BIOLOGICAL TREATMENT OF PETROCHEMICAL EFFLUENT CONTAINING BENZENE, TOLUENE AND XYLENE (BTX) USING *Pseudomonas putida* ATCC49128

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ABSTRAK

Benzena, toluena, dan xilena (BTX) adalah sebahagian daripada hidrokarbon aromatik yang terkandung dalam air buangan petrokimia. BTX dalam bentuk cecair dan wap biasanya ditemukan dalam loji-loji rawatan kumbahan perbandaran. *Pseudomonas putida* ATCC 49128 telah dipilih untuk digunakan dalam kajian ini oleh sebab keupayaannya untuk menyingkirkan sebatian aromatik tanpa menyebabkan penghasilan bahan kimia yang berbahaya dalam proses rawatan kumbahan. Eksperimen-eksperimen yang telah dijalankan di dalam makmal bertujuan untuk mengkaji *P. putida* dari segi (1) corak pertumbuhannya dalam keadaan biasa dan dalam larutan-larutan BTX, (2) keupayaannya untuk menyingkirkan kepekataan BTX yang berbeza-beza dalam kelalang goncang, serta (3) keupayaannya untuk menyingkirkan BTX dari air buangan petrokimia dan kepekatan-kepekatan BTX simulasi. Pengukuran-pengukuran kepekatan BTX dalam salah satu tangki efluen air buangan petrokimia di Semenajung Malaysia telah menunjukkan bahawa bacaan-bacaan bagi benzena, toluena, dan xilena adalah 55.2 ppm, 26.9 ppm, dan 32.8 ppm masing-masing. Seterusnya, keadaan-keadaan yang paling mendorong pertumbuhan *P. putida* adalah kelajuan penggoncang orbital pada 180 rpm, suhu pada 37^{o}C, pH 7 dan nisbah *P. putida*-nutrisi pada 1:9. Selepas itu, *P. putida* telah digunakan untuk menyingkirkan kepekatan-kepekatan BTX simulasi selama 48 jam; langkah ini diulang bagi air buangan petrokimia sebenar pada pH 6, 7, dan 8. Penyingkiran BTX dianalisis menggunakan gas kromatografi (GC-FID). Hasil kajian menunjukkan bahawa *P. putida* ATCC 49128 mampu mengurangkan kepekatan BTX dalam air buangan petrokimia (pH 7) sebanyak 85%, 77%, dan 70% masing-masing. Bagi larutan BTX simulasi, nilai-nilai tersebut adalah 87%, 80%, dan 72% masing-masing. Dalam larutan BTX simulasi tunggal, kuantiti toluena (97%) yang disingkirkan melebihi kuantiti-kuantiti berkenaan bagi benzena (93%) dan xilena (62%). Kesimpulannya, penggunaan *P. putida* ATCC 49128 merupakan kaedah biologikal yang berkesan bagi mengurangkan kepekatan BTX dalam air buangan petrokimia.

ABSTRACT

Benzene, toluene, and xylene (BTX) are some examples of aromatic hydrocarbons that are found in petrochemical wastewater. Liquid and gaseous forms of the same are frequently present in municipal treatment plants. In this research, *Pseudomonas putida* ATCC 49128 was selected for use in the experiments owing to its ability to safely remove the aforementioned aromatic compounds without giving rise to the formation of other hazardous chemicals during the treatment of wastewater. The said experiments were aimed to study *P. putida* in terms of (1) its growth patterns under normal conditions and in concentrations of BTX, (2) its ability to remove different concentrations of BTX in a shake flask, as well as (3) its ability to remove BTX from actual petrochemical wastewater and simulated solutions. Repeated measurements of BTX concentrations at a petrochemical industrial wastewater effluent tank in Peninsular Malaysia revealed that the maximum values for benzene, toluene, and xylene were 55.2 ppm, 26.9 ppm, and 32.8 ppm respectively. Subsequently, the results of the treatment study showed that the optimal conditions for the growth of *P. putida* were an orbital shaker speed of 180 rpm, temperature of 37°C, pH 7, and *P. putida*-nutrient ratio of 1:9. Next, *P. putida* was made to remove different concentrations of simulated BTX for 48 hours; this procedure was repeated for actual petrochemical wastewater of pH 6, 7, and 8 respectively. This process was analysed using gas chromatography (GC-FID). Evidently, the bacteria were able to reduce the concentrations of BTX in actual petrochemical wastewater (of pH 7) by 85%, 77%, and 70% respectively; for the simulated BTX solution, the values were 87%, 80%, and 72% respectively. In the single simulated BTX solution, the reduction in the concentration of toluene (97%) exceeded those of benzene (93%) and xylene (62%). To conclude, the usage of *P. putida* ATCC 49128 is an effective biological method to reduce the concentrations of BTX in petrochemical wastewater.

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LIST OF SYMBOLS

ppb	Parts per billion
π	Pi bond
o-	Ortho
p-	Para
m-	Meta
ppm	Parts per million
hr^{-1}	$Hour^{-1}$
n	Number of generations
μ	Specific growth rate
g	Generation time
k	Growth rate constant
C	Constant
X	Cell concentration
t_d	Doubling time

LIST OF ABBREVIATIONS

BTX	Benzene, toluene, and xylene
CNS	Centre nervous system
OSHA	Occupational Safety and Health Administration
WHO	World Health Organisation
USEPA	United States Environmental Protection Agency
TOL	Monooxygenase pathway
TOD	Dioxygenase pathway
VOC	Volatile organic chemistry
TSS	Total suspended solid
COD	Chemical oxygen demand
BOD	Biological oxygen demand
GC-FID	Gas chromatography- flame ionization detector
IUPAC	International Union of Pure and Applied Chemistry
DNA	Deoxyribonucleic acid
OD	Optical density
NB	Nutrient broth
NA	Nutrient agar
ATCC	American Type Culture Collection
EPA	Environmental Protection Agency
ASTM	American Society for Testing and Materials
NTU	Nephelometric turbidity unit
DO	Dissolved oxygen
DCM	Dichloromethane

CHAPTER 1

INTRODUCTION

1.1 Overview of Research

Aromatic hydrocarbons, which are usually produced by the oil and gas industries, are one of the most environmentally-damaging hazardous compounds such Benzene, toluene, and xylene (BTX) are common aromatic hydrocarbons that endanger human health (Colombo et al., 2004). Currently, no Malaysian health guidelines have designated safe limits of exposure to benzene, toluene, and xylene (Madhoun et al., 2012). In fact, the aforementioned limit has only been only implemented by a few organisations in the world – the US Occupational Safety and Health Administration (OSHA) as well as the World Health Organisation (WHO). One of the petrochemical-based industries in the East Coast of Malaysia has produced higher concentrations of BTX as compared to the standard limits used in most industrial countries. Evidently, the concentrations of benzene, toluene, and xylene in one of the effluent ponds were approximately 50 ppm, 30 ppm, and 18 ppm respectively. The concentrations of BTX in vapour differ slightly from those in wastewater. As BTX are volatile gases which are also soluble in water, their concentrations in vapour can be diminished by reducing the concentrations of the liquid forms of BTX.

Liquid and gaseous forms of BTX are often present in municipal treatment plants. They can be discharged in industrial wastewater and raw effluents (Mrowiec and Suschka, 2009), from small factories, public utilities, as well as domestic sewage. Apart from being polluting agents, they are derived from a variety of sources such as petroleum-like fuel-refining, colorant, coating, and solvent industries, following which they end up in polluted soils, wastewater effluents, and groundwater. BTX represents a serious threat to the groundwater resources and public health since it more toxic and

water-soluble as compared to the other constituents of petroleum (Robledo-Ortíz et al., 2011). With respect to environmental pollution, aromatic hydrocarbons – especially benzene and its aliphatic derivatives – are considered to be very harmful. This is in view of their high levels of toxicities even at very low concentrations (Fan et al., 2010). BTX can cause fatigue, dizziness, headaches, confusion, and even death. Meanwhile, long-term exposure to these volatile organic compounds (VOC), especially benzene, potentially results in excessive bleeding, immune system weakening, anaemias, and leukaemias (Agency for Toxic Substances and Disease Registry, 2005a, 2005b; Robledo-Ortíz et al., 2011). Therefore, the industrial discharge of BTX into the environment requires prevention by efficient and cost-effective treatment technologies.

The process of elimination or reduction of BTX is an important issue that needs to be addressed by the petrochemical industry. There are many different physical, chemical, and biological methods to reduce the concentrations of BTX in industrial wastewater. Most petrochemical industries employ combinations of two or more methods in their wastewater treatment plants. Physical methods – such as liquid-liquid separation, air stripping, adsorption, as well as thermal and catalytic oxidation – are most frequently used to reduce the said concentrations of BTX. Evidently, the major disadvantage of these techniques is the high cost in view of immense energy consumption as well as the need for further treatment or disposal of secondary wastes (Shahna et al., 2010).

Other methods for the abovementioned objective include biological treatment, such as aeration plants or ponds that contain growing natural bacteria. However, this method is rarely used owing to the need for long periods of time to lower the concentrations of BTX. Many researchers have attempted to identify the organisms, especially bacteria, which are capable of reducing such pollutants to environmentally-tolerable forms. Biodegradation by bacteria is particularly suitable in view of the fact that various strains of the same give rise to a wide variety of carbon sources or electron acceptors (Vasileva-tonkova and Galabova, 2003).

Recently, biological treatment processes are being increasingly adopted because of their effective and eco-friendly remediation of polluted areas (Thayer, 1991). *Pseudomonas* species, like *Pseudomonas putida*, are one of the most important microorganisms in these processes (Reardon et al., 2000). Apart from being non-

pathogenic to animals or plants, *Pseudomonas* have the ability to use and metabolise different hydrocarbons as well (Timmis, 2002).

1.2 Problem Statement

Benzene, toluene, and xylene (BTX) are water-polluting agents that are derived from various sources, especially petrochemical industries. These aromatic hydrocarbons can adversely affect human and environmental health because the benzene rings in their molecular structure are hard to break.

VOCs, which are common substances in petroleum-processing industries, have become a cause for worldwide concern, particularly in countries with siseable petroleum sectors. Normally, the concentrations of BTX in the wastewater produced by the said industries are higher than the permissible level as per the international pollution agencies like the USEPA and European Union. According to the former, the maximum average monthly concentrations of benzene and toluene in treated industrial effluents are 37 ppb and 26 ppb respectively. Meanwhile, the maximum permissible concentrations of benzene, toluene, and xylene in water are 0.5 ppb, 100 ppb, and 10,000 ppb respectively (Agency for Toxic Substances and Disease Registry, 2000, 2005a, 2005b).

The methods for removing or reducing BTX are numerous since these compounds endanger human health. Most industries employ air-stripping to separate BTX, but doing so is very costly because there is a need to collect each compound separately following the completion of the process. Another method involves the adsorption of BTX on active charcoal (Torkaman et al., 2010). While the initial two times of the utilisation this technique is very effective for trapping BTX, its efficacy reduces over time because the charcoal has limited adsorption abilities. Thus, the industry will have to frequently replace the charcoal, which in turn increases the maintenance cost.

Currently, Malaysia has no law or regulation detailing the maximum allowable levels of BTX in the air, water and wastewater. Most developed countries – like the USA, England, and Canada – have upper limits of exposure to BTX since they are aware of the hazardousness of the same to human and environmental health. Benzene is

more dangerous as compared to other aromatic compounds because of its toxicity as well as easy access to air from cars and fuel pumps.

1.3 Research Objectives and Scopes

The objectives of this research were:

1. To determine the physical and chemical characteristics of effluents obtained from a petrochemical industry located at the East Coast of Malaysia.
2. To investigate the growth kinetics of *P. putida* in simulated and actual BTX wastewater.
3. To evaluate the contents of BTX before and after biological treatment by *P. putida.*

Scopes of study:

a. To study *P. putida* in terms of growth, acclimatisation time, nutrient requirements.
b. To analyse the initial values of selected parameters (BOD, COD, pH, TSS, and BTX concentrations) of petrochemical industrial wastewater.
c. To investigate the removal of BTX from simulated solutions by *P. putida* in shake flasks at optimal conditions.
d. To study the growth pattern of *P. putida* in petrochemical wastewater
e. To investigate the removal of BTX from actual petrochemical wastewater by *P. putida* in shake flasks.

1.4 Significance of Research

This study has focused on the removal or reduction of benzene, toluene, and xylene (BTX). It has also researched into the pure cultures as well as operating parameters and concentrations of *P. putida,* apart from suggesting the types of enzymes in *P. putida* that were needed to remove BTX from industrial wastewater. The alternative biological method employed in this study has been demonstrated to be efficient, economical, and environmentally-friendly. Also, through the rehydration of

freeze-dried *P. putida*, this research has improved the understanding of the behaviour of the organism in terms of its growth parameters and ideal operating conditions.

Bacterial bioremediation processes are widely used, especially in the treatment of industrial wastewater. The utilisation of *P. putida* is an efficient technology for the decontamination of hydrocarbon-polluted places (Mazzeo et al., 2010). Members of the genus *Pseudomonas* are characterised by their ability to grow in simple media containing a wide variety of organic compounds. They are obligate aerobes and motile (one or several polar flagella are usually present). *P. putida* strains grow best at pH 7, and are easily isolated from the soil and water by enrichment cultures containing mineral media plus various carbon sources (Tsai and Juang, 2006). The aforementioned species was chosen for biological treatment in view of its ability to degrade the aromatic compounds, apart from giving rise to a clean and safe treatment process without the generation of other hazardous chemicals (Tsai and Juang, 2006).

The ability of *P. putida* to reduce BTX has a direct bearing on the development of strategies for dealing with environmental pollution. For example, leaking underground gasoline storage tanks can contaminate groundwater supplies with BTX. Here, *P. putida* can be employed to oxidise all those compounds. Thus, detailed knowledge of the physiology and biodegradational capabilities of *P. putida* are essential to create a scientific foundation for the emerging bioremediation industry (Abuhamed et al., 2004; Brecker et al., 2006).

Bioremediation is largely considered to be environmentally-friendly and cost effective method, so it is gradually making progress in terms of environmental clean-up applications. In this study, *P. putida* has produced the enzyme cathecol-2,3-dioxygenase, which was able to break the benzene rings of BTX using catechol intermediates. The findings of the study could be used a references for future researches on the treatment of wastewater from petrochemical and BTX-related industries.

1.5 Thesis Outline

The outline of this thesis is presented in a schematic form as displayed in Figure 1.1. Brief descriptions of each individual chapters are presented at the end of this chapter.

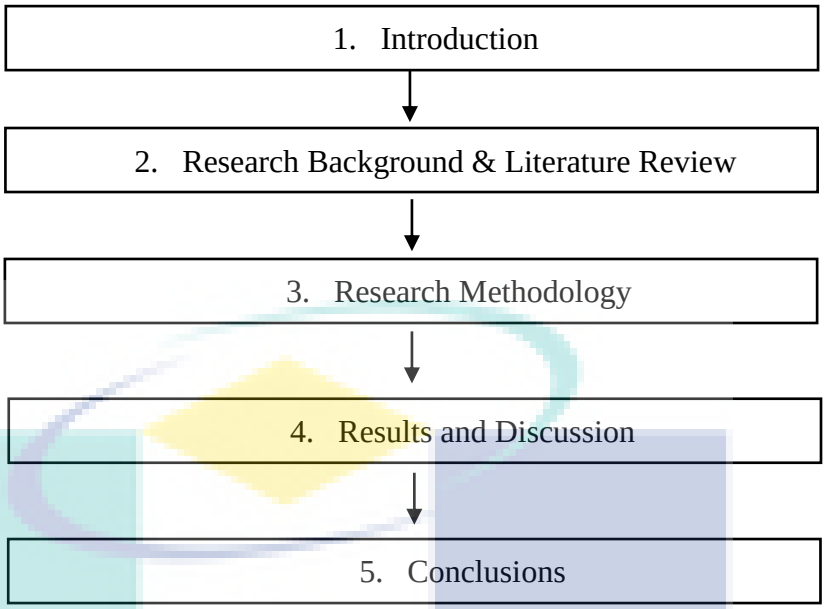


Figure 1.1 Flow of Thesis

Chapter 2 reviews the literature, apart from giving an overview of BTX and its treatment by the petrochemical industry. *Pseudomonas putida* is also discussed as the main subject for the biological treatment method in this research.

Chapter 3 contains detailed descriptions of the research methodology. It elucidates the materials used as well as the methods of characterising industrial wastewater samples and standards. Furthermore, *P. putida* bioremediation of various concentrations of BTX is analysed by gas chromatography-flame ionisation detector (GC-FID).

Chapter 4 presents the results and discussions of this research. It contains detailed reports of the experimental results, along with the elaborations on the ideal concentrations of and conditions for *P. putida* to remove BTX from the simulated samples and actual petrochemical wastewater.

Chapter 5 delivers the conclusions of this research, which takes into account the contributions of the main findings. Recommendations for future works are included as well.

CHAPTER 2

LITERATURE REVIEW

2.1 Benzene, Toluene, and Xylene (BTX) – an Overview

2.1.1 Benzene

In 1825, Michael Faraday was the first person to isolate a colourless compound called benzene from the oily residue in an illuminating gas line in London. Then, in 1862, August Kekulé proposed the first structure of benzene, which consisted of a six-carbon ring with one hydrogen bond attached to each carbon. Ten years later, he proposed a new structure that contained three double bonds that shifted back and forth between the carbons and could not be separated. The evolution of the proposed structures of benzene is shown in Figure 2.1.

Figure 2.1 Benzene formulae by (i) Claus (1867), (ii) Dewar (1867), (iii) Ladenburg (1869), (iv) Armstrong (1887), (v) Thiele (1899), and (iv) Kekulé (1865

Source: http://en.m.wikipedia.org/wiki/Benzene

In the 19th and early 20th centuries, benzene was initially used as an after-shave lotion in view of its sweet smell. As mentioned, it was also frequently used as a solvent in industries, such as the petrochemical industry for the production of styrene. Following its popularisation by Ludwig Roselius in 1903, benzene began to be utilised

for decaffeinating coffee. Additionally, it is the main chemical in various consumer products such as rubber cements, spot/ stain removers, liquid wrench, and other hydrocarbon-containing products.

Benzene is an aromatic hydrocarbon compound comprising six (6) carbon atoms in a ring, with one (1) hydrogen atom attached to each carbon atom. It has a molecular formula of C_6H_6. There are three (3) double bonds rings with a continuous pi (π) bond. Benzene is more stable and less reactive than a typical alkene because of its equivalent carbon atoms, and that all its *p*-orbitals – which are made up of all six (6) pi (π) electrons – are free to move about the entire ring. Benzene is a natural constituent of crude oil and a colorless liquid (Figure 2.2) at room temperature. This highly-flammable compound has a sweet, gasoline-like smell in light of its aromaticity. The systematic name of benzene is cyclohexa-1,3,5-triene; other names include 1,3,5-cyclohexatriene, benzol, and phene. Benzene has a molar mass of 78.11 g/mol[1], density 0.8765 g/cm^3 (at 20^{o}C), melting point 5.53^{o}C, and boiling point 80.1^{o}C. It is slightly soluble in water, but soluble in alcohol, trichloromethane ($CHCl_3$), tetrachloromethane (CCl_4), diethyl ether, acetone, and acetic acid.

Figure 2.2 Colourless liquid benzene

2.1.2 Toluene

Also an aromatic hydrocarbon, toluene, or methylbenzene, is a derivative of benzene, whereby one (1) hydrogen atom of benzene ring is substituted with a methyl group ($-CH_3$). Toluene is a clear liquid which smells like thinner. It was initially isolated in 1837 by Filip Walter through the distillation of pine oil. He named the compound “rétinnaphte-” (Wisniak, 2004); later, it was called toluol and subsequently

toluene (this final name was coined by Jöns Jakob Berzelius). Toluol refers to the aromatic extract of *Myroxylon balsamun* (tropical Colombian tree), or tolu balsam (Stine, 1943). The molecular structure of toluene is shown in Figure 2.3.

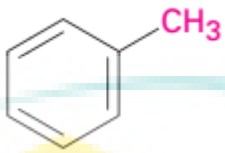


Figure 2.3 Molecular structure of toluene, or methylbenzene

Small amounts of toluene are naturally present in crude oil such as benzene in petrochemical industry and gasoline making process by catalytic reformer. Toluene is widely used as solvents and industrial feedstock. In case of the former, it is employed for dissolving paints, rubber, adhesives, silicone sealants, printing inks, disinfectants, and many chemical reactants (including inorganic non-polar covalent substances such as sulfur, bromine, and iodine). It is also utilised as an octane booster of gasoline fuels in internal combustion engines. Other applications of toluene are (i) cocaine removal from coca leaves in the production of Coca-Cola® syrup, and (ii) coolants in sodium cold traps of nuclear reactor loop systems.

Toluene is an unsaturated hydrocarbon and is 25 times more reactive as compared to benzene because of its methyl side chain which acts an activating group. Toluene reacts via electrophilic aromatic substitutions like a typical hydrocarbon. It can undergo several types of reactions, such as (i) nitration to produce *o*-nitrotoluene, *m*-nitrotoluene, and *p*-nitrotoluene, as well as those typical of alkanes such as (ii) hydrohalogenation, (iii) halogenations, and (iv) oxidation. Toluene can be synthesised in laboratories or industries via different methods like Friedel-Crafts reaction, Wurtz-Fittig reaction, and decarboxylation. In the Friedel-Crafts reaction, benzene reacts with methyl chloride (CH_3Cl) in the presence of an anhydrous aluminium chloride (acyl halide, AlCl) catalyst. Toluene has a molar mass of 92.14 g/mol, density 0.52 g/L (at 20°C), melting point -95°C, boiling point 111°C, and viscosity 0.590 cP. It is slightly soluble in water.

2.1.3 Xylene

Yet another aromatic hydrocarbon, xylene (derived from the Greek word ξύλο, *xylo*, meaning "wood"), or dimethylbenzene, consists of a benzene ring with two (2) methyl substituents. Hence, each molecule contains eight (8) carbon atoms and ten (10) hydrogen atoms (C_8H_{10}). Xylene actually refers to a group of three benzene derivatives; in other words, there are three isomers – *ortho*-, *para*-, and *meta*-xylene – whose methyl groups are attached to different carbon atoms of the benzene ring (Figure 2.4). In 1851, xylene began to be used as a component of wood tar. Other applications of this compound include solvents, paint thinners, as well as cleaning agents in printing, rubber, and leather industries. Small amounts of xylene can also be found in gasoline and airplanes. The world's largest xylene plant, which was constructed by a global consortium, is currently located in Singapore (Tremblay and François, 2012).

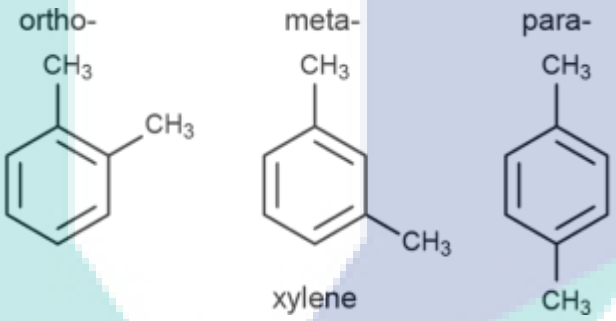


Figure 2.4 Isomers of xylene

Naming of the isomers of xylene involves the determination of the position of the carbon atom containing one of the methyl groups relative to the remaining methylated carbon atom which is otherwise denoted as carbon number 1. The IUPAC names for each isomer are 1,2-dimethylbenzene (*o*-isomer), 1,3-dimethylbenzene (*m*-isomer), and 1,4-dimethylbenzene (*p*-isomer). The aforementioned isomers differ slightly from each other in terms of melting points, boiling points, and viscosities (Table 2.1). Nevertheless, all of them are colourless, have a molar mass of 106.16 g/mol, are soluble in non-polar solvents such as aromatic hydrocarbons, and are practically insoluble in water.

Table 2.1 Properties of BTX

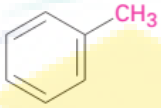
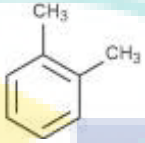
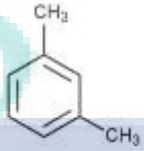
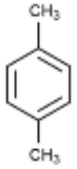
	Benzene	**Toluene**	**o-Xylene**	**m-Xylene**	**p-Xylene**
Molecular formula	C_6H_6	C_7H_8	C_8H_{10}	C_8H_{10}	C_8H_{10}
Molecular Structure					
Molar mass	78.11 $gmol^{-1}$	92.14 g mol^{-1}	106.16 g mol^{-1}	106.16 g mol^{-1}	106.16 g mol^{-1}
Density	0.8765 g/cm^3	0.87 g/cm^3	0.88 g/cm^3	0.86 g/cm^3	0.86 g/cm^3
Odor	Aromatic, Gasoline-like	Aromatic, sweet, pungent, benzene-like odor	Aromatic, sweet, pungent, benzene-like odor		
appearance	Colorless liquid	Colorless liquid	Colorless liquid	Colorless liquid	Colorless liquid
Melting point	5.53ºC	-95ºC	-25 ºC	-48 ºC	13 ºC
Boiling Point	80.1 °C	111 °C	144 °C	139 °C	138 °C
Solubility in water	1.84 g/L (30 °C), 2.26 g/L (61 °C), 3.94 g/L (100 °C)	1.52 g/L (20 °C)	Practically insoluble	Practically insoluble	Practically insoluble
Solubility	Soluble in $CHCl_3$, diethyl ether, alcohol, CCl_4, acetic acid and acetone	Soluble in benzene, ethanol, diethyl ether, glacial acetic acid, acetone, chloroform and carbon disulfide	Soluble in non-polar solvents such as aromatic hydrocarbon	Soluble in non-polar solvents such as aromatic hydrocarbon	Soluble in non-polar solvents such as aromatic hydrocarbon

Table 2.1 Continued

	Benzene	Toluene	o-Xylene	m-Xylene	p-Xylene
Viscosity	0.6076 cP (25 °C), 0.4965 cP (40 °C), 0.3075 cP (80 °C)	0.590 cP (20 °C)	0.812 cP (20 °C)	0.62 cP (20 °C)	0.34 cP (20 °C)

2.2 Risk of Exposure to BTX

Benzene is more hazardous as compared to the other two organic hydrocarbons since it is the most stable among the three. Human exposure to benzene can be classified according to long term adverse effect, acute and disease. This exposure has been classified with a range of acute and long term adverse effects and disease. Exposure to benzene can occur occupationally and domestically, examples of which include its usage in industrial activities, motor fuels, and solvents. Rapid degradation of benzene to a higher atmosphere increases human exposure to the same through inhalation in view of its volatility. The solubility of benzene in water enables it to spread easily from the contaminated areas into the groundwater and rivers, usually with the help of rain. Table 2.2 below shows the effluent pretreatment standards for benzene and toluene, as stated by the USEPA.

Table 2.2 Effluent pretreatment standards in industries

Effluent Pretreatment Standard		
Effluent	**Maximum for any 1 day (µg/L)**	**Maximum for Monthly Average (µg/L)**
Benzene	134	57
Toluene	74	28

Source: 40 CFR chapter 1, part 414, Organic Chemical, Plastics and Synthetic Fibers

The WHO and EPA have stipulated that drinking water should not contain more than 0.01 mg/L of benzene since it is carcinogenic if consumed in the long term. Specifically, it increases the risk of leukemia. Acute exposure to high concentrations of benzene in the air can result in dizziness, skin and eye irritation, headache, confusion, drowsiness, loss of consciousness, as well as neurological and gastrointestinal toxicities.

Toluene can also adversely affect human health. In more detail, its consumption beyond a certain concentration leads to depression of the central nervous system (CNS) and death. Owing to its volatility, the most common route by which toluene enters the body is inhalation. Some researchers have stated that the inhalation of toluene at concentrations of 200 ppm or more can adversely affect the CNS; concentrations exceeding 10,000 ppm usually result in death. Chronic exposure to toluene of less than 200 ppm may give rise to headache, nausea, and fatigue. Meanwhile, certain studies have reported that an exposure to the compound at concentrations of 200 to 500 ppm can cause reversible optic nerve damage, apart from loss of appetite, coordination, and memory.

As mentioned, benzene is more poisonous than toluene as its eponymous ring is difficult to be broken. Benzene has a stable and complete ring structure comprising 6 carbons and 6 hydrogens, unlike toluene that has a methyl group attached to the said ring which easily reacts with other compounds. Hence, chronic exposure to toluene does not result in serious bone marrow injuries. Also, it only gives rise to acute and chronic toxicities in humans, apart from being non-carcinogenic.

Xylene is naturally present in petrochemicals, especially crude oil. It is widely used as solvents in various industries since it is a safer alternative to benzene. Xylene is also utilised as (i) raw materials in resins, coatings, polymers, and rubber, as well as (ii) chemical intermediates in the manufacturing industry. According to the USEPA, the maximum permissible concentration of xylene in drinking water is 10,000 ppb, and that it is a group D substance which is not carcinogenic to humans. Usually, xylene is present in effluents along with benzene and toluene. Thus, there is a need to minimise the concentration of xylene in the same in order to reduce the COD and preserve human health. Direct exposure to this compound, either by inhalation or skin contact, can irritate the eyes, mucous membranes, and skin. The effects of acute exposure include liver dysfunction, renal impairment, transient confusion and memory loss, as well as

pulmonary edema. Meanwhile, chronic exposure can result in headaches, dizziness, confusion, tremors, tinnitus, thirst, renal impairment, anaemia, and reversible ocular damage. Inhalation of high concentrations of vapourised of xylene in a closed area leads to CNS excitation, narcosis, respiratory tract irritation, and non-cardiogenic pulmonary edema. Unknowing indigestion of this compound can also occur, in which case ventricular fibrillation, CNS depression, reversible hepatic and renal toxicities, burning sensation in the stomach, and vomiting are likely.

2.3 BTX Generation in Petrochemical Industries

Petroleum-refining and petrochemical industries account for a major share of the global energy and industrial markets. In many situations, they form the economic backbone of industrial countries such as Malaysia, Brunei, and East Asia. The current volatile market and continuous changes in the customers' demands have given rise to a constant need to seek opportunities that properly align and coordinate the various components of the industries. In particular, the integration of the petroleum-refining and petrochemical industries is gaining a great deal of interest. There are two types of catalytic conversion units in the refineries, namely the cracking and alteration processes. Catalytic cracking converts heavy oils into lighter products that can be blended to produce final products of high quality, such as gasoline, jet fuels, and diesel. Meanwhile, catalytic altering processes convert feedstock to higher quality streams by rearranging their structures. The abovementioned processes include reforming, alkylation, and isomerisation of the units. Catalytic processes produce hydrocarbon molecules with double bonds and form the basis of the petrochemical industry.

2.3.1 Cracking Process

Cracking processes mainly comprise catalytic cracking and hydrocracking. The former involves the breaking down and rearranging of complex hydrocarbons into lighter molecules in order to increase the qualities and quantities of the desired products such as kerosene, gasoline, liquefied petroleum gas (LPG), heating oil, as well as petrochemical feedstock. Catalytic cracking employs a concept similar to that of thermal cracking, except that catalysts are used to promote and control the conversion of the heavier molecules into lighter products under much less severe operating conditions.

Industrially, the most commonly used process is fluid catalytic cracking (FCC), in which oil is cracked in a fluidised catalyst bed where it is continuously circulated between the reactive and regenerative states. On another note, hydrocracking is a process that combines catalytic cracking and hydrogenation. In other words, the feed is cracked in the presence of hydrogen to produce more desirable products. This process mainly depends on the characteristics of the feedstock and the relative rates of the two competing reactions – hydrogenation and cracking. In the case whereby the feedstock has a higher paraffinic content, hydrogen prevents the formation of polycyclic aromatic compounds. Another important role of hydrogen is to reduce the formation of tar and prevent the build-up of coke on the catalyst.

2.3.2 Alteration Process

Alteration processes involve the rearrangement of the molecular structure of feed stream in order to generate products of higher quality. One of the main processes in this category is catalytic reforming. Reforming is important for the conversion of low-octane feedstock into high-octane gasoline blending components called reformate. The kinetics of reforming involve a multitude of simultaneously-occurring reactions such as cracking, polymerisation, dehydrogenation, and isomerisation. Depending on the properties of the feedstock – or their paraffin, olefin, naphthene, and aromatic (PONA) contents – and the catalysts used, reformates can be produced from very high concentrations of toluene, benzene, xylene, and other aromatics. Hydrogen, a by-product of the reforming process, is separated from the products and used as a feed in other refining processes. Another process of alteration is alkylation, which facilitates the production of higher-octane aviation gasoline as well as petrochemical feedstock for explosives and synthetic rubber. Isomerisation can also be employed to produce more alkylation feedstock.

2.3.3 Aromatics

Aromatics are hydrocarbons that contain a stable and saturated benzene ring. Those which are widely used by the petrochemical industry include BTX and ethylbenzene. They are produced by catalytic reforming, whose increase in severity will improve their yields (Gary and Handwerk, 2005). Depending on the chosen technology,

extractive distillation by different solvents can be used to recover the aforementioned compounds. The extraction of BTX entails the utilisation of solvents that enhance the relative volatilities of the preferred compounds, followed by separation of the compounds with respect to the boiling points of the products. Further processing of xylene by isomerisation or separation is commonly required to produce mixtures of *o*-, *m*-, and *p*-xylene, as per the market requirements. Benzene, in particular, is a source of a wide variety of chemical products. It is often converted to ethylbenzene, cumene, cyclohexane, and nitrobenzene, which in turn are further processed into other chemicals like styrene, phenol, and aniline. On the other hand, the production of toluene is mainly driven by the demand for benzene and mixed xylenes. The latter is used to produce para-xylene and polyester, especially in Asia, (Balaraman, 2006). Other sources of aromatics include pyrolysis gasoline (pygas), which is a by-product of the steam cracking of naphtha or gas oil. This presents an excellent synergistic opportunity between refinery, BTX complex and steam cracking for the production of olefins.

2.4 BTX Treatment in Industry

Petrochemical industries in Malaysia produce large volumes of waste and wastewater. Thus, the treatment processes should be improved in order to minimise land and river contamination. Numerous researches have been conducted to reduce pollution and cost, especially during the treatment of wastewater. This system consists of a combination of several processes and designs, depending on the type of wastewater produced. Municipal wastewater treatment systems can be divided into three stages or subsystems, which are primary, secondary, and tertiary. The predominant objective of primary treatment is to separate and remove solid materials. At this stage, the processes involved include comminution, screening, and primary clarification. Subsequently, the secondary stage entails biological equipment such as upflow anaerobic sludge blanket (UASB) reactors, aeration ponds, and trickling filter systems. The aim here is to remove the remaining dissolved or colloidal organic matter. Lastly, tertiary treatment involves a polishing process such as filtration using activated carbon, resins, and membranes.

The three (3) main methods of BTX treatment in industries, especially petroleum-based ones, are physical treatment, chemical treatment, and biological treatment.

2.4.1 Physical Treatment Methods

Physical treatment processes in the treatment of wastewater or raw water include sedimentation, filtration and gas transfer. These basic methods employ specially-engineered systems for the treatment of individual types of wastes. Sedimentation, which is typically carried out in large tanks or ponds, separates particles and suspended solids from the wastewater. Another method of physical treatment is filtration; examples of its processes include filter-pressing, membrane filtration, air sparging, as well as usage of resins or activated carbon.

2.4.1.1 Membrane Filtration or Separation

Membrane separation is a vapour-air or water-solvent segregation method that involves the diffusion of VOCs through a non-porous gas membrane. A membrane is a physical barrier which allows certain compounds or solvents to pass through it according to the concept of negative and positive charges. Usually, membrane filtration employs materials which are hydrophilic and hydrophobic to separate water-soluble compounds from the solvent. In addition, a membrane consists of a porous, thin, and dense support layer whose pore size determines the type of membrane filtration. Figure 2.5 below shows the general concept of membrane filtration.

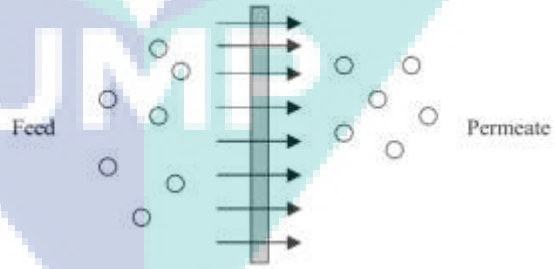


Figure 2.5 General concept of membrane filtration or separation

Two types of membranes can be used to separate vapour and permanent gas. These are rubbery vapour-selective membranes and glassy gas-selective membranes. Almost all commercial plants utilise the former – which are made of silicone rubber (polydimethyl siloxane) – for several reasons. First, rubbery polymers have much higher permeability than their glass counterparts, so a smaller area is needed for the separation

process, which in turn reduces the capital cost. Second, rubbery membranes provide better selective purge, which means that the inert vent gas purge removes more organic vapour components such as benzene. Figure 2.6 below shows the two aforementioned types of membrane filters.

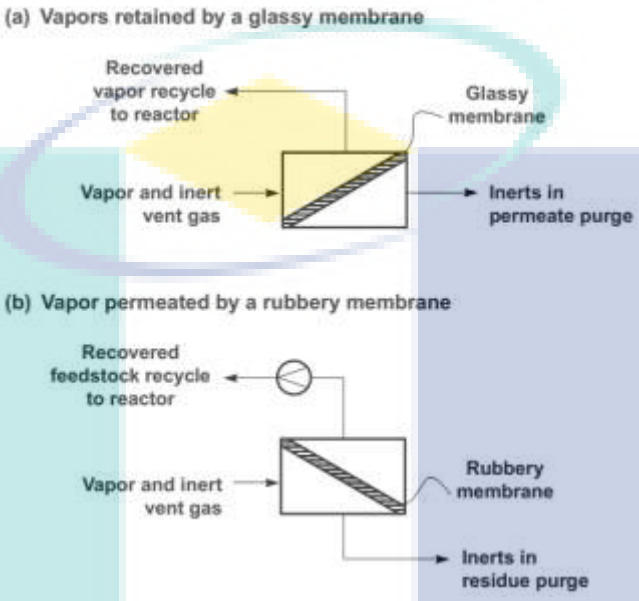


Figure 2.6 Principles of membrane filtration in the separation of vapours/ permanent gases

More than 80% of BTX can be removed by cross-flow filtration systems, which are equipped with (i) a pressure vessel containing the membrane module, (ii) a temperature control box, and (iii) a pressurisation tank (Su et al., 2016). The BTX solution is transferred via compressed air from the tank to the membrane cell within a stainless steel disc.

2.4.1.2 Air Sparging

In the petrochemical industry, air sparging is used to treat BTX-contaminated soil and groundwater (Hendrickx et al., 2006). It is an *in situ* remedial technology that facilitates the volatilisation of organic compounds by the injection of air into BTX-polluted aquifers and soils. This process also encourages natural aerobic biodegradation. Contaminant-free air is injected into the subsurfaces of the contaminated zones, hence promoting the conversion of dissolved hydrocarbon to a vapourised form. The contaminated air is then vented through the uncontaminated zone. This technology is

also known as "*in situ* volatilisation" and "*in situ* air stripping". Usually, the air sparging wells are orientated with respect to the industrial needs and conditions (i.e. either vertically or horizontally). The most effective air sparging method is hinged on two main factors, namely vapour-dissolved phase partitioning and soil permeability. While this technology is employed in view of its lower cost as compared to conventional approaches, it can be difficult to handle, especially in terms of modelling and monitoring. Figure 2.7 below shows the cross-section of a vertical sparging well.

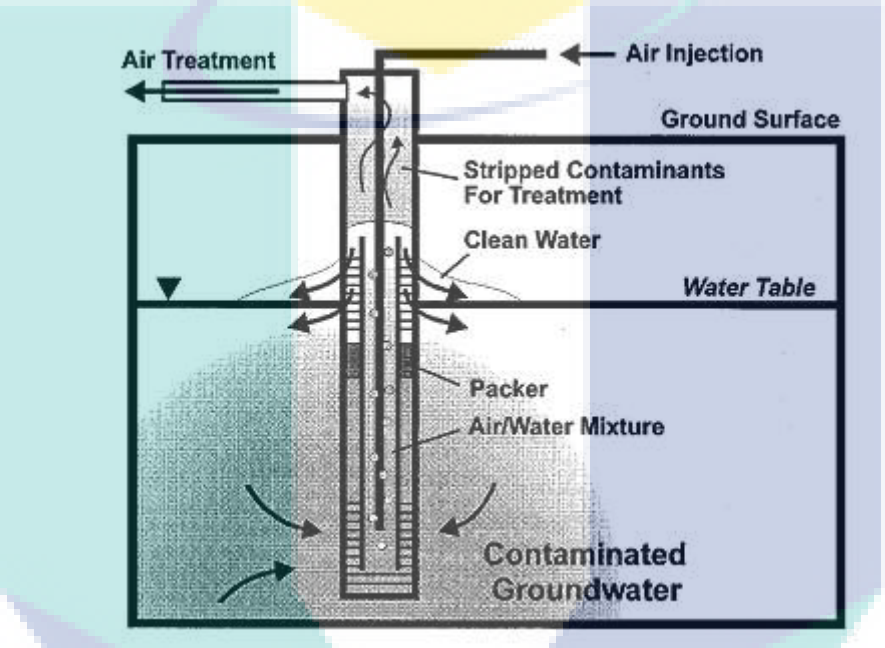


Figure 2.7 In-well vertical air sparging

2.4.1.3 Ex situ Air Stripping

Ex situ air stripping is another physical method for the treatment of BTX-contaminated wastewater and groundwater. This technology requires the usage of stripping towers, such as packed columns which have concurrent flow of liquid and gas. Subsequently, the resultant waste airstream might undergo another treatment method like adsorption (by activated carbon) or incineration. As BTX are volatile hydrocarbons, they have a natural tendency to separate from water or wastewater and evaporate. Thus, contaminated water or wastewater can be stripped by injecting clean into the bottom part, after which it moves up the stripping tower. Evidently, the levels of VOCs can potentially be reduced by as much as 99.99% when a packed air-stripping column is employed. The packed column provides the required surface area and turbulence, along

with a very low pressure, in order to increase the efficiency of reduction. In addition, ex situ air stripping is the most economical method and can give rise to minimal effluent concentration. The removal of substantial amounts of BTX can be achieved by operating the air stripper at high temperatures and relatively low air-water ratios; and vice versa. Evidently, the efficiencies of removal increase non-linearly with respect to an increase in temperature and air-water flow ratio. At a temperature of 50°C and air-water ratios of 100, the efficiencies of removal for BTX were >99%, >93%, and 93% respectively (Abdullahi et al., 2015).

Initially, the contaminated wastewater or water is injected through a packed bed into the upper part of an air stripper column. Usually, plastic media are used for the packing since it provides a large surface area per unit volume. Subsequently, by gravity, the wastewater or water flows down through the packed bed, contrary to the direction of clean air flow. Finally, the air which is injected into the bottom of the column strips the VOC and exits through the top of stripper. Clean water then leaves from the bottom of air stripping column, as shown in Figure 2.8.

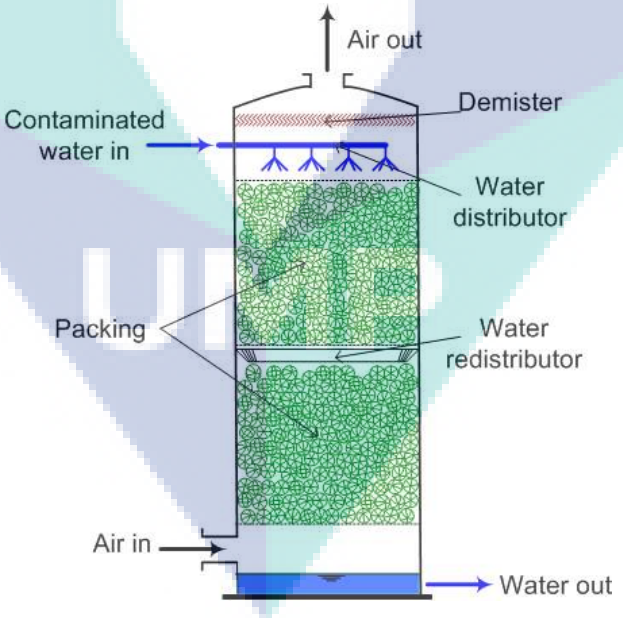


Figure 2.8 Air Stripper

2.4.2 Chemical Treatment Methods

Chemical treatment, as its name suggests, involves the utilisation of chemical products and processes such as coagulation, flocculation, dehalogenation, oxidation, and so on. The most-commonly employed methods for the treatment of BTX include ex situ dehalogenation and ex situ oxidation.

2.4.2.1 Ex situ Dehalogenation

Ex situ dehalogenation is a method whereby contaminated soils subjected to one or two processes like base-catalysed dehalogenation and glycolate dehalogenation. In the former, contaminated soils are mixed with sodium hydroxide (NaOH) in a rotary kiln with the presence of a catalyst. Meanwhile, glycolate dehalogenation involves the dehalogenation of VOC using alkaline polyethelyne glycol (APEG) as a reagent in a batch reactor. Hence, the end products will be less toxic and non-hazardous.

2.4.2.2 Ex situ Oxidation

Usually, ex situ oxidation is used in the treatment of VOC-contaminated groundwater and wastewater. The VOCs are destroyed through oxidation by UV radiation, hydrogen peroxide (H_2O_2), and ozone (O_3) sparging. Generally, VOC off-gases are treated by ozonation. Meanwhile, persulfate is typically employed as an oxidising agent in the treatment of BTX-contaminated groundwater. Owing to radical chain reactions, the rate of decomposition of persulfate increases by as much as 100 times when the concentration of benzene exceeds 0.1 mM. Also, in light of its relatively slow decomposition rate at the subsurface, it may be better to inject persulfate into the groundwater and allow it to migrate to zones of low hydraulic conductivity where clays, metal oxides, and contaminants will accelerate its conversion into reactive oxidants (Liu et al., 2014). Figure 2.9 below shows the mechanism of treatment by oxidation.

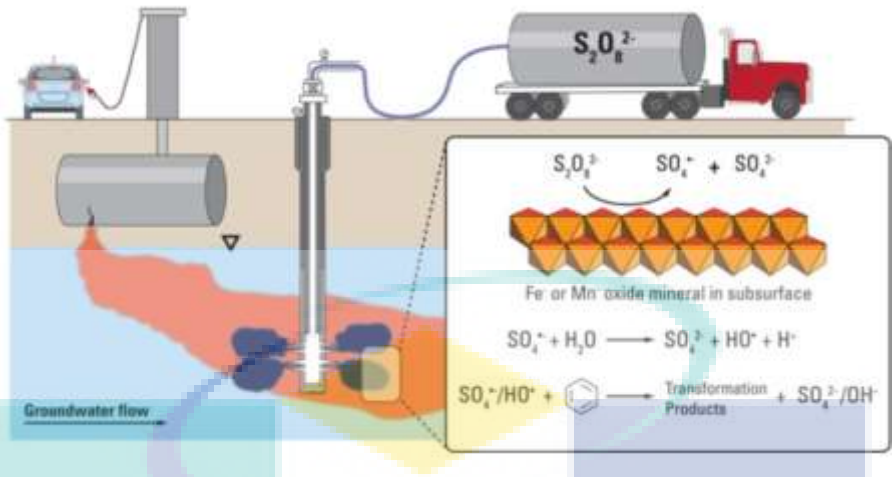


Figure 2.9 Oxidative treatment process

Source: Liu et al (2014)

2.4.3 Biological Treatment Methods

Biological treatment methods employ microorganisms to reduce contaminations, especially those by organic compounds. Organic compounds in the wastewater are utilised by the microorganisms as a food supply or carbon source, following which they convert these into biomass. Most industries use mixed culture which consist of a wide variety of organisms to effect biological treatment. Several techniques in this method include *in situ* bioremediation, biodegradation, bioventing, and biological wastewater treatment.

2.4.3.1 *In situ* Bioremediation

In situ bioremediation of VOC-contaminated soils requires injections of nutrients, oxygen, or surfactant-containing solutions in order to facilitate the breaking down of the contaminants by natural soil microbes. Usually, *Pseudomonas* bacteria are made to produce certain types of enzymes depending on the carbon source or contaminants.

2.4.3.2 Biodegradation

Biodegradation is a generic treatment process in which microorganisms are utilised to biotransform the target pollutants at hydrocarbon-contaminated areas (Mazzeo et al., 2010). The microorganisms are selected with reference to the type of hydrocarbon contaminant, although these take a long time to complete a cycle of the treatment process.

2.4.3.3 Biological Treatment of Wastewater

Biological wastewater treatment is a process in which aromatic hydrocarbons are removed from the wastewater. For easier control of certain parameters (e.g. temperature, pH, and nutrients), this process is performed in closed areas such as tanks. Populations of specialised bacteria – like Aquaficae, Thermomicrobia, and Proteobacteria (*Pseudomonas*) – are also used here in order to optimise the treatment outcomes. These bacteria utilise organic compounds as sources of carbon and energy. In the process of energy generation, oxidised inorganic compounds act as electron acceptors while reduced inorganic compounds electron donors. Different microorganisms have dissimilar characteristics and abilities. Nevertheless, almost all types of bacteria which are used in the biological treatment of wastewater are isolated from the environment such as soil, and sewage treatment plant.

In this study, biological wastewater was treated by a single species of bacteria – *P. putida* ATCC 49128. It is reputed to be the most effective bacteria for the removal of different types of chemical compounds such as phenol, VOCs, aflatoxin, chloroanilines, *p*-cresol, mercury, etc. Its efficacy aside, many studies have shown that this species is not harmful to the human and environment health.

The table below summarises the methods of biological treatment of BTX in petrochemical industry.

Table 2.3 Methods of biological treatment of BTX in petrochemical industry

Microorganism	Type of reactor	Concentration	Removal efficiency (%)	Reference
Free and mixed bacterial strain, Bb5	Batch (Shake flask)	10%(v/v) benzene, 10%(v/v) toluene, 0.5%(v/v) Xylene	100% benzene 80% toluene 53% xylene	(Singh & Celin, 2010)
Free *Pseudomonas* sp., *Yarroia* sp., *Acinetobacter* sp., *Corynebac-terium* sp., *Sphingomonas* sp.	Batch (Shake flask)	15 and 17 mg/L BTX	97% benzene, 93% toluene, 98% xylene	(Jo et al., 2008)
Immobilized *Mycobacterium* sp. CHXY119, *Pseudomonas* sp. YAT0411	Batch (Shake flask)	24.68 mg/L benzene, 23.67 mg/L toluene, 21.97 mg/L xylene	97.8% benzene, 94.2% toluene, 87.4% p-xylene	(Xin et al., 2013)
Free and immobilized *P. putida* F1	Batch (Shake flask)	15, 30, 60, 90 mg/L of B, T, o-Xylene	100% B and T, 60-80% o-xylene	(Robledo-Ortiz et al., 2011)
Free *Bacillus sphaericus*	Continuous, Bench scale corn cob-based biofilter column	0.0970 mL/L benzene, 0.0971 mL/L toluene, 0.0968 mL/L xylene	>99.85%	(Rahul et al., 2013)
Free fungus *Paecilomyces variotii* CBS115145	Batch (Shake flask)	30-60 mg/L	45% benzene, 45% toluene, 30% o- xylene	(Garcia-Pena et al., 2008)

2.5 *Pseudomonas putida*

Pseudomonas, especially *Pseudomonas putida*, is widely used in biological treatment and biotechnology (Wackett, 2003). Other *Pseudomonas* species that are popular among researchers include *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Recently, studies are being conducted on a specific organic solvent-tolerant strains of *Pseudomonas* in the biocatalysis industry (Nijkamp et al., 2006; Park et al., 2007; Ramos-Gonzalez et al., 2003; Rojas et al., 2004; Verhoef et al., 2007; Wierckx et al., 2009).

Pseudomonas putida (*P. putida*) is a Gram-negative bacterium which has a flagellum and a rod-shaped appearance (Figure 2.10). It is found in most water and soil habitats. It can grow in the presence of oxygen and carbon sources, and its optimal

temperature is 27 – 30ºC. Thus, *P. putida* can be easily isolated from its habitat for research purposes. There are several strains of *P. putida*, each with different characteristics and functions (Table 2.4).

The Institute for Genomic Research in Germany was the first organisation to almost complete the sequencing of the genome of *P. putida*. About thirty strains have been fully sequenced, but the work for another seventy five was still in progress (Kowalski, 2002). Through genome analysis, approximately 6.2 million DNA base pairs of *P. putida* have been identified. Likewise, there are almost eighty known circular genes (plasmids) which code for oxidative reductases (enzymes) that are involved in the decomposition of substances in the environment. Furthermore, the majority of these genes can quickly respond to environmental toxins (Marcus, 2003). Important *P. putida* plasmids for the degradation of pollutants include the Octane (OCT) and Toluene plasmids.

Figure 2.10 *Pseudomonas putida*
Source: Dennis Kunkel Microscopy, Inc

P. putida undergoes a highly diverse aerobic metabolism in the process of degrading organic solvents such as benzene, toluene, and xylene, apart from generating the biodegradable plastic polyhydroxyalkanoate (PHA) from styrene oil (Ward et al., 2006). Most genes of *P. putida* are involved in the breaking down of hazardous aliphatic and aromatic hydrocarbons. To most researchers, *P. putida* is a highly useful

"laboratory workhouse" in view of its versatility, strong bioremediation ability, and easy handling, especially in studies on bacterial remediation of soil (Kowalski, 2002; Marcus, 2003).

Table 2.4 *P. putida* strains and their functions

No	*P.putida* strain	Function	Topic
1	CICC 21,906	Phenol	Adsorption–synergic biodegradation of high-concentrated phenolic water by *Pseudomonas putida* immobilized on activated carbon fiber (Ma et al. 2013)
2	ATCC 55687	indene	An immersed hollow fiber membrane bioreactor for enhanced biotransformation of indene to cis-indandiol using *Pseudomonas putida* (Cheng et al., 2014)
3	KT2440	alkyl branched aromatic alkanoic naphthenic acids	Biodegradation of alkyl branched aromatic alkanoic naphthenic acids by *Pseudomonas putida* KT2440 (Johnson et al., 2013)
4	S12	production of monoethanolamine (MEA)	Biological production of monoethanolamine by engineered *Pseudomonas putida* S12 (Foti et al., 2013)
5	P300	p-cresol	Biological treatment of wastewater contaminated with p-cresol using Pseudomonas putida immobilized in polyvinyl alcohol (PVA) gel (Surkatti and El-Naas, 2014)
6	MTCC 4910	Basic Violet 3 and Acid Blue 93	Bioremoval of Basic Violet 3 and Acid Blue 93 by *Pseudomonas putida* and its adsorption isotherms and kinetics (Arunarani et al., 2013)
7	MTCC 4910	Direct Red	Bioremoval of Direct Red from aqueous solution by *Pseudomonas putida* and its adsorption isotherms and kinetics (Deepa et al., 2013)
8	CGMCC3830	cyanopyridine	Characterization and functional cloning of an aromatic nitrilase from *Pseudomonas putida* CGMCC3830 with high conversion efficiency toward cyanopyridine (Zhu et al., 2013)

Table 2.4 Continued

No	*P.putida* strain	Function	Topic
9	MTCC 1274 and 2445 (2014)	aflatoxin B1	Degradation and detoxification of aflatoxin B1 by *Pseudomonas putida* (Samuel et al., 2014)
10	T57 (2014)	chloroanilines	Degradation of chloroanilines by toluene dioxygenase from *Pseudomonas putida* T57 (Nitisakulkan et al., 2014)
11	ATCC 12633	trimethylamine	Degradation of trimethylamine by immobilized cells of *Pseudomonas putida* (ATCC 12633) (Liffourrena and Lucchesi, 2014)
12	KT2440	1,3-dichloroprop-1-ene	Engineering an anaerobic metabolic regime in *Pseudomonas putida* KT2440 for the anoxic biodegradation of 1,3-dichloroprop-1-ene (Nikel and de Lorenzo, 2013)
13	ATCC 49128	Mercury	Mercury Removal Using *Pseudomonas putida* (ATTC 49128): Effect of Acclimatization Time, Speed and Temperature of Incubator Shaker (Azoddein et al., 2015)

Ruiz-manzano et al. (2005) stated that *P. putida* employs a very complex system of metabolism, in which the proteins control specific pathways with respect to the signals received, as well as promoters and regulators present. Following receipt of the signals, it informs the nutrient's cell and oxygen accessibility. In addition, the bacteria are able to change their fatty acid saturation, isomeric forms (cis-trans isomerisation), and cyclopropane fatty acid formation in light of the fact that they have important lipids which act as a mechanism of adaptation to chemical and physical stressors.

All these characteristics allow *P. putida* to thrive in contaminated areas and survive deadly toxins. Also, the abovementioned metabolic mechanisms enable *P. putida* to convert harmful organic solvents into non-toxic compounds.

Many types of soil bacteria possess the metabolic potential to degrade a variety of aromatic hydrocarbons. BTX are highly receptive to microbial attack, and their degradation mostly occurs under aerobic conditions. Toluene has been identified to be the most easily biodegradable among the three compounds. This is possibly due to the

presence of the substituent group on the ring that offers an alternative route of attack on the side chain, or oxidisation of the aromatic ring. Microorganisms such as bacteria, algae, and fungi require aeration or dissolved oxygen as electron acceptors and utilise activation ring and cleavage of the aromatic nucleus.

Most bacteria have evolved to be able to utilise various compounds in the environment via catabolic pathways. Numerous types of aromatic compound metabolic pathways are channeled into a limited number of key intermediates such as catechols and substituted catechols. The degradation of BTX can occur through metabolic pathways which involve either dioxygenases or monooxygenases. In the latter pathway – also referred to as the "tol" pathway – the methyl or ethyl substituents of the aromatic ring are attacked and then transformed via oxidation into the corresponding substituted phenyl glyoxal or pyrocatechol. Meanwhile, the dioxygenase – or "tod" – pathway, involves the attacking of the aromatic ring with the subsequent formation of 2-hydroxy-subtituted compounds. As mentioned, these two metabolic pathways give rise to catechols, and the metabolites generated in this stage proceed to the Krebs cycle.

The first step in the oxidation of benzene involves hydroxylation, which is catalysed by dioxygenases. The presence of a substituent group in the benzene ring allows two possible mechanisms of breakdown, which are (i) oxidation of the benzene ring or (ii) attacking of side chain. The main intermediate product of benzene is catechol; in contrast, toluene degrades via a separate pathway which results in the production of its respective main intermediates: 3-methylcatechol and 3-ethylcatechol (Tsao et al., 1998; Jindrov´a et al., 2002; Johnson et al., 2003; Zhang et al., 2013; El-Naas et al., 2014). Meanwhile, all isomers of xylene are metabolised into mono-methylated catechols. For example, *m*-xylene degrades to 3-methylcatechol (Stephens, 2011), while *p*-xylene degrades into 3,6-dimethylcatechol (Oh et al., 1994).

Subsequently, these catechol intermediates are mineralised by either catechol 1,2-dioxygenase (also termed *ortho*- or intra-diol cleavage or "upper" pathway) or catechol 2,3-dioxygenase (also termed *meta*- or extadiol-cleavage or "lower" pathway). In case of the former, the resulting products and then enter the majority of pathways which contain *β*-ketoadipate (Andreoni, 2007; Jindrov´a et al., 2002; Smith, 1991). The benzene ring is then broken and degraded (Al-Khalid, 2011). Finally, low molecular weight compounds such as pyruvate and acetaldehyde are produced, and these can be

further oxidised in the Krebs cycle. Enzymes that catalyse the key steps in a catabolic pathway can be used for detecting BTX. Examples of these include catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) (Farhadian et al., 2008).

Tsao et al. (1998) reported that enriched soil cultures employ only the "tod" pathway to metabolise benzene. Meanwhile, toluene and xylenes can be oxidised by either the "tod" or "tol" pathways. However, *p*-xylene can only be biodegraded via the "tod" pathway to produce the intermediate 3,6-dimethylcatechol. Similar transformations have been observed in the metabolism of *p*- and *o*-xylenes by *P. putida* PPO1. Figure 2.11 shows the pathway of BTX metabolic by *P. putida* which was proposed by Lee et al. (1994).

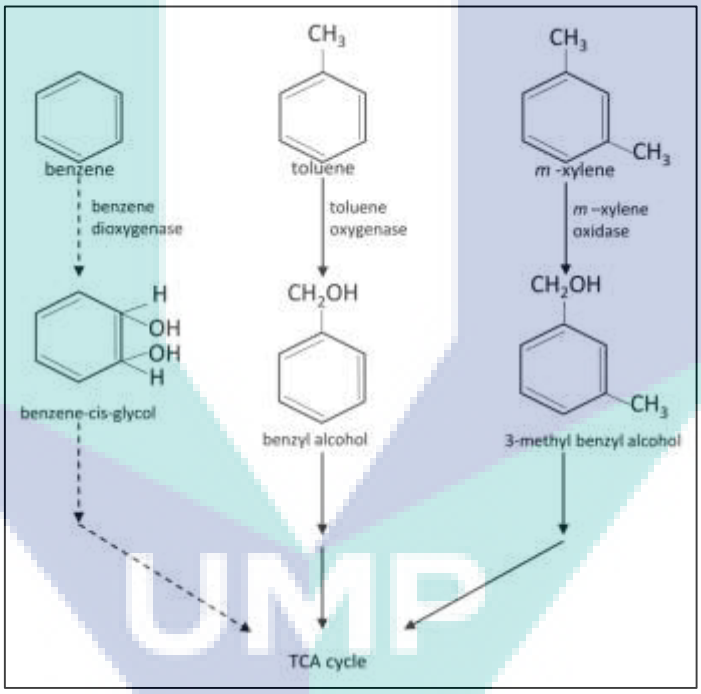


Figure 2.11 Proposed metabolic pathway for the degradation of BTX

Source: Lee *et al* (1994)

Another pathway of BTX metabolism that was proposed by Mesarch et al. (2004) and Farhadian et al. (2008) involved catechol 2,3-dioxygenase (C23O), which is a member of the extradiol dioxygenase superfamily. It catalyses the cleavage of the rings of catechols and substituted catechols. Therefore, C23O is the key enzyme of many bacterial pathways for the degradation of aromatic hydrocarbons. In more detail,

it also mediates the meta-cleavage of catechol-like substances from a wide range of aromatic compounds such as benzene, toluene, xylene, phenol, biphenyl, and naphthalene (Figure 2.12) (Mesarch et al., 2004).

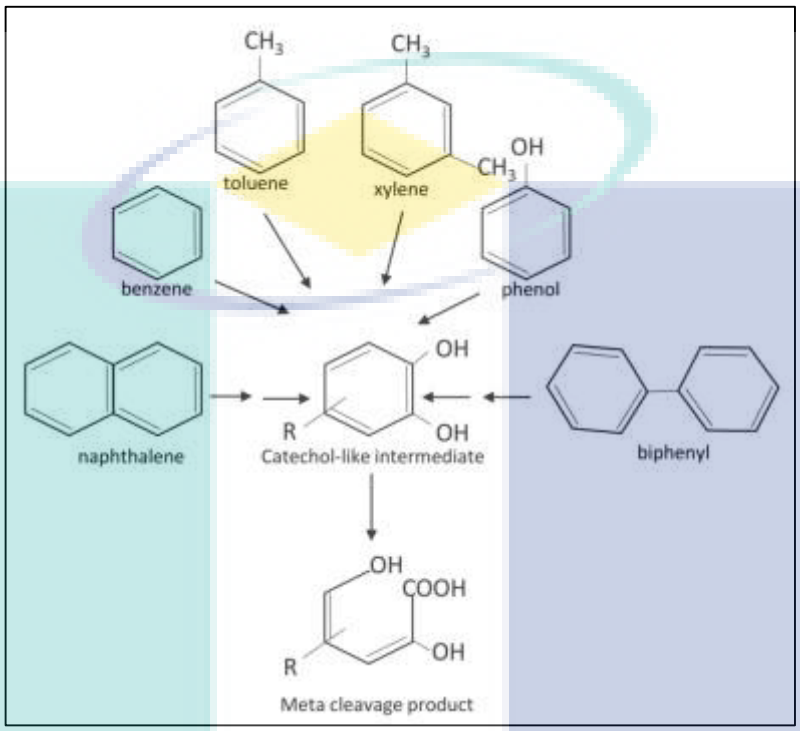


Figure 2.12 Degradation of aromatic compounds via meta-cleavage
Source: Mesarch et al (2004)

2.5.1 Growth Curve of Bacteria in Batch System

Most bacteria reproduce asexually. Their growth involves an orderly increase in cell mass and ribosome count, apart from the synthesis of new cell walls and plasma membranes, duplication of bacterial chromosomes, as well as division of the cells. This process is called binary fission, whereby a parent cell physically divides into two genetically-identical daughter cells.

Bacteria are composed of proteins, lipids, water, carbohydrates, and trace elements. Their genera can be identified or classified with respect to several properties such as specific structures, nutritional requirements, growth patterns, physiology, genetics, biochemistry, and molecular analysis.

Bacteria grow in accordance to a regular cycle which comprises four main phases: lag, growth or exponential, stationary, and death. Their growths can be determined or measured in terms of changes in cell count and cell mass. Usually, the number of colonies is used by the researchers in laboratories to determine the cell counts. Meanwhile, there a several direct and indirect methods to determine the cell mass, for example (i) direct physical measurements (dry weight, wet weight, or volume of cell per volume of medium), (ii) direct chemical measurements (total nitrogen, total DNA content, or total protein), (iii) indirect measurements of chemical activity (rate of O_2 and CO_2 production or consumption), as well as (iv) turbidity measurements (optical density; OD).

Two main aspects are required for the growth of bacteria, namely sources of metabolic energy and environmental factors. Evidently, the synthesis of new cellular components requires nutrients like carbon, nitrogen, hydrogen (donors and acceptors), minerals (sulphur and phosphorus), trace elements (magnesium, iron, and manganese), as well as growth factors (amino acids, vitamins, pyrimidines, and purines). Almost all bacteria are heterotrophs, which mean that they require organic materials like proteins, lipids, and carbohydrates for growth. In addition, the pH and temperature of the media have an effect on bacterial growth as well; most pathogenic bacteria thrive best at neutral pH (6-8) and temperatures from 30 – 40^{o}C.

During the handling or utilisation of bacteria in treatment processes, it is highly important to determine their growth rates. Researchers commonly employ batch systems to monitor this parameter since the pH, temperature and aeration (O_2) can be easily controlled. Doing so can also avoid or reduce contaminations that might otherwise interfere with the growth of the bacteria.

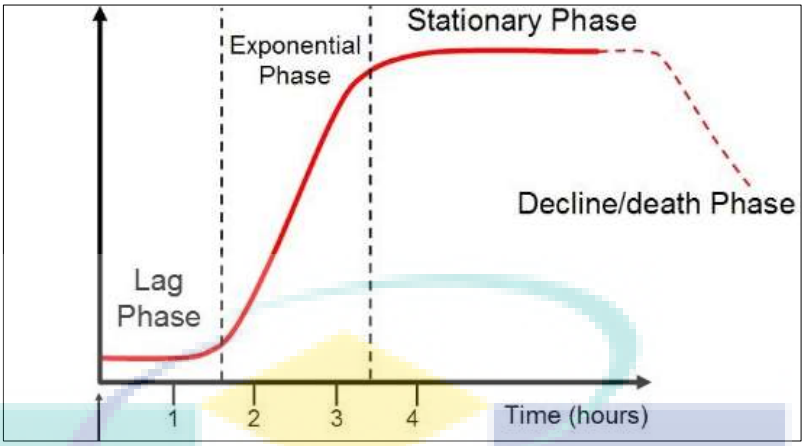


Figure 2.13 Bacteria growth curve in batch system
Source: Widdel (2007)

Figure 2.13 shows the growth curve of bacteria in batch system. The aforementioned curve can be divided into four phases: lag, growth or exponential, stationary, and death or decline.

Lag phase denotes the point in time whereby the bacterial cells attempt to adapt to a new environment. For example, during the transfer of small inoculums of "parent" bacteria into a fresh medium (i.e. a new environment) which contains different nutrients, pH, oxygen feed, temperature, and organic substrates, the organisms need some time to adapt before can duplicate and grow exponentially. Usually, in this phase, the bacterial cells increase in size but not in number. The duration of the lag phase may vary each time the bacteria are transferred into media of different types and contents. Thus, some scientists have concluded that there is no growth ($\mu = 0$) during this phase.

Subsequently, the bacterial growth curve enters the exponential phase, in which the organisms have adapted to the new environment and hence, duplicate as much as they can until the supplies of nutrients and substrates become inadequate. In addition, the harmful by-products of metabolism are yet to accumulate at this point. As such, the growth curve shows a rapid linear increase (μ = c, where c is the constant and c > 0) until the specific growth rate reaches a maximum (μ_{max}), after which it plateaus off.

In the stationary phase, growth ceases completely and the bacteria start to die owing to the exhaustion of substrates and nutrients, as well as accumulation of toxic

substances produced by the dead bacteria. The growth rate gradually approaches 0 in view of a balance between the rates of growth and death ($\mu > 0$, but decreasing to 0).

As proposed by Monod in 1942, the cell concentration (X) is inversely proportional to the specific growth rate (μ) of cells in a batch system. The value of the specific growth rate during the exponential phase is determined by the following formula (Juang and Tsai 2006):

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad 2.1$$

The final part of the bacterial growth curve is the death or decline phase, whereby there is progressive cellular death. Although small amounts of bacteria are still growing, the death rate is much higher.

2.5.2 Exponential growth

In determining the characteristics of bacterial growth, researchers usually focus on the exponential growth phase, whereby the cells successively divide to produce new generations of daughter cells. The duration of this process is called generation time (t_g), or doubling time (t_d), since the cell count doubles in each generation. Some organisms grow relatively slowly as they adapt to new environments, so their generation times are longer. Conversely, many bacteria grows very rapidly; for instance, the generation time of *Escherichia coli (E. coli*) is only 20 minutes.

Usually, the cell count (n) will increase in discrete steps, like 1-2-4-16-… After a period of time, the aforementioned parameter will no longer adhere to these discrete steps; instead, the intermediate numbers will prevail. The process of cell count doubling is shown in Figure 2.14.

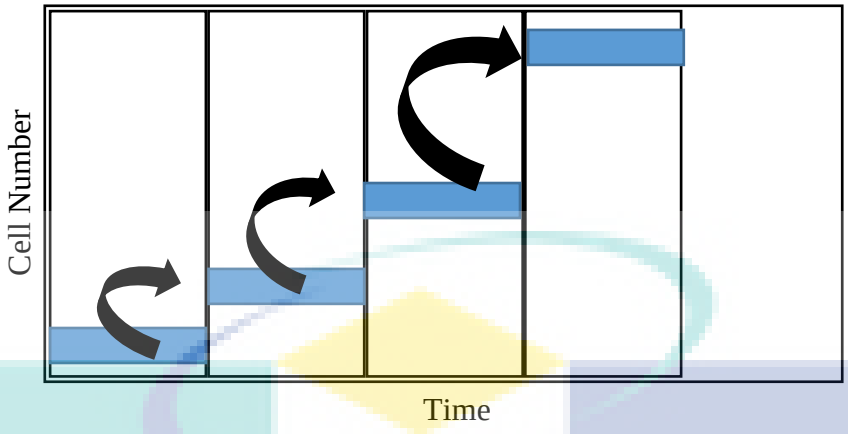


Figure 2.14 Stair-stepped batch bacterial growth

The increase in biomass (X) in a batch culture – which depends on the concentration of cells in the batch bioreactor – can be described as follows:

$$\frac{dX}{dt} \alpha X \qquad 2.2$$

The concentration of biomass can be expressed in terms of any one of the following: (i) mass per volume (g L^{-1}), (ii) dry weight, (iii) volume per volume (m^3 of biomass/m^3 of liquid), as well as (iv) cell turbidity at 600 nm (Shuler and Kargi, 2002).

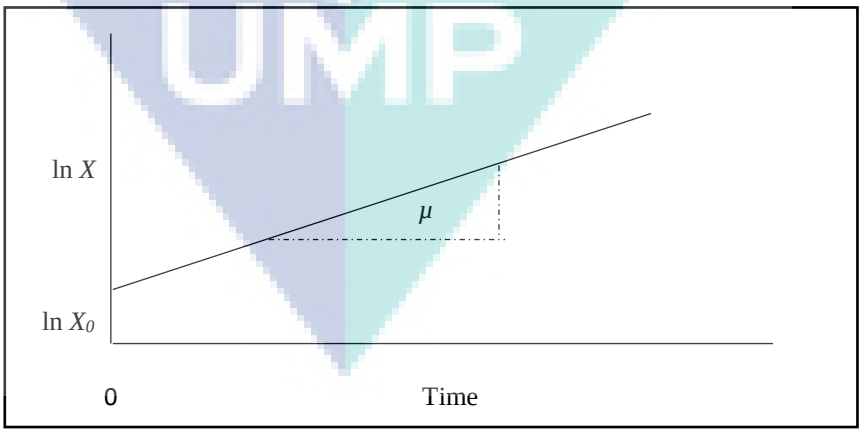


Figure 2.15 Similar plot of exponential growth

Figure 2.15 above shows a linear graph of ln X versus time (t) which depicts exponential growth. X refers to the optical density at 600 nm, while μ the slope of the line between ln X and time taken. Assuming that μ is a constant, differentiating equation (2.2) will give rise to the following formula:

$$\frac{1}{X}\frac{dX}{dt} = \mu \quad 2.3$$

Thus,

$$\frac{dX}{dt} = \mu X \quad 2.4$$

Equation (2.3) shows that μ is directly proportional to the rate at which the concentration of biomass is increasing. Thus, μ can be regarded as the specific growth rate which describes how fast the cells are reproducing. Bacterial growth rates are typically expressed as hours^{-1} (Azoddein, 2013).

When natural logarithms are incorporated into both sides of equation (2.4), the following formula results:

$$In\ X_t - In\ X_0 = \mu t \quad 2.5$$

$$In\frac{X_t}{X_0} = \mu t \quad 2.6$$

Subsequent incorporation of Euler's constant into both sides gives the following formulae:

$$e^{In(\frac{Xt}{Xo})} = e^{\mu t} \quad 2.7$$

$$\frac{X_t}{X_0} = e^{\mu t} \quad 2.8$$

$$X_t = X_o\, e^{\mu t} \quad 2.9$$

Equation (2.9) is useful for calculating the concentration of biomass at a point of time in the near future. With reference to equation (2.5), the specific growth rate (μ) can be simplified thus:

$$\mu = \frac{In\ X_t - In\ X_0}{t} \qquad 2.10$$

The specific growth rate (μ) in equation (2.10) indicates how fast the cells are reproducing. During the exponential phase, the growth rate is relatively constant; the higher its value, the faster the cells are growing. When there is no growth, then the specific growth rate is zero.

During exponential growth, the mathematical relationship involves the following parameters: (i) specific growth rate, μ, which is defined as the increase in cell mass per unit time (h^{-1}), (ii) number of generations, $n = 3.3 \ln X_t/X_o = 3.3 \ln OD/OD_0$, growth rate constant, $k = \ln 2/g = 0.693/g$, which measures of the number of generations present in an exponentially-growing culture, as well as (iv) generation time, g, which is the time taken for the formation of two cells from one doubling time (in other words, $g = t/n$, where t is duration of exponential growth expressed in days, minutes, or hours, depending on the organisms and growth conditions).

All these parameters are used to determine the growths of microorganisms in different culture conditions. They is important for the optimisation of the conditions for bacterial treatment either in negative or positive effect (Shuler and Kargi, 2002; Brock et al., 2004; Azoddein, 2013).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Introduction

This chapter describes the materials and methodology which have been utilised in the experiment to achieve the objectives in Chapter 1. Overall, the trials in this study were related to bacterial growth, cellular dry weight, sample analysis, and bioreduction of BTX by *P. putida*. The glassware and apparatuses were sterilised beforehand to avoid contamination. The sampling process and experiments were conducted in accordance to the method described by the American Society for Testing and Materials (ASTM). Figure 3.1 summarises of methodology of this research in an experimental flowchart.

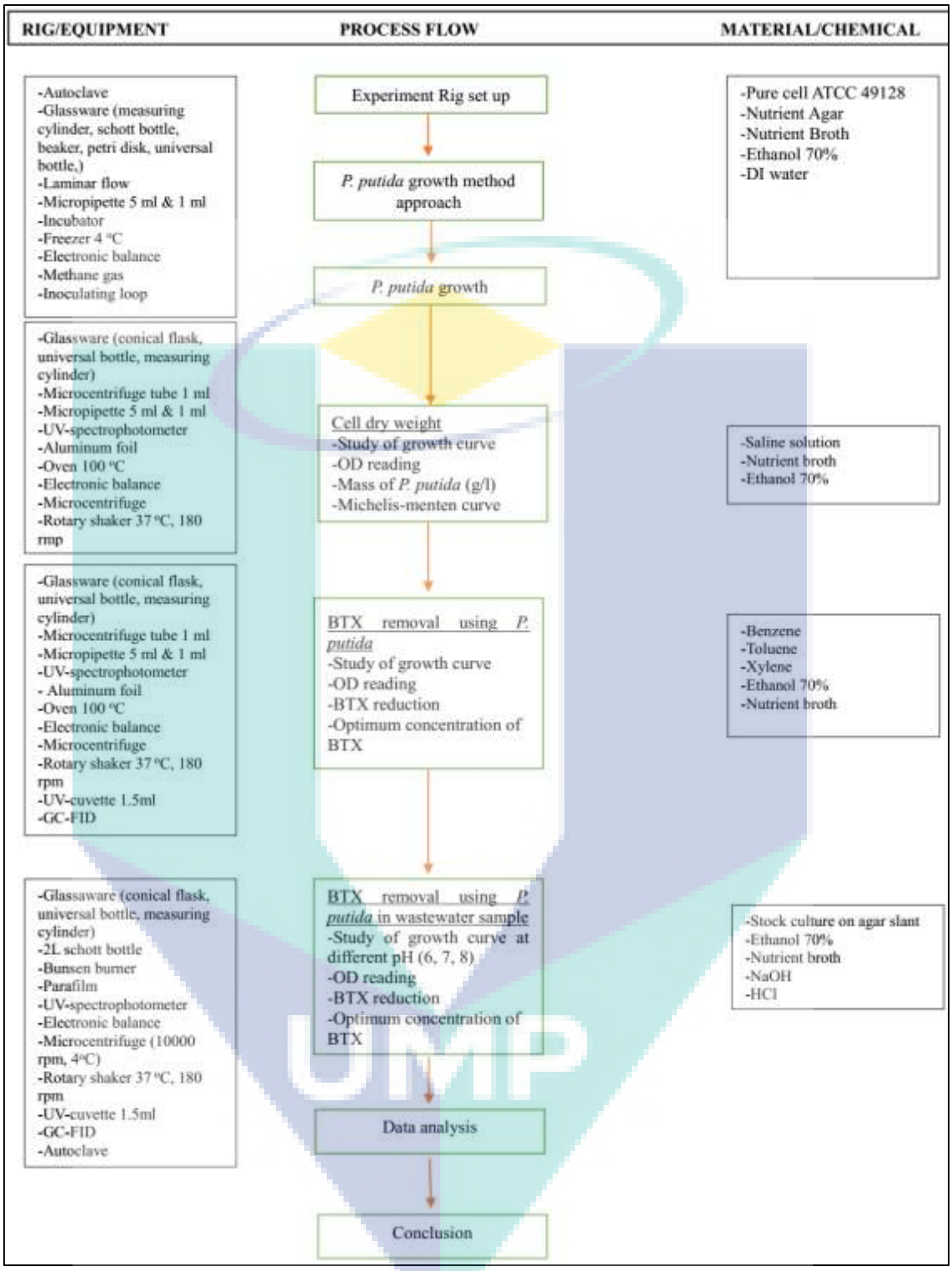


Figure 3.1 Summary of methodology in an experimental flowchart

3.2 Materials

The materials used in this study consisted of *P. putida* strain ATC 49128 as well as the chemicals for the preservation of bacterial growth in agar slants and medium broth slants. Various agents were used in for sampling and glassware-cleaning as well.

3.2.1 Microorganisms

The *P. putida* used in this study was previously used by another researcher for quite severity. The bacteria were obtained from Bio-REV Sdn Bhd. Prior to studying the growth of *P. putida* ATCC 49128, their purity was initially identified. The method and apparatus used in identifying *P. putida* came from the identification kit in the Central Lab of UMP (Appendix 1). The *P. putida* strain was determined using the Biolog microbial identification system (Biolog Inc., USA) (Otenio et al., 2005). In the process, the previous culture was grown in an agar slant for 24 hours before one loopful of bacteria from an agar plate was mixed in a cellulose-containing medium. Subsequently, they were placed in the 99 series of various media to ascertain the characteristics of the bacteria. Then, the pallet was scanned with UV light to compare the bacteria with the library in the equipment.

In the ensuing process, the bacteria were kept in media that were capable of prolonging their lifespan. Again, the materials and glassware were sterilised prior to their usage, with 70% ethanol being the cleaning solvent. Tap water was used for washing and cleaning the glassware, apart from being a cooling agent. Meanwhile, deionised water was used to prepare medium cultures and for analytical purposes.

3.2.1.1 Process of Culturing Freeze-Dried *P. putida*

Freeze-drying is commonly employed in the preparation of microbial cultures despite the fact that conventional methods involve the utilisation of glycerol. Nevertheless, the former method enables the cultures to be maintained for long periods, and hence, easier to store as well as transport. In freeze-drying water is removed while the cell-protectant-suspension concentrates maintained. Hence, the protectant fraction undergoes a transition from a liquid state to a glassy state. Bacterial inactivation during drying and storage is a complex phenomenon (Aschenbrenner et al., 2012).

The bacterial cells were subjected to environmental stress by freezing, drying, prolonged exposure to aridity, and rehydration. Determination of microbial survival is dependent on various factors such as initial concentrations of the microorganisms, intrinsic resistance of the strains, drying media, growth conditions, freezing rates, storage conditions (temperature, atmosphere, relative humidity), protective agents used, as well as rehydration (Edward et al., 2011).

The appropriate culture biosafety protocols were adopted, which included the preparation of cultures in a biosafety cabinet, wearing of suitable eye protective devices, holding of vials away from the face, wearing of gloves, as well as sterilisation of all empty vials and fragments before disposal.

The vial was opened as per the instructions enclosed by the ATCC (Appendix 2). The packaging of the culture (Figure 3.2) was removed using a sharp blade while the ampoule briskly scored once with a sharp file at about one inch (2.5 cm) from the tip. Following disinfection of the ampoule with alcohol-dampened gauze, the latter was wrapped around the former to break the scored area. Care was taken to not make the gauze too wet so as to prevent the alcohol from being sucked into the ampoule when scored area was broken. The cotton was then removed with a sterile forceps, after which 1 ml of nutrient broth was withdrawn from the vial using a micropipette. Then, the cap was closed again before the freeze-dried bacteria were mixed with nutrient by shaking the vial. The bacteria-containing medium broth was transferred into a universal bottle which had approximately 5 – 6 ml of nutrient broth. Several drops of suspension were added to agar plates (Figure 3.3) and agar slants (Figure 3.4). Both types of media were incubated at 30^{o}C for 24 hours.

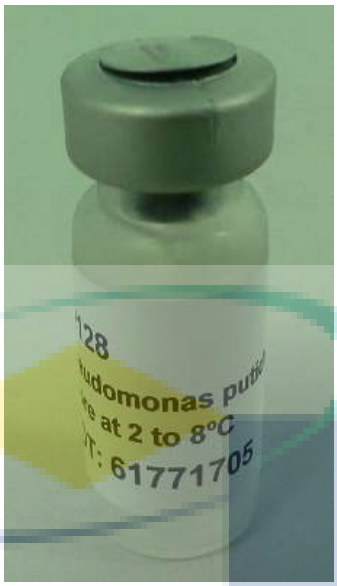


Figure 3.2 Freeze-dried *P. putida* ATCC 4912

Figure 3.3 *P. putida* in agar plate

Figure 3.4 *P. putida* in agar slant

3.2.1.2 Stock Cultures

It was essential to ensure the purity of the stock cultures so that they could be used for longer periods without the loss or reduction of any nutrient. Evidently, nutrient agar can only preserve bacteria for a few weeks, after which the cultures will start to decompose in view of nutrients depletion and toxic by-product accumulation. Hence, in order to maintain the viability of the bacteria for a long time, a stock culture was prepared and stored in a refrigerator at -10^{o}C. This stock culture was able to last for more than six months.

There are several methods to transfer a culture from broth to slanting agar in a laminar-flow cabinet. In this study, an aseptic technique was employed whereby the sterility of the media had to be maintained during the transfer of pure cultures of *P. putida*. First, the inoculating loop was sterilised in a Bunsen flame until it was red-hot. The loop was allowed to cool before being dipped into the broth culture. The test tube which contained the pure cultures was shaken to disperse the cells. Subsequently, the cap of the test tube was removed and the lip of the test tube sterilised in the Bunsen

flame. The culture tube was then held in a slanting position while the inoculating loop inserted into the culture broth.

Next, the culture tube was capped after its lip flamed. The culture tube was then put aside and a test tube which contained NA was opened and its lip flamed as well. The inoculating loop that contained a smear of culture was inserted into the second test tube, after which the loop was gently slid in a continuous streaking motion on the surface of the agar. The loop was again flamed, while the test tube which held the pure culture on NA was incubated at 30°C for 24 hours. It was then kept as a stock culture in a freezer at 4^{o}C (Li et al., 2010).

3.2.2 Chemicals

All chemicals used in this study were of analytical grade, Ethanol (70%), glycerol, nutrient broth (NB), nutrient agar (NA), sodium chloride (NaCl), nitric acid (HNO_3), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Permula Chemicals Sdn Bhd. Meanwhile, benzene (99.7%), toluene (99.8%), and xylene (99.8%) were purchased from Arachem Sdn Bhd. All the aforementioned chemicals were produced by Merck.

3.2.3 Apparatus and Equipment

The equipment used in this study could be divided into two categories: experimental and analytical. Both types had their respective functions in the eponymous activities for data collection.

3.2.3.1 Experimental Apparatus

The experimental apparatus, as their name suggests, are the main equipment for the conduction of the experiments. In this study, they were an autoclave, microbiological incubator (Memmert), freezer (at 4^{o}C), laminar air flow cabinet (ESCO), and oven (Memmert).

3.2.3.2 Analytical Apparatus

The analytical apparatus were directly involved in the analysis and determination of the compounds or substances, as well as the identification of the operating parameters of the processes. In this research, the said apparatus were UV-visible spectrophotometer (U-1800, Hitachi), pH meter (Mettler Toledo), turbidity meter (HACH 2100P), BOD meter (YSI 5000), GC-FID (Perkin Elmer), and analytical balance (Mettler Toledo).

3.3 Methodology

Methodology is an important part of research since the employment of a wrong method used will invalidate the results. Thus, the experimental procedures – which are further detailed in the following sections – were taken from the standard methods stipulated by a few agencies and previous researchers. The aim of the experiment was to achieve and establish the aforementioned objectives. It involved a series of research activities as summarised in Figure 3.4.

3.3.1 Medium

Medium agar was prepared by weighing 23 g of nutrient agar powder (Merck: BD 213020) using digital balance. After placing the powder into a 1.0-litre Schott bottle, 1.0 litre of deionised water was added. The bottle was sterilised at 121^{o}C for 20 minutes in an autoclave and then cooled until its temperature was 50^{o} C (Belzile et al., 2006). Some 15 – 20 ml of solution was poured into Petri plates, each of which was half-filled. The plates were cooled to allow the nutrient agar to solidify prior to their storage in a freezer at 4^{o}C for further processing. Medium broth was prepared by adding 8 g of nutrient broth (Merck: BD 234000) to 1.0 litre of deionised water in a Schott bottle, which was then shaken until the nutrient broth was well-mixed. Subsequently, the medium was sterilised by autoclaving at 121^{o}C for 20 minutes. The agar was ready to use after it was cooled.

3.4 Analysis of Wastewater from Petrochemical Industries

BTX-contaminated soils, groundwater and wastewater are minor polluters of the environment. BTX pollution from petroleum-based industries contributes to the global BTX cycle, whose details are difficult to estimate owing to lack of data and information, especially those pertaining to the wastewater from petrochemical industries in Malaysia.

This paper summarises the case study that was carried out to determine the extent of BTX contamination by a petrochemical industry at the East Coast of Peninsular Malaysia. The petroleum-processing factory in question began its operations in 1984, and was the first to be involved in gas production. It was located approximately 110 km south of Kuala Terengganu in an industrialised area which was dedicated to oil and gas facilities. This industry was designed to accommodate gas and its condensates. With respect to the previous study held at this plant, the concentrations of BTX in its wastewater were higher than maximum levels allowed by the USEPA. In 2006, a BTX test which was conducted at the balancing tank outlet by an external investigator shows that their concentrations were approximately 30 ppm, 17 ppm, and 20 ppm respectively. In 2011, the values at the same site were 50 ppm, 30 ppm, and 18 ppm respectively (Appendix 3).

This time round, the samples were collected from the balancing tank outlet of a wastewater treatment plant of the petroleum- and gas-processing plant. Sampling was conducted for a total of 4 times at random times in order to obtain the average concentrations of BTX over years.

3.4.1 Wastewater Sample Collection

For this investigation, samples from a petroleum-based industrial wastewater plant were collected as per the Standard Methods by the EPA (Appendix 4).

Procedural precautions were observed during the collection of wastewater samples. Examples included storing of the samples at a secure location and wearing of proper personal protective equipment (PPE) to avoid any danger to and contamination of the samples and personnel. As mentioned, the wastewater samples were collected

from the centre of the balancing tank outlet, and only clean containers (bottles, beakers) were used for this purpose. A new, clean pair of disposable gloves was worn during each sampling exercise. The containers were rinsed several times with DI-water prior to their usage. Ideally, the duration from the collection to the analysis of the samples should be minimal. However, depending on the test, special precautions for the handling the samples were necessary to prevent natural interferences such as organic growth or changes in the levels of dissolved gases. Figure 3.5 below shows the petrochemical wastewater in a 500-ml plastic bottle.

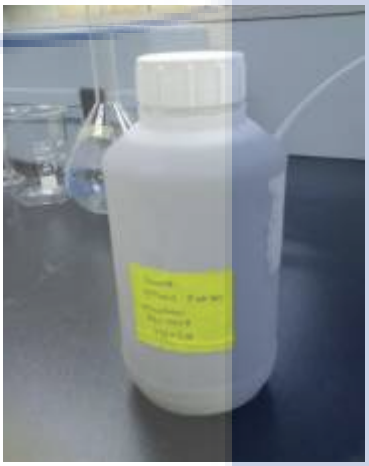


Figure 3.5 Petrochemical wastewater sample

3.4.2 Sample Handling and Preservation

Following sample collection from the effluent, proper storage and preservation was done in accordance with the requirements outlined in 40 Code of Federal Regulation (CFR), part 136.3 (e), Table II, and Figure 3-1 of the USEPA Region 4 Analytical Support Branch Laboratory Operations and Quality Assurance Manual. This measure was taken to ensure the absence of further contamination of the samples. The procedures that were involved were acid-washing, whereby the glassware or plastic items were cleaned using laboratory detergent. After thorough rinsing with tap water, the glassware was rinsed with 1:1 hydrochloric acid (HCl) solution. Subsequently, they were rinsed with deionised water and dried in the oven.

Methods of sample preservation include pH control (with concentrated hydrochloric acid), refrigeration at 4°C, and freezing. In this study, the samples were immediately refrigerated at 4°C for further analysis.

3.4.3 Turbidity

Turbidity analysis was conducted according to Appendix 5 of ASTM D7315. The equipment was calibrated using a set of standard solutions (0.01, 10, 100, and 1000 NTU) prior to the analysis of the actual samples to ensure the turbidity meter was in good condition. The inner and outer surfaces of glass sample tubes have been thoroughly cleaned using HCl and rinsed a few times using distilled water. The glassware was then dried in an oven. From here, 10 ml of sample was filled in the aforementioned tubes. After that, the sample tubes were placed individually in the turbidity meter and the "read" button pressed. The reading was recorded after an unchanging value was displayed. These values were measured in triplicates, after which their averages were calculated.

3.4.4 pH

The pH meter (Mettler Toledo Delta) was used to measure the pH of the samples. These measurements were carried out immediately after sample collection. Prior to its usage, the pH meter was calibrated using buffer solutions of pH 4, 7, and 10 to ensure that it was in good condition. Some 100 ml of sample was placed in a 250-ml beaker after sampling. Then, the electrode of the pH meter was immersed 5 cm below the surface of the sample and stirred for 2 – 5 minutes to ensure sample homogeneity. The stable reading was obtained, and this process repeated three times to obtain the average reading.

3.4.5 Total Suspended Solid (TSS)

This test was conducted with reference to the standard method in Appendix 6 of APHA 2540. The filter disc, with its wrinkled side up, was placed in the filtration apparatus. Then, the vacuum was applied and the filter disc washed three times with 20 ml of distilled water each. Suction was continued until all traces of distilled water (DI) were removed. The vacuum pump was turned off and the washings discarded. The filter disc was removed from the filtration apparatus and transferred to an inert aluminium weighing dish. Subsequently, it was dried in an oven at 103 – 105ºC for 1 hour. The filter disc was removed and placed in the desiccator until it has cooled down to room temperature. After 30 minutes, the filter disc was weighed. The drying, cooling, desiccating, and weighing processes were repeated until the obtained weight was either (i) constant or (ii) having a difference of less than 4% with respect to the previous weight.

The filtering apparatus were assembled as per Figure 3.6, whereby the filter disc was placed after its initial weight was obtained. Then, 10 ml of distilled water was used to wash the filter disc. Subsequently, 100 ml of wastewater sample was stirred by the magnetic stirrer and filtered. Suction was done until about 3 minutes after filtration was completed (i.e. the point of time whereby the sample was able to completely drain between washings). Centrifugal force was used to separate the sample particles by size and density. Next, 10 ml of distilled water was used to wash the filtering apparatus. Suction was continued until the filter disc was completely dry. The filter disc was carefully removed from filtering apparatus and replaced in the aluminium weighing dish for support. The disc was dried in an oven at 103 – 105ºC for 1 hour. Then, it was cooled in the desiccator to attain a stable temperature and weight. The drying, cooling, and weighing processes were repeated until a constant weight was obtained. TSS was then calculated according to equation (3.1):

$$\text{Total Suspended Solid(TSS), mg/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}} \qquad 3.1$$

Where *A* is the combined weight of filter disc and dried residue in mg, while *B* the initial weight of filter disc.

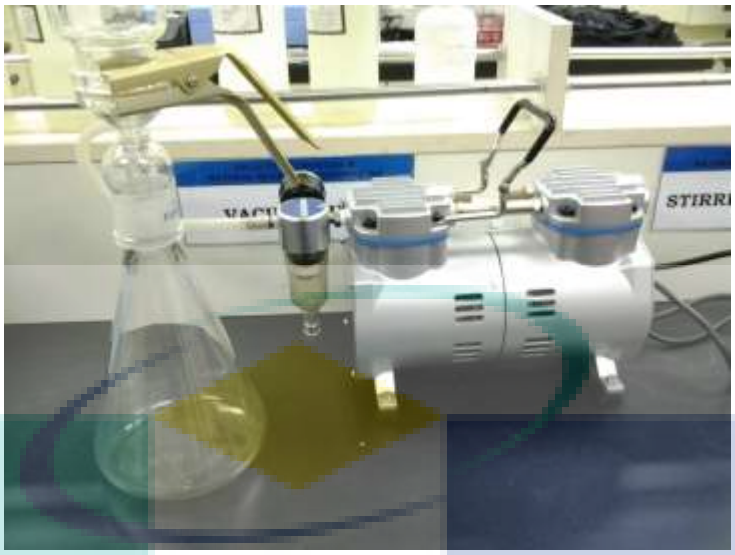


Figure 3.6 Filtration apparatus

3.4.6 Biochemical Oxygen Demand (BOD)

The test was conducted according to the standard method in Appendix 7 of APHA 5120b. This method entailed the filling and overflowing of an airtight bottle of a specified size [Figure 3.7 (a)] with the sample. After that, the bottle was incubated in BOD incubator at 20°C for 5 days [Figure 3.7 (b)]. The wastewater sample was tested within 2 to 24 hours of collection. The samples were also kept at 4°C during compositing in the freezer.

A 300-ml incubation bottle was cleaned using detergent, after which it was rinsed thoroughly and drained before use. First, the dilution water was prepared by adding a desired volume of water into a 1-L volumetric flask. Then, 1 ml each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solutions were added per litre of water. The wastewater sample was checked to ensure its pH range was suitable to proceed to the BOD test. The pH had to be balanced between 6.5 and 7.5. The initial pH of this wastewater sample was 13, which was very alkaline. Thus, the sample was neutralised with sulfuric acid (H_2SO_4) until the pH attained that of the BOD standard.

Some 20 ml of wastewater sample was placed into a 300-ml BOD bottle. Then, the BOD bottle was filled with the pre-preared dilution water until it was completely full. The bottle was capped in such a way that there were no air bubbles within. The

initial reading of DO was measured using the eponymous meter, whereby the DO electrode was dipped into the prepared sample. The bottle containing the BOD sample was stoppered tightly, water-sealed, and incubated in a BOD incubator for 5 days at 20°C (Sridhar et al., 2011). The initial DO value was taken within 30 minutes of sample preparation, as per the abovementioned standard. The final DO value – which was taken after 5 days – was determined according to the equation (3.2):

$$\mathrm{BOD}_5\,, \frac{mg}{L} = \frac{\mathrm{D}_1 - \mathrm{D}_2}{P} \qquad 3.2$$

where D_1 is the DO value (in mg/L) of the diluted sample which was immediately taken after preparation, D_2 the DO value (in mg/L) of the diluted sample after incubation for 5 days at 20°C, and *P* the volumetric fraction of the sample used.

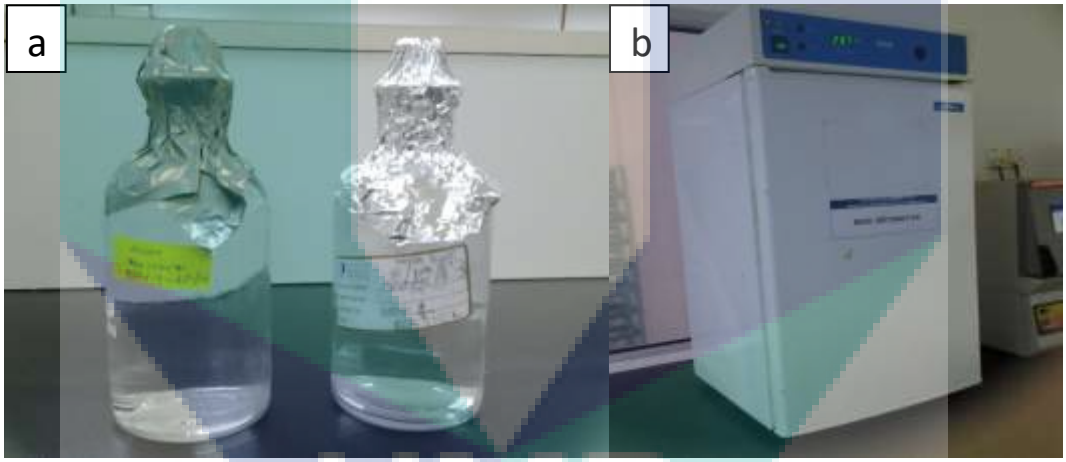


Figure 3.7 (a) 300-ml BOD bottle, (b) BOD incubator at 20°C

3.4.7 Chemical Oxygen Demand (COD)

The test was carried out in according with the standard method in Appendix 8 of USEPA 5220b. Some 100 mL of wastewater sample from the petroleum-based plant was homogenised in a blender for 30 seconds. At the same time, the COD reactor (HACH DRB200) was preheated to 150°C for 30 minutes. Two COD digestion vials were placed on the rack in preparation to be filled with the wastewater sample and blank sample respectively. Then, 2 ml of sample was pipetted into the first vial while 2 mL of

distilled water pipetted into the second vial. Both vials were tightly closed, rinsed with water, and wiped with a clean paper towel. The vials were gently turned over a few times to mix the sample with the reagent. Subsequently, they were placed in the COD reactor and heated to 150°C for two hours. After that, the vials were allowed to cool down to room temperature. They were then inverted and their outer surfaces cleaned. The COD value was determined using the HACH DR/2400 spectrophotometer.

3.4.8 GC-FID of BTX Analysis

The concentrations of BTX in industrial wastewater samples were analysed using capillary column gas chromatography with a flammable ionisation detector (GC-FID). This method was in accordance with that in Appendix 9 of ASTM D5060. The sample was initially filtered to remove the suspended solids. Four calibration standards which contained different concentrations of each organic compound (0.5 – 200 µg/L) were prepared, after which they were transferred to 2-mL GC vials for analysis. The initial calibration standards were analysed as per the abovementioned method. The voltage of the detector (electron multiplier) was adjusted to (i) provide an adequate response to the lowest level of calibration and (ii) avoid saturation at the highest level of calibration. The data for the initial calibration was processed using GC-FID and evaluated in the form of a linear graph (Appendix 10). The said analysis was performed using an Agilent GC–FID system and a 60 m × 0.32 mm id × 0.5 µm HP-INNOWax column (Williams, 2014). Injections were performed in the split ratio 100:1, 0.5 µL, constant carrier gas (helium) flow of 2.5 ml/min, 32 cm/s, and at 19.9 psi (60°C). The injector and interface temperatures were maintained at 220°C while the chromatographic column 50°C during the analyses.

3.5 Growth of *P. putida* in Shake Flasks

Studies on the bacterial growth are a basic form of microbiological analysis. Thus, the optimum conditions for *P. putida* – such as temperature, shaking speed, and pH – were very important to be evaluated before proceeding to the other experimental parameters. Shake flasks were used to growth *P. putida* since the parameters could be easily manipulated.

3.5.1 Sterilisation of Glassware

All glassware was washed with liquid detergent and rinsed with tap water. Then, they were dipped in 1% HCl to neutralise any remaining chemical, after which they were rinsed again using distilled water. An oven was used to dry the glassware at 60^{o}C for 15 minutes. Glassware with open mouths were closed using aluminium foil before being sterilised in an autoclave at 121^{o}C for 20 minutes to avoid any bacterial or chemical contamination before proceeding to the experiment.

3.5.2 Preparation of Bacteria Inoculums

Three loops of *P. putida* which have been cultured in the nutrient agar were placed in 10 ml of nutrient broth in a 20-ml universal bottle, such that the concentration was 23 g/L. The sample was incubated in an incubator for 24 h at 37^{o}C. After that, 20 ml of bacteria was transferred into a 500-ml conical flask which contained 200 ml of medium broth. The volume ratio of medium broth to *P. putida* was 1:9, or 10% of the overall concentration, according to previous studies (Azoddein et al., 2015). The sample was placed in an incubator shaker at 37^{o}C and shaking speed 180 rpm. All preparative processes were conducted in laminar air flow in the presence of a flame.

3.5.3 Determination of Growth of *P. putida*

Every 2 hours, 2.5 ml of sample was taken for UV spectrophotometry until the optical density (OD) decreased (i.e. the decay phase was attained). The sample was transferred using a 5-ml micropipette into a 2.5-ml UV cuvette. A UV spectrophotometer (UV-Vis, U-1800, Hitachi) was used to monitor bacterial growth at 600 nm of absorbance (Juang and Tsai, 2006).

3.5.4 Determination of Dry Weights of Cells

Biomass concentration is one of the most vital parameters of bacterial growth. Cell densities can be quantified in two basic ways: (i) grams of dry or wet weight per litre of sample (Azoddein et al., 2015), or (ii) number of viable/ dead cells per ml. Some

1.5 ml of sample was transferred into a 2-ml centrifuge tube using a 5-ml micropipette. Then, the sample was centrifuged at 5 000 rpm for 10 minutes. The supernatant was then decanted and washed twice with saline solution. Subsequently, the sample was transferred into a weighing boat and dried in an oven for 30 minutes at 103 – 105ºC (Bandaiphet and Prasertsan, 2006). It was cooled in desiccator until room temperature was attained, after which it was weighed using a electronic balance. The weighing boat was weighted before and after transferring of the biomass. The drying, cooling, and weighing processes were repeated until their respective values were stable.

3.6 BTX Removal by *P. putida* in Shake Flasks

The growth characteristics of *P. putida* in a stock solution at optimum conditions were determined before proceeding to the experiments which utilised actual wastewater from petroleum-based industries. Therefore, a series of trials were carried out in order to determine the ideal concentrations of benzene, toluene, and xylene as carbon sources (Otenio et al., 2005) for the growth of *P. putida*. These values were compared with the initial concentrations of BTX in the wastewater sample.

3.6.1 Preparation of Stock Solution

The stock solution of BTX was prepared using standard grades of benzene, toluene, and xylene. They were diluted with distilled water to get 200 ppm of each. The simulation sample was prepared by diluting the stock solution (200 ppm) with nutrient broth (NB) into 5 different concentrations for each solvent: benzene (25 ppm, 35 ppm, 45 ppm, 55 ppm, and 65 ppm), toluene (10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm), as well as xylene (10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm). These values varied according to the concentrations of BTX in the petrochemical wastewater sample (Table 4.1 and Appendix 3).

3.6.2 Determination of *P. putida* Growth in Medium Broth

A simulated solution sample was added to *P. putida* at a ratio of 9:1 or at 10% of *P. putida* concentration in nutrient broth, and the resultant mixture was placed into an incubator shaker at the ideal conditions: pH 7, shaking speed 180 rpm, and 37ºC

(Azoddein et al., 2015). Some 2.5 ml of sample was taken every 2 hours until the bacterial growth decreased (i.e. decay phase). The sample was placed in a-2.5 ml UV cuvette and its OD determined by UV spectrophotometry at 600 nm of absorbance. The bacterial growth curve was plotted to identify the growth pattern. Figure 3.8 shows the stock solution samples containing different concentrations of *P. putida* at (a) 0 h and (b) 24 h of incubation.

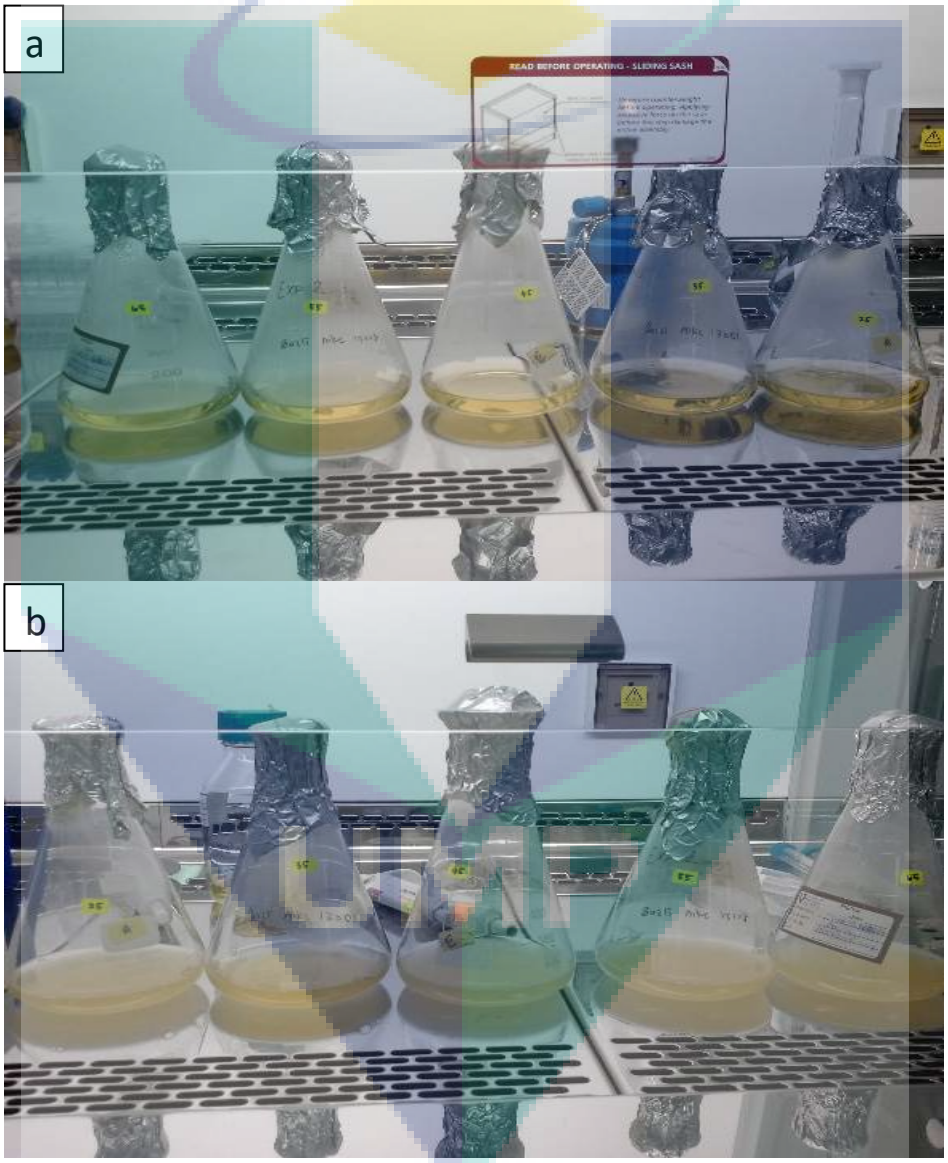


Figure 3.8 Simulated solution sample with *P. putida* at different BTX concentration at (a) 0 h and (b) 24 h after incubation

3.6.3 Preparation of Samples for GC-FID Analysis

Some 10 ml of sample was taken at 12 hours, 24 hours, and 36 hours of incubation to determine the reduction of BTX. Each sample was extracted using dichloromethane (DCM) in a separator funnel to separate water from the BTX solvent. The samples were shaken for 2 min, after which the separator funnel valve was opened to release the pressure. The shaking process was repeated for 15 min, after which the samples were left to settle down. Two layers of liquid were formed, in which DCM was the bottom one (Figure 3.9). The lower layer was then transferred into a GC vial for GC-FID analysis.

Figure 3.9 Liquid-liquid extraction using DCM

3.7 BTX Removal from Wastewater Samples by *P. putida*

A petrochemical wastewater sample was taken from one of the petrochemical plants in the eastern part of Peninsular Malaysia. The sample was analysed in terms of pH, BOD, COD, TSS, and BTX concentrations. This step was important to maximise the removal of BTX using *P. putida* ATCC 49128.

3.7.1 Preparation of Wastewater Samples

Some 200 ml of wastewater samples were prepared by making their pH values 6, 7, and 8 respectively. The pH meter (Metler Toledo) was calibrated using a standard calibration liquid prior to its use. Then, 20 ml of *P. putida* was added to 200 ml of wastewater sample into a 500-ml of conical flask. The prepared sample was incubated in the incubator shaker at 37^{o}C and 180 rpm.

3.7.2 Determination of Growths of *P. putida*

Every 2 hours, 2.5 ml of sample was taken until the decay phase was attained. The sample was placed into a UV cuvette and the optical density (OD) was determined by UV-Visible (U-1800, Hitachi) (Figure 3.10) at 600 nm of absorbance to identify the growth pattern.

Figure 3.10 UV-Visible (U-1800, Hitachi)

3.8 Summary

The research methodology has been described in brief. Samples from petrochemical industries were analysed to identify their parameters like BOD, COD, pH, TSS, and BTX concentration. At the same time, *P. putida* cultures were prepared to determine their growth patterns and the best conditions needed for the same. Then, the simulated BTX solution was prepared with reference to the concentrations of BTX obtained from the initial analysis. Five different concentrations of benzene, toluene, and xylene respectively were prepared, and *P. putida* was added to each so that the bacteria-stock solution ratio was 1:9. Experiments were conducted using these mixtures. The growths of *P. putida* were analysed using a UV-Vis spectrophotometer at 600 nm of absorbance. In each experiment, 10 ml of sample was taken after incubation for 12 hours, 24 hours, and 36 hours to identify the resultant concentrations of BTX. The samples were analysed using GC-FID to determine the same. The data was recorded, and the optimal concentrations of BTX analysed using Microsoft Excel.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Overview

This research presents the outcomes of the study on the removal of benzene, toluene, and xylene from industrial wastewater by *P. putida* ATCC 49128. Industrial wastewater samples were analysed in terms of their concentrations of BTX, pH, BOD, COD, and temperature, all of which had an effect on the removal of BTX. The experiment was then conducted using simulated BTX solutions of different concentrations as per the maximum and minimum values determined earlier. Finally, the experiment was executed using actual petrochemical wastewater sample (which was taken from a petrochemical plant) in a shake flask at ideal conditions.

4.2 Analysis of Petrochemical Wastewater

The petrochemical effluent samples were taken at four different periods from one of the petrochemical plants in the East Coast of Malaysia in order to obtain average values of the parameters (pH, BOD, COD, TSS, turbidity, as well as concentrations of benzene, toluene, and xylene). Table 4.1 shows the results of the four samples which have been taken at four different times at the same sampling point.

Table 4.1 Results of petrochemical wastewater analysis

No.	Parameter	Sample 1	Sample 2	Sample 3	Sample 4	Standard Effluent
1.	pH	13.25@ 21.6 ºC	13.91@ 23.1 ºC	14.00@ 22.7 ºC	14.00@ 24.7 ºC	5.5 - 9.0 (DOE)
2.	BOD	20.78 mg/L	18.10 mg/L	27.17 mg/L	25.21 mg/L	50 mg/L (DOE)
3.	COD	85.6 mg/L	91. 7 mg/L	95.2 mg/L	93.3 mg/L	100 mg/L (DOE)
4.	TSS	11 mg/L	13 mg/L	19 mg/L	16 mg/L	100 mg/L (DOE)
5.	Turbidity	21.6 g/L	25.2 g/L	22.6 g/L	21.3 g/L	50 mg/L (DOE)
6.	Benzene conc.	46.7 mg/L	55.2 mg/L	43.1 mg/L	50.2 mg/L	57 µg/L (USEPA)
7.	Toluene conc.	11.6 mg/L	13.3 mg/L	26.9 mg/L	22.4 mg/L	28 µg/L (USEPA)
8.	Xylene conc.	21.6 mg/L	32.8 mg/L	22.4 mg/L	27.5 mg/L	-

With reference to Table 4.1, the pH of the petrochemical effluent ranged between pH 13 and 14, hence showing that the samples were very alkaline. This value was much higher than the standard effluent pH range of 5.5 – 9.0, according to Malaysia's Environmental Quality Act 1974 and Environmental Quality (Industrial Effluent) Regulation 2009. The alkalinity provides the buffering capacity to maintain a normal pH. Alkalinity is brought about by the carriage water (i.e. sodium and calcium carbonates), proteins, and urea in the wastewater. Microbes need an environment with an average pH of 6 – 8. Thus, the actual sample was neutralised by hydrochloric acid (HCl) to obtain pH values of 6, 7, and 8.

At least the minimum value of DO (1 to 3 mg/L) needs to be present to maintain the biological activity in wastewater. Aerobic bacteria such as *P. putida* require oxygen to decompose the dissolved pollutants. When there are large amounts of pollutants, a lot of bacteria are needed, so the demand for oxygen will increase. BOD determines the quantity of dissolved organic pollutants that can be removed via oxidation by bacteria.

As per the results, the BOD of the petrochemical sample was between 18 and 27 mg/L, which was below the standard BOD for effluents (50 mg/L).

On another note, COD measures the quantity of dissolved organic pollutants that can be removed by chemical oxidants in the presence of strong acids. The COD of the sample was between 85 and 95 mg/L, which was also below that of the standard (100 mg/L). A BOD/COD ratio denotes the fraction of pollutants in the wastewater that are biodegradable; in this case, the ratio was around 0.3.

Suspended solids were another determinant of the required type of treatment process. Normal domestic wastewater contains 200 mg/L total suspended solids (TSS), of which 80% are volatile suspended solids (VSS: 160 mg/L). As per the data, 65% of VSS in raw wastewater are biodegradable, and these accounted for about 100 mg/L BOD. It is important to note that some of the VSS are biodegradable while others are not. BOD – which symbolises the oxygen utilised for the metabolism of biodegradable suspended solids or soluble organic compounds – is hence not independent of the amounts of both types of substances mentioned above. In this study, the average TSS was 14.75 mg/L.

As a conclusion, the initial values of the parameters of the petrochemical wastewater effluent showed that the bacteria were able to grow well. However, the pH was very alkaline and had to be readjusted until the range was between 6 and 8. Other characteristics such as BOD and COD were acceptable in this research.

4.3 Growth Pattern of *P. putida* in Shake Flask

The bacterial growth curve is helps in the identification of the growth pattern of *P. putida* in a laboratory. In this study, the effects of environmental factors on the growth pattern of *P. putida* ATCC 49128 (which will subsequently be referred to as *P. putida*) were determined.

The experiment was carried out in shake flask at 37ºC and shaker speed at 180 rpm. The ratio of *P. putida* to nutrient broth was 1:9, whereby 20 ml of *P. putida* was added to 180 ml of nutrient broth (NB) in a shake flask (Ee, 2004; Azoddein et al., 2015).

Data on the growth of *P. putida* were recorded, and the results are as shown in Appendix 11. Figure 4.1 shows the growth curve of *P. putida* over a period of 60 hours with respect to the OD at 600 nm of absorbance, which denoted the concentration of the cells (Ma et al., 2013).

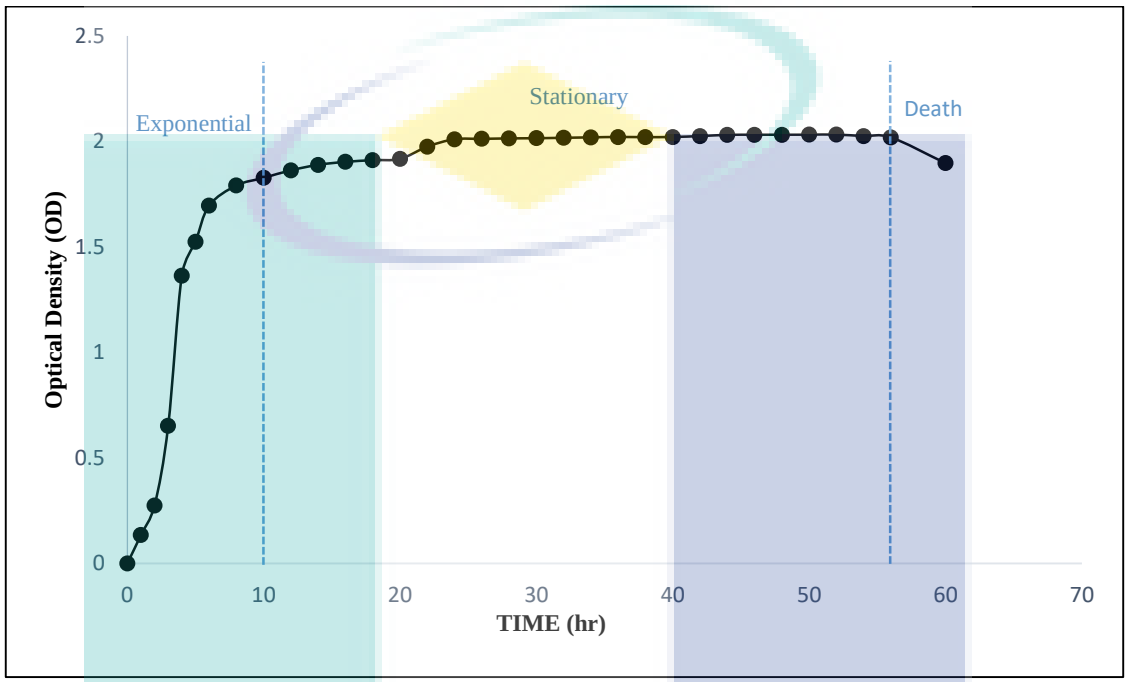


Figure 4.1 *P. putida* growth curve in shake flask

The initial phase of growth is the lag phase, which typically occurs within the 1st hour. However, with reference to Figure 4.1, this phase did not occur, hence indicating that the *P. putida* took a shorter time to acclimatise to the new environment. This result was unlike that of Widdel (2007), whereby the bacteria took several hours to attain the exponential phase. *P. putida* was subjected to new conditions when a small inoculum of "seed" cells, or acclimatised cells, were transferred into a fresh medium. Usually, some time would be needed for the *P. putida* to adapt to the new conditions or environment, thus giving rise to a lag phase prior to exponential growth.

The second phase of growth is exponential ($\mu = c$, where c is greater than 0). Based on the graph, this phase occurred between 1 to 10 hours. At this point, the bacteria have acclimatised to the current environment, which provided optimal conditions for *P. putida* to grow and form as many colonies as possible by degrading the substrates and increasing the bioremediation efforts (Davis et al., 2011).

The stationary phase occurred from 10 to 56 hours. During this phase, the growth rate was equal to the death rate. In other words, the growth rate has declined. In this batch culture, the decreasing supply of substrate supply was responsible for the conversion of the exponential phase to the stationary phase. Besides, *P. putida* was a living organism which produced waste and secondary metabolic products, both of which were accumulated in this phase.

The death phase took place between 56 and 60 hours. Here, the number of living bacteria decreased with time as the death rate was higher than the growth rate. The supply of substrate was depleted, while the toxic waste and by-products resulted in the weakening and subsequent death of the bacteria. Table 4.2 shows the growth parameters of *P. putida* over the course of the 60 hours of the experiment.

Table 4.2 Growth parameters of *P. putida*

Time (h)	Optical Density, OD	Exponential Cell Growth, ln OD/OD_0	Number of Generations, *n*	Generation Time, *g* (h)	Growth rate constant, *k* (h^{-1})
2	0.28	0.71	1.02	1.96	0.35
4	1.36	2.31	3.31	1.21	0.57
6	1.70	2.53	3.63	1.65	0.42
8	1.83	2.61	3.73	2.14	0.32
10	1.89	2.64	3.78	2.64	0.26
12	1.86	2.62	3.76	3.19	0.22
16	1.90	2.65	3.79	4.22	0.16
20	1.92	2.65	3.80	5.26	0.13
26	2.01	2.70	3.87	6.72	0.10
32	2.02	2.70	3.88	8.26	0.08
38	2.02	2.71	3.88	9.80	0.07
44	2.03	2.71	3.89	11.32	0.06
50	2.03	2.71	3.89	12.87	0.05
56	2.02	2.71	3.88	14.44	0.05
60	1.90	2.64	3.79	15.84	0.04

Table 4.3 Summary of growth parameters of *P. putida*

Growth Parameter	Min	Max
Specific Growth Rate, μ (h^{-1})	-	0.91
Optical Density (OD)	0.14	2.03
Exponential cell growth, (ln OD/OD_0)	0.71	2.71
Number Of Generations, n	1.02	3.89
Generation Time, g (h)	1.21	15.84
Growth Rate Constant, k (h^{-1})	0.04	0.57

Tables 4.2 and 4.3 summarise the growth patterns of *P. putida* in optimal conditions for 60 hours or until the death phase. The aforementioned parameters were specific growth rate, μ (h^{-1}), optical density (OD), exponential cell growth, (ln OD/OD_0), number of generations, n = 3.3 ln OD/OD_n, generation time, $g = t/n$ (h), and growth rate constant, k = ln $2/g$ = $0.693/g$ (h^{-1}). The specific growth rate, μ, of *P. putida* was 0.91 h^{-1}, which meant that there was an increase in cell mass per unit time, or exponential cell growth. Meanwhile, the OD was measured using a UV spectrophotometer at 600 nm of absorbance to determine the bacterial growth pattern. The minimum and maximum ODs were 0.14 and 2.03 respectively. On another note, the number of generations, n, reflected the number of cells produced from each cell, and their highest and lowest values were 1.02 and 3.89 repectively. Next, the generation time, g, denoted the duration which was needed for the formation of two cells from one cell of *P. putida* (i.e. doubling time). Its minimum value was 1.21 hours while the maximum 15.84 hours. The growth rate constant, k, was defined as the number of generations present per unit time in an exponential growth, and its value could be calculated when n and t were known. Its lowest and highest values were 0.04 h^{-1} and 0.57 h^{-1} respectively.

4.4 Dry Cell Weight (DCW)

Dry cell weight (DCW) was the most useful parameter to characterise bacterial growth. DCW is commonly used as reference value to calculate specific rates of bacterial growth. It differs substantially from the weight of living cells whose water content is approximately 80%. After the growth curve of *P. putida* was obtained, the experiment on the standard growth curve employed DCW as the chief parameter so that the growth pattern could be directly measured in terms of cell density (OD). In an in-

depth understanding of the growth pattern of *P. putida*, the relationship between DCW and OD needed to be known in order to correlate both parameters. This direct method was most commonly used to determine the cell mass. Basically, a spectrophotometer is used to measure OD, which is dependent on (i) the extent of absorption of light by the suspended cells in sample culture media and (ii) the intensity of the transmitted light (Ee, 2004; Shuler and Kargi, 1992).

The cells were cultured overnight before being centrifuged, filtered, and washed a few times using saline water (Foti et al., 2013). Subsequently, the cells were dried and their weight measured until the value became consistent (Ee, 2004). Appendix 12 and Figure 4.2 display the results for DCW.

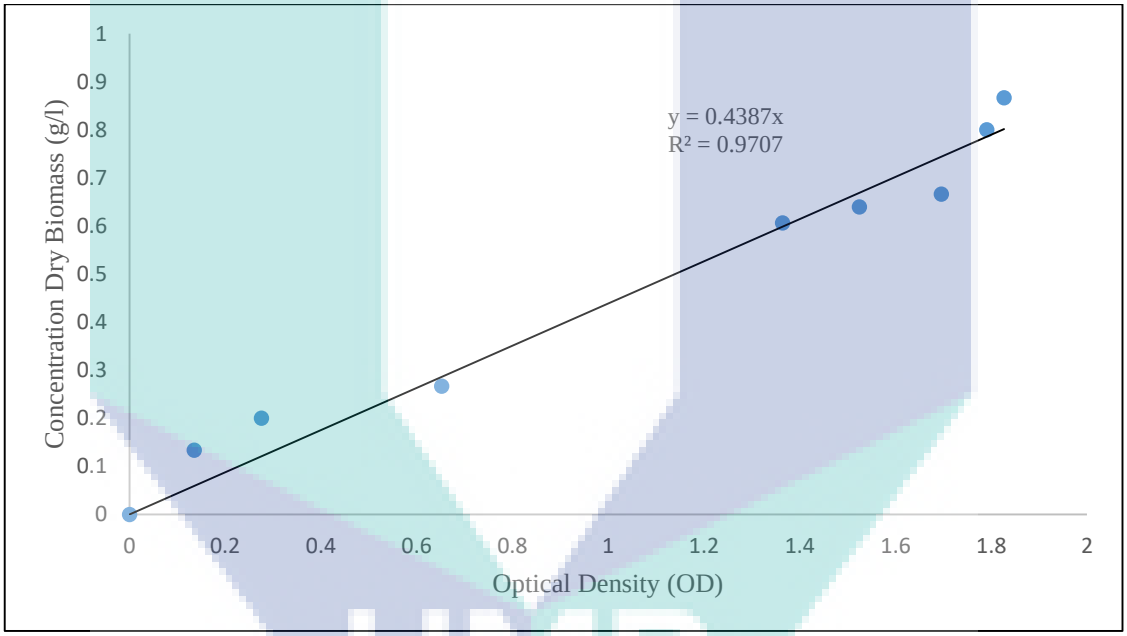


Figure 4.2 *P. putida* standard growth curve – dry cell weight method

As mentioned, Figure 4.2 showed the standard curve for the DCW of *P. putida* in a shake flask. The linear equation of the said curve was $Y = 0.4387X$, where Y was the concentration of the dried biomass while X optical density. It was evident that an increase in the former led to the increase of the latter. Thus, it could be concluded that the concentration of the dry biomass was directly proportional to OD, and that an increase of one unit of OD corresponded to an increase of 0.4347 g of DCW per litre of sample. This result was slightly higher than the DCW of *P. putida* (0.4033 g/L) (Martín et al., 2008) and lower than that of *P. putida* S12, which were 0.47 g/L (Foti et al.,

2013) and 0.451 g/L (Rosas et al., 2013). This scenario could have occurred in light of the usage of different *P. putida* strains, culture conditions, and nutrient types.

4.5 Bioremoval of BTX in Shake Flasks

BTX is a volatile aromatic hydrocarbon that is also very toxic to humans. Benzene itself is considered to be a carcinogenic substance which can result in several types of leukaemias in the event of direct exposure to a certain amount of the same as mentioned by the USEPA (Mazzeo et al., 2013). In the petrochemical industry, the presence of BTX in the effluents is becoming worrisome because of its immense potential to contaminate water resources, particularly groundwater and rivers (Mazzeo et al., 2013).

Biological treatment has been employed as an alternative method to reduce the levels of BTX because it is environmentally-friendly and inexpensive, apart from being able to completely mineralise organic pollutants (Surkatti and El-Naas, 2014). Previous studies have reported complex interactions during the biodegradation of BTX by mixed or pure cultures, despite the similarities in the chemical and structural properties of the said compounds (Jo et al., 2008). Most researchers have studied the interaction between bacteria and BTX (carbon sources), few have evaluated the compounds individually. Thus, there was a need to understand the effects of the interaction between the bacteria and different concentrations of benzene, toluene, as well as xylene respectively (Jo et al., 2008).

In this study, immobilised and suspended *P. putida* cells in batch systems (conical flasks) were utilised to make easy the process of controlling certain parameters (Robledo-Ortíz et al., 2011). To optimise the BTX-bacterial reactions, a few parameters were controlled manually, such as temperature, shaker speed, pH, and BTX concentrations. It is important to note that the enzymes produced in response to the interactions were affected by the environmental factors.

Temperature is one of the most influential environmental factors as it has an effect on all living organisms, including microbes. When the temperature is increased, the rate of bacterial metabolism increases and the enzymatic reactions proceed at a more rapid rate. As a result, the bacterial growth rate increases. In this experiment, 37ºC was

used as it was the ideal temperature for *P. putida* (Azoddein et al., 2015). Higher temperatures might result in the denaturation of certain proteins or enzymes produced by the bacteria, to the extent that it was no longer possible for the cells to functions well (Brock et al., 2004).

Shaker speed is the other parameter that had to be controlled since an increase in speed would increase the interfacial area between the medium and microbe in an aqueous phase (Ishenny, 2006). Evidently, the mass transfer area provided a medium for the bacteria to react, grow, and undergo metabolism (Clarke and Correia, 2008; De-Bashan at al., 2012). Higher shaking speeds could lead to an increase in the mass transfer area and hence, rate of microorganismal growth. At the same time, the concentration polarisation would be reduced, resulting in good mixing and better dispersion of the nutrients and microbes. Thus, the ideal shaker speed of 180 rpm was used in this experiment at (Azoddein et al., 2015; Mortazavi et al., 2005) as well as in the change of oxygen transfer rate (OTR) which can affect the growth rate of *P .putida* (Puthli, 2006).

Generally, pH has an effect on the activities of microbial enzymes. Each organism has its own pH range, which is commonly between 5 and 9 (Srivastava and Majumder, 2008). pH affects the ionisation of chemicals, and this plays a role in the transport of nutrients and toxic chemicals into the cells (Bitton, 2005). Thus, pH 7 was used in this experiment for the optimum growth rate of *P. putida* (Azoddein et al., 2015).

Taking into consideration the partitioning of organic compounds at a gas-liquid equilibrium and constant temperature, Henry's law was used to calculate the amount of benzene, toluene and xylene which had to be added to the media to attain initial liquid concentrations of benzene (25, 35, 45, 55, 65 ppm), toluene (10, 15, 20, 25, 30 ppm), and xylene (10, 15, 20, 25, 30 ppm). The culturing conditions were the same as those which have been used to identify the growth curve of *P. putida* (i.e. 37 ^{o}C, pH 7, 180 rpm, and culture-sample ratio of 1:9 ratio) (Azoddein et al., 2015).

4.5.1 Effect of Benzene Concentration on *P. putida* Growth

Here, benzene was the source of carbon for *P. putida*. Five different concentrations of benzene were used – 25, 35, 45, 55, and 65 ppm. These values were selected with reference to that of the actual wastewater from the petrochemical plant. Evidently, the concentration of benzene in the actual sample during the dry season was 40 ppm to 60 ppm. In certain conditions, it was reported that the aforementioned concentration was lower than the analysis range, which was between 20 and 35 ppm. It was therefore possible that the concentrations were lower during the rainy season since the effluent (benzene) was diluted by the rain.

This experiment was conducted in ideal conditions (37°C, pH 7, and shaker speed of 180 rpm). Table 4.4 presents the results of the mathematical analysis of the experimental data. Meanwhile, Figure 4.3 compares the growth curves of *P. putida* which have been subjected to different concentrations of benzene.

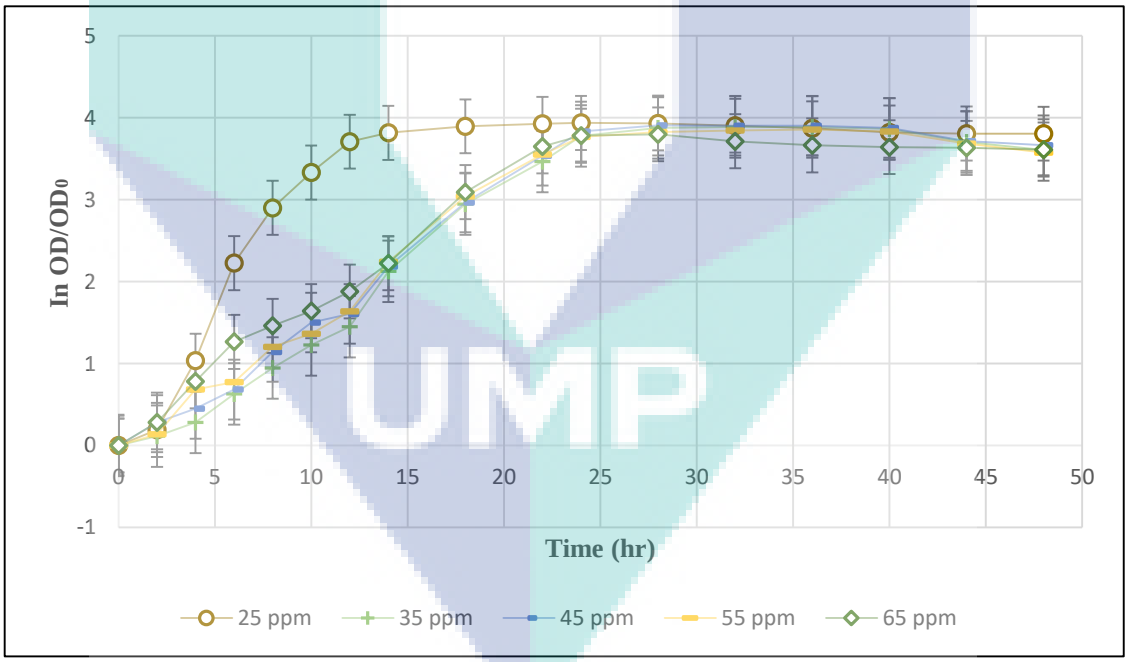


Figure 4.3 *P. putida* growth in different concentrations of benzene in simulated wastewater

Table 4.4 Summary of growth parameters of *P. putida* in benzene

Growth Parameter	**Min (ppm)**					**Max (ppm)**				
	25	**35**	**45**	**55**	**65**	**25**	**35**	**45**	**55**	**65**
Specific Growth Rate, μ (h^{-1})						0.33	0.12	0.15	0.14	0.16
OD	0.02	0.05	0.05	0.05	0.05	2.20	2.39	2.10	2.27	2.01
Exponential cell growth, (ln OD/OD0)	2.31	1.45	1.61	1.64	1.88	4.92	3.89	3.82	3.86	3.78
Number Of Generation, *n*	6.73	2.08	2.30	2.35	2.69	7.06	5.57	5.47	5.53	5.42
Generation Time, *g* (h)	1.78	4.43	5.05	4.45	4.43	5.24	5.77	5.22	5.11	4.46
Growth Rate Constant, *k* (h-1)	0.13	0.12	0.13	0.14	0.16	0.39	0.16	0.14	0.16	0.16

With reference to Figure 4.3, *P. putida* in all concentrations of benzene immediately adapted to a new environment during the lag phases. When the concentration of benzene was 25 ppm, *P. putida* exhibited a slightly different growth pattern in the exponential phase, relative to the other concentrations. At 25 ppm, the bacteria proliferated rapidly for 12 hours, after which the growth rate of the became stationary. For the other concentrations of benzene, *P. putida* needed 24 hours to reach the stationary phase. Based on these observations, lower concentrations of benzene led to an increase in the growth rate, μ, of *P. putida*. Detailed explanations pertaining to the bacteria's growth are shown in Table 4.4. The specific growth rates, μ, of *P. putida* in descending order were 0.33 h^{-1} at 25 ppm, 0.16 h^{-1} at 65 ppm, 0.15 h^{-1} at 45 ppm, 0.14 h^{-1} at 55 ppm, and 0.12 h^{-1} at 35 ppm.

The other growth parameters of *P. putida* in benzene were as follows: number of generations, *n*, generation time, *g*, and growth constant, *k*. With respect to Table 4.4, the highest numbers of generations, *n*, were 7.06 at 25 ppm, 5.57 at 35 ppm, 5.47 at 45 ppm, 5.53 at 55 ppm, and 5.2 at 65 ppm. Also, the maximum generation times, *g*, were 5.24 hr at 25 ppm, 5.77 hr at 35 ppm, 5.22 hr at 45 ppm, 5.11 hr at 55 ppm and 4.46 hr at 65 ppm. The maximum growth rates, *k* are 0.39 h^{-1} at 25 ppm, 0.16 h^{-1} at 35 ppm, 0.14 h^{-1} at 45 ppm, 0.16 h^{-1} at 55 ppm, and 0.16 h^{-1} at 65 ppm. Also, the maximum

concentrations of cells were 0.9636 g/L at 25 ppm, 1.0468 g/L at 35 ppm, 0.9213 g/L at 45 ppm, 0.9958 g/L at 55 ppm and 0.8818 g/L at 65 ppm.

Thus, from the results, it could be concluded that a decrease in the concentration of benzene led to an increase in the growth rate of *P. putida*. The highest maximum specific growth rate, μ_{max}, of *P. putida* ATCC 49128 was 2.39 h^{-1} at 35ppm, which was higher than that of Reardon et al. (2000) at 0.73 ± 0.03hr^{-1}, Abuhamed et al. (2004) at 0.62hr^{-1}, and Robledo-Ortíz et al. (2011) at 0.50 hr^{-1}.

4.5.2 Biological Treatment of Benzene at 12, 24, and 36 h

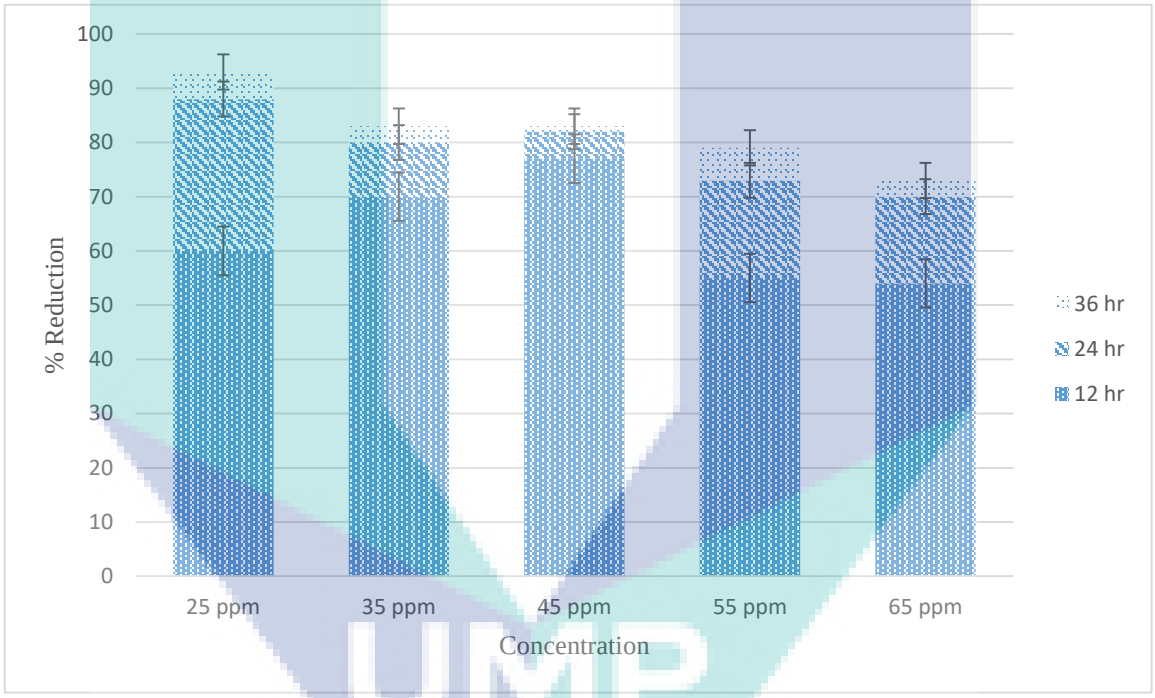


Figure 4.4 Biological treatment of different concentrations of benzene in simulated wastewater

Figure 4.4 shows the trends of biological treatment of benzene by *P. putida* in different concentrations of benzene. In the presence of *P. putida*, nutrient broth, and 25 ppm of benzene, the percentage of benzene concentration reduction was the highest, which was 60% within 12 hours, 88% within 24 hours, and 93% within 36 hours. However, the 12-hour reduction at 25 ppm concentration was lower as compared to 35 ppm and 45 ppm, the values of which were 70% and 77% respectively. The reading could have arisen in light of the rapid adaptation of *P. putida* to both concentrations.

However, for the second phase of reduction (i.e. within 24 hours), the reduction was the highest in 25 ppm as compared to the other concentrations of benzene. Likewise, within 36 hours, the reduction in 25 ppm of benzene was 92%, 35 ppm 82%, 45 ppm 81%, 55 ppm 78%, and 65 ppm 72%. Biological treatment of 45 ppm of benzene gave rise to a rapid reduction in the concentration during the initial 12 hours (77%); the rate of reduction slowed down after that, and at the end of 36 hours, 83% of benzene was removed. The cell count slowly declined henceforth since the rate of cell production was equal to that of cell death. This result was slightly different from the growth pattern of *P. putida*, whereby the bacteria rapidly reproduced in 25 ppm of benzene. The reduction of benzene by *P. putida* was still lower as compared to that of Xin et al. (2103), which had a 97.8% reduction within 24 hours. In conclusion, the growth of *P. putida* did not directly relate to the rate of enzyme production and benzene reduction.

With respect to the outcomes of this experiment, a few metabolic pathways of the enzyme were possible. Jo et al. (2008) claimed that the biodegradation of benzene initially involved its hydroxylation which was catalysed by a dioxygenase. Then, the product (a diol) was converted into a catechol by a dehydrogenase. Both degradation pathways – hydroxylation and dehydroxylation – are common processes in the initial reactions of aromatic hydrocarbons. The main intermediate product of the reduction of benzene is a catechol (El-Naas et al., 2014).

Munoz et al. (2007) stated that the rate of catechol production increased with the initial concentration of benzene. At a very low concentration of the latter, the amount of catechol produced would also be low (Robledo-Ortíz et al., 2011). Thus, the utilisation of 25 ppm of benzene in this study was still acceptable for the production of the catechol intermediate by *P. putida*. However, very high concentrations of benzene (e.g. 100 ppm and above) would be toxic and would cause the death of *P. putida* (Bayraktar and Mehmetog, 2003).

4.5.3 Effect of Toluene Concentration on *P. putida* Growth

Toluene degrades easily under aerobic conditions as compared to other VOCs. Additionally, it can be degraded by all electron acceptors as well (Corseuil et al., 1998).

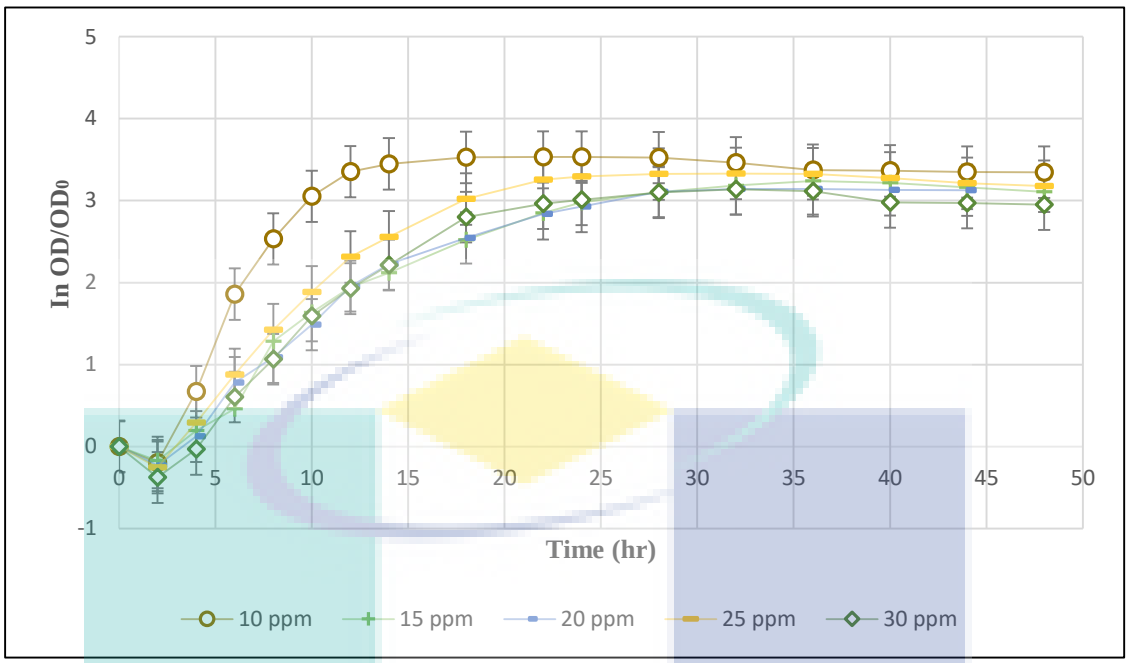


Figure 4.5 *P. putida* growth in different concentrations of toluene in simulated wastewater

Table 4.5 Summary of growth parameters of *P. putida* in toluene

Growth Parameter	**Min (ppm)**					**Max (ppm)**				
	10	**15**	**20**	**25**	**30**	**10**	**15**	**20**	**25**	**30**
Specific Growth Rate, μ (h^{-1})						0.31	0.16	0.15	0.19	0.16
OD	0.06	0.08	0.08	0.07	0.09	2.12	1.91	1.78	1.98	2.01
Exponential cell growth, (ln OD/OD0)	3.35	1.94	1.96	3.30	2.37	3.53	3.24	3.14	3.33	3.12
Number Of Generation, *n*	4.81	2.79	2.80	3.32	3.40	5.06	4.64	4.50	4.77	4.47
Generation Time, *g* (h)	2.50	4.31	4.28	3.62	3.53	7.45	7.75	7.55	7.55	8.06
Growth Rate Constant, *k* (h^{-1})	0.09	0.09	0.09	0.09	0.09	0.28	0.16	0.16	0.19	0.20

Based on Figure 4.5, *P. putida* took about 2 hours to adapt to the new environment (lag phase) in five different concentrations of toluene. Bacteria in the higher concentrations of toluene had greater declines in OD as compared to those of

lower concentrations. In exponential phase, the bacteria multiplied the fastest in 10 ppm of toluene (specific growth rate, μ = 0.31 h^{-1}) vis-à-vis the other concentrations which (0.15 – 0.19 h^{-1}). The aforementioned values were slightly higher than the maximum specific growth rate, μ_{max}, of *P. putida* in toluene reported by Ruegg et al. (2007) (0.26 h^{-1}), but were still lower than specific growth rates of *P. putida* F1 which were 0.58 h^{-1} (Robledo-Ortíz et al., 2011), 0.86 h^{-1} (Reordon et al., 2000), 0.61 h^{-1} (Abuhamed et al., 2004), and 0.78 h^{-1} (Bordel et al., 2007). Bacteria that grew in 10 ppm of toluene entered the stationary phase within 12 hours, while those in the other concentrations did so within 22 hours. Thus, it could be inferred that *P. putida* grew best in low concentrations of toluene when the latter was the sole carbon source. In spite of that, it was observed that the specific growth rates of *P. putida* in toluene were higher than those in benzene (Robledo-Ortíz et al., 2011).

Table 4.5 provided a detailed explanation of the growth pattern of *P. putida* in toluene. The specific growth rate, μ, was the highest (0.31 h^{-1}) at 10 ppm of toluene, followed by 0.16 h^{-1} at 15 ppm, 0.15 h^{-1} at 20 ppm, 0.19hr^{-1} at 25 ppm, and 0.16 h^{-1} at 30 ppm. The maximum numbers of generations, *n*, of *P. putida* in toluene were 5.06 at 10 ppm, 4.64 at 15 ppm, 4.50 at 20 ppm, 4.77 at 25 ppm and 4.47 at 30 ppm. The maximum generation times, *g*, were 7.45 h at 10 ppm, 7.75 h at 15 ppm, 7.55 h at 20 ppm, 7.55 h at 25 ppm and 8.05 h at 30 ppm. The maximum growth rate constants, *k*, for *P. putida* in toluene were 0.28 h^{-1} at 10 ppm, 0.16 h^{-1} at 15 ppm, 0.16 h^{-1} at 20 ppm, 0.19 h^{-1} at 25 ppm and 0.20 h^{-1} at 30 ppm. In conclusion, 15 ppm of toluene was optimal for the growth of *P. putida*. The maximum cell concentration in toluene was 0.9300 g/L at 10 ppm, 0.8379 g/L at 15 ppm, 0.7809 g/L at 20 ppm, 0.8686 g/L at 25 ppm and 0.8818 g/L at 30 ppm.

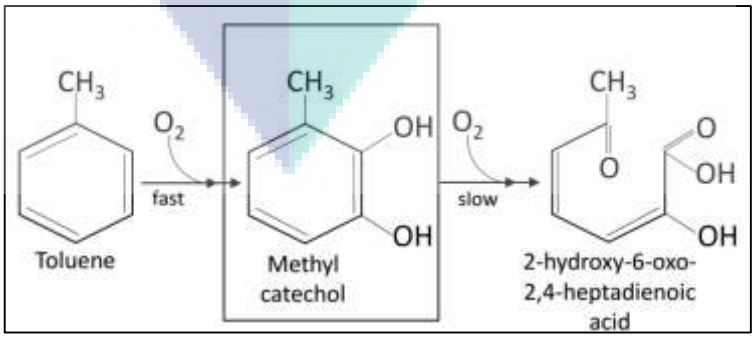


Figure 4.6 Mechanism of action of toluene dioxygenase

Figure 4.6 shows the suggested mechanism of the degradation of toluene, with methyl catechol being the intermediate. When the DO is low, methyl catechol accumulates an electron donor substrate provided after methyl catechol released into the solution to other heterotrophs that catabolise the methyl catechol via another pathway. *P. putida* produces an enzyme called toluene dioxygenase which converts toluene into methyl catechol. This is attained by adding a pair of -OH groups to the former to form an intermediate, or accumulate. Subsequently, methyl catechol changes into 2-hydroxy-6-oxo-2,4-heptadienoic acid. To summarise, the toluene changes into a carboxylic acid, which is a safe compound.

4.5.4 Biological Treatment of Toluene at 12, 24, and 36 h

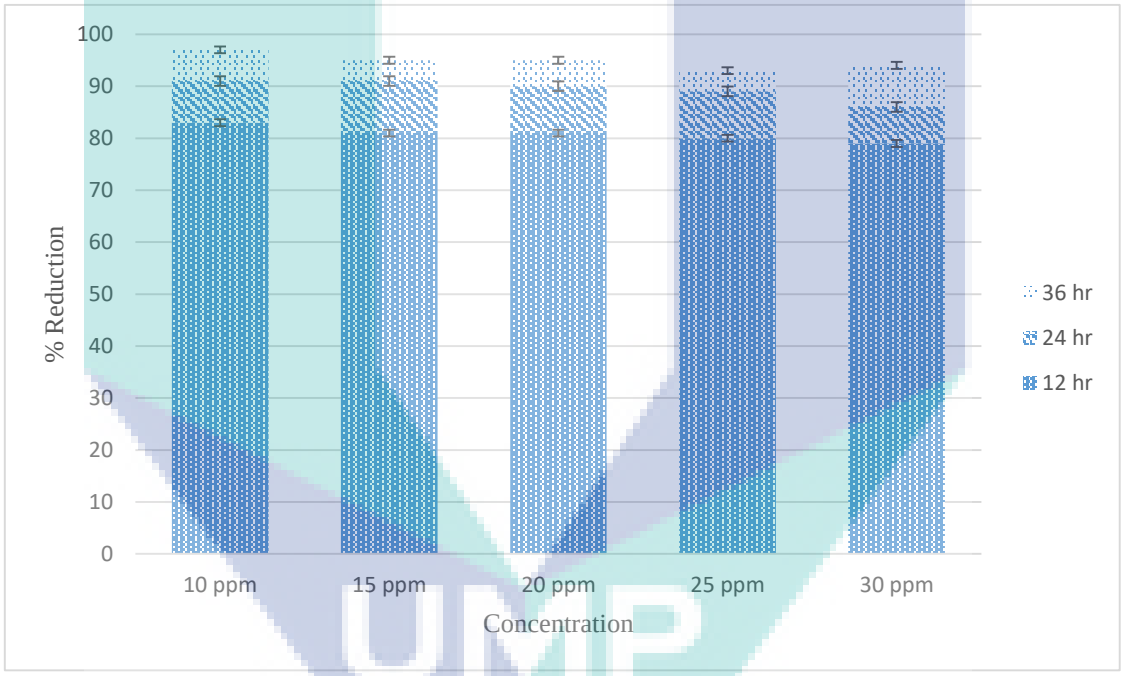


Figure 4.7 Biological treatment of different concentrations of toluene in simulated wastewater

Figure 4.7 shows the extent of biological treatment of different concentrations of toluene by *P. putida*. The experiment was conducted in conical flasks, whereby the optimal parameters for the growth of *P. putida* (pH 7, 37°C, and shaker speed of 180 rpm) were utilised. As with the growth of *P. putida* in toluene, the removal of the compound was also optimal at low concentrations of toluene. During the initial 12 hours of biological treatment of the simulated toluene samples, the greatest reduction in the

concentration of toluene was 83% at 10 ppm, followed by 81% at 15 ppm, 80% at 25 ppm, and 79% at 30 ppm. The results showed a similar trend of toluene reduction within 24 hours, whereby the greatest reduction was 91% at 10 and 15 ppm, followed by 89% at 20 and 25 ppm, as well as 86% at 30 ppm. Between 24 and 36 hours, a slightly different pattern of reduction was observed, whereby the extent of removal of 30 ppm of toluene was higher than those of 20 and 25 ppm. On another note, 97% of 10-ppm toluene was removed over 36 hours, followed by 95% of 15- and 20-ppm, as well as 94% of 25- and 30-ppm. In almost all concentrations of toluene, some 79% to 83% of the compound was degraded within the first 12 hours. Then, a decline in the removal was seen, which correlated to bacterial decay.

These results seemed to suggest that the degradation of toluene involved toluene dioxygenase, which speedily multiplied during the initial 12 hours of the experiment (Colombo et al., 2004). *P. putida* converted toluene into the 3-methyl catechol intermediate (Robledo-Ortíz et al., 2011), which subsequently got converted into benzyl alcohol. To sum up, the greatest degradation of toluene took place at 10 ppm of the compound, and this corresponded to the highest growth rate of *P. putida* (0.28 h^{-1}) at this concentration.

4.5.5 Effect of Xylene Concentration on *P. putida* Growth

The growth of *P. putida* in five different concentrations of xylene in conical flasks at optimum conditions (pH 7, 37°C, and shaker speed of 180 rpm) (Azoddein et al., 2015). Compared to benzene and toluene, xylene (*o*-xylene and *p*-xylene) are less soluble in water, whereby the solubilities of *o*-xylene is 175 mg/L and *p*-xylene 198 mg/L (Rittmann et al., 1994). Thus, the activities of the bacteria in the xylene solutions were also low. Figure 4.8 below shows the graph of *P. putida* growth in xylenes.

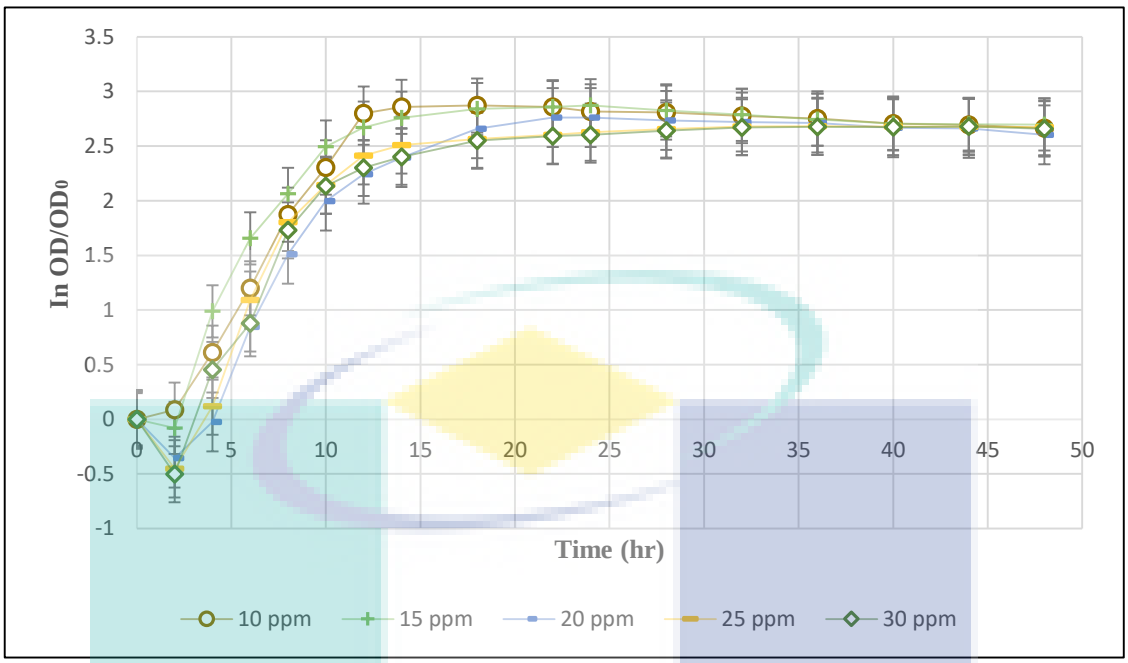


Figure 4.8 *P. putida* growth in different concentrations of xylene in simulated wastewater

Table 4.6 Summary of growth parameters of *P. putida* in xylene

Growth Parameter	**Min (ppm)**					**Max (ppm)**				
	10	**15**	**20**	**25**	**30**	**10**	**15**	**20**	**25**	**30**
Specific Growth Rate, μ (h^{-1})	0.23	0.25	0.20	0.21	0.21					
OD	0.12	0.12	0.12	0.12	0.12	2.07	1.87	1.83	1.52	1.73
Exponential cell growth, (ln OD/OD0)	2.80	2.24	2.67	2.51	2.30	2.85	2.71	2.74	2.56	2.68
Number Of Generation, *n*	4.01	3.21	3.83	3.46	3.30	4.08	3.89	3.93	3.66	3.84
Generation Time, *g* (h)	2.99	3.73	3.14	3.47	3.64	9.88	9.26	9.83	9.83	9.38
Growth Rate Constant, *k* (h^{-1})	0.07	0.07	0.08	0.07	0.07	0.23	0.19	0.22	0.20	0.19

As mentioned, *P. putida* was grown in five distinct concentrations of xylene (10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm). The differences in the said concentrations were based on the actual average value of xylene in petrochemical wastewater sample. It shows that almost all xylene concentrations had an effect on the growth of *P. putida*

in the lag phase, whereby the bacteria needed to adapt to the new environment within the first 24 hours. At 10 ppm of xylene, *P. putida* proliferated rapidly and exponentially in the initial 12 hours and also for this concentration; they took the least time to acclimatise. Evidently, the growth patterns of the bacteria in all concentrations of xylene were similar.

P. putida began to undergo a static growth rate (i.e. rate of cell death = rate of cell growth) in xylene within 12 hours. Detailed descriptions and summaries on the growth patterns of *P. putida* were shown in Table 4.6.

The specific growth rate, μ, of *P. putida* at 10 ppm was 0.23 h^{-1}, 15 ppm 0.25 h^{-1} for, 20 ppm 0.20 h^{-1}, 25 ppm 0.21 h^{-1}, and 30 ppm 0.21 h^{-1}. With respect to the results, *P. putida* grew best in 15 ppm of xylene. As for number of generations, *n*, of *P. putida*, the highest value (4.08) was seen at 10 ppm, followed by 3.89 at 15 ppm, 3.93 at 20 ppm, 3.66 at 25 ppm, and 3.84 at 30 ppm. The generation time, *g*, at 10 ppm was 9.88 h, 15 ppm 9.26 h, 20 ppm 9.83 h, 25 ppm 9.83 h, and 30 ppm 9.83 h. The growth rate constants, *k*, of *P. putida* were 0.23 h^{-1} at 10 ppm, 0.19 h^{-1} at 15 ppm, 0.22 h^{-1} at 20 ppm, 0.20 h^{-1} at 25 ppm and 0.19 h^{-1} at 30 ppm. Also, the cell concentrations were 0.9081 g/L at 10 ppm, 0.8204 g/L at 15 ppm, 0.8028 g/L at 20 ppm, 0.6668 g/L at 25 ppm and 0.75895 g/L at 30ppm. Thus, it could be concluded that 10 ppm of xylene was optimual for the growth of *P. putida*.

4.5.6 Biological Treatment of Xylene at 12, 24, and 36 h

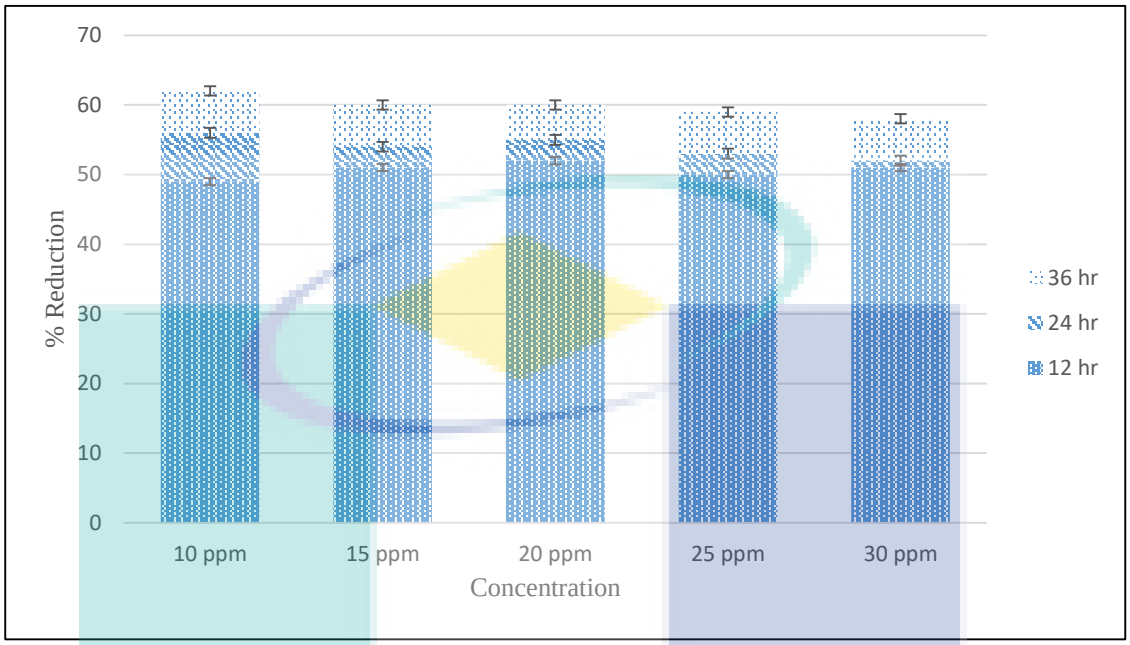


Figure 4.9 Biological treatment of different concentrations of xylene in simulated wastewater

Figure 4.9 above showed the biological treatment of xylenes (*o*-xylene and *p*-xylene) by *P. putida*. The reductions in the concentrations of xylene were much lower than those of the other aromatic compounds (benzene and toluene) owing to its poor solubility in water. It has widely been assumed that when xylene was the sole carbon source, the rate of production of xylene monooxygenase by *P. putida* was very low (Colombo et al., 2004). As per the results, the extent of xylene removal was the greatest at low concentrations of the same. With reference to the graph above, 62% of 10-ppm xylene was removed within 36 h. This was followed by 60% at 15 and 20 ppm, 59% at 25 ppm, and 57% at 30 ppm.

The inconsistency of xylene reduction within the first 12 hours could be due to the inconcistent production of the enzyme by *P. putida*. The greatest reduction of xylene within the first 12 hours was 53% at 20 ppm, followed by 50% at 15, 25, and 30 ppm, as well as 49% at 10 ppm. As for the values within 24 hours, the highest was 56% at 10 ppm, followed by 55% at 20 ppm, 54% at 15 ppm, 53% at 25 ppm, and 52% at 30 ppm. Using the xylene monooxygenase of *P. putida*, the isomers of xylene could be oxidised either via the monooxygenase (tod) or dioxygenase (tol) pathway (Tsao et al., 1998; Jo

et al., 2008) (Colombo et al., 2004). Even though xylene was slowly oxidised, the growing bacteria were supported by the substrates in their attempt to initiate degradation via ring hydrogenation and subsequently transform xylene into dimethyl catechol (Jo et al., 2008).

4.6 Effect of BTX Concentration on *P. putida* Growth

P. putida was grown in a mixture of benzene, toluene, and xylene at a ratio of 5:2:2 in a shake flask, whereby the optimal conditions (pH 7, 37°C, and shaker speed of 180 rpm) (Azoddein, 2013). The initial concentration of each aromatic compound differed with respect to their respective average concentrations in actual wastewater. In the first experiment (experiment 1), the concentrations of benzene, toluene, and xylene were 25 ppm, 10 ppm, and 10 ppm respectively. For the other experiments, the respective concentrations were as follows: experiment 2 – 35, 15, and 15 ppm; experiment 3 – 45, 20, and 20 ppm; experiment 4 – 55, 25, and 25 ppm; as well as experiment 5 – 65, 30, and 30 ppm. The experiments were conducted for 48 hours to analyse the growth patterns of *P. putida*. Sampling was done thrice for each experiment (i.e. at 12, 24, and 36 hours) to analyse the extent of BTX removal during treatment.

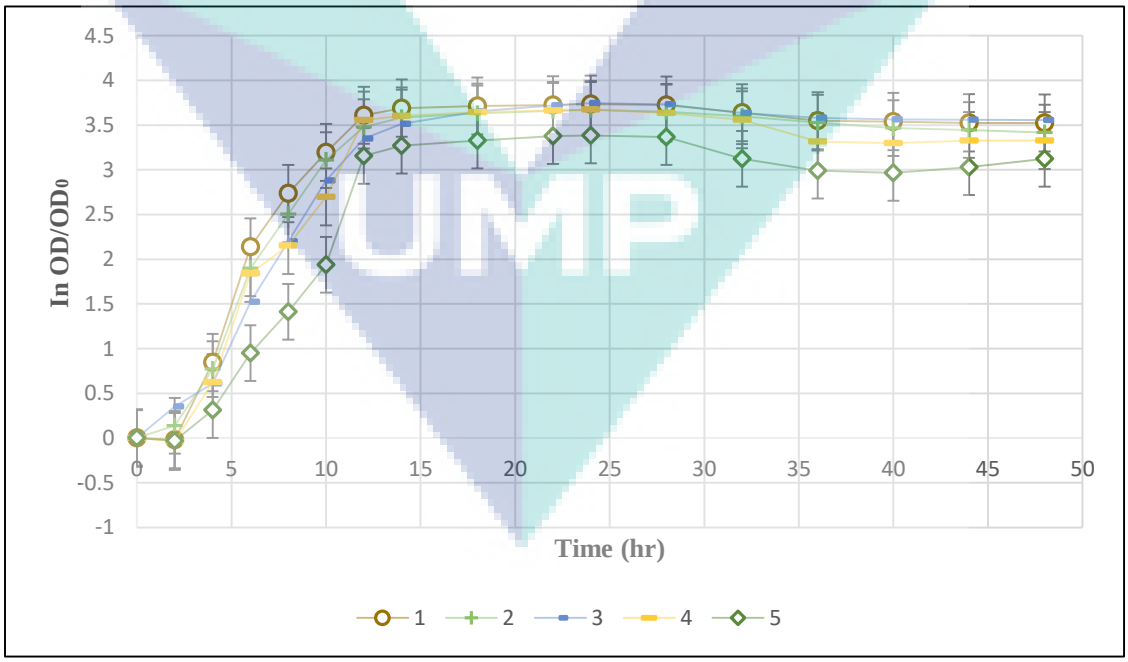


Figure 4.10 *P. putida* growth in different concentrations of BTX

Table 4.7 Summary of growth parameters of *P. putida* in BTX

Growth Parameter	Min					Max				
	1	2	3	4	5	1	2	3	4	5
Specific Growth Rate, μ (h^{-1})	0.32	0.31	0.29	0.27	0.19					
OD	0.05	0.06	0.05	0.05	0.06	2.17	1.87	1.79	1.40	1.26
Exponential cell growth, (ln OD/OD0)	3.63	3.48	3.34	3.67	3.15	3.51	3.53	3.50	3.32	2.99
Number Of Generation, *n*	5.11	4.98	4.78	4.75	4.29	5.38	5.28	5.26	4.75	4.85
Generation Time, *g* (h)	2.31	2.41	2.51	2.35	2.66	7.04	7.12	7.17	7.58	8.40
Growth Rate Constant, *k* (h^{-1})	0.10	0.10	0.10	0.09	0.08	0.30	0.29	0.28	0.29	0.26

Figure 4.10 showed the growth patterns of *P. putida* in a mixture which contained BTX stock solutions of different concentrations. As per the figure, all five growth curves were highly similar. However, the curve in experiment 3 was noticeably different, whereby the OD increased within the first 2 hours. In the remaining experiments, *P. putida* underwent a lag phase, which was the time required for the bacteria to adapt to the new environment. The aforementioned anomaly could have happened because the bacteria could have underwent the lag phase well within the initial 1 hour. Then, in all experiments, the growth rate increased until 12 hours, reflecting the rapid duplication of *P. putida* in the exponential phase. After that, the bacteria entered the stationary phase, which persisted until the end of the experiment. Generally, almost all experiments gave rise to a similar trend in the lag, exponential, and stationary phases. It was evident that BTX concentrations had an effect on the growth pattern of *P. putida*. In experiment 1, the growth rate was lower than those of the other experiments in light of the high concentrations of BTX. Therefore, increments in the concentrations of BTX would decrease the growth rate of *P. putida*.

The basis for the results as per Figure 4.10 could be proven by summarising the outcomes of the parameters of the growth of *P. putida* (Table 4.7). The highest specific growth rate, μ, was seen Exp. 1 (0.32 h^{-1}) while the lowest Exp. 5 at (0.19 h^{-1}); the specific growth rates in Exp. 2, Exp. 3, and Exp. 4 were 0.31 h^{-1}, 0.29 h^{-1}, and 0.29 h^{-1}

respectively. Meanwhile, the highest numbers of generations, *n*, were 5.38 in Exp. 1, 5.28 in Exp. 2, 5.26 in Exp. 3, 4.75 in Exp. 4, and 4.85 in Exp. 5. Also, the maximum generation times, *g*, in Exp.1 was 7.04 h, Exp. 2 was 7.12 h, Exp.3 was 7.17 h, Exp. 4 was 7.56 h, and Exp.5 was 8.40 h. The specific growth rate constants, *k*, for Exp.1, Exp. 2, Exp. 3, Exp. 4, and Exp. 5 were 0.30 h^{-1}, 0.29 h^{-1}, 0.28 h^{-1}, 0.29 h^{-1}, and 0.26 h^{-1} respectively. Thus, it could be concluded that *P. putida* did grow well in high concentrations of benzene, toluene, and xylene; specifically, when the ratio of the three compounds was 65:30:30 ppm.

There were a few factors that had a negative effect on the rate of BTX degradation in mixtures, namely toxicity (Haigler, 1992) and production of toxic intermediates by non-specific enzymes. Nevertheless, in the degradation of BTX mixtures, the isomers of xylene were better removed by co-metabolism. This was because of (i) the production of ring fusion products of different intermediates (catechols) by different enzymes, as well as (ii) accumulation of intermediates such as tolualdehyde, toluic acid, and methylsalicylic acid (Jo et al., 2008; Chang et al., 1993).

Bacteria-produced enzymes are important in the bioremediation of pollutants, as reflected by the different types of carbon and energy sources (e.g. BTX). Dioxygenases are selective in terms of the compound metabolised by them (Bagnéris et al., 2005). Ring-hydroxylating dioxygenases convert aromatic compounds into dihydrodiols in the presence of dioxygen and NADH (Butler et al., 1997). In this study, it was likely that *P. putida* produced catechol 2,3-dioxygenase (C23DO) during the degradation of BTX (Mesarch et al., 2004).

It is suggested that the bacteria produced catechol-2,3-dioxygenase, which broke down the benzene rings of and gave rise to final meta-cleavage product. Thus, the final product was less toxic as compared to BTX.

4.6.1 Benzene, Toluene, and Xylene Removal at 12, 24, and 36 h

The growth patterns of *P. putida* in single VOC compounds were slightly different from those in a mixture of BTX. Thus, the degradation of BTX was also affected by the growth of *P. putida*. Figures 4.11, 4.12, and 4.13 describe the biological treatment different concentrations of benzene, toluene, and xylene in simulated wastewater.

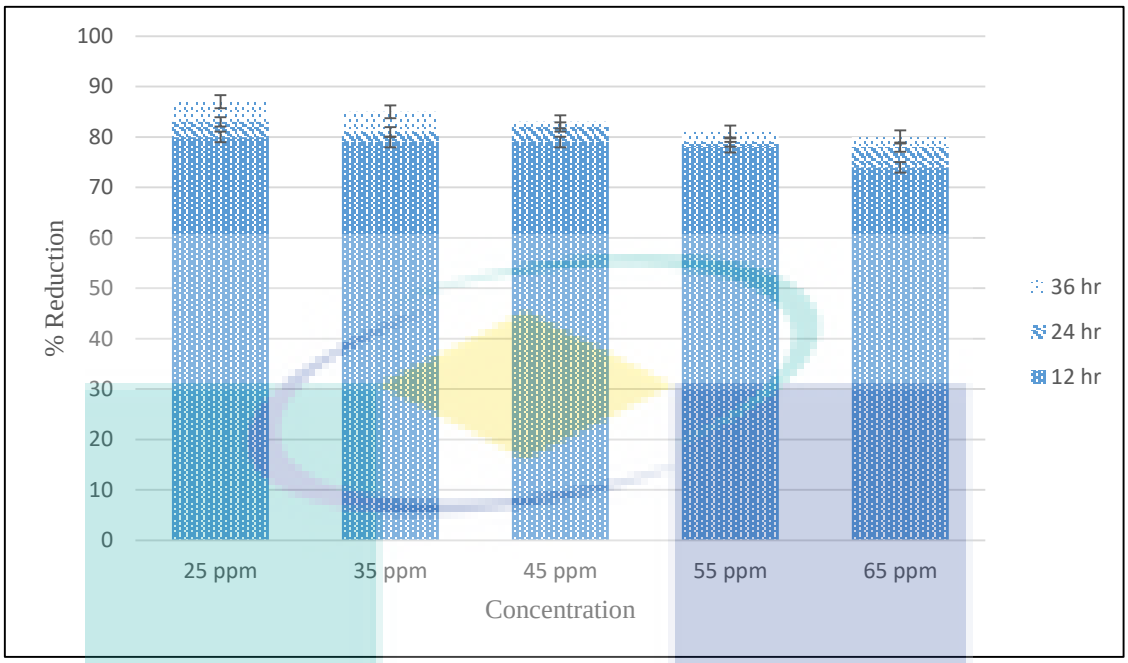


Figure 4.11 Graph of biological treatment of different concentrations of benzene

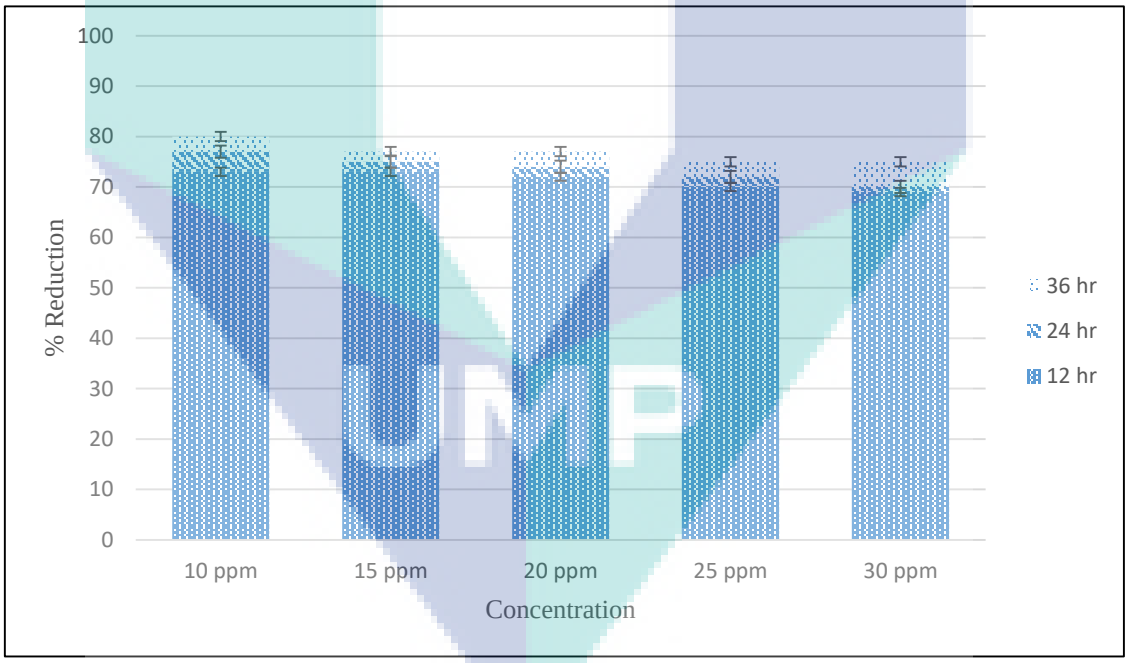


Figure 4.12 Graph of biological treatment of different concentrations of toluene

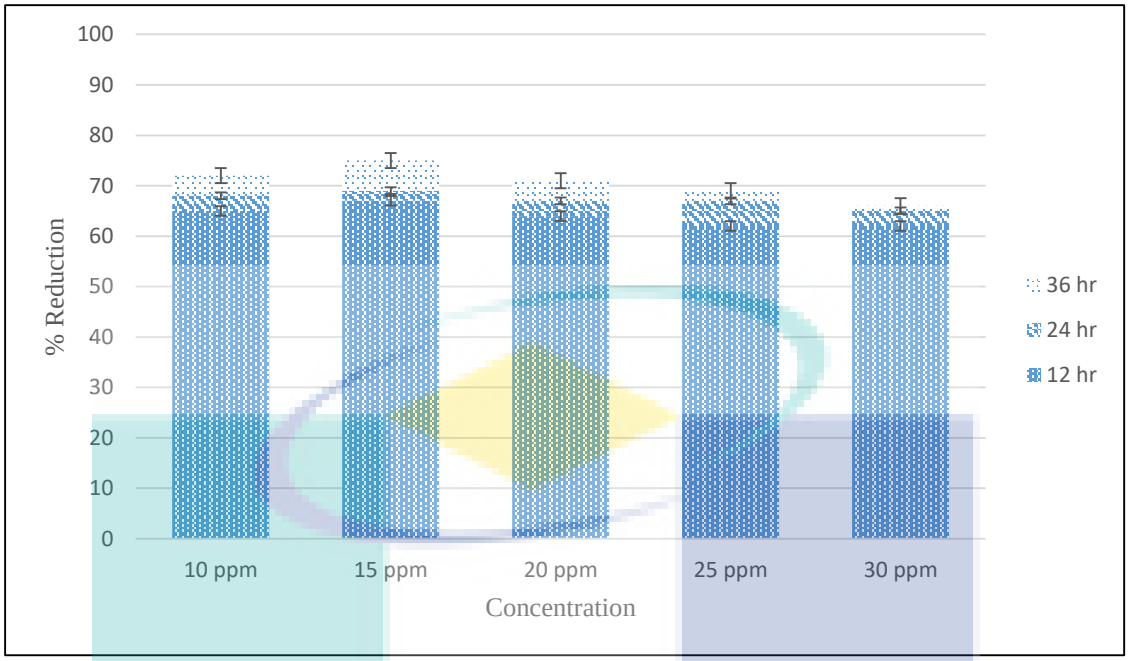


Figure 4.13 Graph of biological treatment of different concentrations of xylene

Figure 4.11 showed the biological treatment of 25, 35, 45, 55, and 65 ppm of benzene in simulated wastewater which contained a mixture of BTX. As per the results, in Exp. 1, 87% of benzene was removed from its initial concentration of 25 ppm within 36 hours – the greatest reduction as compared to the other concentrations of benzene. The percentages of reduction of benzene in Exp. 2, Exp. 3, Exp. 4, and Exp. 5 were 85%, 83%, 81%, and 80% respectively within 36 hours. Evidently, when the initial concentrations of benzene increased from 25 ppm to 65 ppm, the extent of its removal within the first 12 hours showed a decreasing trend concentration (i.e. from 80% to 74%). A similar trend prevailed for the removal of benzene within 24 hours, whereby the highest percentage of reduction (83%) was seen in the lowest initial concentration of benzene. However, the aforementioned percentage was higher at 45 ppm (82%) vis-à-vis 35 ppm (81%). This inconsistency could be attributed to the patterns of enzyme production in the two experiments. For example, the extent of removal of 25-ppm benzene in the BTX mixture was lower than that of benzene alone. The reason for this was that the enzymes produced by *P. putida* were specific for the bonds in toluene and xylene.

Figure 4.12 illustrated the biological treatment of different concentrations of toluene in simulated wastewater. As per the results, 10-ppm toluene was removed to the

greatest extent (80%) within 36 hours. This was followed by 15- and 20-ppm, which were removed by 77%, as well as 25- and 30-ppm by 75%. Similar trends were observed at the end of 12 and 24 hours respectively. Within 12 hours, 73% of 10- and 15-ppm of benzene was removed. This was followed by 72% of 20-ppm, 70% of 25-ppm, and 69% of 35-ppm. As for the initial 24 hours, 77% of 10-ppm toluene was removed, followed by 75% of 15-ppm, 74% of 20-ppm, 72% of 25-ppm, and 70% of 30-ppm. Hence, it could be concluded that toluene alone was degraded to a greater extent than toluene in a mixture.

The percentage of reduction of xylene in a mixture was higher than that of xylene alone. In other words, the extent of degradation of xylene increased in the presence of benzene and toluene, as illustrated in Figure 4.13. As per the results, at the end of 36 hours, 15-ppm xylene was reduced by the greatest extent (75%). This was followed by 10-ppm (72%), 20-ppm (71%), 25-ppm (69%), and 30 ppm (66%). Evidently, these results slightly differed from those of the removal of benzene and toluene. While the percentage of removal was the highest for 15-ppm xylene, the lowest concentrations of both benzene and toluene underwent the greatest extent of removal. This trend applied to the initial 12 and 24 hours. The outcomes of GC-FID showed that the percentages of reduction of 10, 15, 20, 25, and 30 ppm of xylene within 12 hours were 65%, 67%, 64%, 62%, and 62% respectively. Within 24 hours, the percentage of reduction of 15-ppm xylene was the highest (69%), followed by 10-ppm (68%), 20-ppm (67%), 25-ppm (67%), and 30-ppm (65%).

Similarly, the greatest rate of degradation of the mixture of BTX occurred during the initial 12 hours when the bacteria were in the exponential phase. At this time, the amount of enzyme produced was proportional to the growth rate of bacteria, which translated into the rapid removal of BTX. This process slowed down beyond 12 hours, during which the bacteria were in the stationary phase (i.e. growth rate = death rate). Enzyme production also decrease affects the growth of *P. putida* and it decreases the rate of removal of BTX. Thus, according to Figures 4.11 – 4.13, less than 10% of BTX were removed between (i) 12 and 24 hours as well as (ii) 24 and 36 hours.

Sung-Kuk and Lee (2002) reported that the rates of degradation of individual compounds of BTX were different from those of the removal of BTX in a mixture. Also, there was a negative substrate interaction during substrate's growth (Jo et al.,

2008). In a binary mixture which consisted of benzene and toluene, *P. putida* F1 better utilised toluene than benzene, so the former was depleted first (Reardon, 2000). Jo et al. (2008) stated that the presence of toluene restricted the rate of degradation of benzene, while an excess of benzene had little effect on the depletion of toluene.

Previous studies on the degradation of BTX in mixtures by bacteria in batch systems have shown that the presence of *p*-xylene slowed down the removal of toluene (Oh et al., 1994). However, in the presence of toluene and xylene, the rate of degradation of benzene was increased (Okpokwasili and Nweke, 2005). In this study, *P. putida* which grew over 36 hours demonstrated removal rates in the order of benzene > toluene > xylene. Meanwhile, Kelly et al. (1996) observed that in batch systems, toluene depleted first, followed by benzene and toluene (Jo et al., 2008). It is well-known that BTX compounds whose concentrations exceed certain thresholds can inhibit the activities of the microbes owing to complex micro- and macro-level interactions. The most common mechanism of BTX biodegradation is as follows. In the first step, benzene is oxidised via hydroxylation, which is catalysed by a dioxygenase. The product, a diol, is then converted into catechol by a dehydrogenase. These initial reactions – hydroxylation and dehydroxylation – are also commonly seen in the degradation pathways of other aromatic hydrocarbons. The introduction of a substituent methyl group to the benzene ring (toluene) makes possible the commencement of alternative mechanisms to attack the side chains (of toluene) or oxidise its aromatic ring (Jo et al., 2008).

4.7 Bioremoval of BTX from Petrochemical Wastewater by *P. putida*

Biological treatment processes have long been employed by the petrochemical industry. It has been proven that these processes utilised the natural attenuation mechanism in the removal of metal or organic contaminants in total hydrocarbon mass. While numerous species of bacteria, fungi, and yeast are capable of degrading petroleum hydrocarbons, the two major bacterial genera are *Pseudomonas spp* and *Corynebacterium spp* (Fan, 1995). Most of these bacteria effect aerobic biodegradation, whereby enzymes are produced in the presence of oxygen to degrade the compound(s) of interest (Hers et al., 2000). The efficacy of this process can be affected by chemical, physical, and biological factors, such as soil moisture, nutrient (including inorganic

nutrient) availability, nutrient concentration, temperature, salinity, pH, and microbial adaption. (Hers et al., 2000; Singh and Celin, 2010).

VOCs such as benzene, toluene, and xylene can be easily dehydroxylated into *cis*-diols by certain bacteria that produce dioxygenases (Ouyang et al., 2007; Reddy et al., 1999).

4.7.1 Effect of pH on *P. putida* Growth

Every type of microorganism, especially bacteria, has its own optimum pH range for growth. Apart from having an effect on bacterial enzymatic activity, a sutiable pH is also needed for the transport of nutrients and toxic chemicals into the cell (Bitton (2005). *P. putida* activity was maximum between pH 6 and 8, while the said activity was completely inhibited at pH 5, 9, and 10 (El-Naas et al., 2014; You et al., 2013). Lu et al. (2002) showed that from pH 5 – 8, an increase in pH of the nutrient feed led to a proportional increase in the efficiency of BTX removal. However, from pH 8.0 – 8.5, the aforementioned efficiency was reduced. Thus, the efficiencies of the removal of benzene and toluene exceeded 80% when the pH ranged between 7.5 and 8.0 (El-Naas et al., 2014). This result was also consistent with that of Leson et al. (1991), whereby bacterial activity was maximum in the pH range of 7 to 8. As a summary, the optimum pH (6, 7, and 8) for *P. putida* have been selected for use in this study to maximise the bioreduction abilities of *P. putida*.

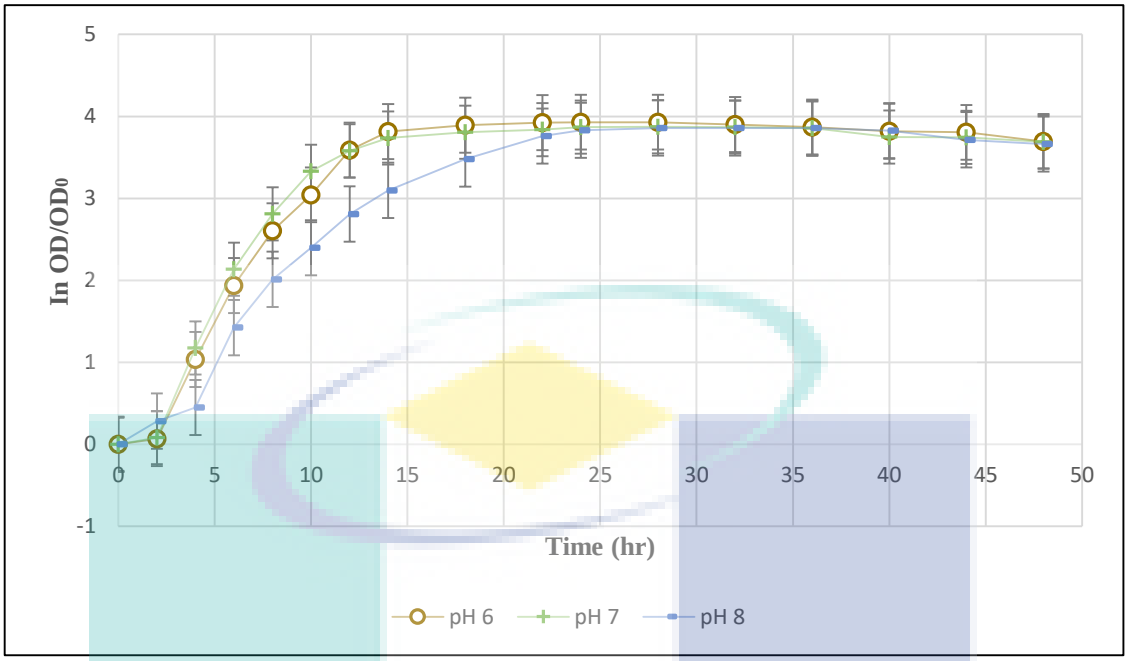


Figure 4.14 *P. putida* growth in actual petrochemical wastewater of different pH

Table 4.8 Summary of growth parameters of *P. putida* in actual petrochemical wastewater

Growth Parameter	**Min**			**Max**		
	pH 6	**pH 7**	**pH 8**	**pH 6**	**pH 7**	**pH 8**
Specific Growth Rate, μ (h^{-1})	0.30	0.33	0.24			
OD	0.04	0.05	0.05	2.19	2.23	2.18
Exponential cell growth, (ln OD/OD0)	3.59	3.58	2.81	3.93	3.87	3.86
Number Of Generation, *n*	5.54	5.13	4.03	5.63	5.55	5.49
Generation Time, *g* (h)	2.33	2.34	2.98	6.49	6.51	6.51
Growth Rate Constant, *k* (h^{-1})	0.11	0.11	0.11	0.30	0.30	0.23

Figure 4.14 showed the growth patterns of *P. putida* in actual petrochemical wastewater samples at different pH. The samples were taken from one of the petrochemical plants in the East Coast of Peninsular Malaysia, after which they were placed in the freezer at a temperature of below 4°C prior to their usage. The initial concentrations of BTX were 50.2, 22.4, and 27.5 ppm respectively. The actual pH of the wastewater samples were 14, these were adjusted to pH 6, 7, and 8 using sulphuric acid. These pH were chosen because *P. putida* grew best in pH 6 to 8 (Azoddein, 2013). The

experiment was conducted in shake flasks, whereby the ratio of *P. putida* to actual wastewater was 1:9 equal to 10% of bacteria concentration in sample. The parameters that were fixed during this experiment were a temperature of 37ºC and shaker speed of 180 rpm.

As per Figure 4.14, the growth curves of *P. putida* were similar at pH 6 and 7. However, the aforementioned curve was slightly different at pH 8, whereby the bacteria multipled slowly during the first 6 hours in light of the effect of pH and the adaption of the bacteria to the new environment. In contrast, *P. putida* only needed 4 hours to acclimatise when the pH was 6 or 7. Subsequently, the bacteria in all three pH values proliferated rapidly until 14 hours before they entered the stationary phase. It appeared that a lag phase was non-existent in all three experiments; otherwise the OD during this phase would have been lower than initial OD of *P. putida*. Evidently, it was obvious that *P. putida* did not grow well in pH 8. Conversely, the growth curves of *P. putida* in pH 6 and 7 were reasonably good and were in accordance with those of Azoddein (2013).

The results of the other growth parameters were shown in Table 4.8. The specific growth rate, μ, was 0.30 at pH 6, 0.33 at pH 7, and 0.24 at pH 8. Meanwhile, the maximum numbers of generations, *n*, were 5.63 at pH 6, 5.55 at pH 7, and 5.49 at pH 8. The longest generation times, *g*, were 6.49 h at pH 6, 6.51 h at pH 7, and 6.5 h at pH 8. Also, the maximum specific growth rate constant, *k*, for pH 6 was 0.30 h^{-1}, pH 7 was 0.30 h^{-1}, and pH 8 was 0.23 h^{-1}. Generally, the growth rate and cell activity at pH 8 were relatively the lowest. Conversely, *P. putida* in actual wastewater at pH 7 demonstrated maximum cell activity and growth rate. Hence, it was confirmed that the pH 7 was the optimum pH for the growth of *P. putida*, and so, the industries should utilise this pH value. This concurred with the results of previous studies (Azoddein et al., 2015).

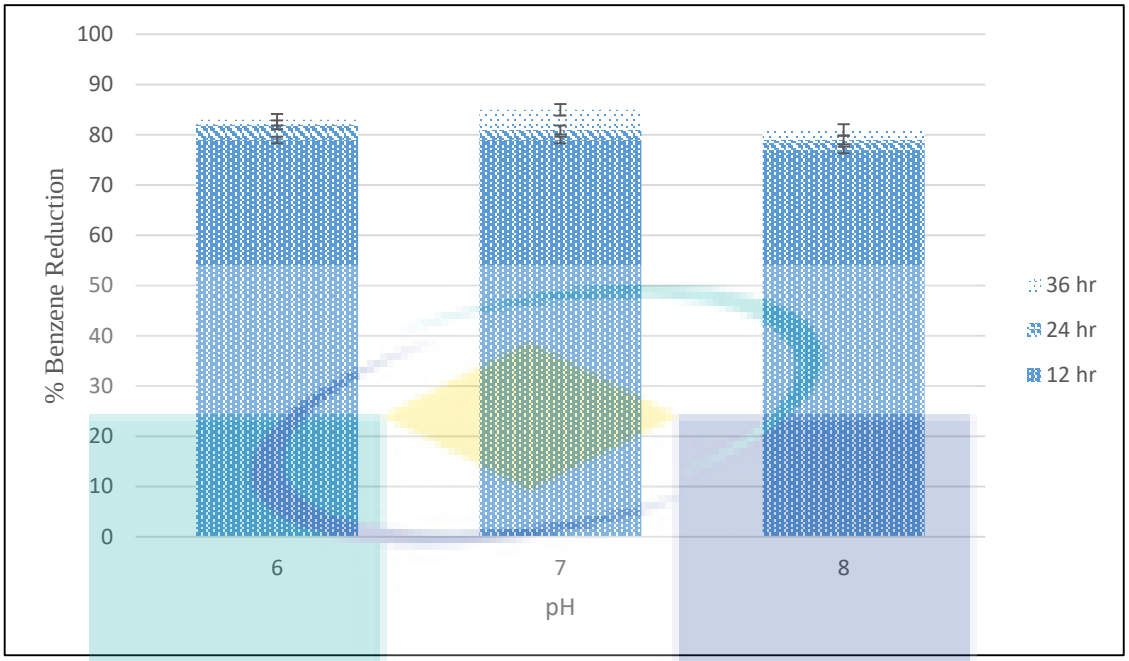


Figure 4.15 Biological treatment of benzene at different pH and concentrations by *P. putida*

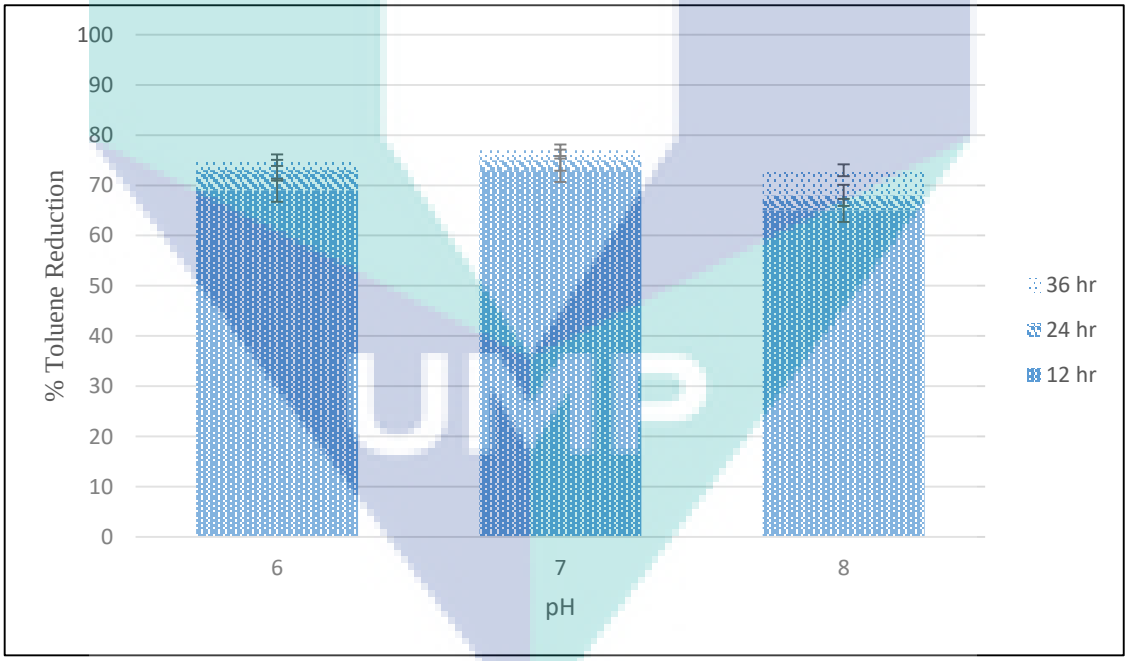


Figure 4.16 Biological treatment of toluene at different pH and concentrations by *P. putida*

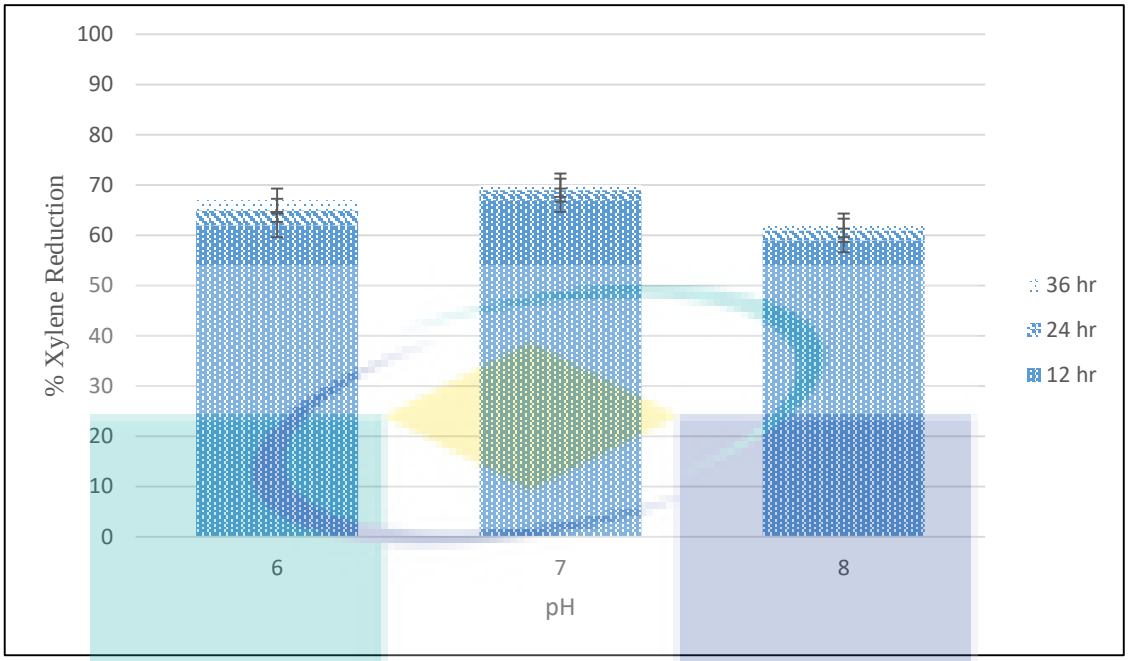


Figure 4.17 Biological treatment of xylene at different pH and concentrations by *P. putida*

Figures 4.15, 4.16, and 4.17 describe the biological treatment of BTX of different concentrations in actual petrochemical wastewater at pH 6, 7, and 8 respectively. As mentioned, the initial pH of the wastewater sample was 14; the value has then been modified into pH 6, 7, and 8 (El-Naas et al., 2014). As per Figure 4.15, within 12 hours, the percentages of removal of benzene were 79% at pH 6 and 7, as well as 77% at pH 8. The results were slightly different at the end of 24 hours, whereby pH 6 led to the highest percentage reduction (82%), followed by pH 7 (81%) and pH 8 (79%). Meanwhile, GC-FID analysis at the end of 36 hours gave rise to completely different results, whereby the greatest extent of benzene removal was 85% at pH 7, followed by 83% at pH 6 and 81% at pH 8. In conclusion, the degree of biological removal of benzene in actual wastewater was slightly lower than that of benzene in synthetic wastewater, be it alone or in a mixture.

As for the biological treatment of toluene in actual wastewater, the highest percentage reduction within 36 hours was 77% at pH 7, followed by 75% at pH 6 and 73% at pH 8. Similar patterns were seen within 12 and 24 hours, whereby the samples at pH 7 had the highest percentage reduction (73% and 75% respectively). In comparison, the values for pH 6 were 69% and 73% respectively, while those of pH 8

were 65% and 68% respectively – these were the lowest values in the experiment. With respect to these observations, toluene in actual wastewater was removed to a lesser extent than that in synthetic wastewater, the latter of which was 95% on average.

As with the previous two compounds, the percentages of reduction of xylene in actual wastewater at pH 7 were the highest, whereby the values were 67% within 12 hours, 69% within 24 hours, and 70% within 36 hours. At pH 6, the values were 62% within 12 hours, 65% within 24 hours, and 67% within 36 hours. The lowest percentages of reduction of toluene were at pH 8, whereby the values were 59% within 12 hours, 61% within 24 hours, and 62% within 36 hours.

Overall, the BTX at pH 7 was removed to a greater extent by *P. putida*, whereby the percentage of reduction of benzene was 85%, toluene 77%, and xylene 70%. At pH 6, the values were 83%, 75%, and 67 % respectively, while at pH 8, the values were 81%, 73%, and 62% respectively. The percentage of removal of xylene in actual wastewater was also lower than that of xylene in a mixture in synthetic wastewater, but higher than that of xylene alone in synthetic wastewater.

With reference to the abovementioned results, it was obvious that pH 7 was optimal for the growth of *P. putida*. The percentages of removal of benzene in all three experiments were higher as compared to toluene. When a sole carbon source was used, the results were slightly different, whereby toluene had the highest degradability relative to benzene and xylene. Thus, it was proven that the enzymes produced for the degradation of carbon sources by *P. putida* were most active at pH 7 (Yeung et al., 2008).

Table 4.9 summarises of overall results of the removal of benzene, toluene, xylene, simulated BTX, and BTX in actual wastewater samples. Meanwhile, Table 4.10 depicts the specific growth rates in each experiment.

Table 4.9 Summary of percentage reduction of BTX within 36 hours

Experiment	**Benzene Reduction (%)**					**Toluene Reduction (%)**					**Xylene Reduction (%)**				
Concentration (ppm)	**25**	**35**	**45**	**55**	**65**	**10**	**15**	**20**	**25**	**30**	**10**	**15**	**20**	**25**	**30**
Simulate Benzene	93	83	83	79	73										
Simulate Toluene						97	95	95	94	94					
Simulate Xylene											62	60	60	59	57
Simulate BTX	87	85	83	81	80	80	77	77	75	75	72	75	71	69	66

pH value	**pH 6**	**pH 7**	**pH 8**	**pH 6**	**pH 7**	**pH 8**	**pH 6**	**pH 7**	**pH 8**
Actual petrochemical wastewater	83	85	81	75	77	73	67	70	62

Table 4.10 Summary of specific growth rates, μ (h^{-1})

Experiment	**Specific Growth Rate, μ (h^{-1})**				
	1	**2**	**3**	**4**	**5**
Simulate Benzene	0.33	0.12	0.15	0.14	0.16
Simulate Toluene	0.31	0.16	0.15	0.19	0.16
Simulate Xylene	0.23	0.25	0.20	0.21	0.21
Simulate BTX	0.32	0.31	0.29	0.27	0.19

pH value	**pH 6**	**pH 7**	**pH 8**
Actual petrochemical wastewater	0.30	0.33	0.24

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

Petrochemical industries in Malaysia give rise to different types of chemicals which largely end up in wastewater effluent and underground water, and these are hazardous to both humans and the environment. This study on the petrochemical wastewater effluent which was extracted from the East Coast of Malaysia has shown aromatic compounds such as benzene, toluene, and xylene were another concern for industry in treating it. Prior to biological treatment by *P. putida* ATCC 49128, the characteristics of the wastewater, including the concentrations of BTX, have been identified. As per the results, the concentration range of benzene was 43.1 – 55.2 ppm, toluene 11.6 – 26.9 ppm, and xylene 21.6 – 32.8 ppm. Also, the pH was 13 ± 1, BOD_5 is 18.1 – 27.17 ppm, COD 85.6 – 95.2 ppm, TSS 11 – 19 ppm, and turbidity 21.3 – 25.2 ppm. The synthetic wastewater was manipulated with reference to the abovementioned data, whereby the concentration of benzene was designated at 25 ppm, toluene 10 ppm, and xylene 10 ppm. There were 5 different concentrations for each aromatic compound, and their concentrations in mixtures were in a ratio of 5:2:2.

The growth pattern and dry cell weight of *P. putida* in optimum conditions have been studied (Azoddein et al., 2015). This research has emphasised on the understanding of the characteristics of *P. putida* prior to its administration on BTX stock solutions and actual wastewater samples. Evidently, the acclimatisation time was 24 h, orbital shaker speed 180 rpm, temperature 37°C, and nutrient concentration 8 g/L. Also, the bacterial growth parameters were OD_{max} = 2.03; number of generations, n = 3.89; generation time, g = 15.84 h; specific growth rate, μ = 0.91 h^{-1}; and growth rate constant, k = 0.57 h^{-1}. The dry cell weight of *P. putida* was another parameter in the

study on the relationship between optical density (OD) and cell biomass. It could be concluded that the concentration of dry biomass was directly proportional to the OD, whereby the linear values were $R^2 = 0.9707$ and $Y = 0.4347X$. Thus, cell biomass was 0.4347 times the OD.

The results of the removal of benzene from synthetic wastewater have shown that *P. putida* grew most actively in low concentrations of the compound (i.e. 25 ppm). The growth parameters were as follows: maximum number of generations, $n_{max} = 7.06$; maximum generation time, $g_{max} = 5.77$ h; maximum growth rate constant, $k_{max} = 0.39$ h^{-1}; and specific growth rate, $\mu = 0.33$ h^{-1}. In accordance with other studies on bacterial growth, the percentage of reduction of 25-ppm benzene by *P. putida* was 92% within 36 hours.

As with benzene, *P. putida* also grew best in low concentrations of toluene. The growth parameters were as follows: specific growth rate, $\mu = 0.31$ h^{-1}; maximum number of generations, $n_{max} = 5.06$; maximum generation time, $g_{max} = 7.45$ h; and maximum growth rate constant, $k_{max} = 0.28$ h^{-1}. Thus, the highest percentages of reduction were seen in 10-ppm toluene, whereby the values at the end of 12, 24, and 36 hours were 83%, 91%, 97% respectively. Also, *P. putida* ATCC 49128 better removed toluene as compared to benzene and xylene when the compounds were present singly.

Xylene was the least hazardous compound relative to the other two because it was less soluble in water (wastewater effluents). *P. putida* did not grow well in synthetic xylene when compared to benzene and toluene. The growth parameters were as follows: specific growth rate, $\mu = 0.23$ h^{-1}, maximum number of generations, $n_{max} = 4.08$; maximum generation time, $g_{max} = 8.88$ h; and maximum growth rate constant, $k_{max} = 0.23$ h^{-1}. The bacterial growth had an effect on the removal of xylene from synthetic wastewater samples. In view of the maximum growth of *P. putida* in 10 ppm of xylene, the percentages of reduction of the same were the highest, whereby the values at the end of 12, 24, and 36 hours were 49%, 56%, and 62% respectively. In conclusion, xylene was not as effectively removed by *P. putida* relative to benzene and toluene.

The investigation clearly showed that the growths of *P. putida* in single aromatic compounds were similar to those in mixtures of aromatic compounds. The optimal operating conditions for all the experiments were 37°C, 180 rpm, and pH 7. As

per the results, the bacteria grew best in experiment 1 which contained the lowest concentrations of BTX (benzene = 25 ppm; toluene = 10 ppm; xylene = 10 ppm). Once again, the growth parameters were as follows: specific growth rate, μ = 0.32 h^{-1}, maximum number of generations, n_{max} = 5.38; maximum generation time, g_{max} = 7.04 h; and maximum growth rate constant, k_{max} = 0.30 h^{-1}. However, the outcomes of the removal of BTX contradicted those of the removal of individual aromatic compounds. In this study, the percentage of reduction of benzene was higher than those of toluene and xylene. It could hence be concluded that low concentrations of BTX in shake flasks were removed to the greatest extent, as reflected by the following values at the end of 36 hours: benzene = 87%, toluene = 80%, and xylene = 72%.

The actual wastewater was very alkaline (pH 14) and thus, not suitable for the growth of *P. putida*. Thus, three different pH values (pH 6, 7, and 8) were used in this experiment as these were the ideal ones. As per the results, *P. putida* grew optimally at pH 7, whereby the growth parameters were as follows: specific growth rate, μ = 0.33 h^{-1}, maximum number of generations, n_{max} = 5.48; maximum generation time, g_{max} = 6.86 h; and maximum growth rate constant, k_{max} = 0.30 h^{-1}. At pH 7, BTX was also removed to the greatest extent, which correlated to the maximum growth rate of *P. putida*. The percentage of reduction of benzene was the highest (85%), followed by toluene (77%) and xylene (70%). In conclusion, BTX removal by *P. putida* ATCC 49128 was ideally conducted at pH 7.

This study has clearly demonstrated that *P. putida* ATCC 49128 in optimum conditions resulted in maximum bioremediation of BTX in petrochemical effluents. This is especially needed for benzene, which is very hazardous and carcinogenic to humans. *P. putida* produces a specific enzyme for each of the aforementioned compounds (which are carbon sources for the bacteria). On another note, previous studies have shown that *P. putida* ATCC 49128 can also treat mercury in petrochemical wastewater, apart from aromatic compounds. Unlike physical treatment methods, bioremediation can reduce the cost of wastewater treatment. Therefore, biological treatment is effective for use in the petrochemical industry.

5.2 Recommendations and Further Studies

The following aspects are recommended for future studies:

i. Study on *P. putida* treatment in high-volume bioreactors which closely mimic the actual biological treatment processes in petrochemical industries
ii. Study on continuous aeration biological treatment
iii. Study on the characteristics of enzymes produced by *P. putida* ATCC 49128 during treatment of BTX
iv. Study on BTX removal by *P. putida* which are isolated from localised areas
v. Study on effects of other parameters such as the presence of other elements in actual petrochemical wastewater in the reduction of BTX by *P. putida*

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APPENDIX 1

BIOLOG **Microbial Community Analysis**

EcoPlate™

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

FIGURE 1. Carbon Sources in EcoPlate

APPENDIX 2

Getting Started with an ATCC Mycology Strain

ATCC mycology strains are shipped either frozen on dry ice, in plastic or glass cryopreservation vials, as lyophilized cultures in glass ampoules or serum vials, or as live cultures in test tubes. Upon receipt of frozen cells, immediately revive cells by thawing and subsequently transferring cells to an appropriate growth medium. If this is not possible, store frozen vials in liquid nitrogen vapor (below -130°C). Alternatively, the frozen material can be stored between -70°C and -80°C for short periods (1 to 5 days); however, viability will decline at temperatures above -130°C. Freeze-dried cultures can be stored safely at 4°C or lower. Upon receipt, rehydrate or dilute freeze-dried cultures using sterile distilled water or the appropriate medium and incubate under the conditions specified on the product sheet. Live cultures should be immediately transferred to tubes or plates containing the recommended medium and incubated under the appropriate growth conditions. Under no circumstances should live cultures be stored at refrigerated or standard freezer temperatures as this may result in the death of the culture.

Product Sheet

ATCC mycology strains are shipped with a product sheet that contains detailed information on the processing and expansion of materials, as well as ideal growth and propagation conditions. This product sheet, as well as additional information, can be found on the ATCC website or can be requested from the ATCC Technical Service Department.

Preparation of Medium

In advance, prepare the appropriate medium and additional supplements necessary for yeast and fungal revival and growth. Additionally, ensure that your incubator is set to maintain the optimal growth conditions of the strain. Information for the formulation and preparation of the media and incubation conditions for these products is available on the ATCC website.

Opening Glass Ampoules

Overview

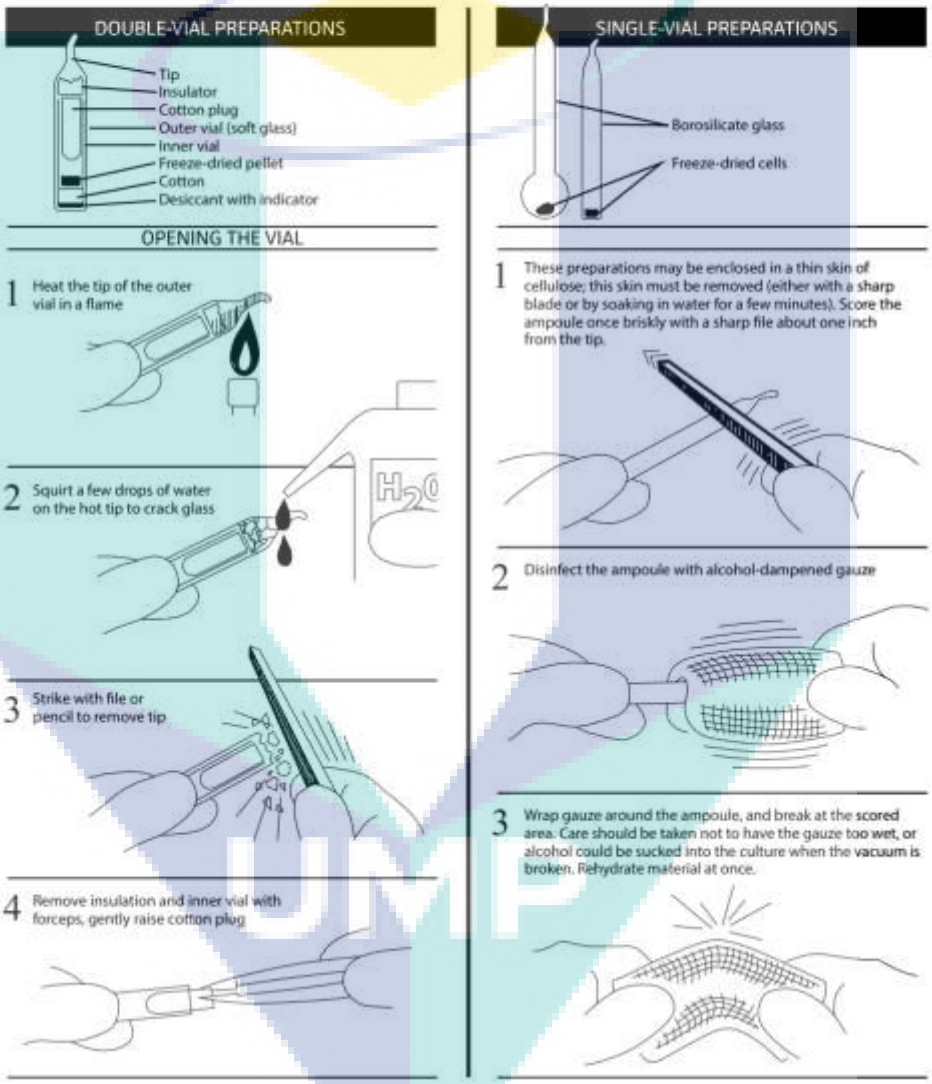
All cultures should be considered potentially hazardous and should be opened by individuals trained in microbiological techniques working in facilities with containment requirements appropriate for the biosafety level of the cultures. ATCC recommends that the handling or opening of glass ampoules be performed in a biological safety cabinet. If this is not possible, wear protective clothing, gloves, a face shield or safety goggles, and hold the vial away from your body. Ensure that all empty vials are sterilized before disposal.

A. Opening Double-Vial Preparations

1. Heat the tip of the vial in a flame.
2. Add a few drops of water on the hot tip to crack the glass.
3. Strike the end of the vial with a file or pencil to remove the tip.
4. Remove the insulation and inner vial with sterile forceps. Gently raise the cotton plug.

B. Opening Single-Vial Preparations

1. To recover the cell suspension from the glass ampoule, score the neck of the ampoule with a small, sterile file.
2. Disinfect the outside of the ampoule with freshly prepared 70% ethanol or dip it into a beaker of freshly prepared 70% ethanol.
3. Wrap the ampoule within several folds of a sterile towel or gauze to dry residual ethanol.
4. Working in a laminar flow hood, hold the vial upright and snap open the vial. Ensure that your gauze does not become too wet with ethanol, or alcohol could be sucked into the culture when the vacuum is broken. Rehydrate the material immediately.



Getting Started with an ATCC Mycology Strain

Initiating Frozen Cultures

1. Prepare a sterile test tube that contains the recommended medium for growth as listed in the product sheet. Ensure that the medium contains all necessary supplements or components and is equilibrated for temperature and pH.
2. Thaw the sample vial via gentle agitation in a water bath that is set to 25°C to 30°C. Thawing will be rapid; approximately 5 minutes or until all ice crystals have melted.
3. Remove the vial from the water bath and decontaminate the outer surface using freshly prepared 70% ethanol. Follow strict aseptic conditions in a laminar flow hood for all further manipulations.
4. Unscrew the top of the vial and transfer the entire contents to a sterile test tube or plate containing the appropriate growth medium. For yeast cells in suspension, cultures can be spread-plated on the medium to promote the growth of individual colonies. Note that many frozen ATCC mycology items contain agar plugs made from fully grown culture.
5. Incubate cultures under the appropriate temperature and atmospheric conditions as recommended on the product sheet.
6. Examine cultures after the recommended incubation period. The incubation period will vary between strains and is listed on the product sheet (See: NOTE 1).

NOTE 1:
Following recovery from cryopreservation or lyophilization, some mycological strains may exhibit a prolonged lag phase. These strains will require an extended incubation period.

Initiating Lyophilized Cultures

1. Using a Pasteur pipette, aseptically add 0.5 to 0.9 mL of sterile water to the freeze-dried material. Mix well.
2. Transfer the entire suspension to a test tube containing 6 mL of sterile water.
3. Allow the culture to soak for 50 to 60 minutes before transferring to solid media. For some strains, prolonged rehydration is recommended.
4. Incubate cultures under the appropriate temperature and atmospheric conditions as recommended on the product sheet. Additional agar medium can be inoculated by transferring 0.5 mL of the primary culture to additional secondary cultures.
5. Most freeze-dried cultures will grow within a few days (See: NOTE 1).

Initiating Test Tube Cultures

1. Upon receipt of the live culture, aseptically transfer the culture from the provided test tube into a test tube or plate containing the recommended medium.
2. Incubate the test tube or plate under the recommended atmospheric conditions and temperature.
3. Examine cultures after the recommended incubation period. The incubation period will vary between strains and is listed on the product sheet.

APPENDIX 3

Data during characteristic study in 2006

Parameters	Units	Day 1		Day 2		Day 3	
		A	B	A	B	A	B
pH	-	6.2	6.8	6.9	6.6	6.5	6.6
BOD_5 @ 20°C	mg/L	1880	1220	426	1230	2140	1460
COD	mg/L	2900	3300	894	2500	4550	3000
Suspended Solids	mg/L	31	34	40	31	26	25
Mercury (Hg)	mg/L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cadmium (Cd)	mg/L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Chromium,Hexavalent (Cr^{6+})	mg/L	<0.05	<0.05	<0.05	<0.05	<0.05	0.14
Copper (Cu)	mg/L	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Arsenic(As)	mg/L	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Cyanide (Cn)	mg/L	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Lead (Pb)	mg/L	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Chromium, trivalent (Cr^{3-})	mg/L	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Manganese (Mn)	mg/L	0.42	0.33	0.28	0.33	0.30	0.30
Nickel (Ni)	mg/L	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Tin (Sn)	mg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Zinc (Zn)	mg/L	0.27	0.18	0.17	0.18	0.19	0.17
Boron (B)	mg/L	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Iron (Fe)	mg/L	18.4	9.99	8.72	8.44	8.60	7.83
Phenol	mg/L	6.0	30.0	35.0	30.0	25.0	24.6
Free Chlorine (Cl)	mg/L	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Sulphide	mg/L	0.03	0.03	0.03	0.02	0.08	0.03
Oil and Grease	mg/L	<1	37	40	21	<1	12
Total Dissolved Solid	mg/L	1610	1320	1470	1380	1470	1330
Chloride	mg/L	516	515	484	464	448	395
Alkalinity	mg/L	117	233	223	164	139	148
Benzene	µg/L	26700	30200	30200	31200	31600	32800
Toluene	µg/L	16000	18600	16600	18700	17000	17000
Ethyl benzene	µg/L	3100	3440	3460	3580	3700	4050
Xylene	µg/L	17550	18830	20460	20510	21240	22680

Date: 3 November 2011

		Lab I.D		155075	155076	155078
		Sample I.D		DOC vessel	Process vessel	Balancing vessel
		Units	LOR			
Method Reference	**Analysis Description**	**Date of analysis**		25/10/2011		
USEPA 5030B,8260B	**BTEX**					
	Benzene	µg/L	1	58900	30900	59100
	Toluene	µg/L	1	30100	18600	31500
	m&p - Xylene	µg/L	1	3090	5470	18400

APPENDIX 4

3. SAMPLE COLLECTION AND TRANSPORT

The objective of sampling is to collect and present for analysis a sample that properly represents the element of the environment, waste or waste discharge of interest.

There are physical, chemical and biological processes that can affect the sample from the time it is collected to when it is analysed. To avoid or minimise the effect of these processes, it is necessary to use the appropriate sampling equipment, select the appropriate container and apply preservation methods in order to maintain the sample's integrity. Samples must also be analysed within stipulated holding time limits.

Care is required to avoid contamination of the sample during sampling, handling and transport to the laboratory.

Health and safety precautions

Safety precautions need to be taken when sampling in the field, and when handling contaminated samples and preservative chemicals. The characteristics and features of each site and sampling point need to be assessed to ensure the safety of the sampler.

The following precautions and warnings must be observed when sampling wastewaters, other wastes or heavily polluted soils, sludges or wastes that may contain harmful chemicals or bacteriological contaminants.

- Skin contact and inhalation of gases from the effluents and polluted samples must be avoided. Wearing disposable plastic gloves is advisable, and if necessary, wearing suitable protective clothing.
- For sewage effluents, hands should be washed with bactericidal soap after sampling such effluent.
- If accidental contact occurs, then rinse the exposed area thoroughly and seek medical advice. If regularly sampling or handling effluents containing sewage, appropriate inoculations should be obtained.
- For hazardous or heavily contaminated samples, the sample label should bear a warning to the analyst.
- Where necessary, designated sampling points, such as a suitable stable platform should be provided. Locations should be readily accessible, and where necessary, safety rails or roping points to which safety harnesses can be attached should be available. The sampler should be either accompanied by another person or make known their sampling location.
- When sampling wastes, care should be taken to avoid inhaling vapours that could be harmful. If necessary, appropriate respiratory protection should be worn by personnel trained in its use. Unless the ignition point of the contents or contaminants is known, precautions should be taken on the assumption that they are flammable.
- Cuts and skin abrasions should be covered with waterproof dressings.
- A suitable change of clothing should be worn during work.
- Eye protection should be used when there is a risk of material entering the eye.
- If dusts or aerosols are considered a problem then masks, conforming to Australian Standard AS 1715 (Standards Australia 1994), should be worn to prevent inhalation. Where possible, work upwind of the application process.

The degree of precaution taken and the type of protective equipment and clothing used should be commensurate with the level of risk. When in doubt, assume the worst case outcome will occur.

When preserving samples in the field, the following precautions should be observed:

- Familiarise yourself with the safety precautions relevant to the preservative(s) to be used.
- The preservation of samples should be performed on a stable surface such as the lid of a portable cooler or a paved surface in order to minimise the chances of spillage.
- Avoid contact with acids, solvents and other preservatives by wearing disposable plastic gloves and safety glasses and by avoiding the inhalation of vapours.
- Addition of acid preservative may release toxic gases such as hydrogen sulphide and hydrogen cyanide. This should always be done in a well-ventilated area, and with due care. After the acid has been added to the sample, time should be allowed for all evolved gas to be vented before placing the lid on the container.

Sampling devices

Sampling devices should be constructed of materials that have minimum interaction with, and do not contaminate, the sample. They should be designed in such a way as to minimise disturbance to the sample.

Sampling devices need to be cleaned appropriately, using the same approach used for sampling containers, as recommended elsewhere in this Guide. Sampling devices should be well cleaned between samples*, particularly when heavy contamination is suspected. In some cases, it may be necessary to collect the final rinsate for analysis, in order to demonstrate that the sampling device has been sufficiently well cleaned. Wherever possible, when sampling waters and wastewaters rinse the container with some of the material to be sampled.

It is not always possible to collect a liquid sample directly into a sample container. Where access is difficult, a scoop or bucket may be used. When a bucket is used, care must be taken to ensure the contents of the bucket remain well mixed while sub-samples are withdrawn. For deep waters, wastewaters and groundwaters, a special sampling device (for example, automatic samplers, Van Dorn bottle) may be needed to collect the sample, which is then transferred into the sample container.

Sample containers

Selecting a sample container

Containers are usually glass, polyethylene or polypropylene, and are selected based on their lack of interaction with analytical parameters. For example, glass is suitable for samples containing trace organics as leaching and adsorption are minimal. However, glass is unsuitable for sampling most trace inorganics because active sites on its surface are capable of binding inorganic ions. For some analytical parameters, fluoropolymer (PTFE) lid liners should be used.

Washing sample containers

To avoid contamination, sample containers need to be specially washed and pre-treated. Suitable containers and special instructions on washing these containers are presented in Appendix A for waters, wastewaters and groundwaters, Appendix B for soils and Appendix C for wastes. Even new containers should be washed and dried, unless specifically not recommended in this Guide.

Containers should only be washed and rinsed with high-grade reagents and solvents. These may need to be retained and submitted to the laboratory for analysis as a blank. Where reagents are added during the preservation step, a sample of the added reagents must be submitted to the laboratory for analysis as a reagent blank.

For waters, wastewaters and groundwaters, rinsing the sampling container with the sample is usually advisable. This minimises any contamination of the container that may have

* When this involves the use of detergents or solvents, the cleaning liquid should be collected and disposed of appropriately.

occurred between washing and sampling. Do not follow this procedure when:

- the analytical parameters are associated with immiscible liquids or suspended particles that will adhere to the sides of the container and may result in higher concentrations in the sample
- sampling for bacterial or other microbiological pollutants, because it is essential that the sterility of the container is maintained before use and the removal of any de-chlorinating agent must be prevented
- containers already contain preservatives (for example solvent or acid).

In the case of waters, wastewaters and wastes, it is occasionally appropriate to overfill containers, particularly when the analytical parameter is potentially oxidisable. For requirements for specific analytical parameters see Appendices B and D.

Sampling waters

Sampling and analysis plans should be devised in accordance with the requirements of Australian Standard 5667.1-12 (Standards Australia 1998a).

Where very low ambient concentrations are expected, special precautions may need to be taken to ensure samples are not contaminated. The integrity of samples must be maintained during sampling, and sources of contamination should be avoided.

Precautions for avoiding or minimising contamination are suggested below.

- Never handle the insides of containers, lids and collection vessels.
- Where preservative is required for the sample, do not allow the device used to add the preservative to make contact with the inside of the container or sample.
- Isolate buffer solutions and preservatives that could cross-contaminate samples. For example, buffer solutions can cross-contaminate water samples being collected for phosphorus analysis.
- Sample containers and chemical preservatives may need to be specially prepared and purified to ensure no cross-contamination occurs. Specialist laboratories have clean rooms to handle the analysis of such samples.

When sampling for volatile species, care should be taken to avoid losses. The sample vial or bottle should be filled gently to reduce agitation that might drive off volatile compounds. Cool the sample immediately on ice for transportation to the analysing laboratory.

Sampling surface waters

When waters are well mixed, a sample taken 100 mm below the surface, well away from the edge, may be adequate. However, deep and stratified waters may require special devices (such as a Van Dorn sampler) and careful handling techniques if the chemical species of interest is unstable. A hand or power-driven pump with an extended inlet tube may be useful to draw water from selected depths.

When sampling shallow waters, contamination of the sample from disturbed sediment should be avoided by using an extended inlet of thin tube on the sample bottle and drawing water into the bottle by suction.

To collect a sample of the surface layer for analysis, the container should be held horizontally in the water, half submerging it. To collect a sample of the water beneath a surface layer, a syringe or other device with an extended inlet tube capable of piercing the surface layer, may be appropriate, depending on the thickness of the surface layer.

In all cases, ensure that the sampling device or method does not contaminate the sample.

Sampling groundwaters

Regular testing of groundwater quality is usually done from bores. Monitoring bores should be constructed in accordance with the guidelines of the Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ 1997).

Groundwater sampling should be undertaken in accordance with *Groundwater Sampling Guidelines* (EPA 2000).

Sampling groundwaters by pumping or bailing the sample to the surface requires special precautions to avoid contamination. All equipment that either enters a bore or carries the water from the bore to the sampling container should be cleaned before each sample is taken. Special care needs to be taken for certain analytical parameters that can be affected by the presence of dissolved gases.

Sampling a waste discharge

The most representative sample of a waste discharge is from a point where the effluent is thoroughly mixed and close to the outlet from the discharging premises. For a licensed discharge, a sampling point will normally be described in the licence, and samples must always be taken from that point.

Use of automatic samplers

Automatic samplers are used to monitor diurnal variations or to collect temporal composite samples from a water body. The probe for these samplers should be placed sufficiently far from both the surface and bottom of the water body so that the sample is not affected by the presence of the air/water or sediment/water interface.

Sampling soils

Sampling and analysis plans should be devised in accordance with the requirements of the *Australian and New Zealand Guidelines for the Assessment of Contaminated Sites* (ANZECC/NH&MRC 1992) and Australian Standard 4482.1 (Standards Australia 1997a) or Australian Standard 4482.2 (Standards Australia 1999). When sampling soils for volatile contaminants, special precautions should be taken to prevent evaporative losses.

Collection of samples should be accomplished with minimal disturbance, using a coring device. The core soil sample should either be immersed into methanol in the field; or the core should be placed into a vial that will also act as a purge vessel in the laboratory. These methods have been shown to provide generally more accurate results than placement of samples into jars (USEPA 1991).

If the soils to be sampled are suspected of being acid sulfate soils or potential acid sulfate soils, the EPA Information Bulletin *Acid Sulfate Soil and Rock* (EPA 1999) provides guidance and further references on sampling and handling.

When sampling from a test pit, samples should be taken from the lowest point first to prevent cross contamination from other sampling points.

Before sampling, vegetation and other non-soil material (including rocks and concrete) should be removed by hand. Any material removed should be weighed and its description recorded.

Sampling sediments

The best locations for sampling sediments are where fine materials accumulate. These are generally confined to areas where there is little or no flow.

Sediment samples can be collected using a number of devices including grabs, scoops, corers, shovels and buckets. When sampling for organic analysis, sampling devices should be constructed from metal. Conversely, when sampling for metals, sampling devices should be constructed from plastic.

Where there is a lack of fine sediment, more than one scoop or grab sample may be necessary to obtain a sufficient amount of material. These samples should be combined and mixed well before processing for analysis.

Sampling wastes

At all times it is important that sampling is carried out so that representative samples are obtained. Sampling wastes can be difficult if the wastes are heterogeneous, contain many different types of waste, or the contamination is not evenly distributed. In these circumstances, it can be useful to keep different types of waste separate (for example by separating the phases in a multi-phase waste), or to separate different portions that contain high levels of contaminants. General guidance on sampling can be obtained from *Pierre Gy's Sampling Theory and Sampling Practice: Heterogeneity, Sample Correctness and Statistical Process Control* (Pitard, 1989) particularly when there are large amounts of waste to be sampled.

Liquid wastes should be handled according to the methods for sampling waters described above. Waste soils should be treated according to the guidelines for soils above.

For solid wastes with particle sizes greater than soils, or non-uniform particle sizes, Australian Standard 1141.3:1996, (Standards Australia, 1996) may be relevant in some cases. Wastes containing biosolids should be handled and treated according to the procedures listed in Appendix C for the individual parameters by which the wastes are to be characterised.

Preserving samples

When biological, chemical or physical changes to the sample may occur between the time it is collected and when it is analysed, it must be either chemically or physically preserved to retard such processes.

Preservation methods vary greatly in their effectiveness and should only be employed when the sample cannot be analysed within a few hours of collection. Preservation should be carried out as soon as possible after sampling.

Recommended preservation methods for waters, wastewaters and groundwaters are given in Appendix A, for soils and sediments in Appendix B, and for wastes in Appendix C.

Freezing

Water and soil samples are best frozen in small amounts sufficient for the determination of one parameter. This procedure avoids repeated thawing and re-freezing if the total analysis is spread over a number of days. Quick freezing with dry ice is recommended.

For water, wastewater and groundwater samples provide sufficient air gap in the container to allow expansion of the liquid.

Thawed samples must be mixed and allowed to reach ambient temperature before any measurements or analysis.

Cooling

Samples that need to be cooled to between 1°C and 4°C should be placed in an insulated container or icebox containing a mixture of water and ice, and checked to ensure that some ice always remains. Alternatively, a maximum/minimum thermometer can be used in the icebox to check that the temperature remained within this range during transport to the laboratory.

Acidification

For water samples, acidification to below pH 2 is used to preserve most trace metals. This reduces precipitation and sorption losses to the container walls. A sample of the acid used, which should be of analytical grade and have low metal content, must be retained for analysis as a blank by the laboratory and correction of the analytical results for any analyte present. For groundwaters, acidification should only be applied to filtered samples.

Reagent addition

Reagents may be added to samples to chemically fix the analytical parameter. Reagents added should be of high grade, and blanks provided to the laboratory so that contamination levels can be checked.

When chemicals are added to a sample to preserve or fix an analytical parameter, it is important to separate procedures for sampling,

sample handling and analysis for each parameter, to minimise any risk of cross-contamination. For example, nitric acid used for container preparation and as a preservative for heavy metals analysis can contaminate samples to be analysed for nitrate. Similarly, copper sulfate used for preservation of phenols may contaminate samples for metals analysis.

For heavily contaminated samples, care needs to be taken when adding chemical preservatives that could release hazardous gases. For example, when adding acid to preserve a sample collected for a heavy metal analysis, care should be taken to ensure that the sample does not also contain high concentrations of cyanide which could result in release of hydrogen cyanide gas.

Solvent extraction

A solvent may, at times, be added to extract the analyte from its matrix. For analysis of organic pollutants such as hydrocarbons, PAHs and some pesticides, an initial on-site solvent extraction in the sample container may be necessary. Samples of the solvent and containers used should be submitted for analysis.

Field filtration

Filtration of water samples in the field may be required in the following circumstances.

- Where organic and inorganic contaminants adsorb onto suspended matter in water, wastewater and groundwater samples.
- Where the concentration of dissolved contaminants or the contaminants associated with suspended matter need to be determined.

Filtering should occur immediately after collection and the analysis conducted on the filtered liquid, the particles, or both.

Filtration can be undertaken under gravity or by applying vacuum or pump pressure.

Filters and filtering devices should be cleaned in a similar manner to sample containers, and care taken that contamination is not introduced in the field. Filters from the same batch as used in the field, and the filtering device should be provided to the laboratory so that blank levels can be determined. On-site (between sample) final rinses from filtration equipment should also be submitted to the laboratory as 'rinsate blanks' for analysis.

Preserving soil samples

Often soil samples are moist when collected. This moisture can accelerate microbial action, which can change the concentration of some contaminants. In these circumstances, it is recommended to store the soil at 4°C or below.

Labelling and logging

Samples should be adequately described and securely labelled at the sampling site.

The sample container must be labelled to uniquely identify the sample, and the time and location of sampling. Use a solvent based marking pen (preferably black) or similar waterproof means of marking. Any changes to a label should be initialled and dated.

The sample log must show all relevant information, including where (precise location and depth) and when (date and time of day) the sample was taken and other relevant information.

Details of preservatives added (the type, concentration before addition and quantity added) and other pre-treatment applied to the sample must be noted on the label.

An example of a label used for samples is shown below:

APPENDIX 5

3. TURBIDITY METHODS & MEASUREMENT

3.1 Introduction

The IESWTR requires systems to measure the turbidity of combined filter effluent and individual filter effluent. Because these measurements are used for reporting and compliance purposes (as described in Chapter 2), accurate measurement and strict adherence to approved methods is of paramount importance. The following chapter describes approved methods, analytical issues associated with turbidimeters, quality assurance and quality control issues, and data collection and management.

3.2 Approved Turbidity Methods

Currently, the Agency has approved three methods for the measurement of turbidity as described in §141.74. Systems must utilize turbidimeters which conform to one of the following methods for compliance purposes. If the instrument does not conform, then it may not be used for monitoring under the requirements of the IESWTR. A brief description of each of the methods is found below.

3.2.1 EPA Method 180.1

EPA method 180.1, "Determination of Turbidity by Nephelometry", is found in the Agency's publication, *Methods for Chemical Analysis of Water and Wastes*. The method is based upon a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension. The higher the intensity of scattered light, the higher the turbidity. Readings, in NTUs, are made in a nephelometer designed according to specifications laid out in the method. A primary standard suspension is used to calibrate the instrument. A secondary standard suspension is used as a daily calibration check and is monitored periodically for deterioration using one of the primary standards. See Appendix B for EPA Method 180.1.

3.2.2 Standard Method 2130B

Standard Method 2130B, found in *Standard Methods* (1995), is similar to EPA Method 180.1. The method is also based on a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension under the same conditions. The higher the intensity of scattered light, the higher the turbidity. Formazin polymer is used as the primary standard reference suspension. See Appendix C for Standard Method 2130B.

APPENDIX 6

Methods B through F are suitable for the determination of solids in potable, surface, and saline waters, as well as domestic and industrial wastewaters in the range up to 20 000 mg/L.

Method G is suitable for the determination of solids in sediments, as well as solid and semisolid materials produced during water and wastewater treatment.

5. Bibliography

THERIAULT, E.J. & H.H. WAGENHALS. 1923. Studies of representative sewage plants. *Pub. Health Bull.* No. 132.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1979. Methods for Chemical Analysis of Water and Wastes. Publ. 600/4-79-020, rev. Mar. 1983. Environmental Monitoring and Support Lab., U.S. Environmental Protection Agency, Cincinnati, Ohio.

2540 D. Total Suspended Solids Dried at 103–105°C

1. General Discussion

a. Principle: A well-mixed sample is filtered through a weighed standard glass-fiber filter and the residue retained on the filter is dried to a constant weight at 103 to 105°C. The increase in weight of the filter represents the total suspended solids. If the suspended material clogs the filter and prolongs filtration, it may be necessary to increase the diameter of the filter or decrease the sample volume. To obtain an estimate of total suspended solids, calculate the difference between total dissolved solids and total solids.

b. Interferences: See Section 2540A.2 and Section 2540B.1. Exclude large floating particles or submerged agglomerates of nonhomogeneous materials from the sample if it is determined that their inclusion is not representative. Because excessive residue on the filter may form a water-entrapping crust, limit the sample size to that yielding no more than 200 mg residue. For samples high in dissolved solids thoroughly wash the filter to ensure removal of dissolved material. Prolonged filtration times resulting from filter clogging may produce high results owing to increased colloidal materials captured on the clogged filter.

2. Apparatus

Apparatus listed in Section 2540B.2 and Section 2540C.2 is required, except for evaporating dishes, steam bath, and 180°C drying oven. In addition:

Aluminum weighing dishes.

3. Procedure

a. Preparation of glass-fiber filter disk: If pre-prepared glass fiber filter disks are used, eliminate this step. Insert disk with wrinkled side up in filtration apparatus. Apply vacuum and wash disk with three successive 20-mL portions of reagent-grade water. Continue suction to remove all traces of water, turn vacuum off, and discard washings. Remove filter from filtration

apparatus and transfer to an inert aluminum weighing dish. If a Gooch crucible is used, remove crucible and filter combination. Dry in an oven at 103 to 105°C for 1 h. If volatile solids are to be measured, ignite at 550°C for 15 min in a muffle furnace. Cool in desiccator to balance temperature and weigh. Repeat cycle of drying or igniting, cooling, desiccating, and weighing until a constant weight is obtained or until weight change is less than 4% of the previous weighing or 0.5 mg, whichever is less. Store in desiccator until needed.

b. Selection of filter and sample sizes: Choose sample volume to yield between 2.5 and 200 mg dried residue. If volume filtered fails to meet minimum yield, increase sample volume up to 1 L. If complete filtration takes more than 10 min, increase filter diameter or decrease sample volume.

c. Sample analysis: Assemble filtering apparatus and filter and begin suction. Wet filter with a small volume of reagent-grade water to seat it. Stir sample with a magnetic stirrer at a speed to shear larger particles, if practical, to obtain a more uniform (preferably homogeneous) particle size. Centrifugal force may separate particles by size and density, resulting in poor precision when point of sample withdrawal is varied. While stirring, pipet a measured volume onto the seated glass-fiber filter. For homogeneous samples, pipet from the approximate midpoint of container but not in vortex. Choose a point both middepth and midway between wall and vortex. Wash filter with three successive 10-mL volumes of reagent-grade water, allowing complete drainage between washings, and continue suction for about 3 min after filtration is complete. Samples with high dissolved solids may require additional washings. Carefully remove filter from filtration apparatus and transfer to an aluminum weighing dish as a support. Alternatively, remove the crucible and filter combination from the crucible adapter if a Gooch crucible is used. Dry for at least 1 h at 103 to 105°C in an oven, cool in a desiccator to balance temperature, and weigh. Repeat the cycle of drying, cooling, desiccating, and weighing until a constant weight is obtained or until the weight change is less than 4% of the previous weight or 0.5 mg, whichever is less. Analyze at least 10% of all samples in duplicate. Duplicate determinations should agree within 5% of their average weight. If volatile solids are to be determined, treat the residue according to 2540E.

4. Calculation

$$\text{mg total suspended solids/L} = \frac{(A - B) \times 1000}{\text{sample volume, mL}}$$

where:

A = weight of filter + dried residue, mg, and
B = weight of filter, mg.

5. Precision

The standard deviation was 5.2 mg/L (coefficient of variation 33%) at 15 mg/L, 24 mg/L

APPENDIX 7

contaminated with soluble organics leached from the resin bed. Use of copper-lined stills or copper fittings attached to distilled water lines may produce water containing excessive amounts of copper (see Section 3500-Cu).

4. Reference

1. YOUNG, J.C. 1973. Chemical methods for nitrification control. *J. Water Pollut. Control Fed.* 45:637.

5. Bibliography

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5210 B. 5-Day BOD Test

1. General Discussion

a. Principle: The method consists of filling with sample, to overflowing, an airtight bottle of the specified size and incubating it at the specified temperature for 5 d. Dissolved oxygen is measured initially and after incubation, and the BOD is computed from the difference between initial and final DO. Because the initial DO is determined shortly after the dilution is made, all oxygen uptake occurring after this measurement is included in the BOD measurement.

b. Sampling and storage: Samples for BOD analysis may degrade significantly during storage between collection and analysis, resulting in low BOD values. Minimize reduction of BOD by analyzing sample promptly or by cooling it to near-freezing temperature during storage. However, even at low temperature, keep holding time to a minimum. Warm chilled samples to 20 ± 3°C before analysis.

1) Grab samples—If analysis is begun within 2 h of collection, cold storage is unnecessary. If analysis is not started within 2 h of sample collection, keep sample at or below 4°C from the time of collection. Begin analysis within 6 h of collection; when this is not possible because the sampling site is distant from the laboratory, store at or below 4°C and report length and temperature of storage with the results. In no case start analysis more than 24 h after grab sample collection. When samples are to be used for regulatory purposes make every effort to deliver

samples for analysis within 6 h of collection.

2) Composite samples—Keep samples at or below 4°C during compositing. Limit compositing period to 24 h. Use the same criteria as for storage of grab samples, starting the measurement of holding time from end of compositing period. State storage time and conditions as part of the results.

2. Apparatus

a. Incubation bottles: Use glass bottles having 60 mL or greater capacity (300-mL bottles having a ground-glass stopper and a flared mouth are preferred). Clean bottles with a detergent, rinse thoroughly, and drain before use. As a precaution against drawing air into the dilution bottle during incubation, use a water seal. Obtain satisfactory water seals by inverting bottles in a water bath or by adding water to the flared mouth of special BOD bottles. Place a paper or plastic cup or foil cap over flared mouth of bottle to reduce evaporation of the water seal during incubation.

b. Air incubator or water bath, thermostatically controlled at 20 ±1°C. Exclude all light to prevent possibility of photosynthetic production of DO.

3. Reagents

Prepare reagents in advance but discard if there is any sign of precipitation or biological growth in the stock bottles. Commercial equivalents of these reagents are acceptable and different stock concentrations may be used if doses are adjusted proportionally.

a. Phosphate buffer solution: Dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in about 500 mL distilled water and dilute to 1 L. The pH should be 7.2 without further adjustment. Alternatively, dissolve 42.5 g KH_2PO_4 or 54.3 g K_2HPO_4 in about 700 mL distilled water. Adjust pH to 7.2 with 30% NaOH and dilute to 1 L.

b. Magnesium sulfate solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 L.

c. Calcium chloride solution: Dissolve 27.5 g $CaCl_2$ in distilled water and dilute to 1 L.

d. Ferric chloride solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in distilled water and dilute to 1 L.

e. Acid and alkali solutions, 1*N*, for neutralization of caustic or acidic waste samples.

1) Acid—Slowly and while stirring, add 28 mL conc sulfuric acid to distilled water. Dilute to 1 L.

2) Alkali—Dissolve 40 g sodium hydroxide in distilled water. Dilute to 1 L.

f. Sodium sulfite solution: Dissolve 1.575 g Na_2SO_3 in 1000 mL distilled water. This solution is not stable; prepare daily.

g. Nitrification inhibitor, 2-chloro-6-(trichloromethyl) pyridine.*#(2)

h. Glucose-glutamic acid solution: Dry reagent-grade glucose and reagent-grade glutamic

APPENDIX 8

Oxygen Demand, Chemical

DOC316.53.01099

USEPA[1] Reactor Digestion Method[2] **Method 8000**

0.7 to 40.0[3] mg/L COD (ULR); 3 to 150 mg/L COD (LR);
20 to 1500 mg/L COD (HR); 200 to 15,000 mg/L COD (HR Plus)

Scope and Application: For water, wastewater; digestion is required

[1] Ranges 3 to 150 mg/L COD and 20 to 1500 mg/L COD are USEPA approved for wastewater analyses (Standard Method 5220 D), *Federal Register*, April 21, 1980, 45(78), 26811-26812.

[2] Jirka, A.M.; Carter, M.J., *Analytical Chemistry*, 1975, 47(8), 1397

[3] The ULR range is not available on the DR 2700 or the DR/2400.

Test preparation

How to use instrument-specific information

The *Instrument-specific information* table displays requirements that may vary between instruments. To use this table, select an instrument then read across to find the corresponding information required to perform this test

Table 310 Instrument-specific information

Instrument	Light shield
DR 6000	—
DR 5000	—
DR 3900	LZV849
DR 3800, DR 2800	LZV646

Before starting the test:

DR 3900, DR 3800, DR 2800 and DR 2700: Install the light shield in Cell Compartment #2 before performing this test.
Some of the chemicals and apparatus used in this procedure may be hazardous to the health and safety of the user if inappropriately handled or accidentally misused. Please read all warnings and associated MSDS sheets.
Run one blank with each set of samples. Run all tests (the samples and the blank) with the same lot of vials. The lot number appears on the container label. See *Blanks for colorimetric determination*.
Spilled reagent will affect test accuracy and is hazardous to skin and other materials. Be prepared to wash spills with running water.
Wear appropriate eye protection and clothing for adequate user protection. If contact occurs, flush the affected area with running water. Review and follow reagent MSDS safety instructions carefully.
Store unused (light sensitive) vials in a closed box.
If high chloride samples are being tested, refer to the Alternate reagents section.

APPENDIX 9

METHOD 5030B

PURGE-AND-TRAP FOR AQUEOUS SAMPLES

1.0 SCOPE AND APPLICATION

1.1 This method describes a purge-and-trap procedure for the analysis of volatile organic compounds (VOCs) in aqueous samples and water miscible liquid samples. It also describes the analysis of high concentration soil and waste sample extracts prepared in Method 5035. The gas chromatographic determinative steps are found in Methods 8015 and 8021. The method is also applicable to GC/MS Method 8260.

1.2 Method 5030 can be used for most volatile organic compounds that have boiling points below 200°C and are insoluble or slightly soluble in water. Volatile water-soluble compounds can be included in this analytical technique; however, quantitation limits (by GC or GC/MS) are approximately ten times higher because of poor purging efficiency. The method is also limited to compounds that elute as sharp peaks from a GC column packed with graphitized carbon lightly coated with a carbowax or a coated capillary column. Such compounds include low molecular weight halogenated hydrocarbons, aromatics, ketones, nitriles, acetates, acrylates, ethers, and sulfides.

1.3 Method 5030, in conjunction with Method 8015 (GC/FID), may be used for the analysis of the aliphatic hydrocarbon fraction in the light ends of total petroleum hydrocarbons, e.g., gasoline. For the aromatic fraction (BTEX), use Method 5030 and Method 8021 (GC/PID). A total determinative analysis of gasoline fractions may be obtained using Methods 8021 GC/PID) in series with Method 8015.

1.4 Water samples can be analyzed directly for volatile organic compounds by purge-and-trap extraction and gas chromatography. Higher concentrations of these analytes in water can be determined by direct injection of the sample into the chromatographic system or by dilution of the sample prior to the purge-and-trap process.

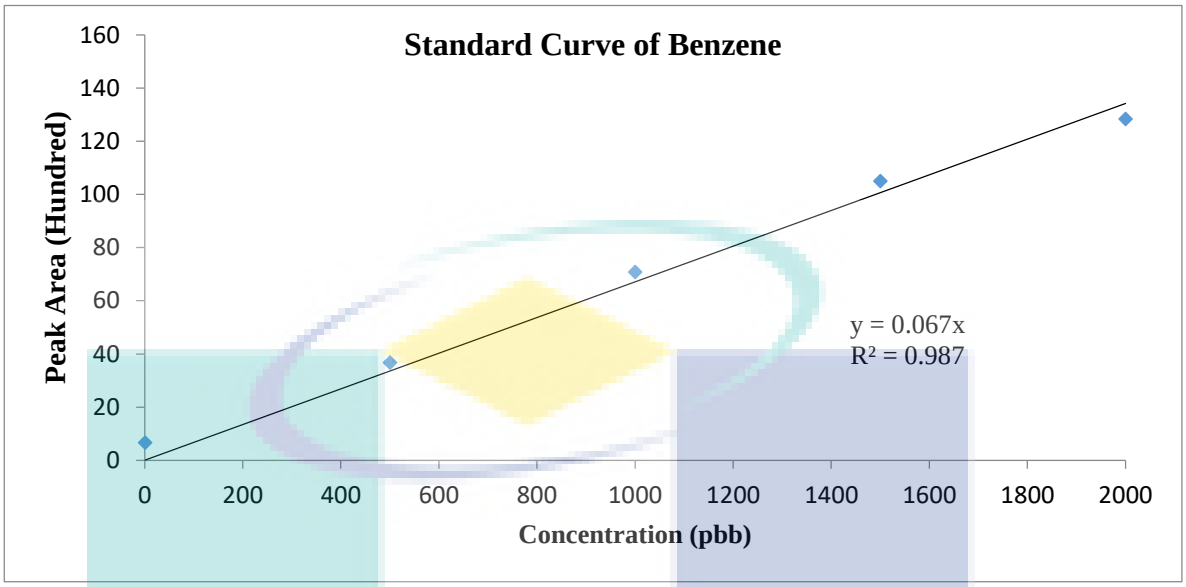
1.5 This method is restricted to use by or under the supervision of trained analysts. Each analyst must demonstrate the ability to generate acceptable results with this method.

2.0 SUMMARY OF METHOD

2.1 Aqueous Samples: An inert gas is bubbled through a portion of the aqueous sample at ambient temperature, and the volatile components are efficiently transferred from the aqueous phase to the vapor phase. The vapor is swept through a sorbent column where the volatile components are adsorbed. After purging is completed, the sorbent column is heated and backflushed with inert gas to desorb the components onto a gas chromatographic column.

2.2 High Concentration Extracts from Method 5035: An aliquot of the extract prepared in Method 5035 is combined with organic free reagent water in the purging chamber. It is then analyzed by purge-and-trap GC or GC/MS following the normal aqueous method.

APPENDIX 10


Standard Curve of Benzene
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Concentration (pbb)
y = 0.067x
R² = 0.987
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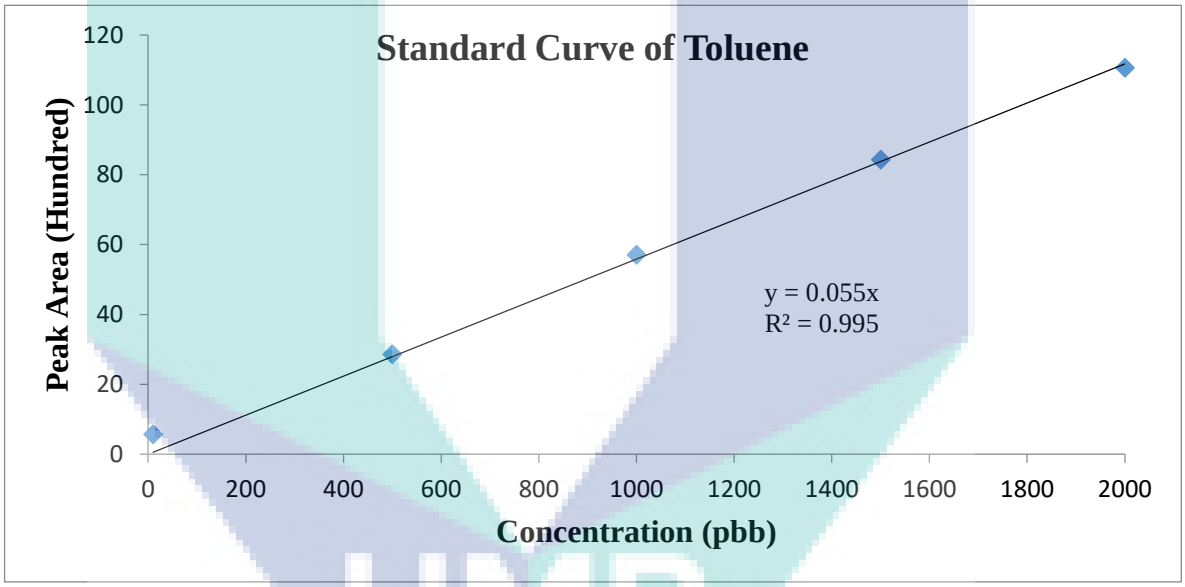
40
60
80
100
120
140
160
0
200
400
600
800
1000
1200
1400
1600
1800
2000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Concentration (pbb)
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R² = 0.995
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0
200
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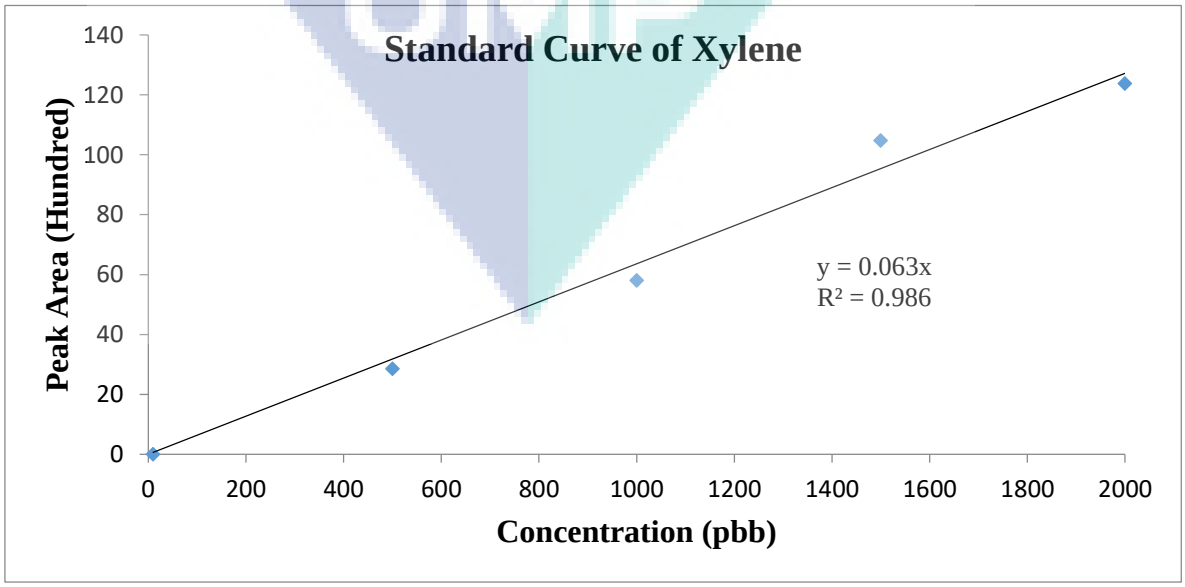
600
800
1000
1200
1400
1600
1800
2000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Peak Area (Hundred)
Concentration (pbb)
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R² = 0.986
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20
40
60
80
100
120
140
0
200
400
600
800
1000
1200
1400
1600
1800
2000

APPENDIX 11

P. putida growth analysis data

Time (Min)	OD	OD/Odo	Log OD/ODo	n	g	k
0	0.135	1	0	0	#DIV/0!	#DIV/0!
1	0.135	1	0	0	#DIV/0!	#DIV/0!
2	0.2755	2.040740741	0.309787835	1.0222999	1.9563732	0.3542269
3	0.652	4.82962963	0.683913827	2.2569156	1.3292477	0.5213475
4	1.364	10.1037037	1.004480602	3.314786	1.2067144	0.5742867
5	1.5245	11.29259259	1.05279366	3.4742191	1.4391723	0.4815268
6	1.6955	12.55925926	1.098964026	3.6265813	1.6544507	0.4188701
7	1.791	13.26666667	1.122761817	3.705114	1.8892806	0.3668063
8	1.827	13.53333333	1.131404779	3.7336358	2.1426836	0.3234262
9	1.863	13.8	1.139879086	3.761601	2.3925983	0.2896433
10	1.889	13.99259259	1.145898189	3.781464	2.6444784	0.2620555
12	1.863	13.8	1.139879086	3.761601	3.190131	0.2172325
14	1.889	13.99259259	1.145898189	3.781464	3.7022698	0.1871825
16	1.903	14.0962963	1.14910502	3.7920466	4.2193575	0.164243
18	1.911	14.15555556	1.150926919	3.7980588	4.7392631	0.1462253
20	1.917	14.2	1.152288344	3.8025515	5.2596263	0.1317584
22	1.975	14.62962963	1.165233331	3.84527	5.7213148	0.121126
24	2.01	14.88888889	1.172862289	3.8704456	6.2008365	0.1117591
26	2.012	14.9037037	1.173294208	3.8718709	6.7151	0.1032003
28	2.014	14.91851852	1.173725698	3.8732948	7.2289876	0.095864
30	2.015	14.92592593	1.173941282	3.8740062	7.7439215	0.0894895
32	2.017	14.94074074	1.17437213	3.875428	8.2571524	0.0839272
34	2.019	14.95555556	1.17480255	3.8768484	8.7700102	0.0790193
36	2.021	14.97037037	1.175232545	3.8782674	9.2824956	0.0746566
38	2.02	14.96296296	1.175017601	3.8775581	9.7999821	0.0707144
40	2.021	14.97037037	1.175232545	3.8782674	10.313884	0.067191
42	2.025	15	1.176091259	3.8811012	10.821671	0.0640382
44	2.031	15.04444444	1.177376155	3.8853413	11.324616	0.0611941
46	2.0316	15.04888889	1.177504436	3.8857646	11.838082	0.0585399
48	2.0316	15.04888889	1.177504436	3.8857646	12.352781	0.0561007
50	2.032	15.05185185	1.177589935	3.8860468	12.866546	0.0538606
52	2.0323	15.05407407	1.177654049	3.8862584	13.380479	0.0517919
54	2.025	15	1.176091259	3.8811012	13.913577	0.0498075
56	2.0189	14.95481481	1.17478104	3.8767774	14.444987	0.0479751
60	1.897	14.05185185	1.147733562	3.7875208	15.841497	0.0437459

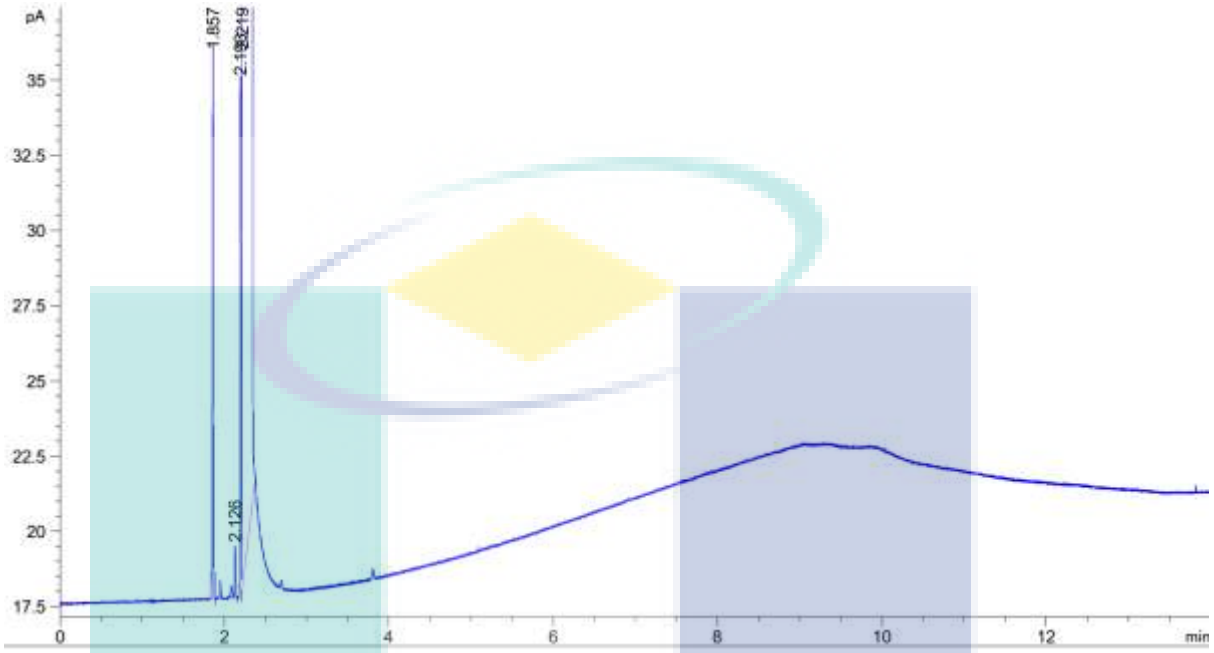
APPENDIX 12

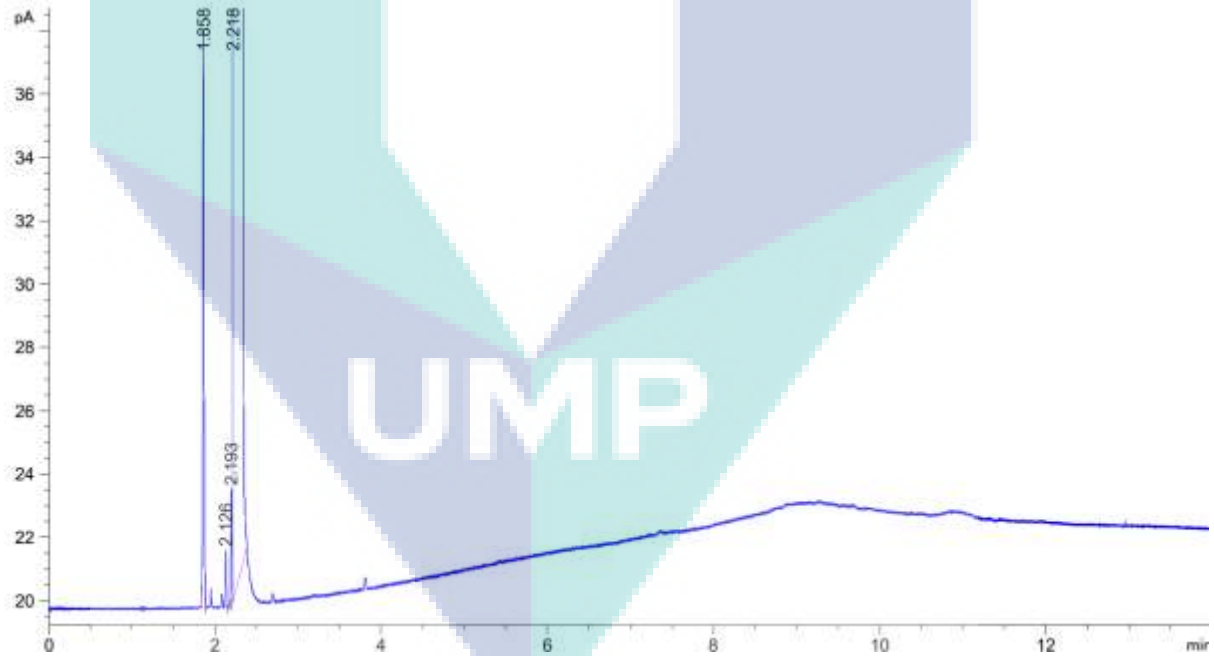
Cell Dry Weight Data Analysis

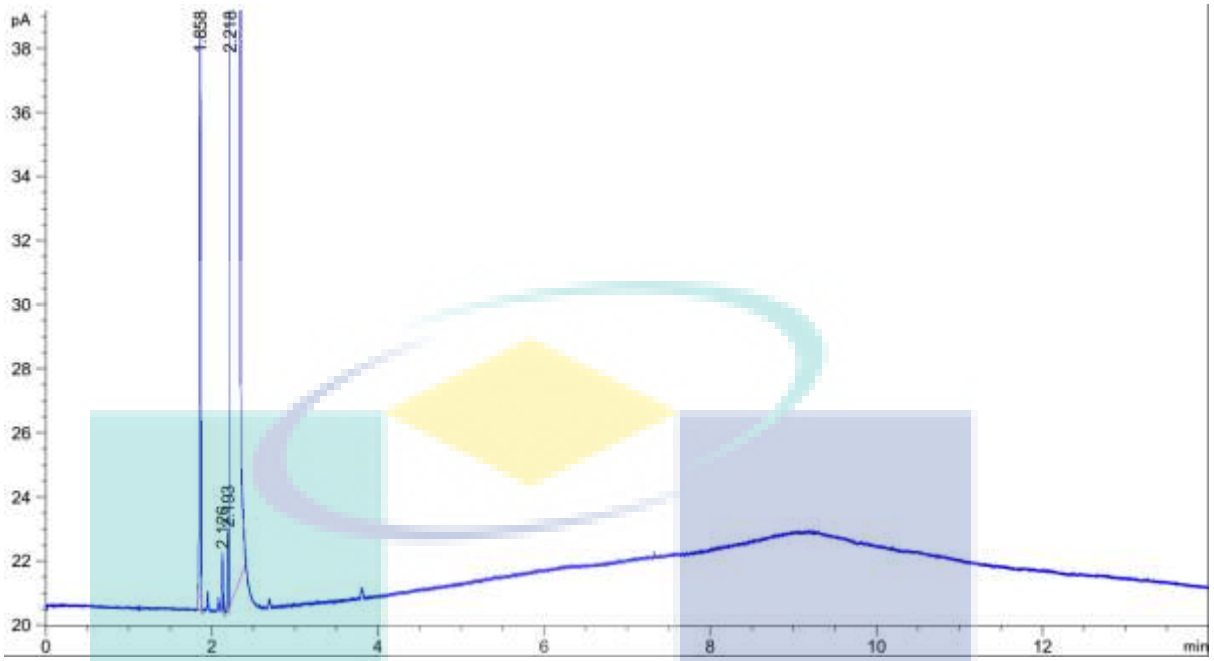
No	Time	*Pseudomonas Putida*				
		Weight Before	Weight after	net	OD	g/L
0	0			0	0	0
1	1	1.003	1.0032	0.0002	0.135	0.133333
2	2	1.0114	1.0117	0.0003	0.2755	0.2
3	3	1.0184	1.0188	0.0004	0.652	0.266667
4	4	1.0131	1.01401	0.00091	1.364	0.606667
5	5	1.0216	1.02256	0.00096	1.5245	0.64
6	6	1.0158	1.0168	0.001	1.6955	0.666667
7	9	1.0109	1.0121	0.0012	1.791	0.8
8	12	1.0025	1.0038	0.0013	1.827	0.866667
9	15	1.0097	1.011	0.0013	1.863	0.866667
10	18	1.0142	1.0156	0.0014	1.889	0.933333
11	21	1.0123	1.0134	0.0011	1.903	0.733333
12	24	1.003	1.0049	0.0019	1.911	1.266667
13	27	1.0131	1.0149	0.0018	1.917	1.2
14	30	1.0218	1.0236	0.0018	1.975	1.2
15	33	1.0187	1.0208	0.0021	2.01	1.4
16	36	1.0136	1.0159	0.0023	2.012	1.533333
17	39	1.0143	1.0166	0.0023	2.014	1.533333
18	42	1.0128	1.0151	0.0023	2.015	1.533333
19	45	1.0214	1.0235	0.0021	2.017	1.4
20	48	1.0149	1.0174	0.0025	2.019	1.666667

APPENDIX 13

GC-FID graph for BTX analysis






pA
38
36
34
32
30
28
26
24
22
20
1.858
2.218
2.129
2.193
0
2
4
6
8
10
12
min

APPENDIX 14

Effect of pH on *P. putida* Growth

Time	pH Value								
	pH 6			pH 7			pH 8		
	Exp 1	exp 2	exp 3	Exp 1	exp 2	exp 3	Exp 1	exp 2	exp 3
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2	0.0674	0.0230	0.0889	0.0417	0.0817	0.2607	0.2822	0.6373	0.1787
4	1.0346	0.9662	1.1291	0.9701	1.1737	1.3810	0.4480	0.7357	0.3145
6	1.9359	1.8592	1.9786	1.8436	2.1363	2.1388	1.4236	1.6138	1.0986
8	2.6036	2.5966	2.6523	2.6391	2.8104	2.8469	2.0120	2.1009	1.8685
10	3.0434	3.0176	3.2151	3.0803	3.3292	3.3927	2.3979	2.4867	2.3347
12	3.5874	3.5534	3.6498	3.5049	3.5817	3.6241	2.8086	2.8892	2.7968
14	3.8149	3.7730	3.8885	3.6387	3.7361	3.7602	3.0979	3.2689	3.0761
18	3.8932	3.8447	3.9886	3.7341	3.8067	3.8086	3.4786	3.5634	3.3598
22	3.9240	3.9236	3.9295	3.8038	3.8397	3.8533	3.7612	3.7708	3.6490
24	3.9291	3.9143	3.9346	3.8465	3.8694	3.8783	3.8296	3.8726	3.7607
28	3.9300	3.9268	3.9409	3.8690	3.8752	3.9030	3.8594	3.8820	3.8267
32	3.9013	3.9295	3.9391	3.8605	3.8703	3.8910	3.8571	3.8515	3.8310
36	3.8688	3.8746	3.8437	3.8501	3.8596	3.8848	3.8561	3.8296	3.8249
40	3.8180	3.7942	3.8387	3.8029	3.7472	3.8086	3.8277	3.8253	3.7662
44	3.8056	3.7900	3.7730	3.6942	3.7447	3.7808	3.7152	3.6495	3.7077
48	3.6953	3.7459	3.6695	3.6493	3.6883	3.7437	3.6619	3.6484	3.6675

PUBLICATIONS

JOURNAL:

1. A. M. Azoddein, F. A. M. Azli, A. B. Bustary, M. H. Sulaiman, A. Z. M. Salehen, M. N. A. Seman, S. Nurdin, M. Sulaiman, Characterization of Enzyme Produced From *Pseudomonas putida* for BTX (Benzene, Toluene & Xylene) Treatment in Petrochemical Industry Wastewater System, *International Journal of Research in Engineering and Science (IJRES),2016.*
2. A. A. M. Azoddein, R. M. Yunus, N. M. Sulaiman, A. B. Bustary, F. A. Azli. Mercury Removal from Petroleum Based Industries Wastewater by P. putida *International Journal of Scientific & Technology Research,* 2015.
3. A. M. Azoddein, R. M. Yunus, N. M. Sulaiman, A. B. Bustary, K. Sabar, Mercury Removal Using *Pseudomonas putida* (ATTC 49128): Effect of Acclimatization Time, Speed and Temperature of Incubator Shaker, *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological,* 2015.
4. A. A. M. Azoddein, R. M. Yunus, N. M. Sulaiman, A. B. Bustary, N. A. M. Basir, Mercury Removal from Actual Petroleum Based Industries Wastewater by *P. putida* in Membrane Bioreactor, *New Directions in Multidisciplinary Research & Practice* (NDMRP), 2015.

AWARD:

1. Silver Medal, Bioremoval of Benzene, Toluene and Xylene (BTX) from Petrochemical Industry Wastewater Using *Pseudomonas putida* ATC 49128. *Creation, Innovation, Technology and Research Exposition* (CITREX), 2015, Universiti Malaysia Pahang (UMP), Gambang, Pahang