EFFECTS OF OPERATIONAL FACTORS ON BIODEGRADATION OF PHENOL AND *p*-NITROPHENOL BY SUSPENDED AND IMMOBILIZED ACTIVATED SLUDGE

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	Alpha
β	Beta
R^2	Correlation Coefficient
C_{crt}	Critical Substrate Concentration
k_1	First-order rate constant
F	Fisher Variation Ratio
K_s	Half-saturation constant
N_i	Initial number of beads
S_o	Initial Substrate Concentration
q_{max}	Maximum Specific Degradation Rate
μ_{max}	Maximum Specific Growth Rate
λ_{max}	Maximum Wavelength
M_w	Molecular Weight
N_t	Number of beads left
rpm	Revolutions per minute
S_t	Substrate Concentration at time interval
K_i	Substrate-inhibition constant
t	Time
w/v	Weight per volume
k_0	Zero-order rate constant

LIST OF ABBREVIATIONS

3D	Three-Dimensional
ANOVA	Analysis of Variance
AS	Activated Sludge
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical Oxygen Uptake
BT	Benzenetriol/Hydroxyquinol
CCD	Central Composite Design
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
HQ	Hydroquinone
MLSS	Mixed Liquor Suspended Solid
MLVSS	Mixed Liquor Volatile Suspended Solid
MNP	*m*-Nitrophenol
NC	Nitrocatechol
NCBI	National Center for Biotechnology Information
NR	Nutrient Rich
ONP	*o*-Nitrophenol
PCR	Polymerase Chain Reaction
PNP	*p*-Nitrophenol
PVA	Polyvinyl alcohol
RMSE	Root Mean Square Error
rRNA	Ribosomal Ribonucleic Acid

RSM	Response Surface Methodology
SBR	Sequencing Batch Reactor
SEM	Scanning Electron Microscope
SOUR	Specific Oxygen Uptake Rate
SV_{30}	Settled Sludge Volume
SVI	Sludge Volume Index
TCA	Tricarboxylic Acid Cycle
UV	Ultraviolet
UV-Vis	UV-Visible
VSS	Volatile Suspended Solids

KESAN FAKTOR OPERASI TERHADAP BIODEGRADASI FENOL DAN *P*-NITROFENOL OLEH ENAP CEMAR TERAKTIF TERAMPAI DAN PEGUN

ABSTRAK

Kehadiran sebatian fenolik dalam efluen industri mengakibatkan masalah alam sekitar yang serius disebabkan oleh kadar ketoksikan dan bioakumulasi yang tinggi. Kajian ini bertujuan untuk mengkaji kecekapan dan kinetik biodegradasi aerobik fenol dan *p*-nitrofenol (PNP) masing-masing oleh enap cemar teraktif terampai dan pegun dalam keadaan operasi yang berlainan. Biodegradasi phenol oleh enap cemar teraktif mematuhi model kinetik tertib sifar ($R^2 > 0.9$), dengan kadar biodegradasi maksimum diperhatikan pada kepekatan fenol 50 mg/L. Kinetik biodegradasi PNP mematuhi model Haldane dengan kepekatan PNP kritikal, C_{crt}, 85.14 dan 216.8 mg/L masing-masing diperoleh bagi kepekatan aklimatisasi 250 dan 500 mg/L. Kesan perencatan substrat terhadap kinetik pertumbuhan kedua-dua enap cemar teraktif yang teraklimatisasi kepada fenol dan PNP telah dihuraikan dengan baik oleh model Haldane. Memandangkan nilai K_s/K_i yang lebih tinggi, enap cemar teraktif yang teraklimatisasi kepada PNP memaparkan rintangan yang lebih rendah ke arah perencatan substrat berbanding dengan enap cemar yang teraklimatisasi kepada fenol walaupun melalui proses aklimatisasi. Strain pengurai fenol (*Acinetobacter* sp USM dan *Rhodococcus* sp. USM1) dan strain pengurai PNP (*Caballeronia* sp. USM2) telah berjaya diasingkan daripada enap cemar teraktif. Gen dioksigenase dikesan dalam bakteria pengurai fenol dan laluan metabolik yang berpotensi untuk biodegradasi fenol adalah laluan orto-fisi gelangan melalui katekol atau protokatekuat. Di samping itu, kajian ini mengkaji pemodelan dan pengoptimuman pemboleh ubah proses bagi

pemegunan enap cemar teraktif dalam kriogel polivinil alcohol (PVA)/alginat menggunakan kaedah permukaan sambutan (RSM). Manik kriogel PVA/alginat-AS yang disintesis digunakan untuk biodegradasi PNP. Keputusan ANOVA menunjukkan bahawa model yang diperoleh adalah berwibawa dan ketara dalam meramalkan kadar biodegradasi PNP dan pemecahan manik. Kesan interaksi oleh lima pemboleh ubah iaitu bilangan kitaran pembekuan-pencairan, saiz manik, kepekatan PVA, alginat dan $CaCl_2$ telah dianalisis secara statistik melalui reka bentuk komposit pusat (CCD). Kepekatan alginat memberi kesan yang paling ketara kepada kedua-dua sambutan. Interaksi kepekatan alginat dengan $CaCl_2$, kepekatan alginat dengan bilangan kitaran pembekuan-pencairan dan kepekatan alginat dengan saiz manik didapati menjejaskan prestasi kryogel PVA/alginat-AS dengan ketara terhadap kadar biodegradasi PNP dan pemecahan manik. Hasil yang diramalkan menunjukkan bahawa kadar biodegradasi PNP maksimum sebanyak 7.4 mg/L·h dan 0% kepecahan minimum boleh dicapai dengan 8.0% PVA, 1.411% alginat, 3.012% $CaCl_2$, 3.659 mm manik dan 3 kitaran proses pembekuan-pencairan. Pada 100 mg/L PNP, kryogel PVA/alginat-AS boleh diguna semula sebanyak 20 kitaran biodegradasi PNP secara berturut-turut dan tiada kehilangan ketara dalam kecekapan biodegradasi.

EFFECTS OF OPERATIONAL FACTORS ON BIODEGRADATION OF PHENOL AND *P*-NITROPHENOL BY SUSPENDED AND IMMOBILIZED ACTIVATED SLUDGE

ABSTRACT

The presence of phenolic compounds in industrial effluents give rise to serious environmental problems owing to their high toxicity and bioaccumulation rate. This study aims to investigate the efficacy and kinetics of aerobic biodegradations of phenol and *p*-nitrophenol (PNP), respectively, by suspended and immobilized activated sludge at different operational conditions. Phenol biodegradation by suspended activated sludge fitted well into zero order kinetic model ($R^2 > 0.9$), with maximum degradation rate observed at initial phenol concentration of 50 mg/L. The PNP biodegradation kinetics was well described using Haldane model with critical PNP concentration, C_{crt}, of 85.14 and 216.8 mg/L obtained for acclimatization concentration of 250 and 500 mg/L, respectively. The effect of substrate inhibition on the growth kinetics of both phenol- and PNP-acclimated activated sludge was well described by Haldane model. In view of its higher value of $K_s/K_{i.}$, the PNP-acclimated activated sludge exhibited lower resistance towards the substrate inhibition in comparison to phenol-acclimated activated sludge albeit the acclimation process. Phenol-degrading (*Acinetobacter* sp. USM and *Rhodococcus* sp. USM1) and PNP-degrading (*Caballeronia* sp. USM2) strains were successfully isolated from the activated sludge. Respective dioxygenase genes were detected in phenol-degrading bacteria and the potential metabolic pathways of phenol biodegradation was ring *ortho*-fission pathway through catechol or protocatechuate. In addition, this study investigated on the modelling and optimization of process variables for the

immobilization of PNP-acclimated activated sludge in polyvinyl alcohol (PVA)/alginate cryogel using response surface methodology (RSM). The performances of PNP biodegradation using PVA/alginate-AS cryogel beads produced were assessed. The ANOVA results showed that the obtained models were reliable and significant in predicting PNP biodegradation rate and bead breakage. The interactive effects of five variables, namely number of freeze-thaw cycle, bead size as well as PVA, alginate and $CaCl_2$ concentrations, were statistically analyzed via central composite design (CCD). Alginate concentration contributed the most significant effect on both responses. The interaction of alginate with $CaCl_2$ concentration, alginate concentration with number of freeze-thaw cycle and alginate concentration with bead size were found significantly affected the performance of PVA/alginate-AS cryogel beads on PNP biodegradation rate and bead breakage. The predicted results showed that maximum PNP biodegradation rate of 7.4 mg/L·h and minimum bead breakage of 0 % could be achieved with 8.0 wt % of PVA, 1.411 wt % of sodium alginate, 3.012 wt % of $CaCl_2$, 3.659 mm of bead and 3 cycles for freeze-thaw process. At 100 mg/L PNP, the PVA/alginate-AS cryogel beads could be reused up to 20 consecutive cycles of PNP biodegradation with no significant loss in biodegradation efficacy was observed.

CHAPTER 1

INTRODUCTION

1.1 Background study

Water is undeniably important for all organisms on earth. However, the world is facing the clean and fresh water crisis due to water pollution in this era of science and technology development. Water pollution by toxic organic compounds such as phenolic compounds became a prominent environmental issue over the decades due to their extensive use in industries. Wastewater containing these compounds gives rise to serious discharge problems due to their high bio-accumulation and toxicity. Therefore, the stringent regulation standard coupled with the increasing concern for sustainable water resources had triggered all in seeking efficient wastewater treatment technologies.

In comparison with physicochemical methods, biological treatment processes for toxic organic compounds, especially phenolic compounds, have been shown to be more cost-effective, practical, reliable and low possibility of by-product formation [1]. The biological approach using pure or mixed cultures in batch or continuous process is extensively employed for phenolics-containing wastewater treatment [2–4]. Activated sludge process is one of the widely used biological approaches in treating wastewater using mixed cultures. With regards to biodegradation of phenolic compounds, mixed cultures are more efficient and practical compared to pure culture in attaining complete mineralization of organic pollutants.

The performance and efficiency of biodegradation can be further improved by using acclimated activated sludge [4–6]. Acclimatization promotes the growth of new biomass population that is adapted to the targeted compounds and enhances their biodegrading ability [6]. Numerous studies had been worked on the biomass

acclimatization for phenols removal, however, there is lack of information on the effect of acclimation concentration of phenolic compounds towards the performance of activated sludge. Hence, a comprehensive study on the performance and dynamics characteristics during the phenols biodegradation under different operational conditions using acclimated activated sludge was further explored in this study.

It is also of great significance to identify the potential degrading microorganisms that can endure at relatively harsh conditions. Various bacterial strains have been reported in exhibiting the ability of outcompeting non-indigenous microorganisms during the treatment of polluted environment [7–9]. The biodegradation pathway of phenolic compounds by bacteria is well known. The biodegradation pathway can be verified through identification of intermediates or gene expression for better understanding on biochemical steps in degradation [10]. The present study worked on both chemical analysis and molecular technique to elucidate the biodegradation mechanisms of phenol and PNP for a comprehensive knowledge about the degradation processes.

Besides, immobilization technologies are becoming a promising alternative to help in advancing the tolerance against harsh environment and achieving more efficient operation systems [11–13]. The biomass entrapment method for cryogelation involving the use of macroporous polymeric materials is of great interest nowadays. One of the most attractive features of cryogels is their unique macroporosity properties for processing suspension of microbial cells [14,15]. Among porous polymer materials with good chemical and physical stabilities, polyvinyl alcohol (PVA) and alginate with their non-toxic and mild gelling characteristics were the most frequently used for cryogel synthesis [16,17].

A range of processing parameters can be tailored to alter the properties of cryogel and therefore its application. Thus, it is necessary to establish an optimum condition for biomass immobilization to be employed in biodegradation process. The optimization for biomass immobilization condition using multivariate statistical approach is preferred compared to conventional sequential optimization approach in order to avoid overlook the interactive effects of each process variable and minimize the errors [18,19]. In this regard, statistical design of experiment (DOE) such as response surface methodology (RSM) helps in analyzing and determining the optimum condition of interactive factors within the design space of the experimental study [20]. The information about the optimization for biomass immobilization conditions in cryogel through multiple responses is still lacking. Hence, further exploration on this optimization and interactions of each process variable is deemed necessary.

1.2 Problem statements

Numerous technologies designed to treat wastewater containing toxic organic compounds before discharged to the environment have been adopted. The physicochemical methods such as adsorption and oxidation are frequently used in reducing the amount of contaminants present, however, these methods will cause the formation of secondary by-products. Bioremediation that employs biological activities by microorganisms is becoming promising alternative, especially with acclimatization process which could alleviate the inhibition at high initial concentration of toxic organic compounds. The extent of biodegradation is affected by numerous operational parameters such as initial substrate, biomass and acclimation concentrations. Initial acclimation and biomass concentrations are always be constant parameters in most of the studies. As such, the interactive effects among these parameters are not understood

in depth. Besides that, it is of great interest to identify the potential degrading microorganisms that can endure at harsh environment and their respective degradative pathways in order to mitigate the inhibition. Furthermore, the biomass immobilization in cryogel for wastewater treatment is also receiving interest in advancing the tolerance against harsh environment. The ability of altering the properties of cryogel is deemed important to employ in biodegradation process. However, to date, the research on phenol and PNP degradation by immobilized biomass is still lacking, especially in optimization of biomass immobilization condition. Besides that, most of the researches worked on optimization studies through the conventional sequential approach instead of multivariate statistical approach. Hence, the further exploration on the optimization using statistical approach is worth for discussion since it is more helping in analyzing and determining optimum condition of interactive factors.

1.3 Research objectives

In order to address the aforementioned problems, the following objectives were aimed to be achieved:

(i) To evaluate the effect of operational factors including acclimatization concentration, biomass concentration and initial substrate concentrations on the removal efficiency of phenol and PNP.

(ii) To investigate the kinetics of biodegradation of phenol and PNP under different operational conditions.

(iii) To identify the potential degrading bacteria strains and mechanism pathways for biodegradation of phenol and PNP.

(iv) To optimize the effect of bead size, PVA concentration, sodium alginate concentration, calcium chloride concentration and freeze-thaw cycles on formation

of PVA-alginate cryogel immobilized activated sludge using response surface methodology method (RSM)

1.4 Scope of study

This study focuses on the effects of different operational and immobilization conditions towards phenol and PNP biodegradations, including initial acclimation, biomass and substrate concentrations using suspended and immobilized activated sludge. Their growth and biodegradation kinetics were evaluated simultaneously in order to be more accurate in analyzing and interpreting their interactive effects in biodegradation. Besides that, further insight on the identification of potential degrading microorganisms and their preferred degradative mechanisms for phenol and PNP were also studied through the genotype data in microorganisms. In order to enhance the bioprocess control and tolerance against harsh environment, the immobilization technique was employed, in which entrapped the acclimated activated sludge in polymer matrix. Cryogelation using PVA and alginate polymers was used in this present study. The process variables (bead size, PVA concentration, sodium alginate concentration, calcium chloride concentration and freeze-thaw cycles) were studied and optimized for the synthesis of PVA/alginate cryogel beads using multivariate statistical approach (eg. RSM) as well as their interactive effects. The assessment for reusability of PVA/alginate cryogel beads was also done in PNP biodegradation.

1.5 Overview of thesis

This thesis consists of five chapters, including the background, experimental procedures and research findings of the projects. Chapter 1 is the introduction highlighting the significance of acclimatization and microbial identification in improving biodegradation process. Besides, it also briefs about the biomass immobilization using cryogelation and optimization approach.

Chapter 2 is a comprehensive literature review, which elaborates the source and effect of phenolics, microbial dynamics, metabolic pathways and immobilization technique. Some detailed information about cryogel fabrication and the fundamental for optimization method (response surface methodology) had also been discussed here.

In Chapter 3, both phenol and PNP biodegradation studies was conducted, employing activated sludge acclimated to low and high phenol and PNP concentrations. Their biodegradation kinetics or performance under different operational conditions were compared, which is in line with objectives (i) and (ii). Additionally, this chapter also relates objective (iii) discussing the identification of phenol- and PNP- degrading microbes and further insight for the biodegradation mechanisms for both phenol and PNP from the information of genotype data in microorganisms.

Chapter 4 focuses on the modelling and optimization of process variables for synthesis of PVA/alginate-AS cryogel beads in enhancing both the PNP biodegradation rate and the mechanical stability using response surface methodology. The interaction effects of all process parameters: bead size, number of freeze-thaw cycle, PVA, alginate and calcium chloride concentration, were analyzed and discussed.

The experiments are associated to objective (iv). Moreover, the reusability of the optimized cryogel beads is also tested and further studied.

Chapter 5 concludes the research findings for the project. Recommendations for the future work of this research are also had been provided to broaden the prospect of using potential degrading strains for the wastewater treatment as well as the immobilized cryogel beads which potential in other applications.

CHAPTER 2

LITERATURE REVIEW

2.1 Phenol and *p*-nitrophenol (PNP)

Phenol, also known as carbolic acid, benzophenol or hydroxybenzene, is a colourless and crystalline substance with characteristic odour. It is an aromatic hydrocarbon with the chemical formula of C_6H_5OH. The phenol molecule that consists of a benzene ring bonded to a hydroxy group (-OH) is a basic structural unit for various synthetic organic compound. Phenol is soluble in water and organic solvents because of its ability to form strong hydrogen bonds.

Nitrophenols are xenobiotic organic compounds produced by extensive urbanisation and industrialisation in the past few decades. They are nitrated aromatic compound consisting of benzene ring, nitro ($-NO_2$) and hydroxyl (-OH) groups. Mononitrophenols (chemical formula: $C_6H_5NO_3$) are the simplest form of the nitrophenols, which appeared as three isomers: (i) *o*-nitrophenol (ONP), (ii) *m*-nitrophenol (MNP) and (iii) *p*-nitrophenol (PNP). PNP is the most common isomer of mononitrophenols and forms colorless to slightly yellow odorless crystals at room temperature with sweetish and burning taste. Figure 2.1 shows the chemical structures of phenol and PNP; while the physical and chemical properties of phenol and PNP are shown in Table 2.1 [21,22].

(a) (b)

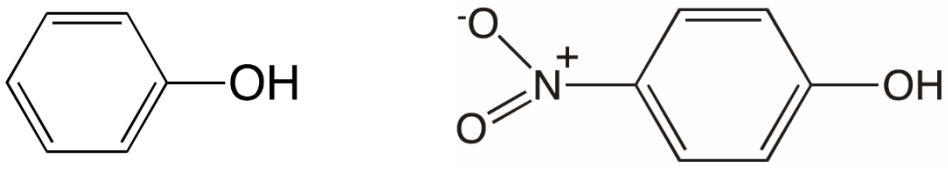

Figure 2.1 Chemical structures of (a) phenol and (b) *p*-nitrophenol

Table 2.1 Physical and chemical properties of phenol and PNP

Parameters	Unit	Phenol	PNP
Molecular mass	g/mol	94.1	139.11
Melting point	°C	42.8	113-114
Boiling point	°C	182	279
Density (at 20 °C)	g/cm^3	1.05	1.48
Water solubility (at 20 °C)	g/L	83.0	14.8
Acidity in water (pKa)	-	9.95	7.15
Toxicity (EC_{50})	mg/L	270	64.0

2.1.1 Sources and effects

Natural sources of phenols in water pollution include animal wastes and decomposition of organic matter (dead plants and animals), while industrial, domestic and agricultural activities constitute the anthropogenic sources in water. These phenols usually present in the effluents or wastewater at different concentrations. For instance, coal processing (9-6800 mg/L), petrochemical plant (2.8-1220 mg/L), refineries (6-500 mg/L) and coking operations (28-3900 mg/L). Besides, plastic, wood and pharmaceutical industries produce 0.1-1600 mg/L phenol in their wastewater [7,23].

The pollution by phenols with high degree toxicity in aquatic environment is of great concern as their presence modified the environment biota. The exposure to phenols causes acute and chronic deleterious health effects. Phenol and PNP are rapidly adsorbed through contact with skin or eyes (dermal adsorption), inhalation and ingestion [24,25]. Long-term and uncontrolled exposure to phenols can lead to irregular breathing, muscle weakness, tremor, blood disorder, methemoglobinemia,

nausea, neuropsychiatric disturbances and irritation [22,24]. Animal studies for phenol and PNP exposure to the rat, rabbit and guinea pig showed fatal body weight loss, growth retardation, abnormal development in the offspring. The acute dermal and oral LD_{50} values in rats was found to be 1024 and 667 mg/kg, respectively [22].

In view of these adverse effects exhibited by phenol and PNP, the international regulatory bodies have set a stringent discharge limits for a sustainable environment. Phenol and PNP were designated as toxic pollutant (1977 Amendments to the Clean Water Act) and inscribed into the List of Priority Pollutants by US Environmental Protection Agency (US EPA) due to their high usage and potential toxicities. The water purify standard of less than 1 µg/L of phenol was set for surface water and the PNP concentration in natural water source is restricted to below 10 ng/L. The legislations in the UAE limit the total phenols of 0.1 mg/L in industrial waste discharged to marine environment. Besides, World Health Organization (WHO) prescribes 1.0 µg/L as the maximum permissible phenol concentration in drinking water [26–29]. In Malaysia, the recommended standard for total phenols in raw water is limited to 0.2 µg/L.

2.2 Biological treatment for phenolic compounds removals

Owing to the high toxicities of phenol and PNP, treatment of the organic wastewater becomes a mandatory requirement to safeguard the life of human and aquatic organisms. Deployment of appropriate methods for effective removal of these compounds will eliminate problems of possible harm associated with pollutants and waste disposal. Various techniques of physical, chemical and biological treatment processes have been employed to effectively remove phenolic compounds from wastewater prior to their final discharge into water bodies. However, physical treatment such as adsorption and extraction only concentrate the pollutants and further

mineralization is required. On the other hand, chemical treatment, for example Fenton and coagulation, is fast and efficient but it is expensive and may result in the formation of undesired and hazardous by-products [7,30]. Biological treatment using enzymatic or microbial processes is therefore a preferred alternative in pollution control because of the possibility of complete mineralization. In addition, biological methods are relatively cost-effective as well as eco-friendly compared to physicochemical methods [7,31,32]. In biological methods, pure or mixed microbial culture could be used to carry out the biodegradation process [33]. With regard to the treatment of wastewater containing phenolic compounds, biodegradation using mixed culture was found to be more practical and effective compared to pure culture [34,35]. Mixed culture was found to be more stable with greater tolerance to the inhibitory effect by toxic compounds.

2.2.1 Sequencing Batch Reactor (SBR) system

Activated sludge (AS) consists of biological flocs with matrices of non-living organic matter, inorganic matter and microorganisms such as bacteria, fungi, protozoa and higher form of animals (worms or insect larvae) [1]. An activated sludge process can be clarified as a high complex biological system in which these biological flocs are continuously circulated to come into contact with each other and undergo bio-oxidation [36]. Over the years, this process has evolved into many types of wastewater treatment systems such as conventional, tapered aeration, step aeration, extended aeration, sequencing batch reactor (SBR) and oxidation ditch [33,36].

In comparison with other biological methods, SBR system is highly flexible in operation and easier to control filamentous growth and settling problems [37]. This system commonly incorporates five basic steps: filling, aeration, settling, decantation

and idling, that take place sequentially in a batch reactor. The SBR system is a promising system to obtain versatile microorganism population in developing different metabolic pathways to remove bioresistant compounds found in wastewater [38]. Studies have shown the efficacy of SBR system in successfully biodegrading various phenolic compounds. As an example, biodegradation of PNP was successfully adapted in lab-scale SBR by Tomei *et al.* [38] in which 320 mg/L of PNP was utilised as the carbon source. The results in Monsalvo *et al*. [39] study show that both phenol and *p*-chlorophenol (PCP) can be completely removed in SBR within a wide range of PCP influent concentrations (525 and 105-1470 mg/L for phenol and PCP, respectively) at temperatures between 20 and 35 °C. Low-strength activated sludge acclimated to 140 mg/L of phenol was successfully cultured in SBR in Lim *et al*.'s [4] study. Albeit the low acclimation concentration, complete degradation of phenol with high COD removal efficiency (> 95%) was achieved up to 1050 mg/L of initial phenol concentration in their study.

2.3 Factors affecting biodegradation

Biodegradation is a multifaceted process and its performance is affected by various abiotic and biotic factors [33]. Wastewater treatment became difficult in the presence of inhibitory substrate. Their toxicities may cause deflocculation and inhibition or interferences during the bioprocess treatment. Hence, it is necessary to investigate the physicochemical and biological factors affecting the biodegradation process to understand the limitations of microbes in different environments and thus optimize the working efficacy.

2.3.1 Acclimatization

To attempt the biodegradation of inhibitory substrate especially at high initial concentration, investigations on the factor of acclimatization or pre-adaption were conducted by researchers. Wiggins *et al.* [40] suggested that selection and multiplication of specialized microorganisms occur during acclimatization process. Physiological transformations i.e. alteration at the enzyme level, regulation and production or mutation, occurring in the metabolic system of microorganisms result in enhanced biodegrading ability.

Studies have been done for investigating the potential of using the acclimated activated sludge on various types of inhibitory organic pollutants. Biodegradation of *o*-chlorophenol and *o*-nitrophenol were reported fail using unacclimated biomass [41]. However, successful biodegradation at initial substrate concentrations as high as 300 mg/L occurred by employing acclimated biomass. Similar results were reported by Janecko and Oleszkiewicz [42] that immediate degradation of *o*-nitrophenol without any lag phase was shown using acclimated activated sludge, whereas a 170 to 230 h lag time was observed using unacclimated activated sludge. Phenol-acclimated activated sludge was also proved to be able degrade phenol, cresols and 4-chlorophenol with 100, 95 and 38% of efficiencies, respectively [43]. Besides, Tomei *et al.* [38] suggested that the supply of feed with increasing fraction of PNP as carbon source improved biomass ability to degrade the xenobiotic compounds as indicated by the increasing trend of removal rates. Acclimatization of activated sludge towards increasing phenol concentration (500 to 3000 mg/L) was also conducted by Hussain *et al.* [44]. They found that the length of acclimatization was dependent on phenol concentration and bioactivity of biomass. Complete mineralization of phenol was

achieved for influent phenol concentration of 500 – 1500 mg/L. Beyond that, phenol removal efficiency decreased indicating an inhibitory effect exerted by the phenol.

2.3.2 Chemical compound toxicity

The effect of the chemical structure of the targeted compound on biodegradation can be reflected by the type of substituents, position of substituents, number of substituents or branching degree in the aromatic ring [33]. This contributes to the differences in inhibitory and toxicity intensity towards the reactions of microorganisms. Unsubstituted phenol is more degradable compared to those substituted phenols such as mono, di- or trichlorophenol or nitrophenols. The different positions of the same substituents in the aromatic ring would produce different intermediates during the metabolic process that might inhibit their own degradation at different levels.

These effects have been reported in several works in the past few decades. In Lim *et al.* [43] study, the toxicity exerted by each phenolic compound which inhibited its own degradation followed the trend of *o*-chlorophenol > *m*-chlorophenol > *p*-chlorophenol > *o*-cresol > *m*-cresol > phenol. This observation concurred with Andreozzi *et al*. [45] work that reported the biodegradation slowed down caused by the deactivation of aromatic ring by electron-withdrawing chlorine-substituted group. Megharaj *et al*. [46] also studied the toxicity of phenol and its nitro-substituented derivatives towards the growth and metabolic activities of *Nostoc linckia* isolated from soil. They found out that the nitrophenols significantly inhibited the cell production, CO_2 uptake, activities of nitrate reductase, nitrogenase and glutamine synthetase compared to phenol, following the toxicity trend of phenol < PNP < MNP < ONP.

2.3.3 Initial substrate concentration

The effect of substrate concentration on biodegradation process is significantly important [47]. At extremely low concentration, there is no noticeable effect on gross measure of metabolic activity. By increasing the substrate concentration, the stimulation of metabolism starts and thus the physiological activity increases. Eventually when the concentration reaches a point at which further increase in concentration does not affect the physiological activity due to the limitation of microbes themselves. Further increment in concentration will result in severe inhibition and thus inhibit the microbial growth and distort their metabolism [33,47]. The inhibition is plausibly caused by the physical disruption of microbial cell structure or hindrance in enzymatic activity [48].

A study of effects of varying initial phenol concentrations on the degradation efficiency of a novel *Pseudomonas* sp. NBM11 was conducted by Mohanty and Jena [49]. The results showed that phenol degradation percentage decreased with increasing initial concentration of phenol. Similar observation was also reported by Hussain *et al.* [44], in which the rate of phenol biodegradation by activated sludge decreased when the phenol concentration was increased from 500 to 3000 mg/L. The substrate inhibition at high PNP concentration was found to be a major limiting factor for aerobic PNP degradation. *Rhodococcus wratislaviensis* strain 9 was found to degrade 360 µM PNP within 8 h but required 50 h to completely mineralized 3.60 mM PNP [8]. Besides, an aerobic degradation of PNP by acclimated activated sludge in a bubble column was studied at varying initial PNP concentrations (12.8 to 128 mg/L) in Salehi *et al*. [50]. They found that higher PNP loadings were not degraded effectively whereas low loading (<128 mg/L) were readily degraded.

2.3.4 Inoculum concentration

Bacteria abundance is another factor affecting the biodegradation performance. Therefore, the selection of inoculum size is of great significance in order to optimize the biodegradation process. The toxic effect of substrate could be counteracted when higher number of cells or concentration is employed. In the study of Najafpoor *et al.* [51], the tolerance towards phenol toxicity at higher phenol concentration was found increased by inoculating higher amount of baker's yeast. This is corroborated with the result reported by Bhattacharya *et al*. [7] and Shourian *et al.* [30] on phenol biodegradation by *Acinetobacter* sp. B9 and *Pseudomonas* sp. SA01, respectively. In the study conducted by Salehi *et al*. [50], it was observed that higher activated sludge concentration enhanced the PNP biodegradation, in which only about 100 min were required to degrade 69 mg/L of PNP using 6300 mg/L of activated sludge compared to 130 min for 3530 mg/L of activated sludge.

2.4 Immobilization of activated sludge

Although the complete mineralization of organic pollutants using suspended activated sludge was widely reported [4,32], it experiences some limitations such as excess sludge production and substrate inhibition on microbial activities at high toxic contaminant concentration [12,26]. Consequently, immobilization technologies are receiving interests to minimize those limitations in order to achieve more effective operation system. Immobilization refers to the physical localization and confinement of microbial cells or enzymes to a defined space with a simultaneous preservation of their viability and catalytic functions [11,52]. This technology provides more convenient handling and separation of cells or enzymes, protection from adverse environmental condition and improves the bioprocess control [33,53–55].

Technically, the principal components of an immobilized system consist of microbial cells or enzymes, matrix and mode of attachment. There are five techniques for immobilization: physical adsorption, electrostatic binding, covalent binding, flocculation, entrapment and encapsulation (Figure 2.2) [11]. Among these, the entrapment of microorganisms is extensively used in bioremediation. It is an irreversible method where microbial cells are entrapped in a matrix media that allows the transportation of substrate and product [56]. The most common and easiest method for entrapment is gelation of polyanionic or polycationic polymers by adding the multivalent counter-ions [52].

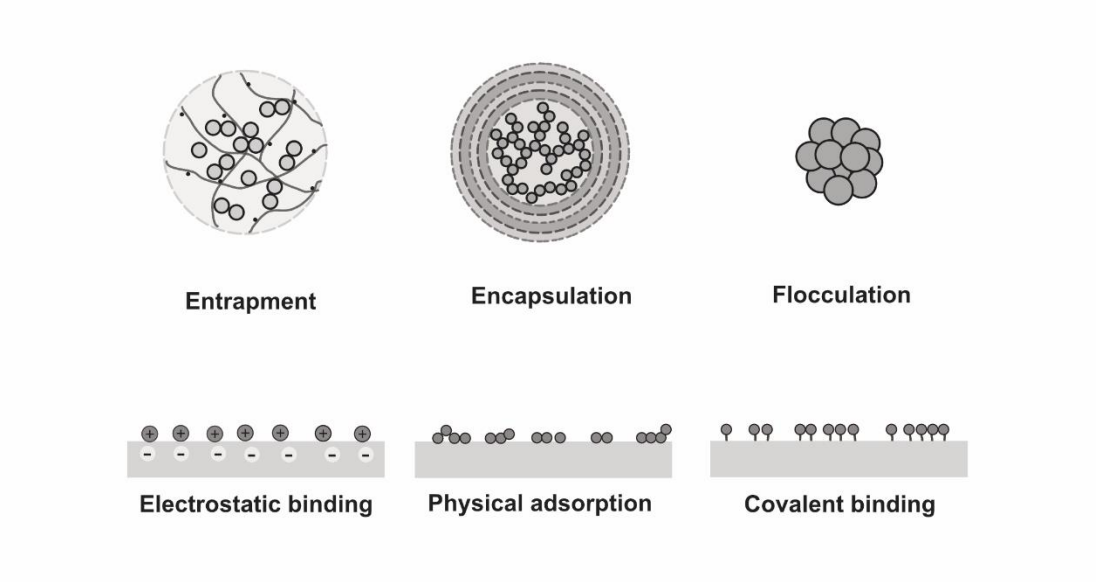

Figure 2.2 Techniques for immobilization

Selection of materials for immobilization is important. An ideal matrix material should exhibits some specific properties, such as hydrophilicity, biocompatibility, resistance or inertness towards cells or enzymes, resistance to compression and accessible at a low cost [26,57]. Besides that, the porous materials with large surface area, allowing for high microbial loading is also desired for immobilization. The nature of bioremediation is another important aspect on the choice of the materials. For

example, the support media should possess high mechanical resistance in wastewater treatment since it might expose to different kind of physical forces [58]. Commonly, it involves the use of inorganic silica material and organic biopolymeric to create the matrixes for the microorganisms. The latter is of great interest as they are renewable and sustainable in aspect of production cost and environment.

Polymeric matrixes, i.e. natural and synthetic polymers, are normally used for entrapment of microorganisms. Natural polymers such as alginate and chitosan, are biocompatible and inexpensive but might suffer from low mechanical stability and resistance to biodegradation [11]. While for synthetic polymers, they are possible to regulate their structure that favour the viability at the macromolecular level by proper selection of molecular weight, spatial structure and arrangement order of each active function group in chain [11]. The combination use of both natural and synthetic polymers is established and studied over the years to synthesize better polymeric matrix.

The study conducted by Toh *et al*.[12] clearly demonstrated that the merits of using PVA/alginate hydrogel-immobilized biomass in better tolerance towards higher initial concentration of *o*-cresol and good separation of biomass and treated solution. The reusability of immobilized biomass beads could be used up to three cycles at relatively lower initial *o*-cresol concentration. Another study showed the complete phenol removals up to 2 g/L were achieved and more effective with immobilized biomass in hydrogel beads of calcium alginate and cross-linked poly(N-vinyl pyrrolidone) in the continuous reactor containing alternate P and N sources with hydraulic time of 12.5 h, an influent pH of 5 and < 0.25 for food/microorganism ratio [59]. In other recent work that compared the organic load removal of suspended activated sludge with hydrogel-immobilized sludge, it was reported that the latter

exhibited two to three times larger organic load removal [60]. Based on these works, polymeric hydrogel is popular choice for entrapment matrix in wastewater treatment. Polymeric hydrogels are material which comprises an insoluble network of hydrophilic polymers that can take up biological fluids and water, and consequently formed a three-dimensional structure [61]. However, there is still few shortcomings; i.e. inadequate mechanical strength, low osmotic stability and possibility of syneresis during storage [61]. Therefore, the utilization of supermacroporous polymer hydrogels could overcome those limitations. One of the most accessible technologies that permits the formation of macroporous hydrogel (or cryogel) is cryogelation. Cryogelation process involves freezing, storage in the frozen state for a definite time and defrosting [15]. Their unique structure of cryogels with large interconnected pores and spongy morphology make them meet all aforementioned criteria. Besides, it allows unhindered diffusion of solutes of any size or even nano- and microparticles. They can withstand extensive deformation caused by cryo-concentration effect (accumulation of dissolved solutes in the unfrozen liquid microphase), resulting dense pore walls.

Mild conditions for cell immobilization and high mechanical stability of cryogels permits for the fabrication of effective biocatalysts to be employed in many potential applications. Based on the literature, cryogels can be used as a matrix in the detoxification process from wastewater, in which the cryogels are synthesized in the various forms such as membrane, bead and column [14,15]. Poly(2-hydroxyethyl methacrylate) (PHEMA)-based cryogels were used as carrier matrix for *Trichoderma spp*. to use in cyanide removal from wastewater by varying affecting factors including pH, temperature and initial cyanide concentration [62]. Cryogel-M (M = Cu, Ni and Co) composites were also employed as superporous reactor for the catalytic reduction

of toxic phenol compounds ONP and PNP as well as some dyes such as methylene blue and Eosin Y in the study carried out by Sahiner *et al.*[63]

2.5 Factors for cryogel fabrication

The structure and physicochemical characteristics could be altered by tuning the synthesis conditions, especially polymer concentration in the initial solution and process of freeze-thaw [64,65]. Polymer solution concentration significantly affected their properties and structure. Number of freeze-thaw cycles which involved in the freeze-thaw process, attribute directly to the crystallite formation mechanism and surface micromorphology [64]. Significant works have been reported on effect of processing parameters towards mechanical stability of PVA cryogels [66,67].

2.5.1 Polymeric solution concentration

2.5.1(a) Polyvinyl alcohol

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer composed of secondary alcohol group attached to a linear carbon chain, with the formula of $[CH_2CH(OH)]_n$. The use of PVA as immobilization matrix began about 20 years ago due to its desirable properties (biologically compatible and non-toxic) for biotechnology and pharmaceutical applications as well as for wastewater treatment.

The mechanism of gelation of pure PVA involves the formation of cross-linking points where individual polymer chain are related with weak hydrogen bond or electrostatic interaction resulting a three-dimensional network [68]. To alter the structure and properties of PVA cyogel, the initial PVA concentration is the first processing parameter to investigate. It was reported that higher concentration of PVA solution produced a more ordered or crystalline structure with less porosity and low

equilibrium swelling [69]. In turn, it led to an increase in tensile strength and tear resistance [70]. The increasing in cryogel rigidity with increasing PVA concentration used was resulted from the greater number of hydroxyl groups and thus increased intermolecular hydrogen bonding [66]. Wang and Campbell [71] had studied the mechanical properties of PVA cryogels under physiological conditions. They found that the mechanical strength such as Young modulus increased with an increase in PVA concentration. See *et al.* [72] reported that the PAC-hydrogel beads containing 2.5 %(w/v) PVA exerted weak mechanical stability, while higher PVA content, 9.5 %(w/v), beads caused diffusion limitations in their adsorption study for o-cresol.

2.5.1(b) Alginate

Alginate belongs to family of anionic linear copolymer composed of 1,4'-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) in different proportions and sequential arrangement (Figure 2.3) [73,74]. Carboxylate, ether and hydroxyl are generally the most abundant acidic groups in the alginate polymer [75]. It can form hydrophilic gel by crosslinking the carboxyl group of the α-L-guluronic acid with a cationic crosslinker. The crosslinking of the cation occurs primarily at the GG blocks binding to the negatively charged oxygen atom, forming "egg-box" structure [74,76], leading to gelation. The affinity of alginate towards different cations has been demonstrated. It was reported that calcium ion has the lowest binding capacity of the alginate and smaller cation size compared to other divalent ions [74,76].

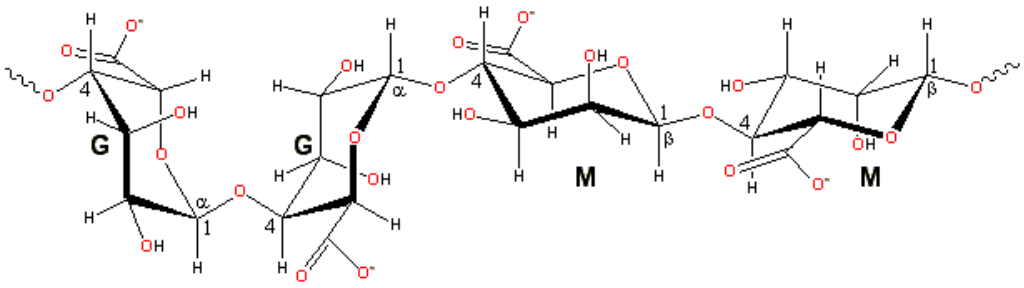

Figure 2.3 Alginate chemical structure (G: guluronic acid, M: mannuronic acid)

The alginate concentration has been shown to have significant effect on the physicochemical properties and structure in formation of gel. The higher the concentration of alginate was, the stronger the gel strength [67,77]. In a study conducted by Dong *et al.* [67], it was reported that 1.1% (w/v) of alginate was the optimum concentration to produced stable immobilized beads with high ammonia oxidation ability. The surface response analysis in Lotfipour *et al.* [77] study revealed that the alginate concentration was the important factor in deciding size, shape and encapsulation efficiency (> 98%) of the alginate beads.

As pointed out above that the gelation mechanism involves crosslinking process, it was observed that the number of apparent crosslinking point increased as the alginate concentration in producing gel increased [78]. The crosslinker was also found to have optimum operative concentration. Employment of higher ion concentration allowed greater penetration of ions giving complete crosslinking and less swelling water ability of the polymer matrix [76]. Dong *et al.* [67] presented that higher osmotic pressure was found as increasing the $CaCl_2$ content in PVA-alginate bead, resulting cell dehydration and reduction of microbial activity on ammonia nitrogen removal. Won *et al.* [16] also reported that an increase in alginate and $CaCl_2$ concentration will make it difficult for enzyme to leak out of the polymer network.

2.5.2 Freeze-thaw cycling process

The freeze-thaw cycling process contributes to the formation of polymer matrix structure consist of both amorphous and crystalline regions. A gelation mechanism for the formation of cryogel through freeze-thaw cycling process was proposed by Willcox *et al.* [79] and Lozinksy *et al.* [15]. During freezing, the growing ice crystals expel amorphous polymer segment and form regions of high polymer concentration, in which bring the molecular chains are brought closer to each other and this allows the formation of crystallites or crosslinks. The crystallites remain intact upon thawing as "junction zone" to provide rigid constraints. The final thawing of the ice crystals leaves macropores filled with solvent. Further freeze-thaw cycles were then induced the formation of secondary crystallites in amorphous region between the original crosslinks.

The number of freeze-thaw cycle has been shown to affect the mechanical properties of cryogel [70,80]. The reinforcement of crystallites within the cryogel structure after each additional freeze-thaw cycle was demonstrated in several studies. A linear relationship between compressive strength and number of freeze-thaw cycles for the first sixth cycles using various PVA concentrations was observed by Holloway *et al*. [80]. Beyond cycle 6, the strength did not increase and reached the plateau values. Similar result was observed by Millon and Wan [70], where the strength of pure PVA hydrogel increased monotonically with increasing freeze-thaw cycle up to cycle 6. However, in their study, it was found that the strength of PVA-bacterial cellulose nanocomposite exhibited significant increase from cycle 1 to 3, but no more increment was observed beyond third cycles due to limited surface area of bacterial cellulose nanofibers. Work reported by Stauffer and Peppast [81] showed that a diffusion coefficient decrease of 62% occurred between the second and fifth freeze-thaw cycles.

Similar observation was reported by Li *et al*. [82] where the drug release rate dropped when the number of freeze-thaw cycles increased attributed to difficulty of drug movement through the amorphous zone resulted from the increase in the volume fraction of crystalline regions.

2.5.3 Bead size

The influence of bead size on biodegradation could be associated with the extent of diffusion limitation [12,83], in which the diffusion limitation increases with increasing the bead size. This was reported by Aksu and Bulbul [84] in their phenol degradation study using Ca-alginate-immobilized cells. The bead size was found to affect the effectiveness factor that measure the extent of diffusion limitation. Therefore, the effect of bead size on biodegradation rate was very significant and should not be ignored in any engineering consideration. This could be envisaged that the interconnecting pathway of polymeric entanglement within the bead increase the path length for the movement of substrate to the biomass for degradation, resulting in diffusive mass-transfer resistance [12,84]. This is consistent with the results which had been reported by several researchers. The larger the beads size, the slower the phenol biodegradation rate for initial phenol concentration of 1730 mg/L using immobilized cells in alginate matrix (alginate, clay and PAC) [85]. In Toh *et al.*'s [12] finding, faster biodegradation rate of *o*-cresol (7.99 ± 0.25 mg/L h) was noted when using smaller hydrogel beads (3 mm) compared to other bead sizes (4 to 6 mm) with the ranges of rate from 3.23 and 6.95 mg/L h. Similar observation was reported by Ng *et al.* [26] that faster biodegradation rate of 25 mg/L PNP was observed when using smaller hydrogel bead size within the range investigated (3 to 5 mm).