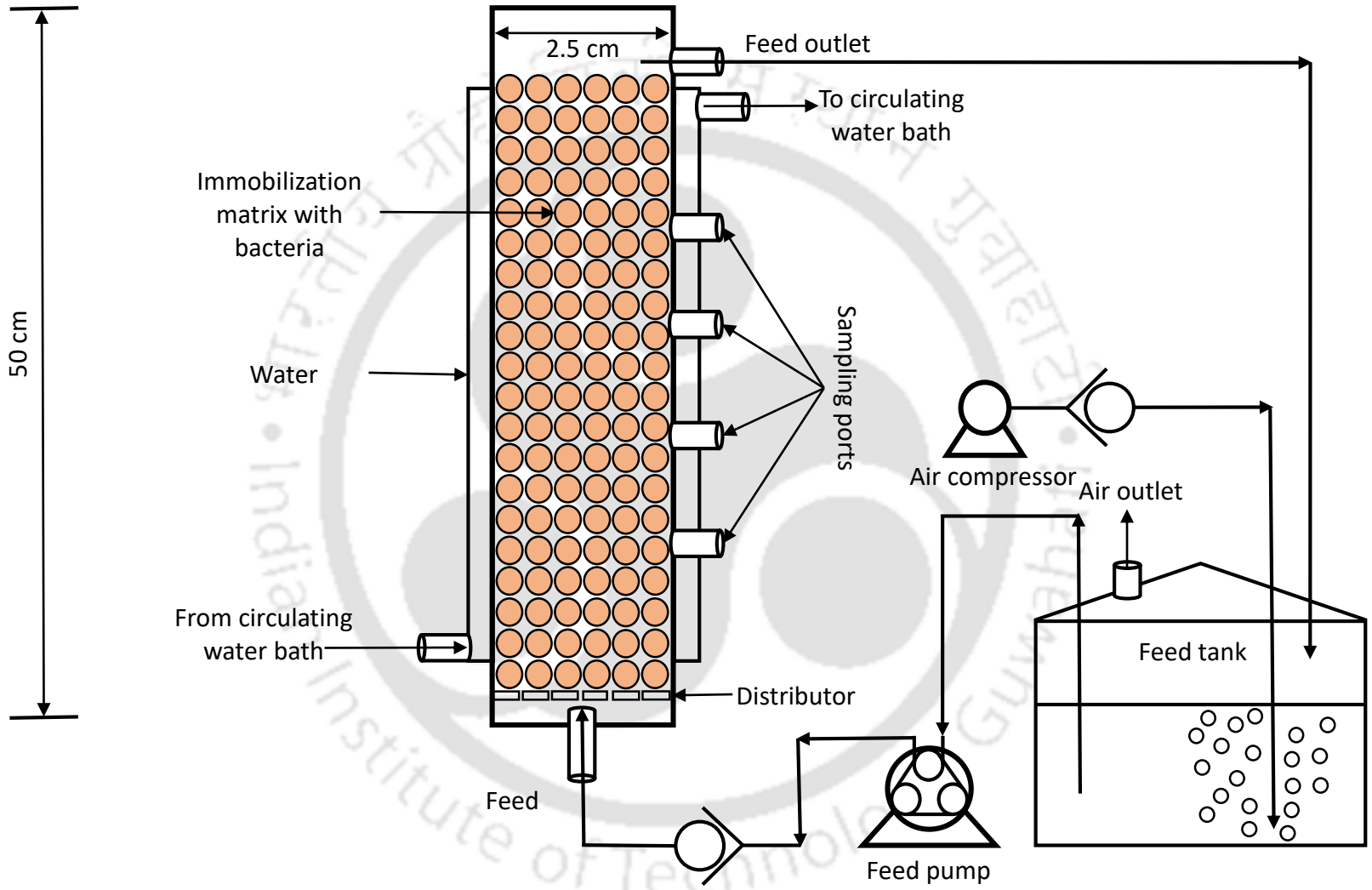
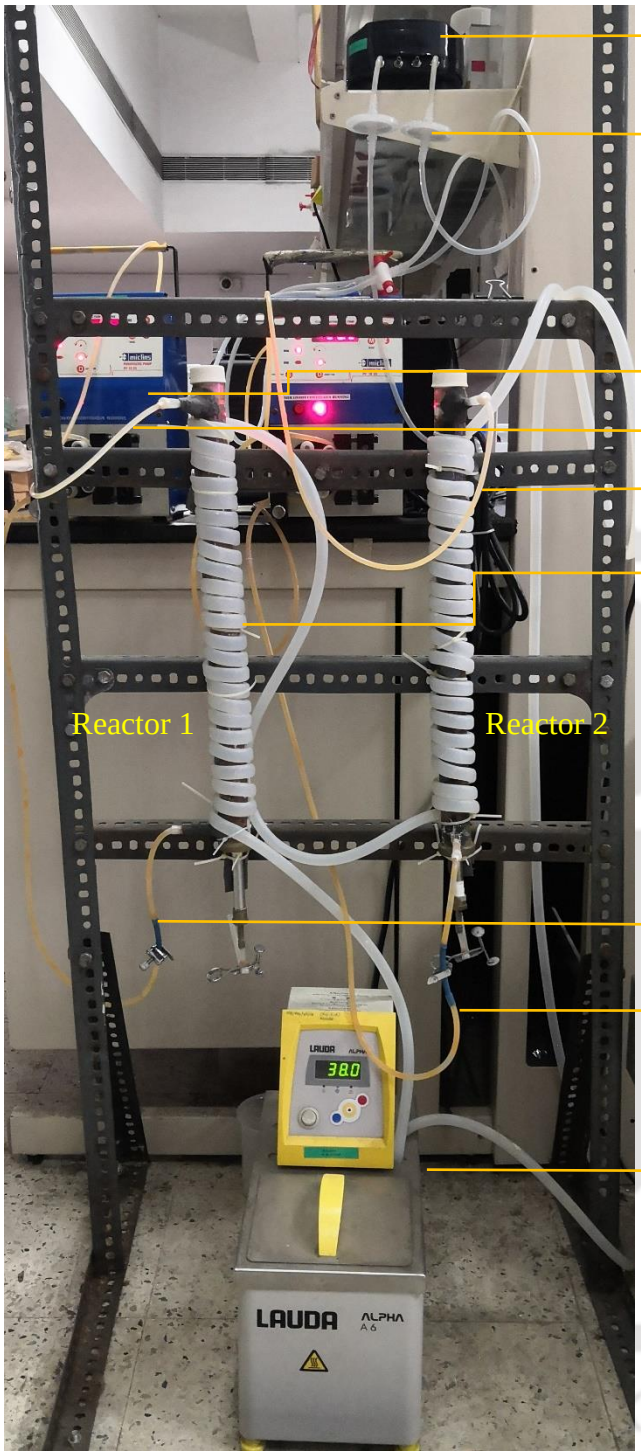


Figure 3.1. Schematic of the packed bed reactor setup used for the present study



Air pump

Air filters

Peristaltic pumps

Feed outlet - Reactor 1

Feed outlet - Reactor 2

Water jacket from the circulating bath

Feed inlet - Reactor 1

Feed inlet - Reactor 2

Circulating water bath

NOTE:

- Reactor 1 and 2 are independent of each other.
- Reactor 1 and 2 are packed with Luffa sponge fibers and Areca nut husks respectively.
- The synthetic phenol feed bottles are kept behind the peristaltic pumps (hence not visible in the setup).

Figure 3.2. Actual photograph of the reactor setup

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CHAPTER 4

BIODEGRADATION OF PHENOL BY A NATIVE MIXED BACTERIAL CULTURE ISOLATED FROM CRUDE OIL CONTAMINATED SITE

This chapter contains the results and discussions about the first part of the study which includes sampling, isolation and identification of culturable bacterial strains from various sites with a rich history of petroleum hydrocarbon contamination. It also depicts the phylogenetic tree of the three bacterial strains in the mixed consortia used in the study. The chapter discusses in details about the optimal parameters required for growth and efficient degradation of phenol by the isolated mixed culture. Apart from this the chapter also discusses about the growth kinetics. Phenol degradation pathway was identified and reported in this chapter.

Chapter 4

BIODEGRADATION OF PHENOL BY A NATIVE MIXED BACTERIAL CULTURE ISOLATED FROM CRUDE OIL CONTAMINATED SITE

4.1 Isolation and identification of the bacterial strains

A phenol tolerant mixed bacterial culture was isolated from a petroleum refinery sludge sample. It was enriched in high concentrations of phenol (1000 mg L^{-1}) for a month. On isolation of bacteria present in the mixed culture in MSM agar plates with yeast extract as the carbon source, three distinct colonies were obtained. Analysis of the 16S rDNA sequences by online BLAST tool revealed that the strains had >99% sequence identity with *Stenotrophomonas acidaminiphila*, *Brevibacterium sp*. and *Brucella sp*. respectively. The strains were designated as DBK (GenBank Accession no. KC992293), DBK1 (GenBank Accession no.KP231222) and DBK2 (GenBank Accession no.KP231223) respectively. The neighbor joining phylogenetic trees of these three isolates are shown in Figure 4.1. The isolate DBK showed highest sequence similarity with *Stenotrophomonas acidaminiphila* SR50-5, DBK1 showed highest sequence similarity with *Brevibacterium sp*. FXJ8.052 and the isolate DBK2 showed highest sequence similarity with *Brucella sp*. ADB-7.

For observation of the bacterial cells scanning electron microscopy of 24 h old phenol grown mixed culture was done. The scanning electron micrographs (Figure 4.2) revealed the presence of rod shaped bacterial cells of different sizes.

Several biochemical tests and 16S rDNA identification was done for all the culturable bacterial isolates obtained from the collected samples. Their GenBank accession numbers and results of the biochemical tests are presented in Table 4.1.

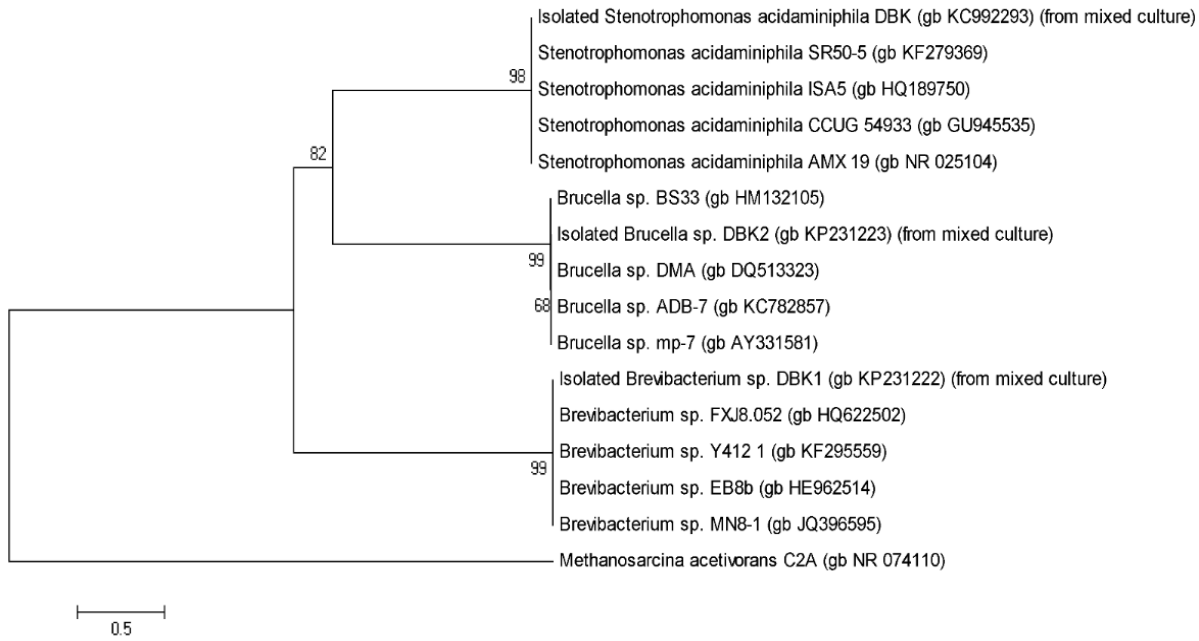

Figure 4.1. Neighbor-joining Phylogenetic Tree based on 16S rDNA gene sequences (1000 bootstrap iterations)

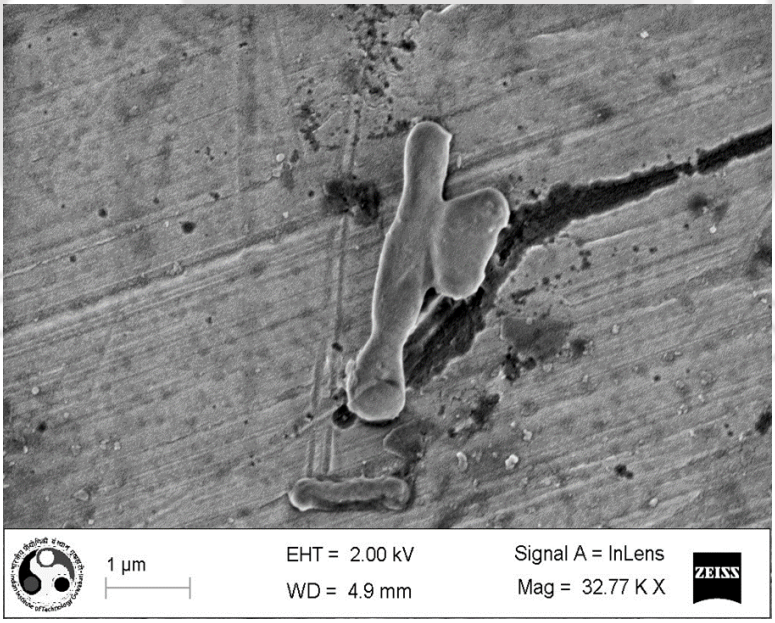

Figure 4.2. Scanning electron micrograph of the mixed bacterial culture showing rods of various sizes

Table 4.1. Characterization of the bacterial isolates

Sampling Site	Isolates	Morphological Characteristics				Biochemical Characteristics						Growth in different Hydrocarbons				
		Form	Margin	Elevation	Pigmentation	Gram Staining	Gelatin Hydrolysis	Starch Hydrolysis	Catalase Test	E_{24} Assay	Oil spread assay	Phenol	Benzene	Toluene	Ethyl benzene	Xylene
												100 ppm				
Location 1 (DB1, DB2, DB3)	DBA(*Stenotrophomonas maltophilia*)(KC992288)	Circular	Entire	Raised	Pale white	+	-	-	+	-	-	+	Poor growth	Poor growth	Poor growth	-
	DBC (*Brevibacillus parabrevis*)(KC992290)	Circular	Entire	Raised	White	+	-	-	-	-	-	+	+	-	Poor growth	Poor growth
	DBD (*Brevibacillus parabrevis)* (KC9922910)	Circular	Entire	Raised	Milky white	+	-	-	+	-	-	+	+	++	Poor growth	++
	DBK (*Stenotrophomonas acidaminiphila*)(KC992293)	Circular	Entire	Raised	Green	+	-	+	+	+	+	++	+	+	Poor growth	+
	DBK1 (*Brevibacterium sp.*)(KP231222)	Circular	Entire	Raised	White	+						+	-	-	-	-
	DBK2 (*Brucella sp.*)(KP231223)	Circular	Entire	Raised	Pale white	-						+	-	-	-	-
	DBH (*Stenotrophomonas maltophilia*) (KC992292)	Circular	Entire	Raised	Pale Yellow	-	-	-	+	+	+	+	-	-	Poor growth	Poor growth
	DBB (*Achromobacter xylosoxidans*) (KC992289)	Circular	Entire	Raised	Dirty white	-	+	-	+	+	+	+	Poor growth	+	Poor growth	++
Location 2 (DJ1, DJ3, DJ4)	DJ5 (*Bacillus tequilensis*) (KC992298)	Circular	Entire	Raised	Yellowish Green	+	-	-	+	-	-	+	++	Poor growth	++	Poor growth
	DJ1 (*Pseudomonas aeruginosa*) (KC992294)	Circular	Entire	Raised	White	+	+	-	+	+	+	+	Poor growth	Poor growth	Poor growth	+
	DJ6 (*Brevibacillus parabrevis*) (KC992299)	Circular	Entire	Raised	White	-	-	-	+	-	-	+	++	+	Poor growth	+
	DJ3 (*Ochrobactrum anthropi*) (KC992296)	Circular	Entire	Raised	White	-	-	-	+	+	+	+	+	-	Poor growth	-
	DJ4 (*Brevibacillus parabrevis*) (KC992297)	Circular	Entire	Raised	White	+	-	-	+	-	-	+	++	++	Poor growth	++
Location 3 (J1, J3)	JJ5 (*Stenotrophomonas maltophilia*) (KC992308)	Circular	Entire	Raised	Greenish white	+	-	-	+	+	+	+	++	++	Poor growth	++
	JJ14 (*Brevibacillus parabrevis)*	Circular	Entire	Raised	Yellow	+	+	+	+	-	-	+	++	++	+	++
	JJ16 (*Stenotrophomonas acidaminiphila*) (KC992305)	Circular	Entire	Raised	White	+	-	-	+	+	+	+	+	Poor growth	Poor growth	+
	JJ8 (*Brevibacillus parabrevis*) (KC992310)	Circular	Entire	Raised	White	-	-	-	+	-	-	+	Poor growth	+	Poor growth	+
	JJ2 (*Brevibacillus parabrevis*) (KC992307)	Circular	Entire	Raised	White	+	-	-	+	-	-	+	-	+	Poor growth	Poor growth
Location 4	GRC (*Lysinibacillus sphaericus*) (KC992301)	Circular	Entire	Raised	Dirty white	+	-	-	+	+	+	+	Poor growth	Poor growth	+	+
	GR4 (*Bacillus tequilensis*) (KC992300	Circular	Entire	Raised	Dirty white	-	-	-	+	-	-	+	+	+	Poor growth	+
	GRG (*Brevibacillus parabrevis*) (KC992303)	Circular	Entire	Raised	White	+	-	-	+	-	-	+	++	++	Poor growth	+

"+" indicates less growth; "++" indicates moderate growth; "-"indicates no growth

4.2 Optimization of bacterial growth and phenol degradation

The effect of different inoculum dosages on degradation of phenol was studied. Figure 4.3 depicts the growth profiles of the mixed bacterial culture for different doses of inoculum (0.5, 1.0, 1.5, 2.0 and 2.5% v/v). It clearly indicates that the biomass growth in 2.0% v/v and 2.5% v/v inoculum volume were more as compared to the other inoculum sizes. The lag phase for the inoculum doses 2.0% and 2.5% v/v were also short in comparison to the other inoculum sizes. The phenol degradation curves for the optimum inoculum dosage experiments are depicted in Figure 4.4. It is clear from the graph that both the inoculum volumes of 2.0 % v/v and 2.5% v/v takes about 96 h to degrade the phenol, whereas, the other inoculum volumes take much longer. Based on the degradation data, the optimum inoculum dosage was determined to be 2.0% v/v. This was due to the fact that, increasing inoculum concentration results in shortening of the lag phase in growth and this helps the system gain the exponential growth phase quickly (Bandyopadhyay et al., 1998). The inoculum concentration used for the experiments was 2.684 (OD_{600}) equivalent to 2.32 g L^{-1} dry cell weight (DCW) (1 OD corresponds to 0.866 g L^{-1} of dry biomass). 2.0% v/v inoculum dosage contained approximately 4.65 mg of dry biomass.

Studies on effect of initial phenol concentration on growth was also undertaken in the present study. Phenol concentration was varied from 75 mg L^{-1} to 1000 mg L^{-1} (75, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg L^{-1}). Figure 4.5 shows the phenol degradation profiles of the aforementioned phenol concentrations (75 mg L^{-1} not shown). At first degradation experiments were performed for three random concentrations of phenol (75, 300 and 900 mg L^{-1}) for development of degradation model in MATLAB. In the second step of experiments, studies were carried out with the remaining concentrations of phenol for validation of the developed model (will be discussed later in *Section 4.3.*). The mixed culture could also degrade a maximum of 1000 mg L^{-1} phenol within 96 h (Figure 4.5). The lag phase was much shorter in lower phenol concentrations (100 mg L^{-1} to 400 mg L^{-1}) as compared to higher concentrations. This shows that the inhibitory effect of phenol becomes quite significant after 400 mg L^{-1} of phenol. However, the mixed culture was able to attain much increased biomass growth in higher concentrations of phenol when compared to lower concentrations owing to availability of a higher amount of substrate for utilization. The time taken to utilize phenol was much longer for the higher concentrations (96 h for 1000 mg L^{-1}) when compared to lower concentrations of phenol (22 h for 100 mg L^{-1} at 37 °C and pH 7.0). The degradation time for highest tolerated phenol concentration (1000 mg L^{-1}) was superior when compared to

many reported pure and mixed bacterial cultures (Table 4.2) (Chung et al., 2003; Gallego et al., 2003; Kumar et al., 2005; Tziotzios et al., 2005; Agarry et al., 2008; Saravanan et al., 2008; Dastager et al., 2009; El-Naas et al., 2009; Christen et al., 2012; Haddadi and Shavandi, 2013). The complete utilization of phenol at 1000 mg L^{-1} within 96 h and a high biomass concentration indicated that the mixed bacterial culture was fairly efficient in utilization of phenol. Similar outcomes have also been reported in earlier studies (Saravanan et al., 2008). Figure 4.6 illustrates the variation of the specific growth rate of the mixed culture with changing phenol concentrations.

pH plays a crucial role for optimal utilization of any substrate by microorganisms. Activity and stability of the phenol degrading enzymes are significantly susceptible to pH changes. Most of the enzymes of neutrophilic organisms tend to denature and lose their catalytic activity completely under extreme pH conditions (Banerjee and Ghoshal, 2010). Hence, the metabolic activities are halted causing rapid death of biomass. Therefore, it is essential to maintain an optimum initial pH so that the bacterial cells can utilize phenol efficiently. In this study effect of initial pH of the culture media was studied over a wide range (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5). The experiments were conducted at a temperature of 37 °C and a pre-determined phenol concentration of 100 mg L^{-1}. Figure 4.7 shows the growth profiles of the mixed culture at various initial pH. It can be observed that at pH 5.5 the lag phase is longer as compared to the others. Also, there is no significant growth in pH 5.5. At pH 7.5, 8.0 and 8.5 there is an initial drop in the biomass concentration. A very sharp rise in biomass concentration after 6^{th} hour is observed for pH 6.5. Specific growth rates were calculated from the exponential phase of these growth curves (Figure 4.8). It was noticed that growth was severely inhibited at pH 5.5 and highest specific growth rate was observed for pH 6.5. Hence, pH 6.5 was considered as the optimum pH for growth.

Like pH, temperature is also an essential parameter that needs to be optimized for efficient utilization of any substrate. Figure 4.9 depicts the specific growth rate profiles of the mixed culture at various incubation temperatures (25 °C, 30 °C, 37 °C and 45 °C). It was observed that the growth rate increased with increasing temperatures. Maximum growth rate was observed at 37 °C. At 25 °C and 45 °C the growth rate was very less which indicates that the microorganisms could not possibly have survived at that temperature (Banerjee and Ghoshal, 2010). This suggested that the mixed culture was mesophilic in nature with an optimum growing temperature of 37 °C.

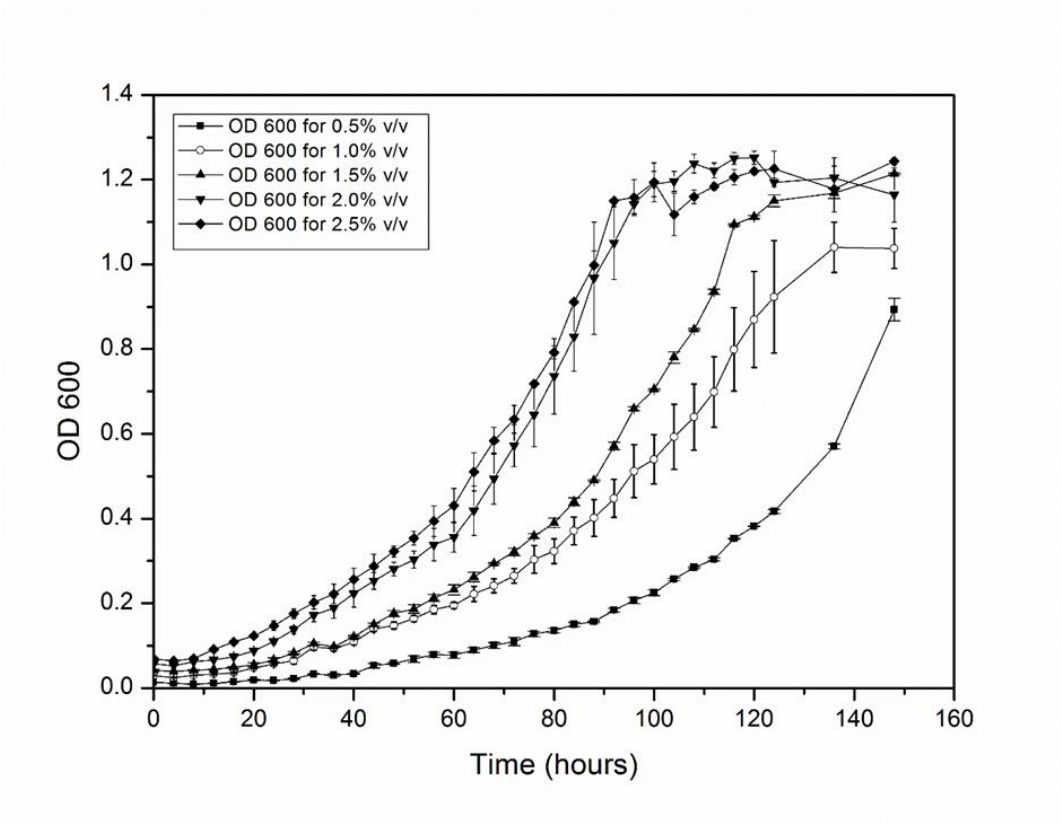

Figure 4.3. Growth at different inoculum doses (Initial phenol concentration 1000 mg L^{-1} at pH 7.0 and 37°C)

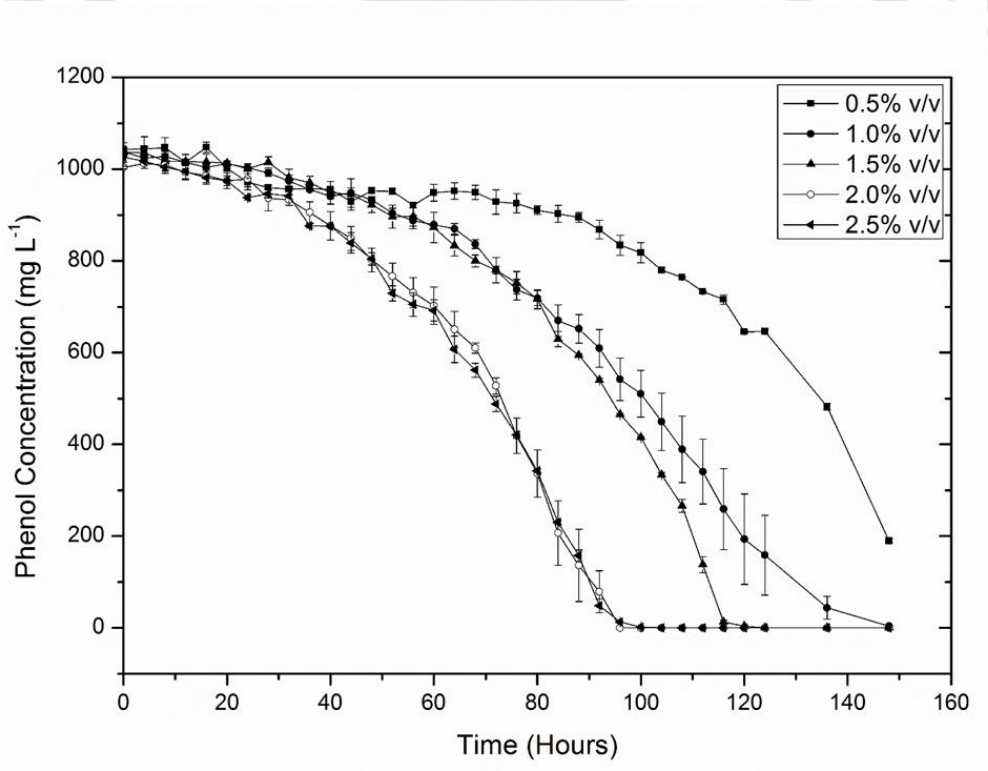

Figure 4.4. Degradation curves at different inoculum dosages for initial phenol concentration of 1000 mg L^{-1} at pH 7.0 and 37°C

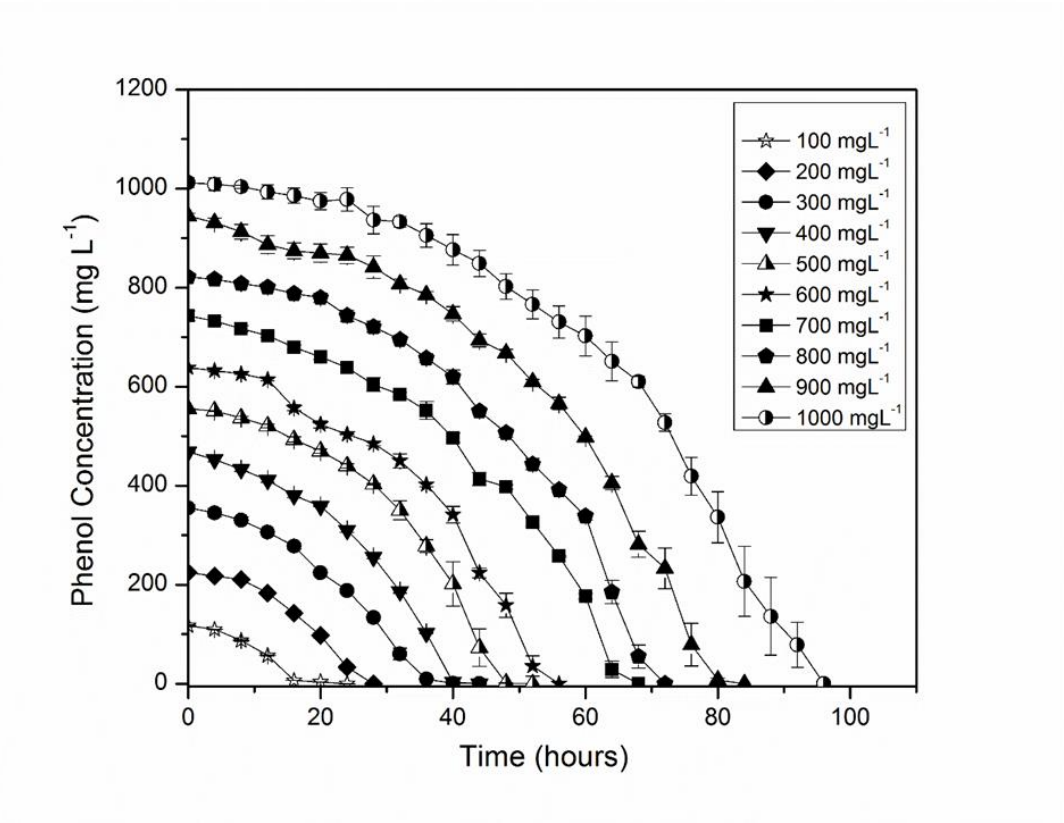

Figure 4.5. Degradation curves when phenol was used as a sole source of carbon (concentrations 100 to 1000 mg L^{-1})

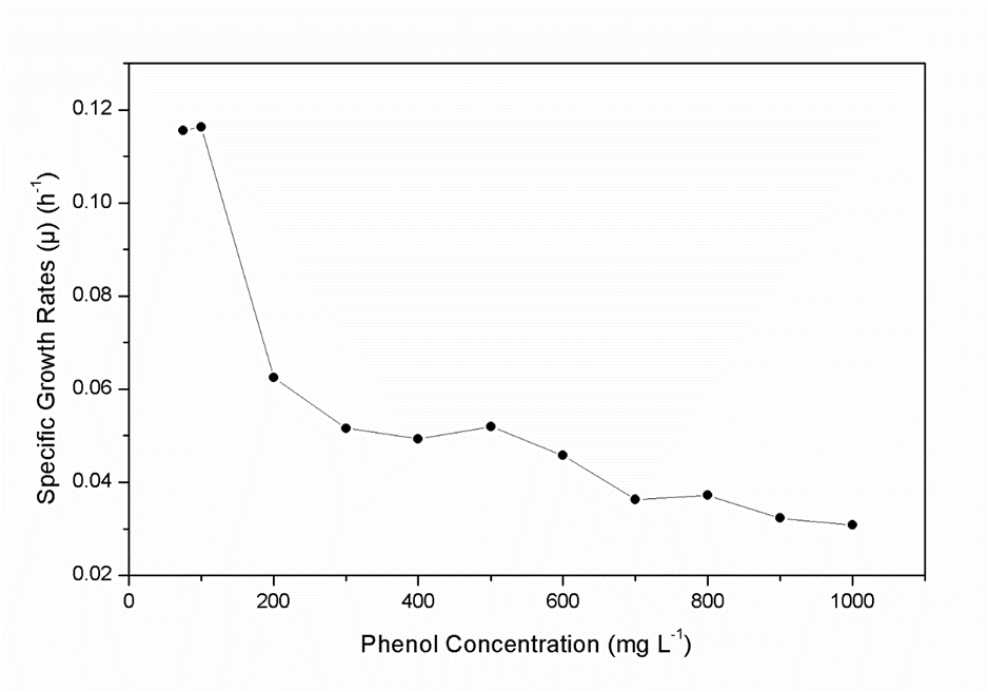

Figure 4.6. Plot showing the maximum specific growth rate for determining the optimum substrate (phenol) concentration

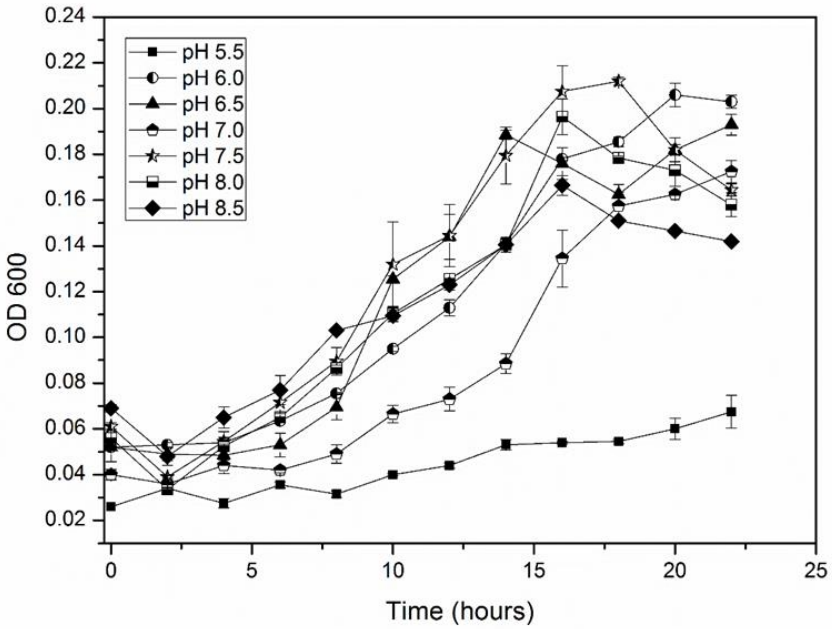

Figure 4.7. Growth in different initial pH for an initial substrate concentration of 100 mg L^{-1} (pH 5.5 to 8.5)

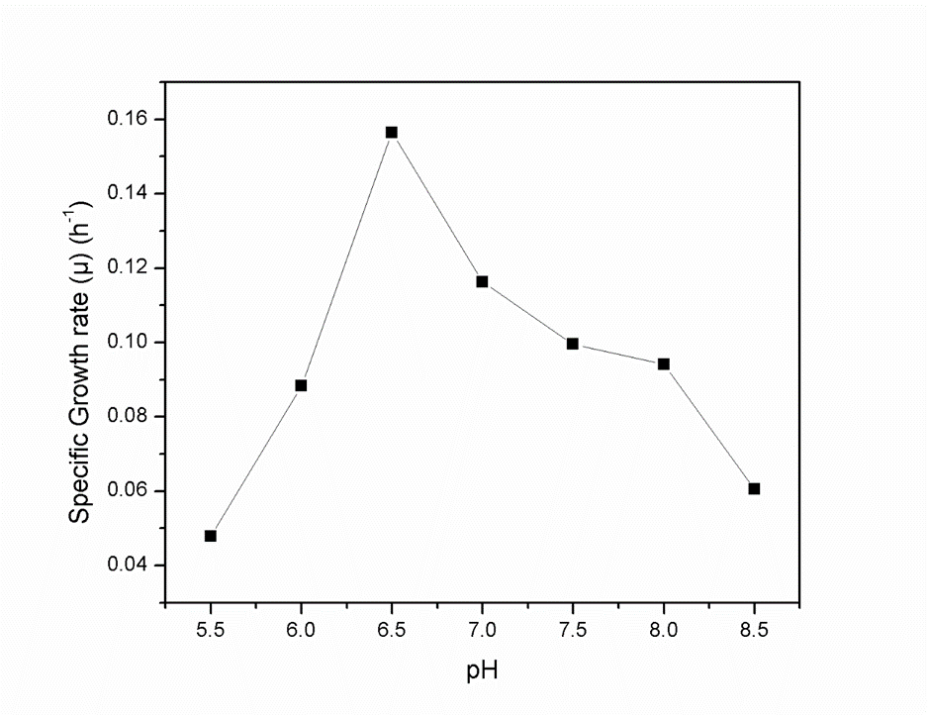

Figure 4.8. Plot showing the maximum specific growth rate for determining the optimum initial pH of the culture media (at an initial substrate concentration of 100 mg L^{-1})

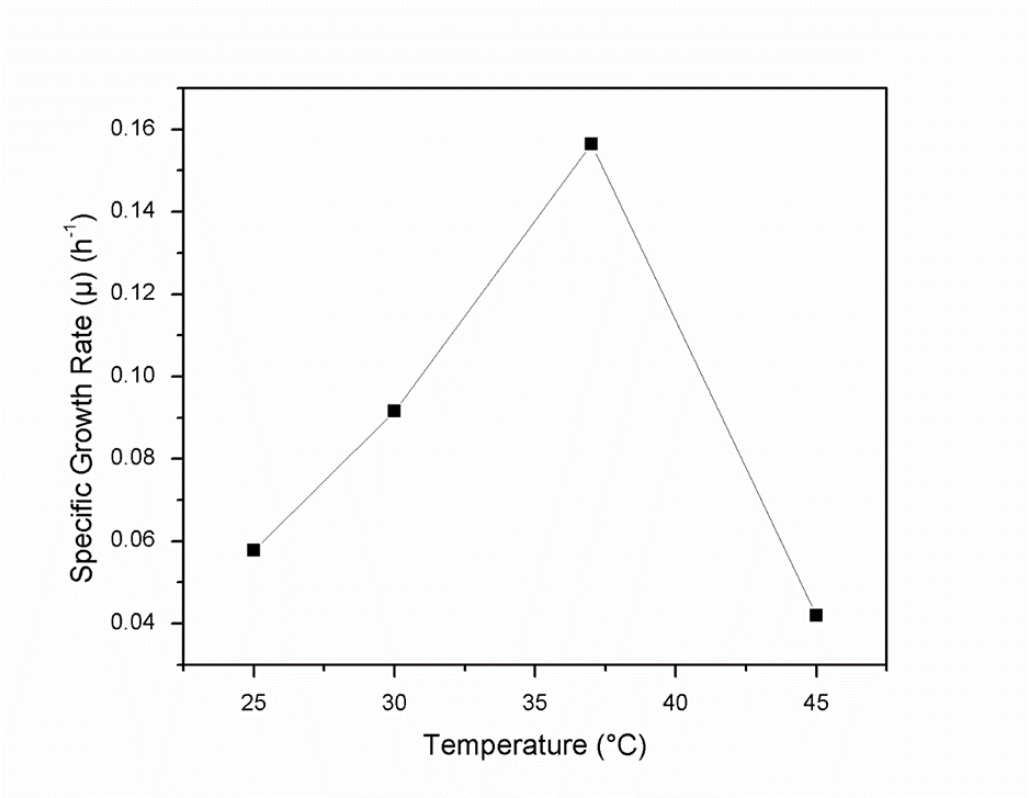

Figure 4.9. Plot depicting the maximum specific growth rate for determining the optimum incubation temperature (at an initial substrate concentration of 100 mg L^{-1})

Table 4.2. Comparison of growth kinetic parameters and degradation times of various pure and mixed bacterial cultures

Sl. No.	Organism(s)	Concentration range (mg L^{-1})	Residence time of maximum tolerated phenol concentration (unless mentioned otherwise)	Haldane's Model			Reference
				μ_{max}	K_S	K_I	
1.	*Pseudomonas aeruginosa* and *P. fluorescence* (Binary Mixed Culture)	100 – 500	96 h	-	-	-	(Agarry et al., 2008)
2.	*Pantoea* strain	100 – 900	64 h for 600 mgL^{-1}	-	-	-	(Dastager et al., 2009)
3.	*Halomonas* sp.	100 – 1600	168 h for 1100 mgL^{-1}	-	-	-	(Haddadi and Shavandi, 2013)
4.	*Sulfolobus solfataricus* 98/2	51 – 745	146 h for 745 mgL^{-1}	0.047	77.7	319.4	(Christen et al., 2012)
5.	Mixed indigenous bacterial culture from olive pulp	300 – 1850	300 h (in draw-fill suspended growth flask bioreactor)	-	-	-	(Tziotzios et al., 2005)
6.	*Pseudomonas putida* CCRC14365	25 – 600	18 h for 400 mgL^{-1} *couldn't degrade 500 and 600 ppm completely	0.33	13.9	669	(Chung et al., 2003)
7.	*Pseudomonas putida*	Up to 300 mg L^{-1} *optimized concentration is 75 mg L^{-1}	5 h for 150 mgL^{-1}	-	-	-	(El-Naas et al., 2009)
8.	Indigenous Mixed bacterial Culture	mixture of 2-clorophenol (100 mg L^{-1}), phenol (50 mg L^{-1}) and m-cresol (50 mg L^{-1})	36 h	-	-	-	(Gallego et al., 2003)
9.	*Pseudomonas putida* MTCC 1194	10 – 1000	162 h	0.305	36.33	129.79	(Kumar et al., 2005)
10.	Mixed Microbial Consortium	100 – 800	69 h	0.3085	44.92	525	(Saravanan et al., 2008)
11.	*Pseudomonas putida Q5*	14 – 1000	-	0.099	5.27	377	(Kotturi et al., 1991)
12.	*Alcaligenes faecalis*	10 – 1400		0.15	2.22	245.37	(Bai et al., 2007)
13.	*Pseudomonas putida F1* ATCC 700007	750 – 1750	-	0.051	18	430	(Abuhamed et al., 2004)
14.	Mixed Culture	25 – 1450	-	0.143	87.45	107.06	(Nuhoglu and Yalcin, 2005)
15.	Mixed Bacterial Culture	75 – 1000	96 h	0.155	11.5	400	**Present study**

4.3 Growth kinetics studies – Model fitting, model validation and calculation of bio-kinetic parameters

Since phenol is an inhibitory substrate, Monod's model was not taken into consideration. Instead, a substrate inhibition model (Haldane model, the most widely used inhibition model) has been used to describe the growth kinetics of the mixed culture in this study. Degradation studies were carried out using optimum inoculum concentration in various increasing concentrations of phenol. In the present study, the kinetic studies were carried out in a different way unlike the traditional way reported in previous literatures, involving various inhibition models like Haldane, Yano, Edward, Aiba and Webb models. Here, data from three batch experiments with phenol concentrations of 75, 300 and 900 mg L^{-1} respectively were used for model development. Bio-kinetic parameters were estimated using simulated dynamic profiles of growth and phenol utilization with their corresponding experimental values. The predicted model was finally validated by cultivating the mixed culture under the remaining phenol concentrations. All the experimental data were then fitted against the simulated growth and substrate utilization profiles and coefficient of regression was calculated for each of the phenol concentrations to represent the goodness of fit (with R^2 values ranging from 0.86 – 0.98). Figure 4.10 shows the fitting of growth and substrate utilization curves against their corresponding simulated curves for substrate concentrations of 75, 300 and 900 mg L^{-1} respectively (for the fitted curves of the other concentrations of phenol refer Figure 4.11-4.18 at the end of this chapter). The values of bio-kinetic parameters like μ_{max}, K_S and K_I were determined using constrained based linear optimization algorithm '*fmincon*' available in MATLAB. Depending on the phenol concentrations, the mixed culture had varying lengths of lag and exponential phases. The value of μ_{max} was found to be comparable to the previously reported literatures (Nuhoglu and Yalcin, 2005; Bai et al., 2007) and superior to a few studies reported in literature (Kotturi et al., 1991; Abuhamed et al., 2004; Christen et al., 2012) (Table 4.2). The value of K_I was also found to be higher than many earlier reported literatures (Table 4.2). This reflects the fact that the mixed culture isolated in the present study is resistant to high concentrations of phenol and this characteristic feature could be attributed to possible genetic modifications owing to long term exposure to crude oil contaminated wastes containing toxic concentrations of petroleum hydrocarbons (Banerjee and Ghoshal, 2010).

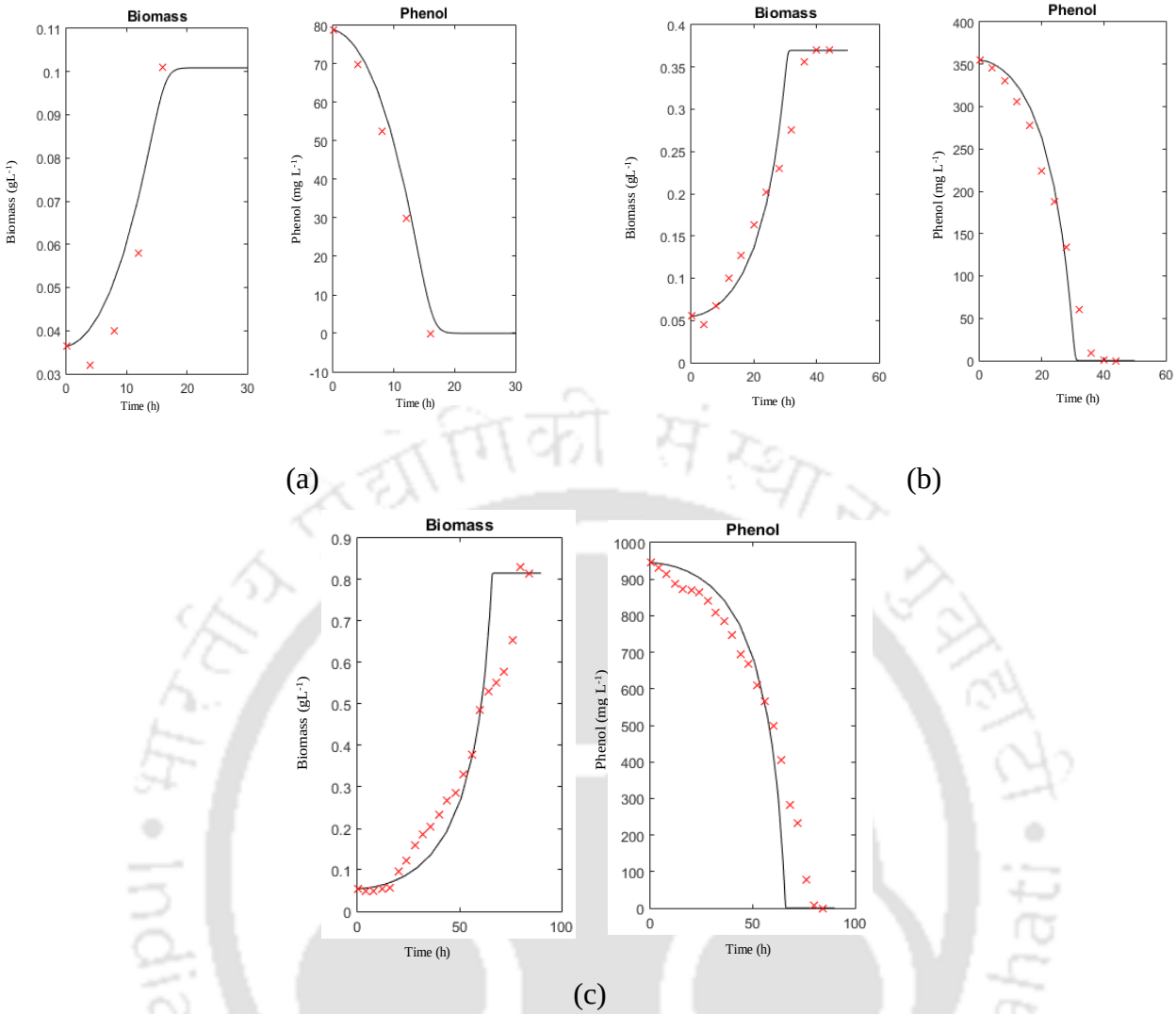

Figure 4.10. Dynamic Profiles of growth and substrate utilization for phenol concentration of (a) 75 mg L^{-1}, (b) 300 mg L^{-1}, and (c) 900 mg L^{-1} at 37°C and pH 7.0; Simulated profile = (—), Experimental value = (×)

4.4 Phenol degradation pathway

For detection of the mechanism of phenol degradation by the mixed culture, both *ortho*-cleavage and *meta*-cleavage pathways were examined. Enzyme assays were carried out for detection of the intermediate products of catechol 1, 2-dioxygenase and/or catechol 2, 3-dioxygenase of the degradation pathway (Banerjee and Ghoshal, 2010). The absence of absorbance at 375 nm indicated that, the intermediate, 2-HMSA (2-hydroxymuconic semialdehyde) was absent in the reaction mixture and that the phenol degradation did not follow the *meta*-cleavage pathway. In other words, the enzyme, catechol 2, 3-dioxygenase was absent in the mixed culture. However, the presence of absorbance at 260 nm indicated the occurrence of *cis*, *cis*- muconate in the reaction mixture as a result of oxidation of catechol by catechol 1, 2-dioxygenase suggesting that the phenol metabolism followed the *ortho*-cleavage pathway.

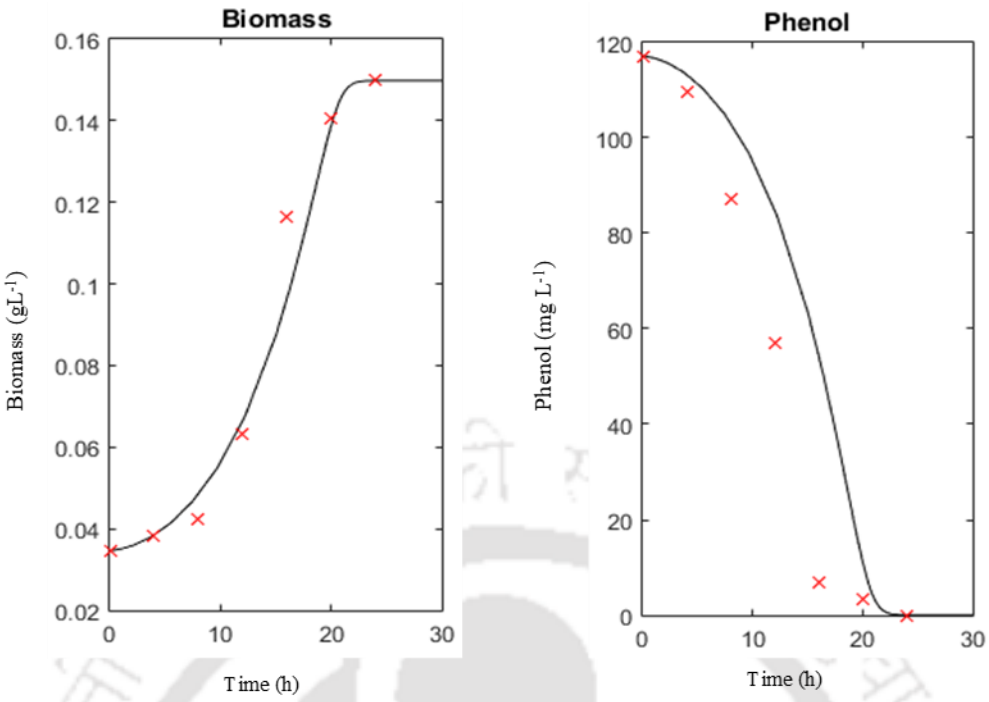

Figure 4.11. Dynamic Profiles of growth and substrate utilization for phenol concentration of 100 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

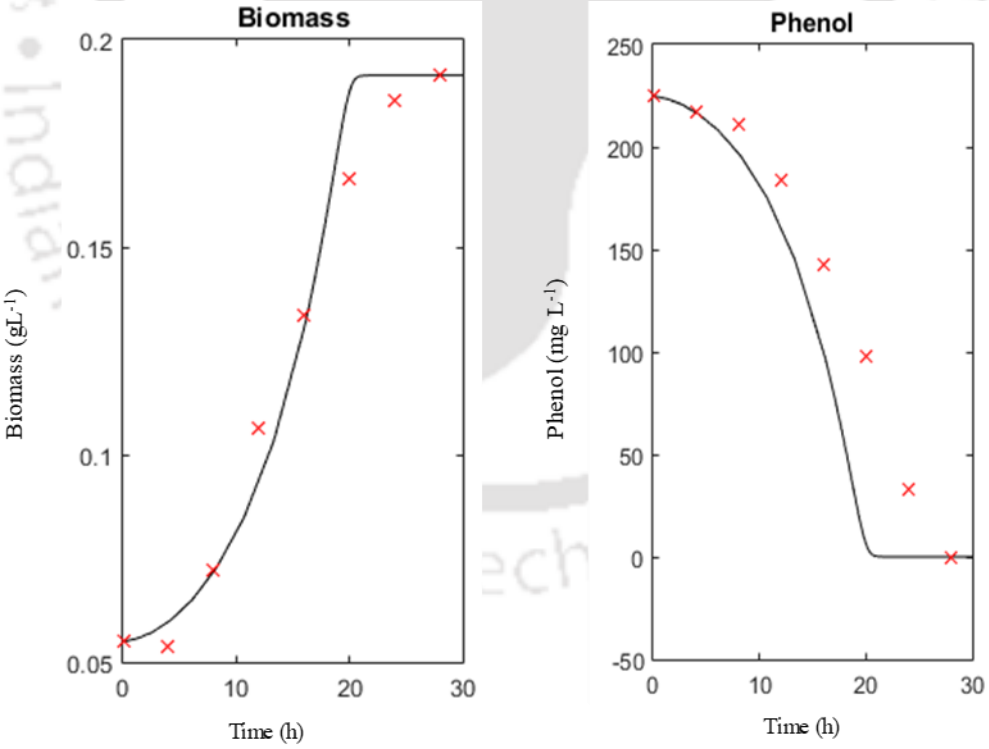

Figure 4.12. Dynamic Profiles of growth and substrate utilization for phenol concentration of 200 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

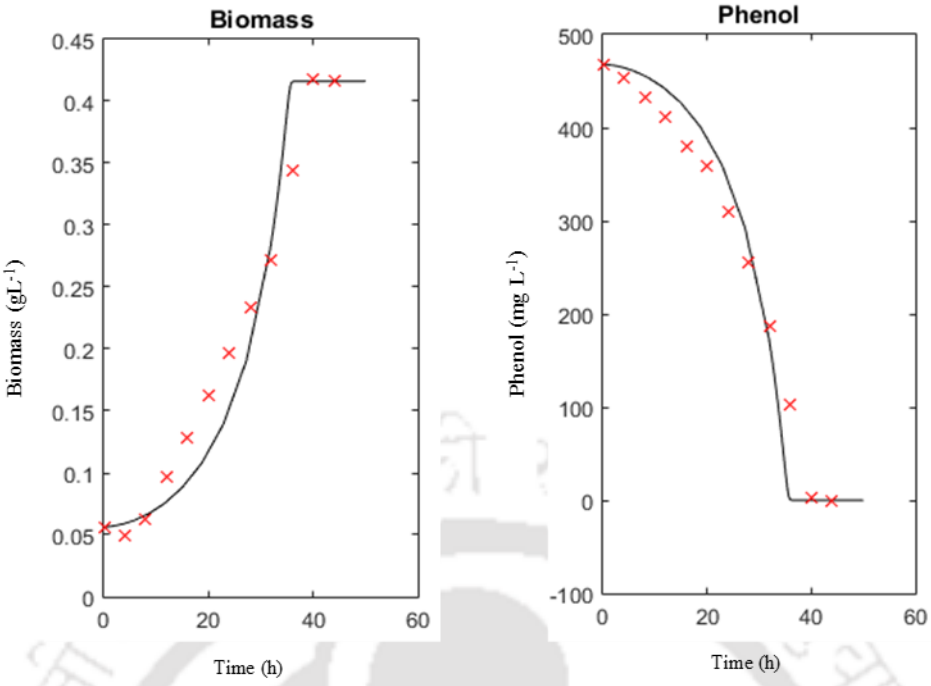

Figure 4.13. Dynamic Profiles of growth and substrate utilization for phenol concentration of 400 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

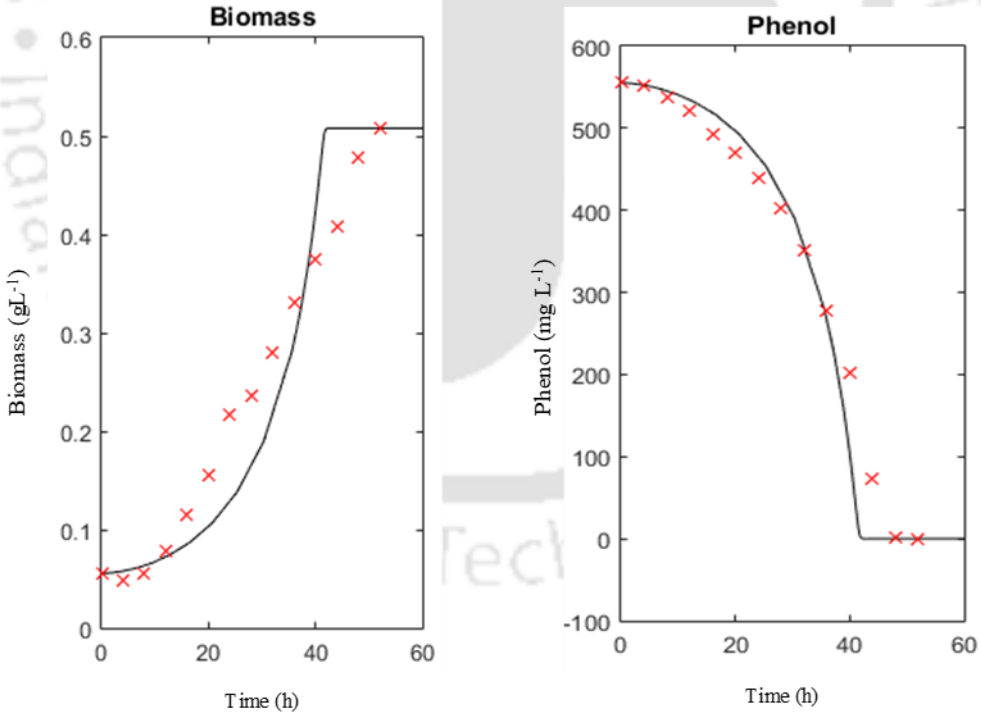

Figure 4.14. Dynamic Profiles of growth and substrate utilization for phenol concentration of 500 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

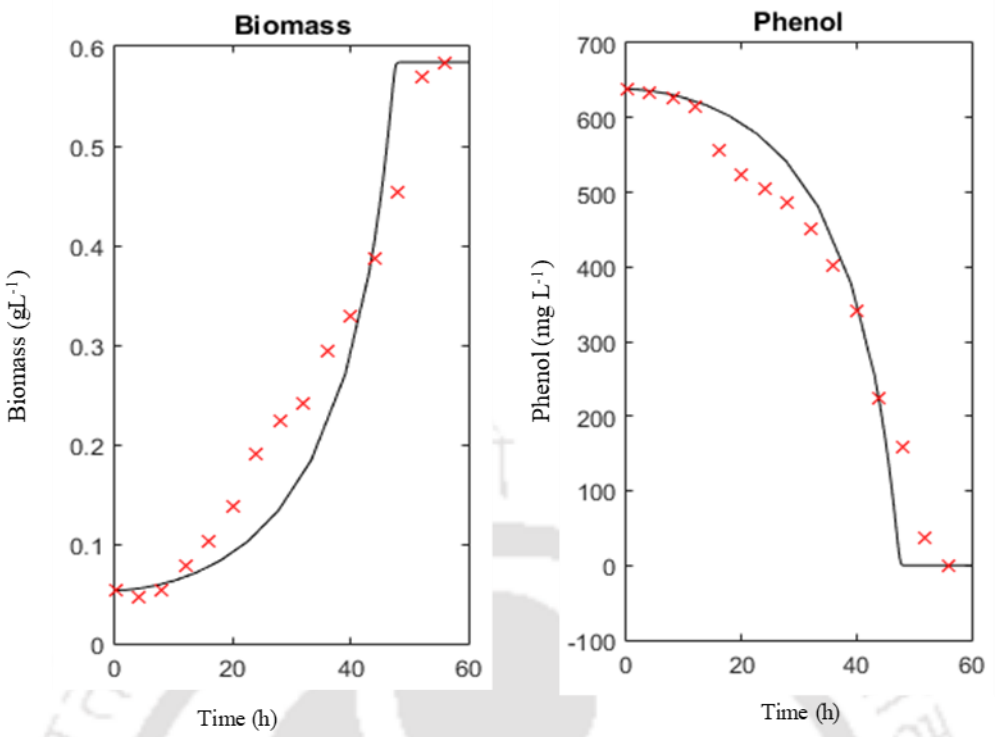

Figure 4.15. Dynamic Profiles of growth and substrate utilization for phenol concentration of 600 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

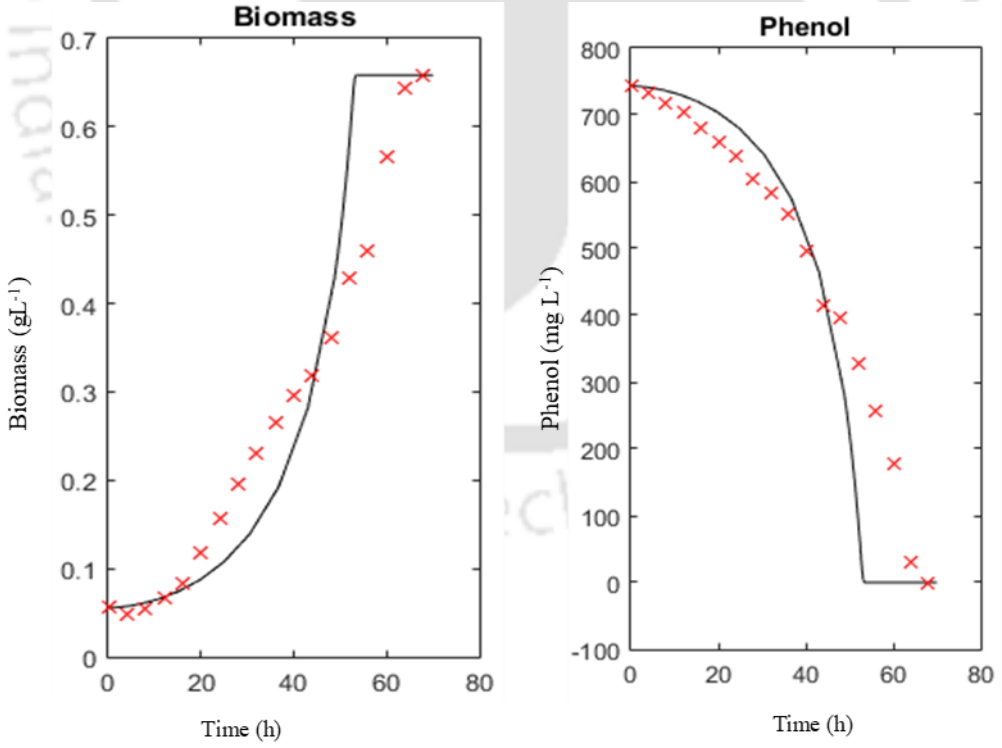

Figure 4.16. Dynamic Profiles of growth and substrate utilization for phenol concentration of 700 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

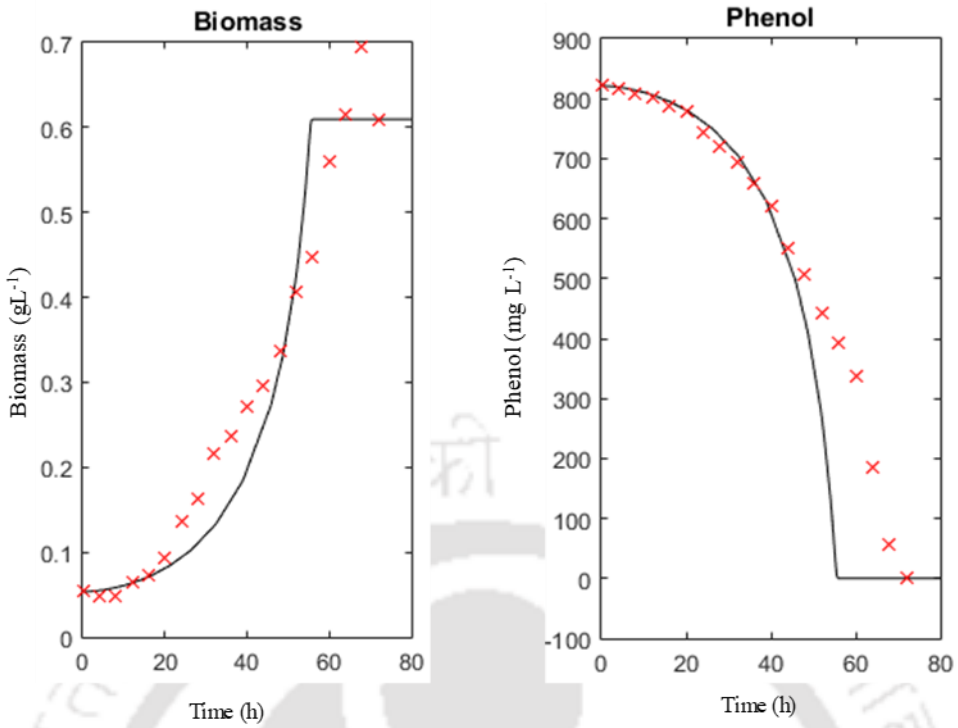

Figure 4.17. Dynamic Profiles of growth and substrate utilization for phenol concentration of 800 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

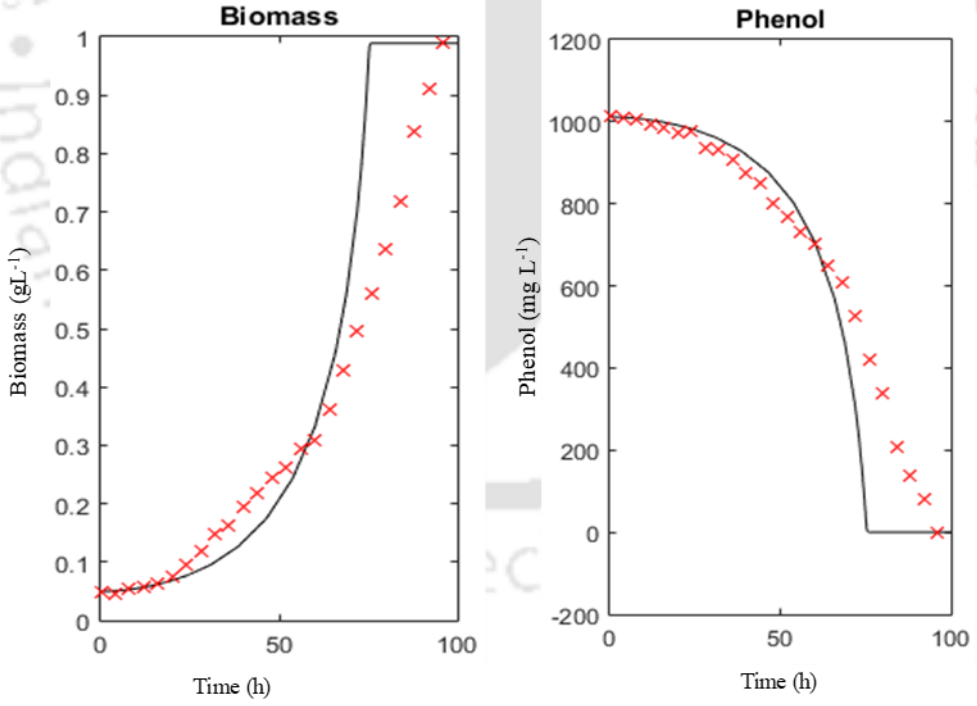

Figure 4.18. Dynamic Profiles of growth and substrate utilization for phenol concentration of 1000 mg L^{-1} at 37 °C and pH 7.0; Simulated profile = (—), Experimental value = (×)

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CHAPTER 5

ARECA NUT (*Areca catechu*) HUSKS AND LUFFA (*Luffa cylindrica*) SPONGE AS MICROBIAL IMMOBILIZATION MATRICES FOR EFFICIENT PHENOL DEGRADATION AND THEIR USE IN PACKED BED REACTOR WITH VARYING FLOW RATES

This chapter reports two dried lignocellulosic biomass (dried areca nut husk and mature luffa sponge fibres) as potential candidates for bacterial immobilization. These biomasses have not been reported earlier as microbial immobilization matrices used for phenol bioremediation. The chapter also discusses about optimization of various physicochemical and environmental parameters for efficient phenol degradation by the immobilization systems. The degradation kinetic studies were also reported in this chapter. Storage and reusability studies regarding the immobilization matrices were also reported. The chapter also reports the use of these immobilization matrices in packed bed reactors for the degradation of synthetic phenol feed at varying feed flow rates.

Chapter 5

ARECA NUT (*Areca catechu*) HUSKS & LUFFA (*Luffa cylindrica*) SPONGE AS MICROBIAL IMMOBILIZATION MATRICES FOR EFFICIENT PHENOL DEGRADATION AND THEIR USE IN PACKED BED REACTOR WITH VARYING FLOW RATES

5.1 A brief introduction to the immobilization matrices of choice

Over the years researchers have shown that bioremediation is a cost-effective and eco-friendly alternative to the traditional physicochemical methods used for phenol removal (Wang et al., 2007; Al-Zuhair and El-Naas, 2011; Banerjee and Ghoshal, 2011; Basak et al., 2014). Although sustainable, the inhibitory effect of phenol at high concentrations pose a serious challenge for wastewater remediation by microorganisms (Banerjee and Ghoshal, 2016). Hence, several strategies have been explored to overcome this problem. Most common approaches include use of genetically modified organisms, acclimatization of organisms to lethal phenol concentrations, and immobilization of organisms (Mollaei et al., 2010). Immobilization of microbial cells offer several benefits over other approaches for treatment of phenolic wastewaters: (1) fortifies microbes against the toxic effects of phenol and harsh environments, (2) helps reach a high microbial concentration hence achieving a high contaminant degradation rate, (3) helps easy recovery and re-use of microbial cells for repeated degradation of target pollutants and (4) helps microbial cells stay viable during storage for extended durations with little or no loss of degradation capacity hence ensuring stable long-term operation (Chen et al., 2007, 2017; Basak et al., 2014). Immobilization by entrapment of microbial cells in matrices such as calcium-alginate beads (Keweloh et al., 1989; Banerjee and Ghoshal, 2016) or a more mechanically stable PVA (poly vinyl alcohol) (Wang et al., 2007; Liu et al., 2009; Al-Zuhair and El-Naas, 2011) were reported earlier. Entrapment of whole cells using chitosan beads (Chen et al., 2007) or hybrid systems involving alginate-chitosan, PVA-alginate and glycerol-alginate (Mollaei et al., 2010) were also implemented. Immobilization studies were carried out using supports such as polyurethane foam (PUF) (Kureel et al., 2017), Celite R-635 (Kim et al., 2002) and Liapor clay beads (Bajaj et al., 2008). A major drawback of systems such as calcium-alginate is its instability against phosphates and disruption of gel particles due to CO_2 evolution (Yu et al., 2007).

In recent years, the pursuit for finding a natural, renewable, non-toxic and biodegradable immobilization matrix has brought lignocellulosic biomass to limelight.

Microbial cells immobilize onto lignocellulosic biomass via physical adsorption (Wang et al., 2012). Owing to its abundance on planet Earth, a variety of lignocellulosic supports such as bagasse, sawdust, wood shavings/chips, rice husk, straw and dry coconut husk were tested for fermentation and wastewater treatment studies (Yu et al., 2007; Wang et al., 2012; Basak et al., 2014). Table 5.1 describes various reported immobilization matrices used for phenol degradation. In the current study, we have used dried areca nut husks and dry mature luffa vegetable fibers as potential candidates to immobilize bacteria for phenol degradation.

Areca catechu (Family: Arecaceae) is a species of palm, which grows lavishly in Asian countries. The fruit is known as areca nut or betel nut. India is the largest producer of areca nut in the world with Karnataka topping the list of producing states followed by Kerala and Assam (Sasmal et al., 2011). The fruit is protected by a fibrous husk, which is separated before consumption of the areca nut. This husk is discarded and is considered as agricultural waste (Sasmal et al., 2011). Owing to the considerable amount of sugars (21%) present in these husks, possibility of using them as low-cost substrates for citric acid production by solid state fermentation was investigated (Narayanamurthy et al., 2008). However, there are no reports on areca nut husk used as a matrix for bacterial immobilization for treatment of phenolic wastewater.

Luffa cylindrica (Family: Cucurbitaceae) is a vegetable crop that grows abundantly in tropical and sub-tropical climates of African and Asian countries. The fruit is edible at a younger stage. Once ripened, the fruit becomes very fibrous. The mature fruit is left to dry to a point where the fleshy part fades away leaving a skeleton of randomly interconnected network of highly fibrous matrix. The dried fruit is widely used as bath scrubber and for manufacturing bath mats, table and door mats (Behera et al., 2011). These fibers are highly porous and are used as a potential support for immobilization of microbial cells for ethanol production (Ogbonna et al., 1997; Behera et al., 2011). The use of luffa sponge for immobilization of algal and fungal cells for removal of heavy metals was also reported (Sriharsha et al., 2017). However, to our knowledge, there are no reports on bacterial immobilization on luffa fibers for treatment of phenolic wastewater.

Therefore, the potential of these two lignocellulosic biomass as carrier matrices for bacterial immobilization and their use for treatment of synthetic phenolic wastewater have been explored. A mixed bacterial culture, which was previously acclimatized to high phenol concentrations, was immobilized onto these two biomass. The effect of factors such as pH of

the growth medium, temperature of incubation and initial phenol concentration were studied and optimized. The experimental degradation data were fitted to various established substrate inhibition models via MATLAB R2018A and various bio-kinetic parameters were estimated.

Table 5.1. Comparison of reported immobilization matrices for phenol degradation

Sl. No.	Immobilization matrix	Pollutant/ concentration	Organism/s used	Experimental degradation rate, q (h^{-1})	Reference
1	Microfiltration membrane capsule	Phenol@100 mg L^{-1}	*Pseudomonas putida* F1	1.05	(Kurzbaum et al., 2017)
2	PVA Gel	Phenol@800 mg L^{-1}	*Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03 (1:1 ratio)	1.2	(Liu et al., 2009)
3	PVA Gel	Phenol@500 mg L^{-1}	*Acinetobacter* sp. strain PD12	1.249	(Wang et al., 2007)
4	Calcium Alginate	Phenol@500 mg L^{-1}	*Bacillus cereus* MTCC9817	0.26	(Banerjee and Ghoshal, 2011)
5	Calcium Alginate	Phenol@100 mg L^{-1}	*Bacillus cereus* MTCC 9818	0.15	(Banerjee and Ghoshal, 2011)
6	Areca nut husk	Phenol@200 mg L^{-1}	Mixed culture	0.37	**This work**
7	Luffa fibers	Phenol@200 mg L^{-1}	Mixed culture	0.21	**This work**

5.2 Lignocellulosic matrices, immobilization of the mixed culture and electron microscopic imagery

Usually, microorganisms are immobilized onto solid supports by recirculating a concentrated microbial culture suspension through a reactor where the lignocellulosic materials act as solid supports. One major drawback of this process is cell desorption, which restricts operational stability (Yu et al., 2007). In this study, a semi-solid state approach was adopted to achieve maximum cell immobilization and enhanced stability. Adding fresh media with phenol after 24 h and 48 h (as discussed in *Section 3.9*) resulted in enhanced cell growth, causing microbial cells to be immobilized thoroughly on the supports (Yu et al., 2007). Scanning electron microscopy (FESEM) was performed to visualize the lignocellulosic matrices with and without immobilization of the mixed bacterial culture. Figure 5.1a and Figure 5.1b portrays the surface architecture of the areca nut husks and luffa sponge fibers respectively without immobilized microorganisms. It can be easily visualized that the surface of the areca nut husks is a lot rougher and porous in texture when compared to the luffa sponge fibers. Figure 5.1 (c-d) depicts the immobilization of the microbial cells on the areca nut husks. Copious amounts of bacterial cells can be seen immobilized on the surface of the areca nut husks. The surface architecture of the husks appears to be smooth (in contrast to the surface without microorganisms), which is possibly due to formation of biofilms. Figure 5.1 (e-f) illustrates the immobilization of microbial cells on the surface of the luffa sponge fibers. It can be observed that, when compared with areca nut husk fibers, the intensity of immobilized bacterial cells on the luffa sponges has declined. This impairment in immobilization intensity can be attributed to the fact that the surface of the luffa sponge fibers is smoother compared to the areca nut husks. The rougher surface of the areca nut husks might be responsible for enhanced cell adsorption. The bacterial cells were adsorbed on the lignocellulosic biomass naturally, which can be ascribed to covalent bonding or electrostatic forces between the surface of the lignocellulosic biomass and the bacterial cells (Basak et al., 2014).

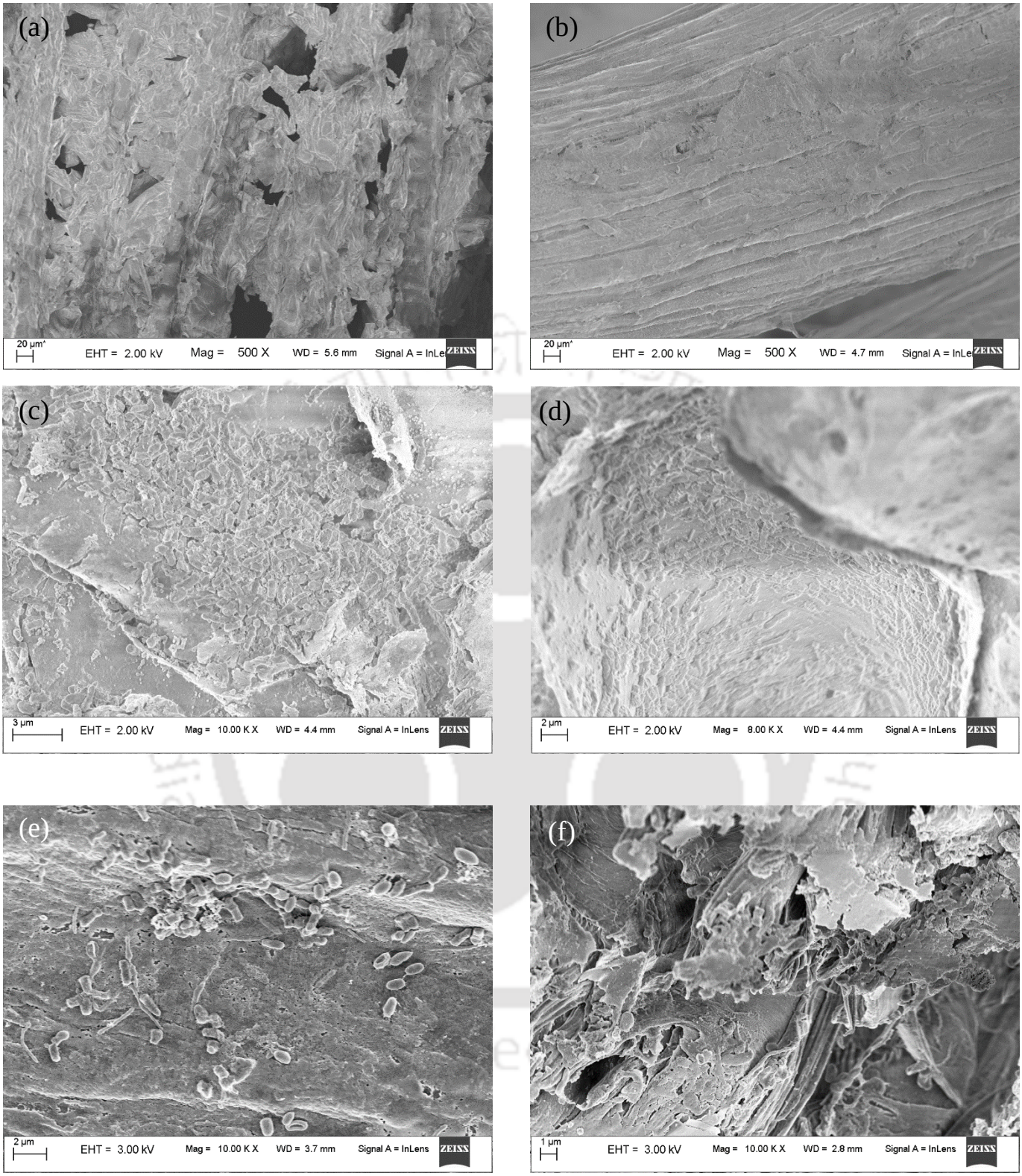

Figure 5.1. (a) FESEM image of areca nut husk; (b) FESEM image of luffa sponge fiber; (c) & (d) Mixed bacterial culture immobilized on areca nut husks; (e) & (f) Mixed bacterial culture immobilized on luffa sponge fibers

5.3 Effect of initial pH on the degradation of phenol

pH plays a vital role in utilization and degradation of phenolic compounds by microorganisms. Maintaining an optimum pH of the culture media activates enzymes that are responsible for degradation of phenol. Extreme changes in pH renders these enzymes non-functional causing denaturation and loss of catalytic activity (Bera et al., 2017). Denaturation of enzymes impairs metabolic activity of the microorganism eventually leading to rapid obliteration of bacterial biomass. Hence, an optimum initial pH should be imperatively maintained for efficient phenol degradation.

Figure 5.2a represents the phenol degradation curves at different pH values in case of areca nut husk immobilization system. It can be observed that phenol was completely degraded in 6 h for pH 7.0 and pH 8.0. Whereas, complete degradation of phenol was achieved in 8 h for pH 5.0 and pH 6.0. For higher pH values of 9.0 and 10.0, more than 99% of the pollutant was degraded in 10 h. Figure 5.2b represents the phenol degradation percentage and degradation rates for 200 mg L^{-1} phenol at different pH values in areca nut husk immobilization system. The degradation rate reached a maximum value of 0.37 h^{-1} at pH 8.0. Although complete degradation of phenol was achieved at pH 7.0, the degradation rate was lower at a value of 0.23 h^{-1}. It may be noted that when compared to the suspension cell culture reported in our previous study (Bera et al., 2017), there is a marked improvement in phenol degradation. Phenol was degraded completely in pH values of 5.0, 6.0, 7.0 and 8.0. Besides, more than 99% of phenol was also degraded at pH 9 and pH 10 during the study duration. Whereas, in our previous study using free cells (Bera et al., 2017), at pH 5.5 and pH 8.5, a severe inhibition of growth was observed which indicated minimal or no degradation of phenol.

Similarly, Figure 5.3a represents the phenol degradation curves at different pH values in luffa-sponge immobilization system. Here, it can be observed that 200 mg L^{-1} of phenol was completely degraded at pH 8.0 in 8 h. At pH 7.0, complete degradation was achieved in 10 h. Complete phenol degradation for pH 9.0 and pH 10.0 was achieved at 12 h and 14 h respectively. At pH 6.0, complete degradation of phenol took 20 h. However, at pH 5.0, phenol was not completely degraded in the duration of the study, and about 79% degradation could be achieved in 24 h. Figure 5.3b represents the phenol degradation rates and corresponding degradation percentages for luffa immobilization system. The phenol degradation rate attained a maximum value of 0.21 h^{-1} at pH 8.0.

Hence, an initial pH of 8.0 was determined to be optimum for efficient phenol degradation in both the immobilization systems, since it was at this pH value that maximum phenol degradation rates were attained. A substantial improvement of the capability of immobilized bacterial cells in tolerating harsher pH conditions can be attributed to the fact that, immobilization on the lignocellulosic supports provided ample resistance and protection (Liu et al., 2009). Moreover, immobilization makes larger number of bacterial cells available for utilization of substrate compared to the freely suspended counterparts which were not able to tolerate highly acidic or alkaline pH (Bera et al., 2017). Areca nut husk system was able to tolerate and degrade phenol over a wider range of pH when compared to luffa sponge system. This could be attributed to the fact that the luffa sponges were less porous than areca nut husks and more bacterial cells were immobilized on the areca nut husks compared to luffa sponges. The surface morphology and porosity of the husks possibly provided superior protection to the bacterial cells compared to the luffa sponges, enabling the bacterial cells to tolerate harsher conditions.

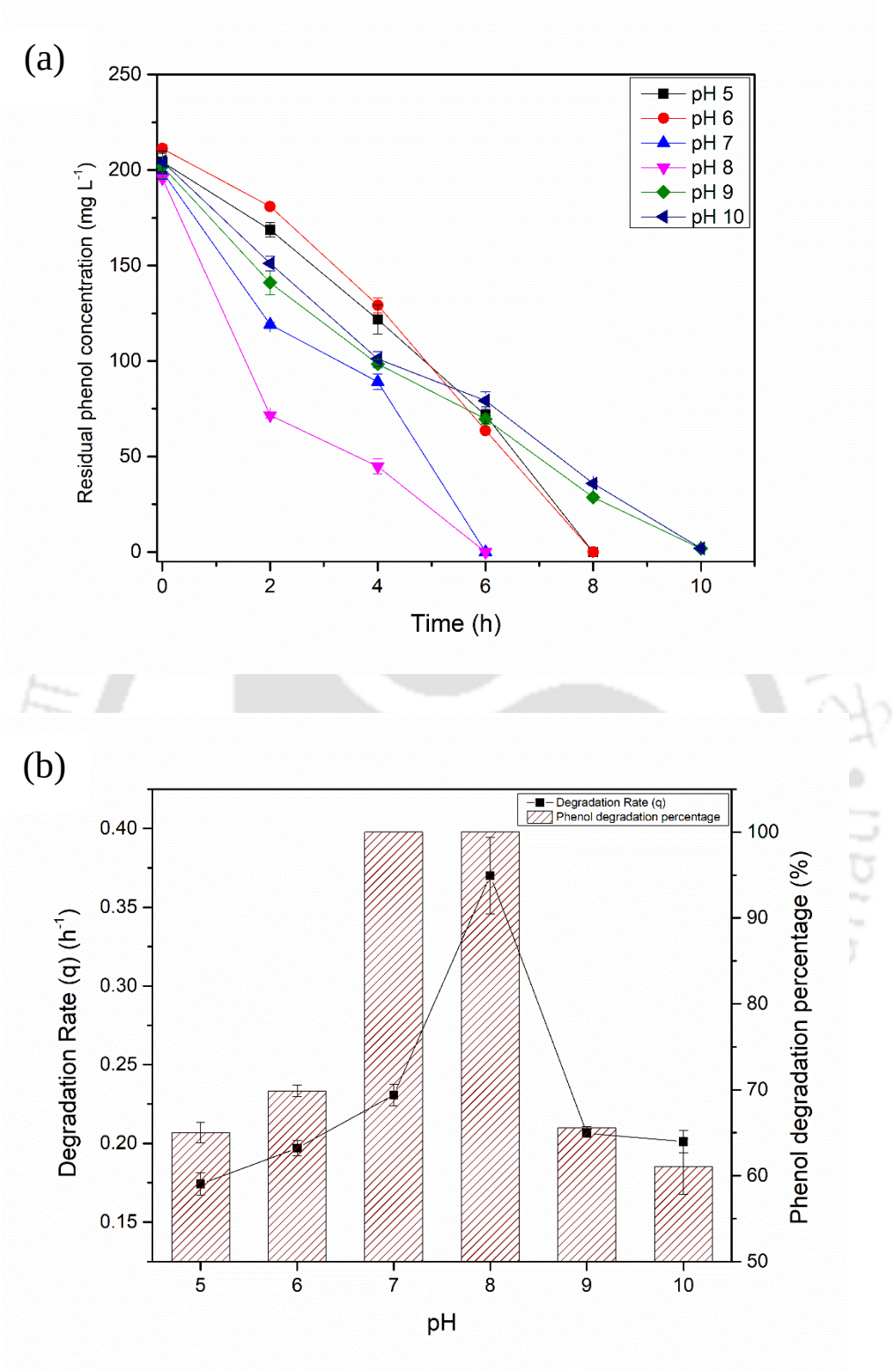

Figure 5.2. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different pH values for areca nut husk system (Phenol = 200 mg L^{-1}; Temperature = 37 °C for an experimental duration of 6 h)

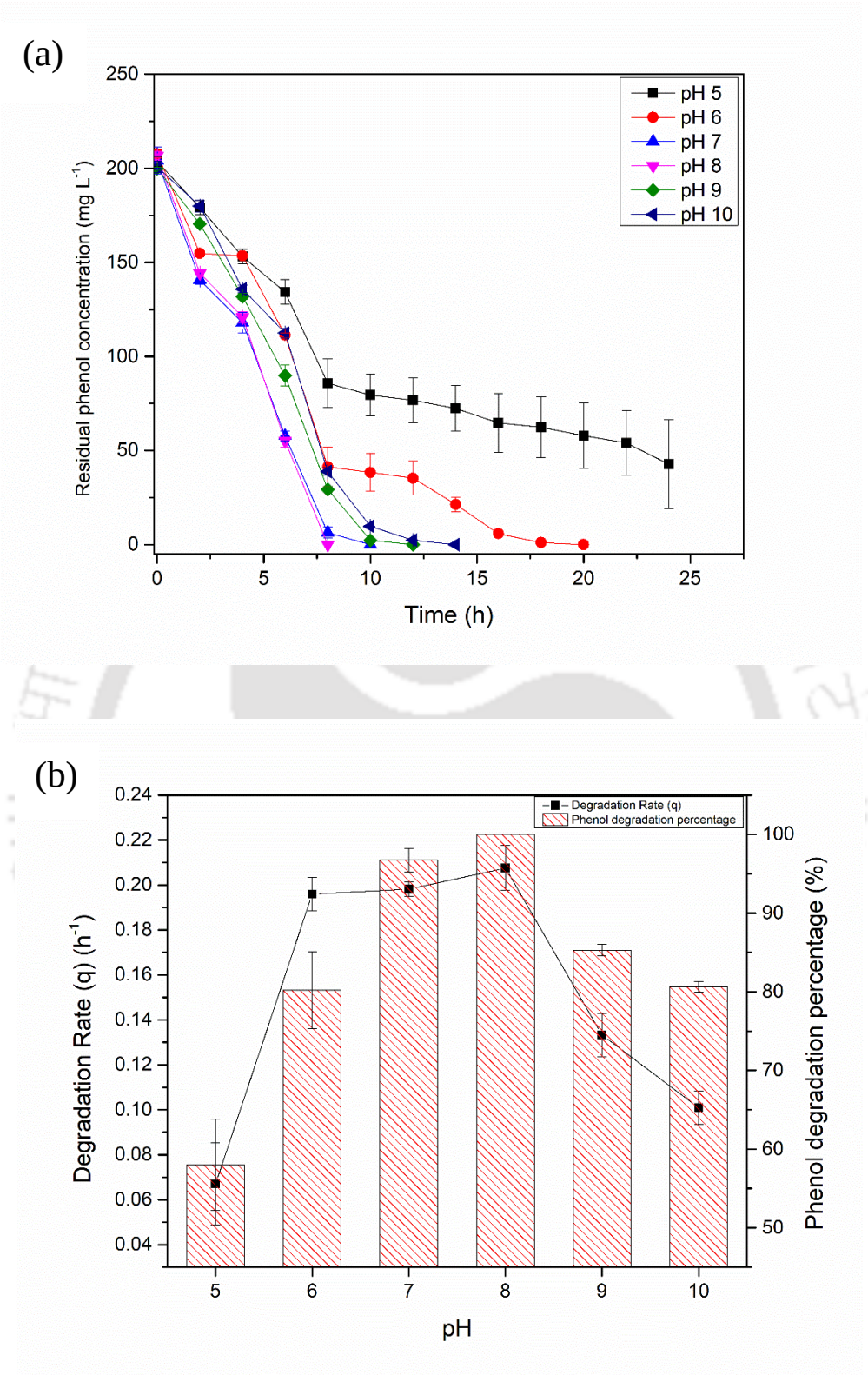

Figure 5.3. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different pH values for luffa sponge system (Phenol = 200 mg L^{-1}; Temperature = 37 °C for an experimental duration of 8 h)

5.4 Effect of incubation temperature on phenol bioremediation

Enzymes responsible for phenol degradation are vulnerable to temperature changes. Hence, an optimum temperature must be maintained for efficient utilization of a substrate. Suboptimal temperatures might lead to impaired functioning of enzymes, eventually slowing down organismal metabolism, leading to slower and inefficient consumption of phenol. Figure 5.4a and Figure 5.4b represents the degradation rates and degradation percentages of phenol at different temperatures of incubation for areca nut husk and luffa-sponge immobilization systems respectively. All experiments were performed at pH 8.0. Both cases, witnessed an improvement in degradation rate with rise in temperature. A peak degradation rate was achieved at 37 °C for both the immobilization systems. A maximum degradation rate of 0.37 h^{-1} was observed in case of areca nut husks, whereas, the maximum degradation rate was 0.21 h^{-1} in case of luffa sponges. Complete phenol degradation was achieved in 6 h and 8 h for areca nut husks and luffa sponge immobilization systems respectively. The degradation rate declined at 45 °C. In both cases, tolerance towards temperatures on either side of the optimum point was quite remarkable. For both immobilization systems, percentage degradation and degradation rate of phenol was more at 45 °C compared to 25 °C and 30 °C. Areca nut husk system could degrade 71.34% of phenol at 45 °C during the experimental duration (data not shown here). Whereas, luffa fiber system could achieve 65.18% phenol degradation at 45 °C (data not shown here). Phenol degradation rates at 30 °C for both immobilization systems were also acceptable. However, the degradation percentages of phenol at 25 °C was very low suggesting very slow substrate utilization rates. When compared to our previous study involving free cells (Bera et al., 2017), there was a remarkable improvement in phenol degradation at 45 °C. The mixed culture in free suspension exhibited a stunted growth featuring very low growth rates and almost no phenol degradation (Bera et al., 2017). The above results verified that immobilization of microorganisms improved tolerance towards temperature changes.

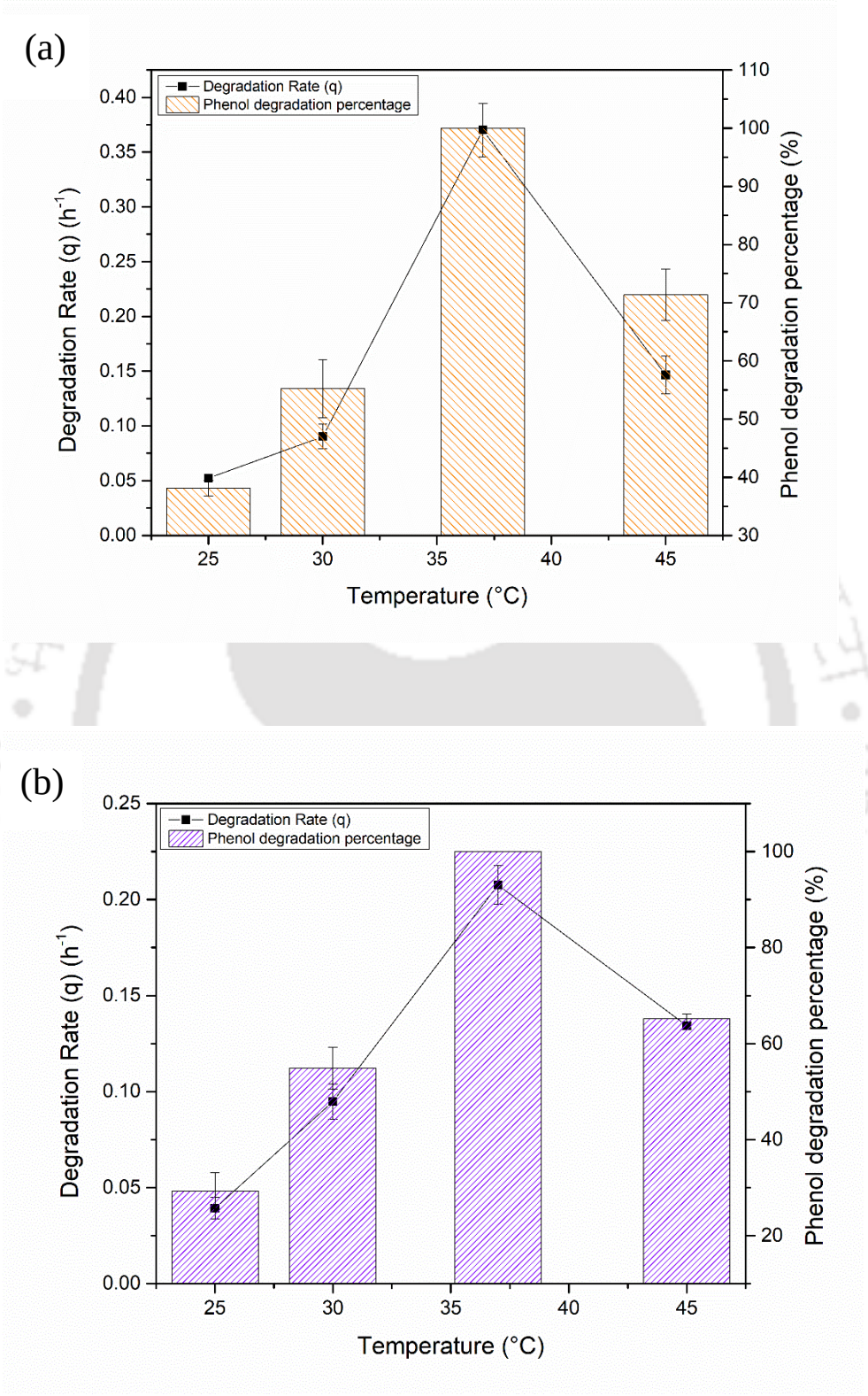

Figure 5.4. (a) Phenol degradation rates and degradation percentages @ different temperatures for areca nut husk system (Phenol = 200 mg L^{-1}; pH = 8.0 for an experimental duration of 6 h); (b) for luffa sponge system (Phenol = 200 mg L^{-1}; pH = 8.0 for an experimental duration of 8 h)

5.5 Effect of initial substrate concentration on the degradation of phenol

Initial concentration of phenol plays a crucial role in the bioremediation process, as phenol is a very potent growth inhibitor. Experiments were carried out for both lignocellulosic biomass with different initial phenol concentrations (200 mg L^{-1}, 400 mg L^{-1}, 600 mg L^{-1}, 800 mg L^{-1} and 1000 mg L^{-1}) at an optimized pH and temperature of 8.0 and 37 °C respectively.

Figure 5.5a represents the phenol degradation profiles at various initial phenol concentrations for the areca nut husk system. Figure 5.5b represents the phenol degradation rates and degradation percentages for the same. The maximum phenol degradation rate (0.37 h^{-1}) was observed at 200 mg L^{-1} phenol. The degradation rate declined with increase in phenol concentrations indicating the inhibitory effect of the substrate. The degradation rate at 1000 mg L^{-1} phenol was 0.096 h^{-1} and the areca nut husk immobilized microbes could degrade only about 21% of the initial substrate concentration. However, the experiment was continued to monitor the time taken for the depletion of phenol entirely in all the batches considered in the study. 1000 mg L^{-1} of phenol was completely depleted in 28 h.

Likewise, Figure 5.6a depicts the phenol degradation profiles for the luffa sponge immobilization system at varying phenol concentrations. Figure 5.6b represents the phenol degradation rates and degradation percentages for the luffa immobilized organisms. The maximum degradation rate was 0.21 h^{-1} and it was achieved by the organisms growing in 200 mg L^{-1} phenol. The trend of decline of the degradation rates were similar to the areca nut husk immobilization system indicating substrate inhibition. Organisms exhibited a minimum degradation rate of 0.089 h^{-1} at 1000 mg L^{-1} of phenol. However, 1000 mg L^{-1} phenol was degraded completely in 30 h by the microbes in the luffa sponge system.

The above results when compared to free cell culture from our earlier reported study revealed promising results. A free suspension of the mixed culture could degrade 1000 mg L^{-1} phenol in 96 h (Bera et al., 2017). For both our immobilization systems, there is a remarkable improvement in phenol degradation times. The phenol degradation times reduced by more than three times in both cases. This enhancement of degradation can be attributed to the fact that immobilization shields the microorganisms from the toxic effects of phenol (Wang et al., 2007). Moreover, reports also suggest that immobilization of bacterial cells enhance cellular activity by altering their physiological features in metabolism such as amplified enzyme induction, reduced specific cell growth and cell yield (Wang et al., 2007; Ali et al., 2013).

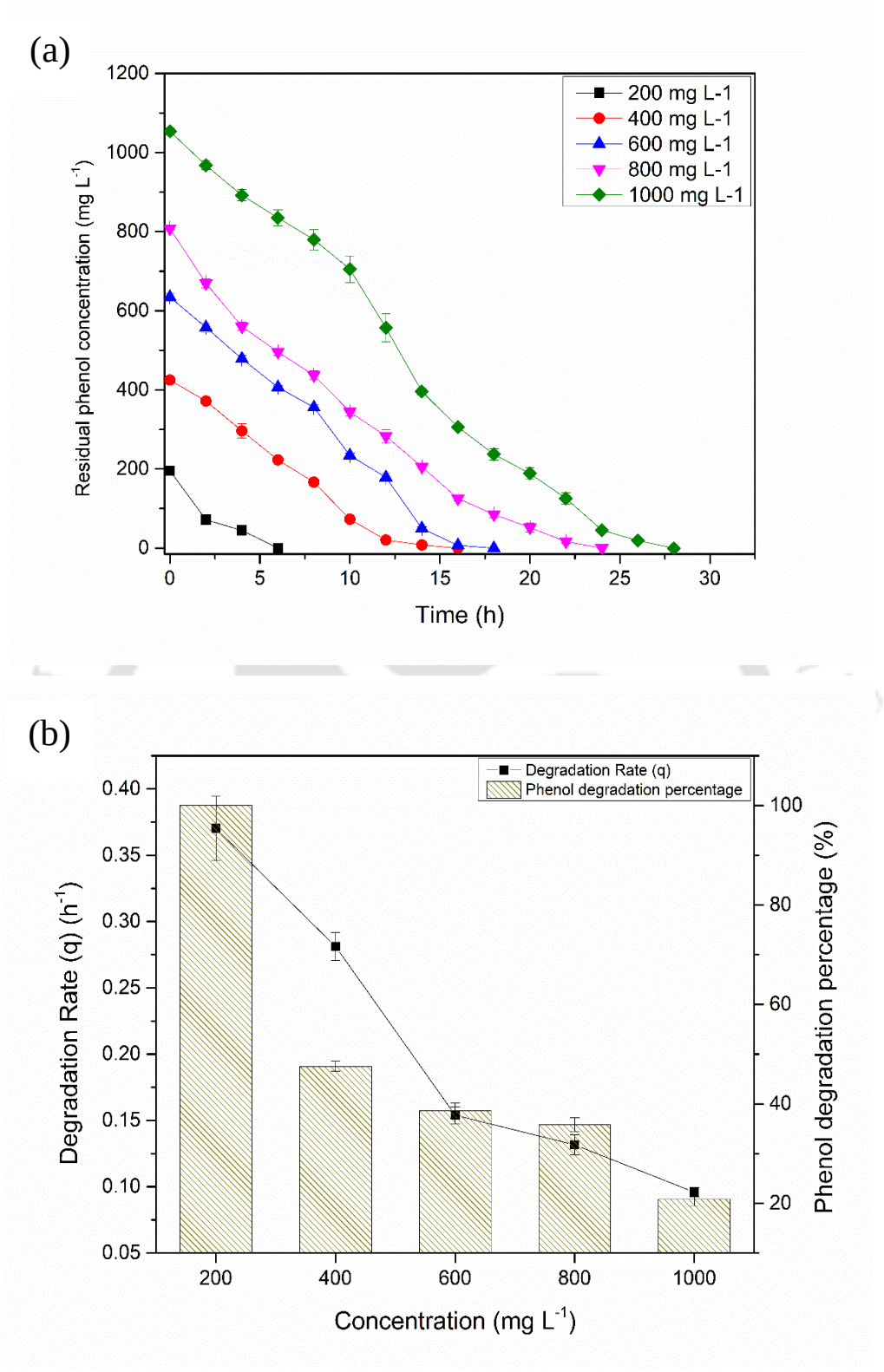

Figure 5.5. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different initial phenol concentrations for areca nut husk system (pH = 8.0; Temperature = 37 °C for an experimental duration of 6 h)

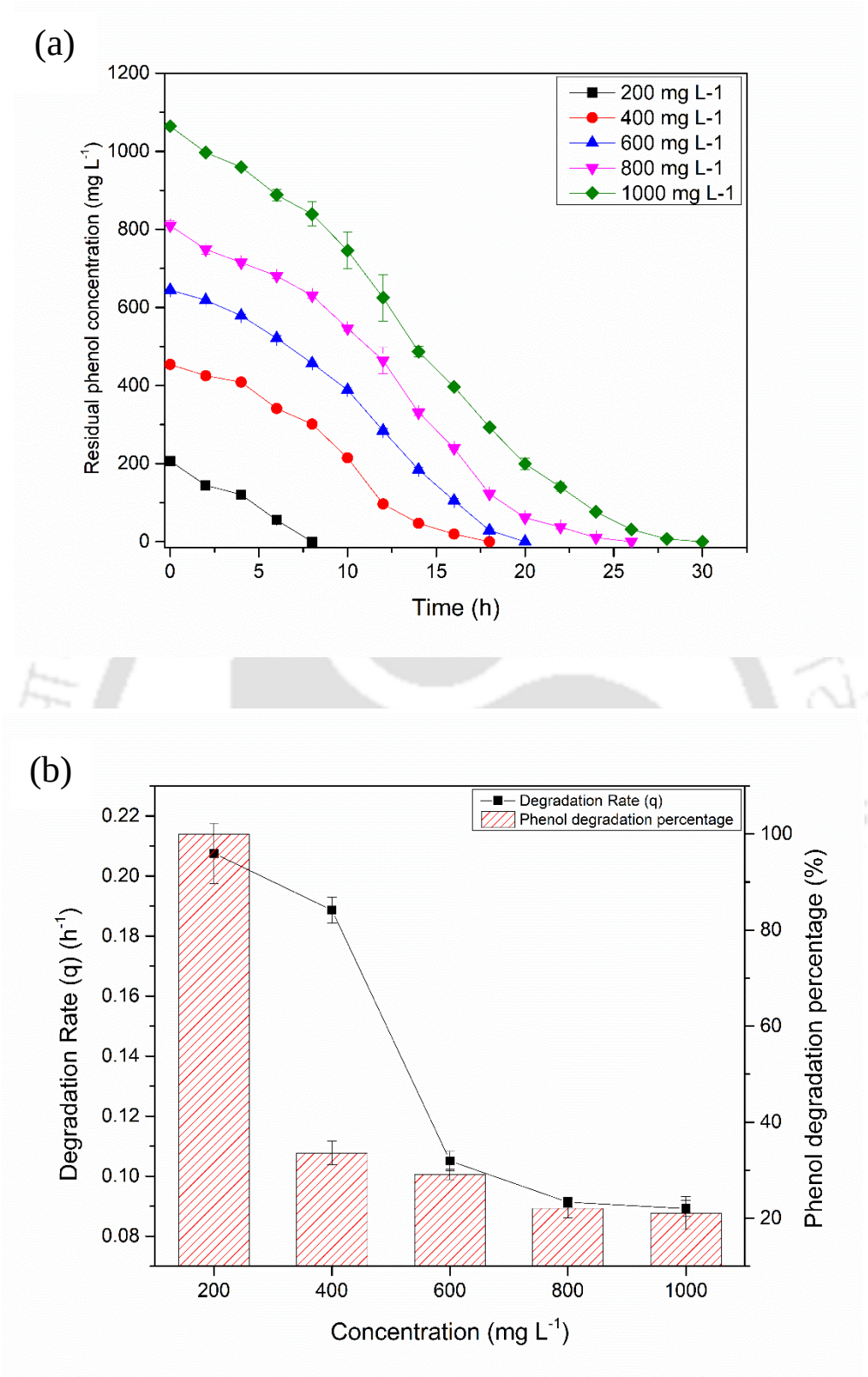

Figure 5.6. (a) Phenol degradation profiles; (b) Phenol degradation rates and degradation percentages @ different initial phenol concentrations for luffa sponge system (pH = 8.0; Temperature = 37 °C for an experimental duration of 8 h)

5.6 Modeling and kinetics of phenol degradation

Phenol degradation kinetics by the bacterial consortium immobilized on areca nut husks and luffa sponges was studied at different starting phenol concentrations (S_o) (200 - 1000 mg L^{-1}) of the growth medium. For each initial phenol concentration, experimental substrate degradation rates (q, h^{-1}) were calculated as per the description in *Section 3.13*. A maximum experimental degradation rate was achieved at a pollutant concentration of 200 mg L^{-1} for both areca nut husk and luffa sponge immobilization systems. The experimental degradation rates were comparable to the reported literatures which made use of synthetic matrices (Table 5.1). Thereafter, the degradation rates started declining, indicating substrate inhibition. The plot of experimental degradation rates versus their corresponding initial substrate concentrations was fitted to several available inhibition kinetic models via MATLAB R2017b. Figure 5.7a and Figure 5.7b depicts the fitting of experimental q with model simulated values of different inhibition models for areca nut husk and luffa sponge system respectively. Several bio-kinetic parameters were estimated by nonlinear regression analysis and portrayed in Table 5.2. The corresponding values of the coefficients of correlation (R^2) are also reported. The experimental degradation rates demonstrated by the mixed bacterial culture immobilized on the areca nut husks (0.37 h^{-1}) were found to be higher than the bacteria immobilized on luffa sponges (0.21 h^{-1}). Based on the value of correlation coefficient (R^2 = 0.9858) the best fit in case of areca nut husk system was given by Edward model with a predicted maximum degradation rate (q_{max}) of 0.0042 h^{-1}. The values of K_S and K_I were predicted to be 3.58 mg L^{-1} and 231 mg L^{-1} respectively. In case of luffa fibers, however, the best-fitted model was Aiba model with a R^2 value of 0.9903 and a q_{max} of 0.3413 h^{-1}. The values of K_S and K_I were calculated to be 39.5 mg L^{-1} and 663 mg L^{-1} respectively. The values of K_S points to the ability of the organisms to grow at a fairly higher concentration of phenol (Liu et al., 2009). Apart from this, however, other models also fitted the experimental data fairly well in case of both the immobilization systems (owing to the value of R_2). Interestingly, Haldane model, which is the most widely used inhibition model, exhibited a very low value of K_I for both the immobilization systems (although R^2 is > 0.9). K_I value indicates inhibitory concentration of phenol, which is toxic to the organisms (Liu et al., 2009) and an inferior predicted K_I value (compared to K_S value) renders the Haldane model unsuitable.

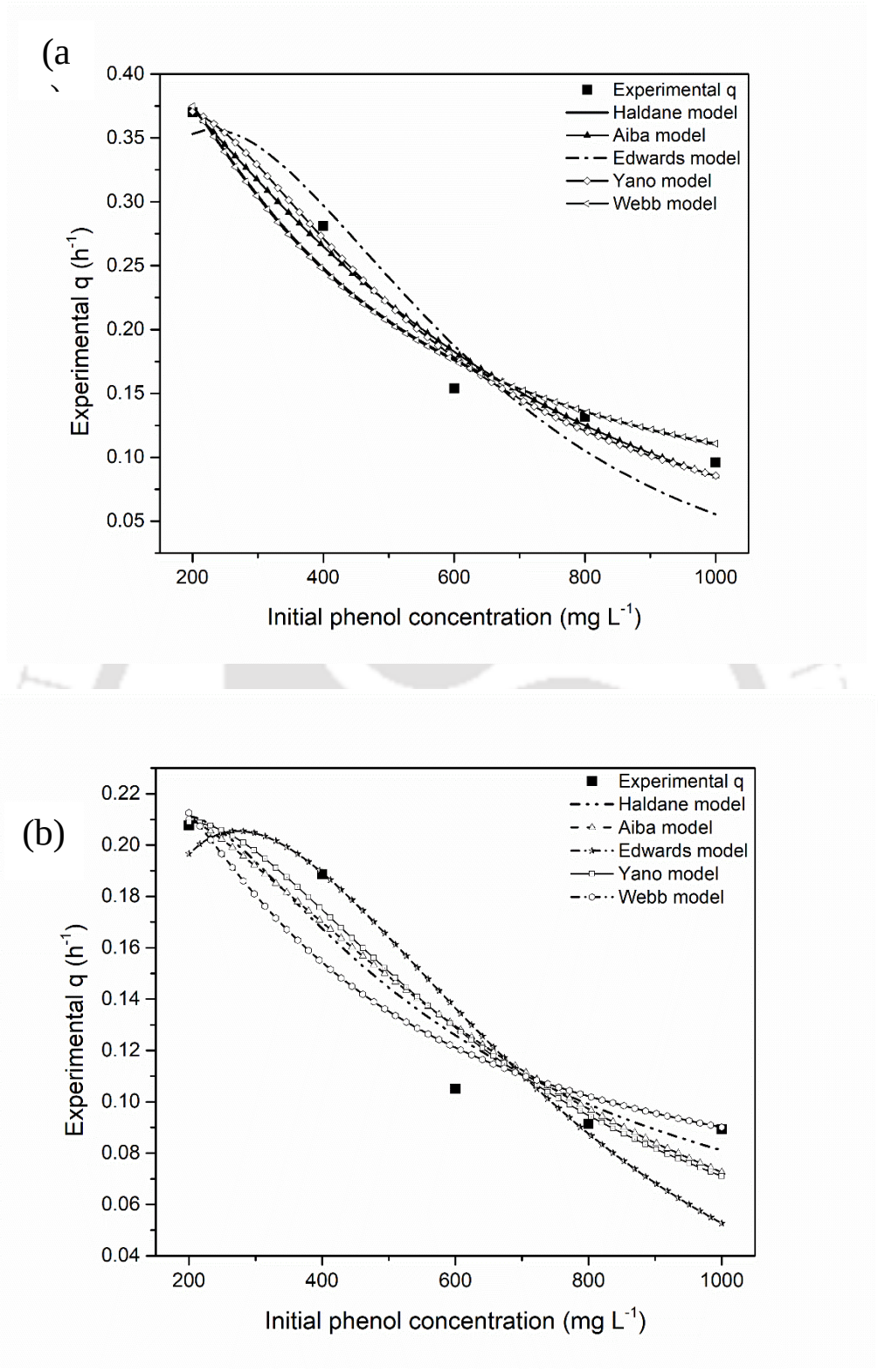

Figure 5.7. Model prediction and fitting of experimental degradation rates at various initial phenol concentrations in: (a) areca nut husk system; (b) luffa sponge system (pH = 8.0; Temperature = 37 °C)

Table 5.2. Model fitting and calculation of bio-kinetic parameters

Immobilization matrix	Model	q_{max} (h^{-1})	K_S (mg L^{-1})	K_I (mg L^{-1})	K (mg L^{-1})	R^2
Areca nut husk	Haldane model: $q = \frac{q_{max}S}{K_S+S+\left(\frac{S^2}{K_I}\right)}$ (Haldane, 1965)	11.1632	1805.71	10	-	0.9218
	Aiba model: $q = \frac{q_{max}S}{K_S+S} exp\left(\frac{-S}{K_I}\right)$ (Aiba et al., 1968)	0.6053	20.53	515	-	0.9706
	Edward model: $q = q_{max}S\left[exp\left(\frac{-S}{K_I}\right) - exp\left(\frac{-S}{K_S}\right)\right]$ (Edwards, 1970)	**0.0042**	**3.58**	**231**	-	**0.9858**
	Yano model: $q = \frac{q_{max}S}{K_S+S+(S^2/K_I)[1+S/K]}$ (Yano et al., 1966)	0.7446	105.02	893	173.17	0.9626
	Webb model: $q = \frac{q_{max}S[1+S/K]}{S+K_S+(S^2/K_I)}$ (Webb, 1963)	162.1371	27031.92	1	22632.45	0.9183
Luffa fibers	Haldane model	5.2247	2298.7	16	-	0.9681
	Aiba model	**0.3413**	**39.5**	**663**	-	**0.9903**
	Edward model	0.002	5.6	273	-	0.9815
	Yano model	0.3958	104.78	832	368.32	0.9877
	Webb model	4.1883	1224.26	13	1436.41	0.9233

5.7 Storage and reusability of the immobilized microorganisms

Stability of the immobilized cells during long-term storage is an indispensable aspect to be considered for the feasible application of the immobilized cell system (Wang et al., 2007). The effect of storage of the immobilized microorganisms at 4 °C on degradation of phenol was studied for a period of 6 weeks. Both lignocellulosic biomass (with microorganisms immobilized) were stored for a period of 7, 14, 21, 28, 35 and 42 days at 4 °C. Figure 5.8 portrays the variation of the phenol degradation rates of areca nut husk and luffa sponge systems at optimal degradation conditions for 200 mg L^{-1} phenol. It was noticed that there were minimal changes in degradation rates over the course of 6 weeks. The time taken for complete depletion of 200 mg L^{-1} of phenol was also noted for each batch. It was observed that, each batch of experiments took precisely 6 h and 8 h for complete degradation of 200 mg L^{-1} phenol (degradation data not shown) for areca nut husk and luffa sponge supports respectively. These results demonstrated that the immobilized cells had a very strong storage stability and could successfully be reused for a long time. The obtained results were at par with earlier reported studies (Wang et al., 2007; Liu et al., 2009; Banerjee and Ghoshal, 2011). It is worth mentioning that the storage of immobilized cells is possible in sterilized glass containers or flasks at 4°C without any special care and any significant deterioration of degradation efficiency.

Similar to storage, the stability of immobilized cells in long-term operation is also an important aspect, which must be considered for practical applicability of the immobilized cell system (Ali et al., 2013). In order to investigate this, areca nut husk and luffa sponge supports containing the immobilized microbes were exposed to 15 consecutive batch degradation experiments. Each batch consisted of 200 mg L^{-1} of phenol and, 6 h and 8 h of incubation for areca nut husk and luffa sponge supports respectively. It was observed that there were negligible shifts in the degradation efficiencies in case of both the supports. Moreover, 200 mg L^{-1} of phenol was biodegraded completely in the duration of the experiment for each batch. The results are comparable to earlier reported literatures (Wang et al., 2007; Liu et al., 2009) and better than a few others (Banerjee and Ghoshal, 2011; Basak et al., 2014). Structural integrity in terms of lignocellulosic biomass composition was studied after the completion of reusability studies (results discussed in the next *Section 5.8*).

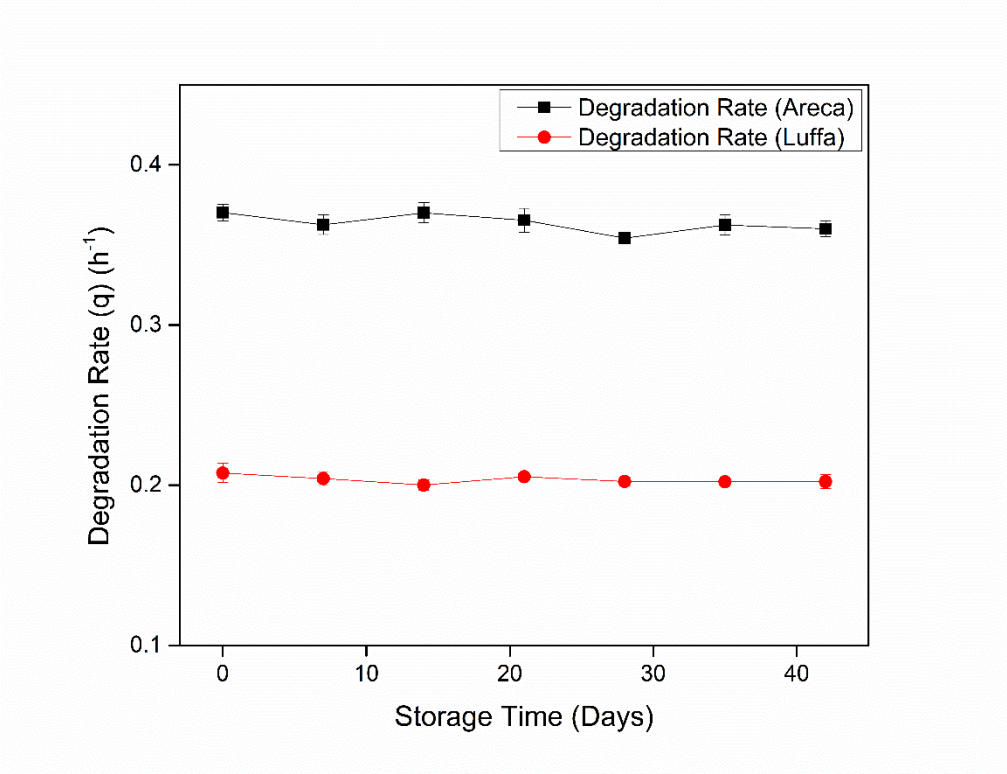

Figure 5.8. Degradation rates of the immobilized bacterial culture when stored @ 4 °C for 0, 7, 14, 21, 28, 35 & 42 days (for both areca nut husk and luffa sponge systems)

5.8 Studies on compositional analysis of the lignocellulosic biomass

Compositional analysis studies were carried out to determine the degradation of structural components of the lignocellulosic biomass (if any) due to repeated batch degradation experiments. Table 5.3 summarizes the results of the compositional analysis studies. It can be clearly observed that there were no noticeable shifts in the structural composition of both the lignocellulosic biomass even after 15 consecutive batch biodegradation experiments. These results indicate the resilience of both the lignocellulosic biomass and their resistance towards degradation by the mixed culture that was immobilized on them. Hence, it can be concluded that both lignocellulosic biomasses can be used successfully as immobilization supports for the mixed bacterial culture to achieve effective and enhanced phenol degradation efficiencies.

Table 5.3. Compositional analysis of lignocellulosic biomass

Biomass (without bacteria)	Hemicellulose content (wt%)	Cellulose content (wt%)	Lignin content (wt%)
Areca nut husk	26.8 ± 2.5	44.4 ± 1.0	14.0 ± 0.5
Luffa fiber	8.7 ± 1.2	65.1 ± 1.5	6.5 ± 1.6
Biomass (after 15 repeated batch experiments)			
Areca nut husk	24.5 ± 2.2	46.65 ± 1.3	16.2 ± 1.4
Luffa fiber	8.1 ± 1.5	63.05 ± 1.2	7.2 ± 0.7

5.9 Operation of packed bed rector for degradation of synthetic phenol feed

The two packed bed reactors setup for the two different immobilization matrices were run in batches with a fixed feed volume of 1000 mL. The feed contained 1000 mg L^{-1} of phenol as the sole source of carbon. The reactors were operated with feed recycle as shown in Figure 3.1 (in the *Chapter 3*). The reactors were fed synthetic phenol feed containing 700 mg L^{-1} of phenol continuously at a flow rate of 7 mL min^{-1} to stabilize them. Thereafter, experiments were conducted at various flow rates (3.6, 7.2, 10.8 & 14.4 mL min^{-1}) of the synthetic phenol feed. The peristaltic pumps were pre-calibrated for this purpose. Effluents were collected from the top of the reactor and analysed for residual phenol. Both the reactors showed no detectable phenol concentrations in the effluents after 48 h for the flow rate of 3.6 mL min^{-1}. At the feed flow rate of 7.2 mL min^{-1}, the effluent of the reactor packed with areca nut husk was able to degrade 94.5% of phenol at the end of 48 h. The reactor packed with the luffa fibers could however, degrade 90.5% of phenol in the same time frame. As the flow rates of the feed was increased to 10.8 mL min^{-1}, the percentage degradation of phenol at 48 h, fell to 84% and 78% for the areca nut husk and luffa fibers packed reactors respectively. Phenol degradation was highly restricted to 55% and 44% for areca nut and luffa packaged reactor respectively at a flow rate of 14.4 mL min^{-1}. The complete removal of phenol at a lower feed flow rate is most probably due to the higher retention time of the substrate. The higher percentage of phenol degradation in the areca nut husk reactor when compared to the luffa fiber reactor in the same time frame must be due to the fact that areca nut husks are a lot denser compared to the luffa fibers. This property of the areca nut husks enables them to harbor a larger population of the mixed culture. Larger the microbial population, higher is the quantity of carbon source required

by them. Therefore, phenol is used up a lot faster when compared to luffa fiber system. The graph of percentage removal of phenol against time have been portrayed in Figure 5.9.

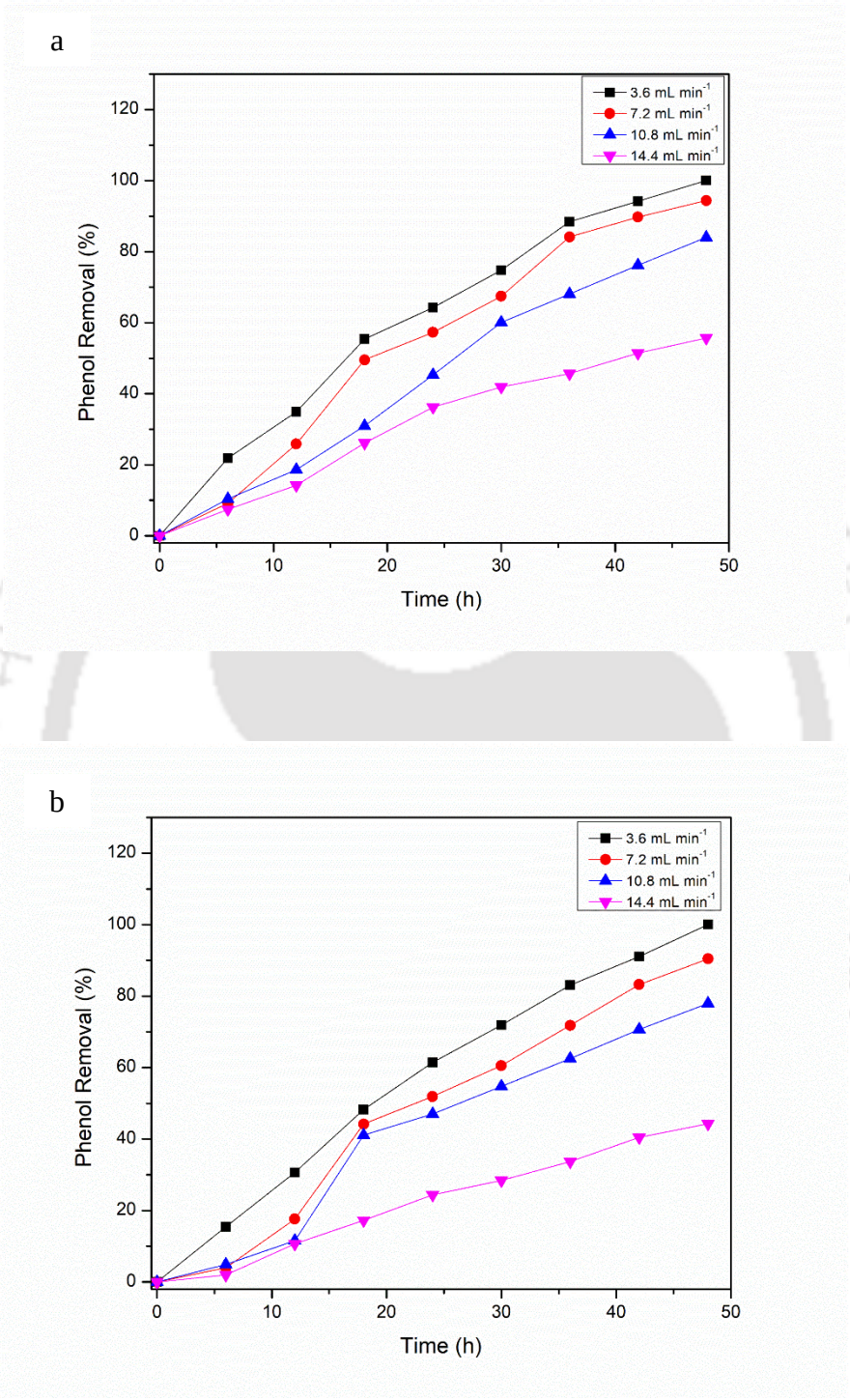

Figure 5.9. Percentage degradation of phenol at different flow rates of feed for the (a) areca nut husk packed bioreactor; (b) luffa fiber packed bioreactor

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CHAPTER 6

CONCLUSIONS AND FUTURE SCOPES

This chapter summarizes the inferences drawn from the current research and recommendations for future work.

Chapter 6

CONCLUSIONS AND FUTURE SCOPES

6.1 Conclusions

In the present research work, oil exploration sites, rigs, petroleum refineries and their fall out sites were visited. Environmental samples contaminated with petroleum crude oil were collected from these sites and phenol degrading bacteria were isolated from them. Further, the application of these isolated bacteria for the treatment of synthetic phenolic wastewater was investigated. The major conclusions drawn from this study are summarized below.

A mixed bacterial culture which was capable of degrading a phenol concentration of 1000 mg L^{-1} was isolated from petroleum crude oil contaminated sludge collected from a refinery located in Assam, India. The bacterial culture could degrade 1000 mg L^{-1} of phenol as a sole source of carbon and energy within 96 h. The mixed bacterial culture was found to primarily contain three culturable bacterial isolates namely, *Stenotrophomonas acidaminiphila* DBK (GenBank Accession no. KC992293), *Brevibacterium sp.* DBK1. (GenBank Accession no. KP231222) and *Brucella sp*. DBK2 (GenBank Accession no. KP231223). Lab scale optimization of growth parameters were carried out in several batch experiments. The optimal pH and temperature for efficient degradation of phenol was found to be 6.5 and 37 °C respectively. A maximum experimental specific growth rate was achieved at an initial phenol concentration of 100 mg L^{-1}. The mixed culture was able to efficiently consume phenol as a sole source of carbon in the concentration range of 75 mg L^{-1} to 1000 mg L^{-1}. Phenol was metabolized via the *ortho-cleavage* pathway. Growth kinetic studies were carried out and the biokinetic parameters were determined to be: μ_{max} = 0.155 h^{-1}; K_I = 400 mg L^{-1}; K_S = 11.5 mg L^{-1}.

Dried lignocellulosic biomass were the immobilization supports of choice in the present study. Dried areca nut husk and dry mature luffa sponges were chosen as the potential candidates for carrying out immobilization studies. The mixed bacterial culture was immobilized on the supports via natural adsorption. When compared to the freely suspended degradation system, the immobilized system could degrade phenol a lot faster under optimized pH and temperature conditions. Environmental factors affecting growth and phenol degradation were optimized for both areca nut husk and luffa sponge immobilization systems.

The optimum pH and temperature for efficient phenol degradation were determined to be 8.0 and 37°C respectively. The enhanced tolerance of the immobilized systems towards adverse conditions have also been demonstrated in this study. Degradation kinetic studies were carried out using different substrate inhibition models and bio-kinetic parameters were estimated via nonlinear regression analysis in MATLAB. The best-fitted model for areca nut husk and luffa sponge system was Edward model and Aiba model respectively. Interestingly, the immobilized microorganisms could be stored at 4 °C for up to 6 weeks without any noticeable loss in degradation efficiency. The immobilized microorganisms could also be used for successfully conducting up to 15 consecutive batch biodegradation experiments. Compositional analysis studies of both the lignocellulosic biomass before immobilization and after 15 successive batch experiments revealed that the structural components of the biomass were not degraded, which establishes the fact that these can be successfully used for long-term operation of reactors. These results establish the fact that areca nut husks and luffa sponge fibers have the potential to be efficiently used as inexpensive immobilization matrices for the bioremediation of environmental pollutants. Two similarly designed tubular packed bed reactors were operated with synthetic phenol feed with recycle to study the phenol degrading ability of both the immobilization supports. Flow rates of the feed were varied via peristaltic pump. At a flow rate of 3.6 mL min^{-1}, 1000 mL of synthetic phenol feed (1000 mg L^{-1}) could be treated efficiently for both the immobilization supports.

The present work has demonstrated the potential of a mixed bacterial culture consisting primarily of three less-reported bacterial species towards the degradation of phenolic wastewater. The mixed culture degraded phenol via the *ortho-cleavage* pathway. The possibility of using lignocellulosic biomass as carrier matrix for immobilization of microbes was explored in this study. Microbial cells were immobilized onto the surface of lignocellulosic materials by physical adsorption. Dried areca nut husk and mature luffa sponge fibers were selected as the immobilization matrices. Immobilization of the mixed culture improved degradation efficiency, batch repeatability and storage stability. The immobilization matrices were also used for conducting biodegradation experiment in packed bed reactors and yielded satisfactory results.

6.2 Future scope

Scope for future work can be summarized in the following points:

- Potential of these matrices in stirred tank reactor and continuous reactors can be studied.
- An extensive study of the enzymes and reaction intermediates of the phenol metabolic pathway by the mixed culture could provide an interesting insight of how the bacterial community can act in synergy for metabolizing the pollutant.
- Future work can also include studies in laboratory microcosms and field mesocosms.
- The spent media from reactors are rich in nitrates and phosphates and can be further used as nutrient supplement for algal plants and ponds. This is a very important aspect and need to be researched and implemented.
- Spent biomass from reactors can be used for the production of bio-fuels and specialty chemicals (furfural, levoglucosan etc.) by thermochemical degradation. The bio-char which is formed as a byproduct from the process can be used in soil amendment to improve crop yields.
- Further research can explore the molecular biology of the enzyme responsible for phenol degradation. Upon sequencing the gene for the enzyme, suitable primers can be designed and expression levels can be checked by quantitative PCR.

LIST OF PUBLICATIONS

Peer reviewed journals (from thesis):

- Sounak Bera, Abhijit Sarma Roy, Kaustubha Mohanty, Biodegradation of phenol by a native mixed bacterial culture isolated from crude oil contaminated site, ***International Biodeterioration & Biodegradation***, Volume 121, July 2017, Pages 107-113, ISSN 0964-8305, https://doi.org/10.1016/j.ibiod.2017.04.002.
- Sounak Bera, Kaustubha Mohanty, Areca nut (Areca catechu) husks and Luffa (Luffa cylindrica) sponge as microbial immobilization matrices for efficient phenol degradation, ***Journal of Water Process Engineering***, Volume 33, 2020, 100999, ISSN 2214-7144, https://doi.org/10.1016/j.jwpe.2019.100999.

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- Sounak Bera, Abhijit Sarma Roy, Aloke Kumar Ghoshal, Kaustubha Mohanty, Debasish Das. Phenol degradation by a native mixed bacterial culture isolated from crude oil contaminated sites of Assam, India. National Conference on Challenges in Environmental Research (NCOCER-2015) organized by Center for the Environment, IIT Guwahati, Assam, India.
- Sounak Bera, Abhijit Sarma Roy, Debasish Das, Kaustubha Mohanty. Optimization of phenol degradation by a native mixed bacterial culture isolated from crude oil contaminated sites in Assam. 57th Annual Conference of Association of Microbiologists of India & International Symposium on "Microbes and Biosphere: What's New What's Next" November 2016, organized by Gauhati University, Guwahati, Assam, India.

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Biodegradation of phenol by a native mixed bacterial culture isolated from crude oil contaminated site

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ABSTRACT

An efficient phenol degrading mixed bacterial culture was isolated from sludge sample collected from one of the refinery located in Assam, India. The mixed culture was found to consist of three bacterial strains. These were identified as *Stenotrophomonas acidaminiphila*, *Brevibacterium sp.* and *Brucella sp.* Batch phenol biodegradation experiments were carried out for a wide range of initial phenol concentrations after pH and temperature optimization. It was found that the mixed culture was able to degrade a maximum phenol concentration up to 1000 mg L^{-1} within 96 h while the maximum specific growth rate (μ_{max}) was observed at 100 mg L^{-1}. The pH and temperature required for optimal phenol degradation was 6.5 and 37 °C respectively. The mixed culture degrades phenol via *ortho*-cleavage pathway by formation of an intermediate (*cis, cis*-muconate) which was detected spectrophotometrically at 260 nm. The experimental data were validated by fitting the growth and substrate utilization curves with their corresponding simulated dynamic profiles obtained by solving Haldane's equation via MATLAB R2015a with $\mu_{max} = 0.155\ h^{-1}$ and $K_I = 400$ mg L^{-1}.

1. Introduction

Crude oil drilling and refining activities in North Eastern region of India, particularly Assam, dates back to early part of the nineteenth century. The natural environment in and around these sites have been continuously exposed to different hydrocarbon components present in crude oil since those days. The various hydrocarbons present in crude oil can be classified into aliphatic, aromatic and polycyclic aromatic hydrocarbons (PAHs). Among the different aromatic hydrocarbon contaminants present in crude oil, phenol is of utmost importance due to its recalcitrant nature and widespread prevalence in soil and water ecosystems near the drilling sites and refinery fallouts. This is attributed to its high solubility in water with reports of up to 10,000 mg L^{-1} (Bajaj et al., 2009) whereas its permissible limit in potable water is 10^{-3} mg L^{-1} as recommended by World Health Organization (WHO) (Kumaran and Paruchuri, 1997). Apart from oil drilling sites and refinery fallouts, high concentration of phenol has also been reported in wastewater discharges of other industries like phenol-formaldehyde resin, coal conversion, coking plant, leather, textiles, pharmaceutical etc by various research groups across the world (Huang et al., 2014; Kumaran and Paruchuri, 1997; Pinto et al., 2003; Wang et al., 2014). Phenol is toxic to a host range of beneficial soil and water microbes, aquatic life and plants and has adverse effect on human health even at low concentrations. It has been reported to cause liver and kidney damage, cardiac toxicity, reproductive and developmental toxicity, neurotoxicity, cardiac depression and reduced blood pressure in humans and therefore must be removed from the environment (Huang et al., 2015; Nuhoglu and Yalcin, 2005). Several research groups across the world have reported the degradation of phenol and its derivatives by various physical and chemical methods. However, these methods are quite energy consuming and are not cost effective and also cause secondary pollution (Shourian et al., 2009). On the other hand, biological methods involving potent microorganisms with phenol degrading ability are becoming increasingly popular, as they are inexpensive, eco-friendly and do not cause secondary pollution (Liu et al., 2016). Numerous approaches like immobilization of microbial cells, addition of readily utilizable carbon source as co-substrate or adaptation of microbial cells to high phenol concentrations have been put forward in order to resist the toxicity. However, adaptation of microbial cells to high phenol concentration has been

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Areca nut (*Areca catechu*) husks and Luffa (*Luffa cylindrica*) sponge as microbial immobilization matrices for efficient phenol degradation

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ABSTRACT

Immobilization of microorganisms is a widely adopted strategy for the efficient degradation of hazardous organic compounds like phenol. Microorganisms can be immobilized in synthetic or natural matrices. In this work dried areca nut (*Areca catechu*) husks and luffa (*Luffa cylindrica*) sponge fibers were used as alternative and inexpensive natural matrices for microbial cell immobilization. The potential of these immobilization systems for the effective bioremediation of phenolic wastewater was explored. A bacterial consortium was isolated by enriching a sludge sample from a petroleum refinery in high phenol concentrations. The mixed bacterial culture was capable of degrading 1000 mg L^{-1} of phenol in suspension cultures. The bacterial consortium was immobilized on the lignocellulosic matrices. Phenol degradation studies were performed in batches to optimize the physicochemical parameters. Optimum pH and temperature for phenol degradation was found to be 8.0 and 37 °C. At an optimum pH and temperature, the areca nut husk and luffa sponge systems immobilized with the mixed culture could degrade 1000 mg L^{-1} phenol in 28 h and 30 h respectively. The highest experimental degradation rates in areca nut husk and luffa sponge systems were 0.37 h^{-1} and 0.21 h^{-1} respectively at 200 mg L^{-1} phenol. Degradation kinetic studies were carried out using several inhibition models. Further studies revealed that both matrices with immobilized microbes could be reused for several successive batch degradation experiments and stored at 4 °C for several weeks without any noticeable loss in degradation efficiency.

1. Introduction

Phenols or phenolics are a class of organic chemical compounds well known for their toxic effects on microbes, aquatic flora and fauna, animals and humans even at low concentrations. The simplest of them is phenol, which is the most sought-after pollutant due to its recalcitrant and ubiquitous nature. Phenolic compounds are very frequently found in run-offs and effluents of various industries such as petrochemicals, coal conversion, coking plant, leather, textiles, pharmaceutical, olive oil, paint, polycarbonate resin, ink, paper, perfume etc. [1]. Phenol has been categorized in the list of significant contaminants by the US Environmental Protection Agency (USEPA) due to its high solubility in water sources and toxicity [2]. The permissible limits of phenol in potable waters has been set to 1 μg L^{-1} by the World Health Organization (WHO) [3]. Hence, removal of phenol from wastewater is essential before its release into the environment.

Over the years researchers have shown that bioremediation is a cost-effective and eco-friendly alternative to the traditional physico-chemical methods used for phenol removal [4–7]. Although sustainable, the inhibitory effect of phenol at high concentrations pose a serious challenge for wastewater remediation by microorganisms [8]. Hence, several strategies have been explored to overcome this problem. Most common approaches include use of genetically modified organisms, acclimatization of organisms to lethal phenol concentrations, and immobilization of organisms [9]. Immobilization of microbial cells offer several benefits over other approaches for treatment of phenolic wastewaters: (1) fortifies microbes against the toxic effects of phenol and harsh environments, (2) helps reach a high microbial concentration hence achieving a high contaminant degradation rate, (3) helps easy recovery and re-use of microbial cells for repeated degradation of target pollutants and (4) helps microbial cells stay viable during storage for extended durations with little or no loss of degradation capacity hence ensuring stable long-term operation [5,10,11]. Immobilization by entrapment of microbial cells in matrices such as calcium-alginate beads [8,12] or a more mechanically stable PVA (poly vinyl alcohol) [4,6,13] were reported earlier. Entrapment of whole cells using chitosan beads [10] or hybrid systems involving alginate-chitosan, PVA-alginate and glycerol-alginate [9] were also implemented. Immobilization studies

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