CHARACTERIZATION OF ENZYME PRODUCED FROM *PSEUDOMONAS PUTIDA* FOR BTX (BENZENE, TOLUENE & XYLENE) TREATMENT IN PETROCHEMICAL INDUSTRY WASTEWATER SYSTEM

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ABSTRACT

One of the big challenges in petrochemical industries is waste management. Currently, huge money was spending on the disposal of the waste. Industries are trying hard to find an alternative method to reduce the cost and improve the effectiveness of current waste management including treatment efficiency. Most of petrochemical wastes are containing benzene, toluene and xylene (BTX) which are very harmful to environment and living organisms. Common method used to separate the BTX from the waste are by using liquid-liquid extraction and stripping process. One of the alternative to treat BTX is biological treatment method that used the natural capability of microorganisms to degrade to less harmful product is been applied. Some of examples are *Pseudomonas Putida.* (*P. putida*), *Rhizobium*, and *Agrobacterium*. *P. putida* is selected in this study for the biological treatment of BTX in petrochemical wastewater because it can produce an enzyme that has the capability of breakdown the aromatic hydrocarbon to carbon dioxide (CO_2) and water (H_2O). The main objective of this study is to produce and extract the enzymes produce, characterised the enzymes. This study also to investigate the effect of different concentration on the treatment as well as the growth of the bacteria. The enzyme is purified using salt precipitation and analysed using SDS-PAGE technique. UV-Vis is used to study the growth of the bacteria in the culture stock by measuring its optical density. The concentration of BTX was varied to determine the effect of the concentration on the percentage removal and the growth of *P. putida*. Enzymes detected or purified in this study was benzene reductase. Other expected enzymes were not able to be purified or analysed. It was found that in this experimental study, the removal of benzene is at 74% to 80%. The removal of toluene is at 62% to 75%. The removal of xylene is at 23% to 42%. Increasing the concentration of contaminants will reduce the removal capabilities.

ABSTRAK

Salah satu cabaran besar dalam industri petrokimia adalah pengurusan sisa. Pada masaini, jumlah wang yang besar dibelanjakan untuk pelupusan sisa sahaja. Industri berusaha keras untuk mencari kaedah alternatif bagi mengurangkan kos dan meningkatkan keberkesanan pengurusan sisa semasa termasuk kecekapan rawatan. Kebanyakan sisa petrokimia mengandungi benzena, toluena dan xilena (BTX) yang amat berbahaya kepada alam sekitar dan organisma hidup. Kaedah biasa yang digunakan untuk memisahkan BTX daripada bahan buangan adalah dengan menggunakan pengekstrakan cecair-cecair dan proses pelucutan. Salah satu alternatif untuk merawat BTX adalah kaedah rawatan biologi yang mengunapakai keupayaan semulajadi mikroorganisma untuk mencerna bahan cemar kepada produk kurang berbahaya. Beberapa contoh bakteria adalah *Pseudomonas Putida* (*P. putida*), rhizobium dan agrobacterium. *P. putida* dipilih dalam kajian ini untuk rawatan biologi BTX dalam sisa air petrokimia kerana ia boleh menghasilkan enzim yang mempunyai keupayaan mencerna hidrokarbon aromatik kepada karbon dioksida (CO_2) dan air (H_2O). Objektif utama kajian ini adalah untuk menghasilkan dan menulenkan enzim, serta mencirikan enzim tersebut. Kajian ini juga untuk mengkaji kesan kepekatan yang berbeza pada rawatan dan juga pertumbuhan bakteria. Enzim ditulenkan menggunakan mendapan garam dan dianalisis menggunakan teknik SDS-PAGE. UV-Vis digunakan untuk mengkaji pertumbuhan bakteria dalam stok kultur dengan mengukur ketumpatan optik. Kepekatan BTX dimanipulasi untuk menentukan kesan kepekatan ke atas peratusan penyingkiran dan pertumbuhan *P. putida*. Enzim dikesan atau ditulenkan dalam kajian ini adalah reductase benzena. Enzim lain yan idjangka dapat dihasilkan tidak dapat ditulenkan atau dianalisis. Telah didapati bahawa dalam kajian ini, penyingkiran benzena adalah pada 74% - 80%. Penyingkiran toluene adalah pada 62% -75%. Penyingkiran xylene adalah pada 23% - 42%. Meningkatkan kepekatan bahan cemar akan mengurangkan keupayaan penyingkiran..

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LIST OF ABBREVIATIONS

DOE	Department of Environment
BTX	Benzene, toluene, xylene
OSHA	Occupational Safety and Health Administration
IPCS	International Programme on Chemical Safety
ATSDR	Agency for Toxic Substances and Disease Registry
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
DHHS	Department of Health and Human Services
NPI	National Provider Identifier
DNA	Deoxyribonucleic Acid
FID	Flame ionization detector

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Due to high demand of chemical product in the global market, many chemical plants are built to ensure the consistent production. Most of the wastes are not properly treated due to various reasons including no effective method and capital cost involve. Lack of enforcement and concern in handling industrial waste to abide the rule requirement stated by the Department of Environment (DOE) may affect the environment and living organisms including human safety. Thus finding effective method in term of removal efficiency and cost is important to ensure sustainable development for us and future generation.

Nowadays, benzene, toluene, xylene (BTX) are among the frequent hazardous chemical presence in petrochemical wastewater. Based on their toxicity and carcinogenic potential that will harm human, animal and environment a proper treatment is needed to reduce the hazard of BTX before discharging petrochemical wastewater into the water body. The standards of discharge of petrochemical wastewater need to follows the guide and regulation that by Department of Environment (DOE) and Occupational, Safety and Health Administration (OSHA).

Petroleum industries' production stages such as extraction and refining commonly produce large amount of waste that need to be discarded. Even though petrochemical industries' wastewater contains high concentration of biodegradable component it is also consist of toxic material or activity inhibitor to biological unit (Davarnejad, et al., 2014). Hydrocarbon such as BTX in petrochemical wastewater is highly toxic and considered as risky to the environment due to their high solubility (Courseuil., et al., 1998). BTX occurs

naturally in crude oil and still exist in gasoline despite undergoing various processes. It may be found on the surface of water due to leakage from the storage tanks or pipelines, improper disposal practices, accidental spilling, and leaching from landfills area (Heider, et al., 1998).

Benzene has been widely used as multipurpose organic solvent but it has been discouraged due to its high toxicity. High exposure of benzene may cause cancer because of its carcinogenic effect. Benzene also can cause serious poisoning depend on the amount and length of exposure as well as the age of individual exposed. Signs of exposure of benzene include dizziness, headaches, rapid and irregular heartbeat and may lead to death (IPCS, 2004). Toluene is a major constituent of gasoline in which usually used as paint thinner and solvent based cleaning agent. Exposed to low or moderate level of toluene can give symptoms such as sleepiness and headaches. High exposure may cause death (Agency for Toxic Substance and Disease Registry (ASTDR), 2000). As for xylene, it is commonly found in pesticides, synthetic fibres and in manufacturing of plastics. Short-term exposure to mixed xylenes in humans can results in irritation of the eyes, nose, and throat, gastrointestinal effects, and neurological effects. Long-term exposure can cause headache, dizziness, fatigue, tremors, and incoordination of respiratory, cardiovascular, and kidney (Agency for Toxic Substances and Disease Registry (ATSDR), 2007).

The waste water treatment process is the process of removing harmful materials or contaminants form the water or effluent discharge form certain process in order to make it safe to discharge the water to the environment. There are many methods that can be used to remove BTX in the petrochemical waste. Usually physical treatment is needed as the primary treatment in wastewater to remove most of the solid materials before it proceeds to the biological and chemical treatment. Physical treatment may apply electrical attraction, gravity and Van der Waal forces as well as using physical barrier (Woodard, 2001).

Chemical treatment utilised chemical reaction or reactions to improve the water quality. A commonly used chemical process in many industrial wastewater treatment operations is neutralization. Neutralization consists of the addition of acid or base to adjust the pH levels back to neutral (Shokrollahzadeh, et al., 2008). Biological treatment

methods use microorganisms, mostly bacteria, in the biochemical decomposition of wastewater to stable the end products (Gupta, & Mittal, 2016). Some natural microorganisms have the capabilities of degrading BTX. They are extensively presence in the activated sludge of wastewater treatment plants as well as contaminated soil (Mazzeo, et al., 2010). Examples of microorganisms that can be used in biological treatment are *Pseudomonas putida*, *Rhizobium*, and *Agrobacterium*.

Pseudomonas putida (*P. putida)* is described as Gram-negative rod-shaped and has one or more polar flagella that help them to move faster (Givskov, et al., 1994). It is fluorescent, aerobic, non-spore forming and oxidizes positive bacteria. *P. putida* mostly found in moist environments, such as soil and water habitat in the present of oxygen since it is an aerobic microorganism. The optimum growth condition was usually at room temperature or in temperature range of 25-30^{o}C. *P. putida* have the ability to grow by breaking down many dangerous pollutants and aromatic hydrocarbons such as toluene, benzene, and xylene (Hannaford, & Kuek, 1999).

The enzyme from *P. putida* is needed to be purified. There are several method involved in enzyme purification such as affinity chromatography, immunoaffinity chromatography, chromatographic methods and salt precipitation. Affinity chromatography is separation of the enzyme based on specific biological interaction such as its substrate recognition (Maxwell, et al., 2014). Other method is salt precipitation. Salt precipitation is one of useful and ideal method to be used in a laboratory scale. This is because it increases the ionic strength of the solution cause a reduction in the repulsive effect between identical molecules of a protein. It's also reducing the forces holding the solvation shell around the protein molecules. Hence, when these forces are sufficiently reduced, the protein will precipitate. Ammonium sulphate is effective in salt precipitation because of its high solubility, lack of toxicity to most enzymes and its stabilizing effect on some enzymes (Chaplin, 2014).

1.2 Objective

The objectives of this research to produce and purify the enzyme from *P. putida* produced in the treatment of BTX from petrochemical wastewater.

1.3 Scopes of this research

The following are the scopes of this research:

i. To study *P. putida* growth curve
ii. To determine the cell dry weight of *P. putida*.
iii. To extract enzyme produced from *P. putida* used in treatment of BTX
iv. To purify enzyme produced from *P. Putida* used in treatment of BTX
v. To investigate the effect of concentration of BTX on the production of enzyme

1.4 Main contribution of this work

This work will provide an alternative way to treat BTX in petrochemical wastewater. As conventional method, it can be expensive or unable to remove low concentration of BTX, retrofitting biological method will greatly safe the treatment cost as well ensuring the effluent discharge abiding local rule and regulation. This method will greatly reduce petrochemical waste effect on environment and more importantly, it will not produce additional or secondary pollutant. Side contribution of this work is to give more exposure to the industries regarding the dangerousness of BTX in petrochemical industries and its need to be treated.

1.5 Organisation of this thesis

The general outline of this report is as follows:

Chapter 1 provides a background of the study. This chapter will give a brief explanation about petrochemical wastewater treatment containing BTX that are harmful to environment and living organism. *P. putida*, is one example of organism use in biological treatment for the waste water treatment by using its enzyme. Next, the objectives and scope of this research are also mentioned here. Chapter 1 ends with the outline of this report.

Chapter 2 is literature review. First part explaining about aromatic hydrocarbon studied in this research; benzene, toluene and xylene. Second part provides description about

method available for wastewater treatment followed by part three explaining about bacteria and its growth curve. Fourth, fifth and sixth parts introduce *P. putida*, enzymes and enzymes purification respectively. This chapter ends with the summary of the literature review.

Chapter 3 is explaining materials and methodology. This chapter gives information about list of chemicals and equipment used in this study. Methodology involve growth of *P. putida*, preparation of bacteria, stock solution of BTX, cell free extract, enzyme purification, treatment, and analysis equipment. This chapter ends with the summary of materials and methods used.

Chapter 4 is for results and discussion. The subchapters include *P. putida* growth, cell dry weight, effect of concentration, growth curve at different concentration, and enzymes purification.

Chapter 5 is the conclusion and recommendation of this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter is presenting the review of literature for this study. Literature is part of preliminary study as well as theoretical study to understand the relevant information before proceeding with experimental study. The first subchapter is a review about aromatic hydrocarbon involve in this experiment; benzene, toluene, and xylene. Study on this compound gives understanding regarding the toxicity and why it is dangerous to human and environment as well as to predict possible reaction and mechanism in biological treatment. Literature also includes the general information regarding petrochemical wastewater treatment. Bacteria can be sensitive to certain chemicals thus knowing the possible contaminants can be useful to discuss the possible outcome of the treatment. Several types of existing treatment also briefly explain such as physical and chemical treatment as well as current application of biological treatment. Next subchapter is literature on bacteria and its growth curve, *P. putida*, enzyme and enzymes purification. Understanding biological reaction is paramount important for this research as it studies directly involving the utilization of bacteria in treatment of BTX.

2.2 Aromatic Hydrocarbon

"Aromatic" term first introduced by August Wilhelm von Hofmann; a German chemist in 1855 to several compound. It is commonly applied to compounds contain phenyl radical in their molecular structure. In organic chemistry, the structures of some rings of atoms are unexpectedly stable. Aromaticity is a chemical property in which a conjugated ring of unsaturated bonds, lone pairs, or empty orbital exhibit stabilization

stronger than would be expected by the stabilization of conjugation alone. It can also be considered a manifestation of cyclic delocalization and of resonance (mohamed khir, 2012).

2.2.1 Benzene

Benzene is the simplest aromatic compound comprising of six carbon atoms. The structure of Benzene was a cyclic planer six carbon unit structure with alternate single and double bonds. Each carbon atom is attached to one another by covalent bond. Benzene is very stable compound because it can exist in different forms based on the positioning of double bond. This also become one of the reasons why benzene does not undergo addition reactions readily but it undergoes substitution reactions. **Figure 2.1** below shows the structure of benzene ring (Clark, 2013)

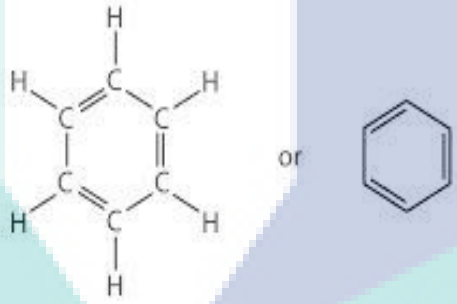

Figure 2.1: Structure of benzene ring (Clark, 2013)

Benzene is a colourless liquid with a sweet odour. It evaporates into the air very quickly and dissolves in water. In some industries, benzene is used to produce other chemicals such as plastics, resins and nylon. It is highly flammable and is formed from both natural processes and human activities. According to Gunatilaka (2003), benzene has properties of highly volatile, colourless, highly flammable liquid and sweet odour. This property makes benzene as a dangerous compound and unsafe for exposure on human, biodiversity or ecosystem. **Table 2.1** shows list of the physical properties of benzene (Budavari, et al., 2001).

Table 2.1: Physical Properties of Benzene (Budavari, et al., 2001)

Properties	Value
Appearance	Colourless
Molecular formula	C_6H_6
Molecular Weight	78.11 g/mol
Melting point, ^oC	5.533 °C
Boiling point, ^oC	80.10 °C
Density at 25 °C	0.879 g/cm^3
Solubility in water at 25 ^oC, g/100g water	0.180
Solubility of water in benzene at 25 ^oC, g/100g benzene	0.05
Viscosity, at 20 ^oC, Cp	0.6468

Exposure to human may cause adverse health effect such as cancer, aplastic anaemia, eye and skin irritant. Chronic exposure can damage the immune system, change blood levels of antibody and loss of white blood cell (Gunatilaka, 2003). Based on the report from (World Health Organization (WHO), 2003). The major source of benzene are industrial processes such as processing of petroleum products, coking of coal and heating oil. Indoor or residential air may contain benzene emitted form building materials as well as smoke from cigarette.

Benzene is identified as one of the causes of cancer in human by the Department of Health and Human Services. Long-term exposure to high levels of benzene in the air can cause leukaemia; cancer of the blood-forming organs. Among the signs that the person is exposed to benzene are dizziness, headaches, rapid and irregular heartbeat and also death (IPCS, 2004).

2.2.2 Toluene

Toluene is a mono-substituted benzene derivative, consisting of a methyl group (CH_3) attached to a phenyl group. **Figure 2.2** shows the molecular structure of toluene where CH_3 molecules attached to the ring to form toluene compound.

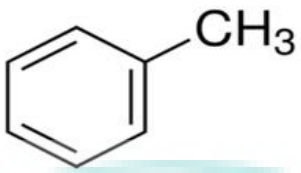

Figure 2.2: Structure of Toluene (Lee, et al., 1994)

Toluene is a colourless, flammable, non-corrosive liquid with a benzene-like odour. It is insoluble in water and soluble in acetone, absolute alcohol, ether, chloroform, benzene, petroleum ether, glacial acetic acid, and carbon disulphide. **Table 2.2** shows the physical properties of toluene (World Health Organization (WHO), 2000).

Table 2.2: Physical properties of toluene (World Health Organization (WHO), 2000)

Properties	Value
Appearance	Colorless liquid
Molecular formula	C_7H_8 or $C_6H_5CH_3$
Molecular Weight	92.13 g/mol
Boiling Point	110.7 ^oC
Melting Point	-95 ^oC
Flash Point	40 ^oF (closed cup)
Vapor Density	3.2 (air = 1)
Vapor Pressure at 30 ^oC	36.7 mm Hg
Density at 25° C	0.8623 g/mL

Toluene is major constituent of gasoline and usually used for paint thinner and solvent based cleaning agent. Exposed to low or moderate levels of toluene can give symptoms of sleepiness, headaches, and death if it is occurred at higher levels of exposure (Agency for Toxic Substance and Disease Registry (ASTDR), 2000).

2.2.3 Xylene

Xylene is an aromatic hydrocarbon consisting of a benzene/phenyl ring with two methyl groups at various substituted positions; ortho-, metha- and para. **Figure 2.3** shows three structure of xylene with different position of methyl group attach to the phenyl group.

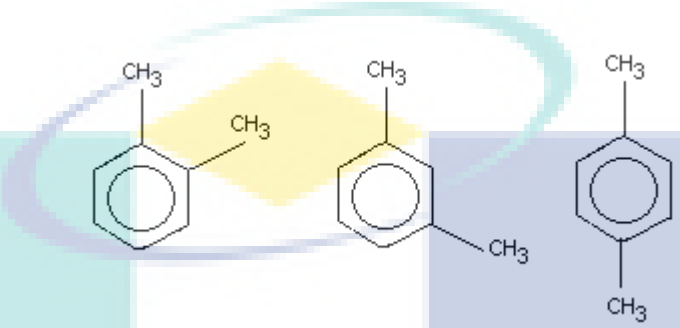

Figure 2.3: Structure of Xylene (Lee, et al., 1994)

Xylene is slightly greasy and colourless liquid. It is also has very strong sweet odour. It is practically insoluble in water and only soluble in non-polar solvents such as aromatic hydrocarbons (Kandyala, et al., 2010). **Table 2.3** shows the physical properties of xylene

Table 2.3: Physical Properties of Xylene (Kandyala, et al., 2010)

Properties	**Value**			
	p-xylene	**m-xylene**	**o-xylene**	**Ethyl benzene**
Molecular weight g/mol	106.167	106.167	106.167	106.167
Density, 25°C, g/cm^3	0.8610	0.8642	0.8802	0.8671
Boiling point °C	138.37	139.12	144.41	136.19
Refractive index @ 25°C	1.4958	1.4971	1.5054	1.4959
Viscosity	0.34 cP, 30°C	0.62 cP, 20°C	0.812 cP, 20°C	

Xylene is commonly found in pesticides, synthetic fibres and in manufacturing of plastics. Short-term exposure to mixed xylenes in humans can results in irritation of the eyes, nose, and throat, gastrointestinal effects, eye irritation, and neurological effects. For long-term exposure to humans can cause headache, dizziness, fatigue, tremors, and

incoordination of respiratory, cardiovascular, and kidney (Agency for Toxic Substances and Disease Registry (ATSDR), 2007).

2.3 Petrochemical Wastewater Treatment

The wastewater treatment process is a process of removing harmful contaminants from the waste water prior to it being discharged to the environment. Wastewater treatments involve applying known technology that helps to improve or upgrade the quality of a wastewater. These wastewater treatment processes are commonly carried out on continuous flowing wastewaters or a series of periodic treatment processes in which treatment is carried out on parcels or "batches" of wastewaters because it involve large volumes of wastewater. Wastewater treatment can be categorised into physical treatment, chemical treatment and biological treatment.

The monoaromatic hydrocarbon; benzene, toluene and xylene (BTX) is a common environmental problem commonly produced from oil and gas industry. This industry will spend immense amount of money just the BTX from their effluent. The U.S. Environmental Protection Agency has established maximum permissible level in water at 0.5 ppb, 0.1 ppm, and 10 ppm for benzene, toluene, and xylene, respectively (Agency for Toxic Substance and Disease Registry (ASTDR), 2000). This stringent legislation and impact of BTX on health and environment makes effective removal technique as an important development in a country with vigorous petrochemical industries.

Biological treatment is a modern alternative in wastewater treatment with a low operational cost. It is basically the same biological activities that would occur naturally in the receiving water except that it is practiced under controlled condition. Most biological treatment use bacteria as primary microorganisms and degradation of organic compound. The treatment work when waste is utilised as food by microorganism during their growth process to produce protoplasm for new cell (Azoddein, et al., 2015). The study choose *P. putida* the biological treatment of BTX in petrochemical wastewater due to its capability to breakdown the aromatic hydrocarbon to carbon dioxide (CO_2) and water (H_2O) since *P. putida* is aerobic metabolisms. Besides, *P. putida* is non-pathogenic which is not causing any dieses while the reaction occur (Kudela, et al., 2010).

An enzyme produced from *P. putida* reacts with BTX as a catalyst that will break down the bond between carbon-carbon atoms. Benzene dioxygenase enzyme mediated the meta-cleavage of benzene-like metabolism from wide range of aromatic compounds. The specificity of an enzyme for its substrate is generally a function of the enzyme's "active site" or binding site. The improvement of the techniques in the enzyme production, purification and characterization will affect the reaction with BTX. As a consequence, enzymes are highly specific and are able to discriminate between slightly different substrate molecules. In addition, enzymes exhibit optimal catalytic activity over a narrow range of temperature, ionic strength and pH. They have no ability to adapt to changing conditions or substrate sources. Their level of activity is a function of these conditions. If they are not in optimal conditions, their activity decreases or stops. Thus, the characterization of enzyme is needed to identify the maximum condition of an enzyme react with BTX.

2.3.1 Physical Wastewater Treatment

Usually, physical treatment is use as a primary treatment in wastewater to remove solid materials before it proceeds to the biological and chemical treatment. The physical treatment is removal of substances by using natural occurring forces. For examples, electrical attraction, gravity and Van der Waal forces as well as using physical barrier (Woodard, 2001). Filtration is one of example for physical wastewater treatment. Here wastewater is passed through a filter medium to separate solids. An example would be the use of sand filters to further remove entrained solids from the wastewater.

2.3.2 Chemical Wastewater Treatment

Chemical treatment consists of using some chemical reaction or reactions to improve the water quality. There are several distinct chemical unit processes, including chemical coagulation, chemical precipitation, chemical oxidation and advanced oxidation, ion exchange, and chemical neutralization and stabilization, which can be

applied. An example of chemical process commonly used in many industrial wastewater treatment operations is coagulation. Coagulation produce end product in form of insoluble that serves to remove substances from wastewater through a chemical reaction. Most commonly used chemicals in coagulation is polyvalent metals and typical coagulants are lime and other certain compound such as ferric chloride (Henze, & Comeau, 2008).

2.3.3 Biological Wastewater Treatment

Biological treatment methods use microorganisms, mostly bacteria, in the biochemical decomposition of contaminants into stable end products. Generally, biological treatment methods can be divided into aerobic and anaerobic methods, based on availability of dissolved oxygen. Aerobic method takes place in the presence of oxygen and by microorganisms which convert organic matter into carbon dioxide, water and biomass. While for anaerobic treatment processes, it takes place in the absence of oxygen by the microorganisms to assimilate organic impurities (Gupta, & Mittal, 2016). Some natural microorganisms have the capabilities of degrading BTX. They are extensively present in the activated sludge of wastewater treatment plants as well as contaminated soil (Mazzeo, et al., 2010). Example of bacteria is called *Pseudomonas putida, Rhizobium*, and *Agrobacterium.*

2.4 Bacteria

Bacteria are single celled microbes. The cell structure is simpler than that of other organisms as there is no nucleus or membrane bound organelles. Instead, their control centre or their genetic information contained a single loop of DNA. Some bacteria have an extra circle of genetic material called a plasmid. The plasmid often contains genes that give the bacterium some advantage over other bacteria. Bacteria are classified into 5 groups according to their basic shapes which are spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes) as showsn in **Figure 2.4**. They can exist as single cells, in pairs, chains or clusters (Snyder, et al., 2013).

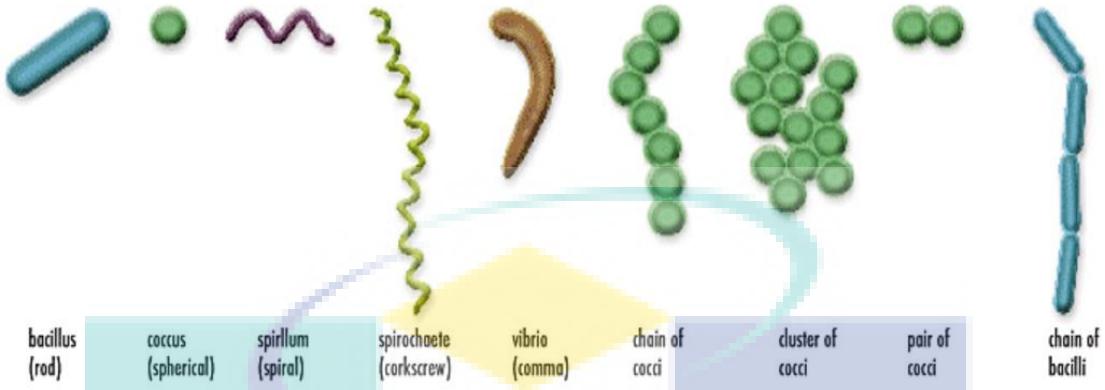

Figure 2.4: Classification of Bacteria (Snyder, et al., 2013)

Bacteria reproduce by binary fission. In this process the bacterium, which is a single cell, divides into two identical daughter cells. Binary fission begins when the DNA of the bacterium divides into two (replicates). The bacterial cell then elongates and splits into two daughter cells each with identical DNA to the parent cell. Each daughter cell is a clone of the parent cell (Alexander, et al., 2015). The stage of bacteria production is shown in **Figure 2.5** below.

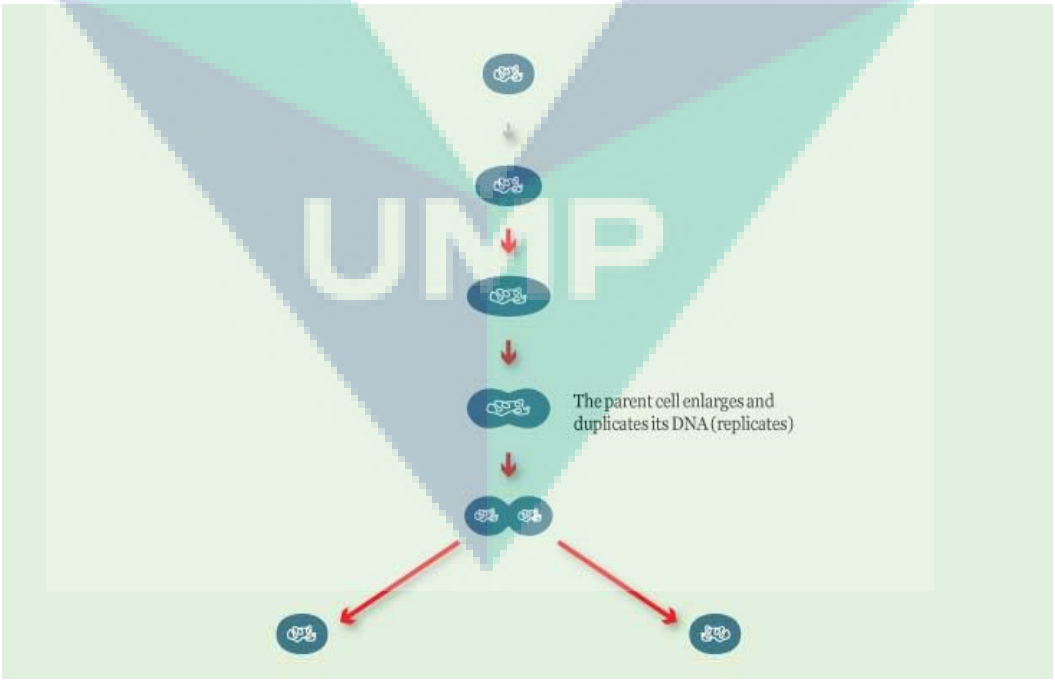

Figure 2.5: Binary fission of Bacteria (Alexander, et al., 2015)

The schematic growth curve in **Figure 2.6** below shows batch culture conditions that growth with fixed volume and amount of nutrient (Maier, 2010).

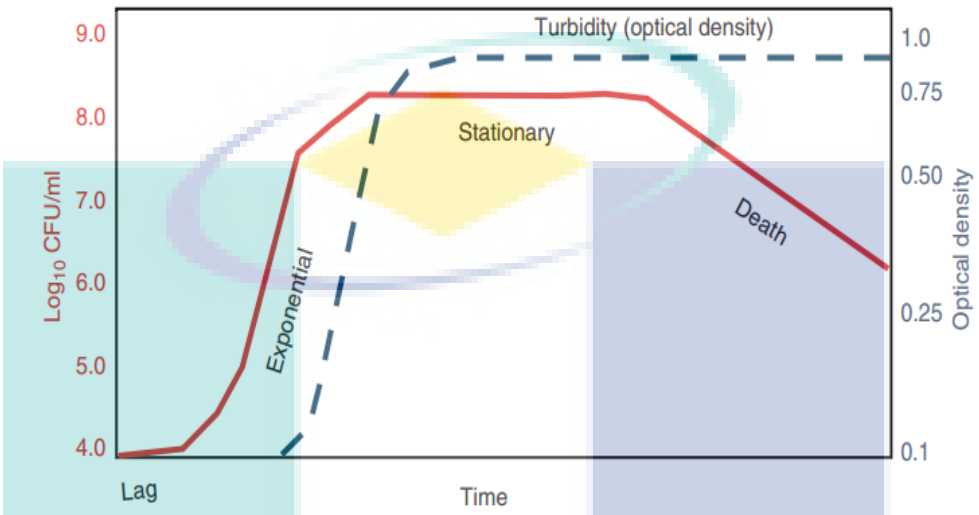

Figure 2.6: A typical growth curve for a bacterial population (Maier, 2010)

The first stage is termed as lag phase, which at this point the microorganisms are introduced into fresh medium, and take time to adjust to the new environment. At this phase the cellular metabolism is increased, where the cells increase in size but does not able to not able to replicate resulting in slightly or no increase in cell mass (Maier, 2010)

Second phase is log phase where the bacterial population are continuing to growth. The organisms continue DNA replication by binary fission at constant rate due to increase in their metabolic activity and reaches the maximum growth rate at this phase (Maier, 2010).

The third phase is stationary phase. As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilized (Maier, 2010).

The last phase is the death phase, at which the bacterium completely lose its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions and the

death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells (Maier, 2010).

2.5 Pseudomonas putida

Pseudomonas putida is described as Gram-negative rod-shaped and has one or more polar flagella that help them to move faster (Givskov, et al., 1994; Azoddein, et al., 2015). It is fluorescent, aerobic, non-spore forming, oxidize positive bacteria. They are mostly found in moist environments, such as soil and water habitat in the present of oxygen. The optimum growth condition is usually at room temperature or in range of 25-30^{o}C. Every bacterium has certain strains. This bacteria is very unique because it has the most genes involved in breaking down aromatic or aliphatic hydrocarbons which are hazardous chemicals caused by burning fuel, coal, tobacco, and other organic matter (Hannaford, & Kuek, 1999). **Figure 2.7** shows the microscope image of *P. putida*.

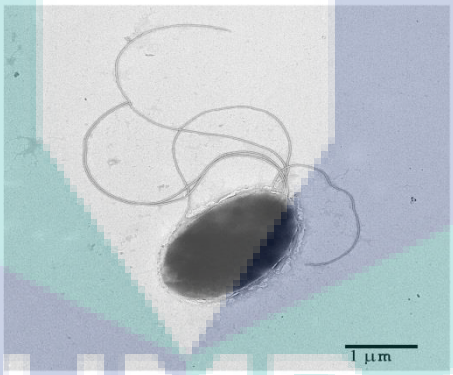

Figure 2.7: Structure of *P. putida* under Microscope (Hannaford, & Kuek, 1999)

2.6 Enzyme

Enzyme is a biocatalyst that widely uses in industries and medicine. The microbial enzymes are also more active and stable than plant and animal enzymes. In addition, the microorganisms represent an alternative source of enzymes because they can be cultured in large quantities in a short time by fermentation (Abada, 2014). Otenio, M. H. *et al* (2005) stated that there are several possible enzymes that can be produce from *P. putida* in the treatment of BTX such as benzene dioxygenase, toluene oxygenase and xylene oxidase, and catechol 2, 3-dioxygenase as shown in **Figure 2.8**. Dotted lines represent the

TOD Pathway and solid lines, the TOL pathway. **Table 2.4** shows the characteristic of benzene dioxygenase (Otenio, et al., 2005).

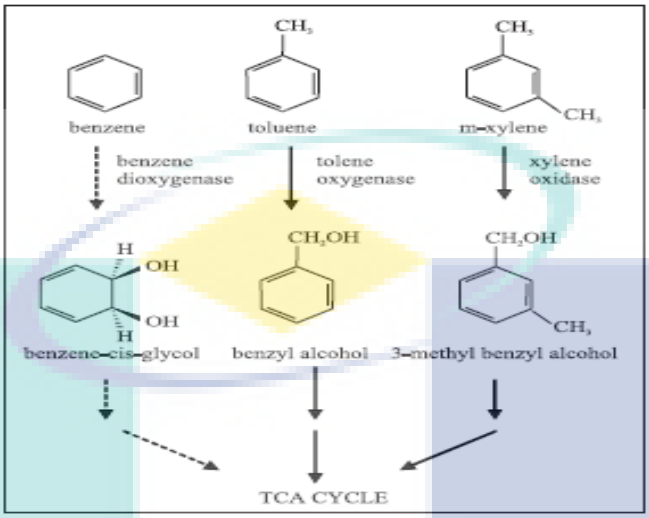

Figure 2.8: Metabolic Pathway for the Degradation of the BTX Mixture by *P. putida* (Otenio, et al., 2005)

Table 2.4: Benzene Dioxygenase (Chang, 2006)

EC Number	**1.14.12.3**
Systematic Name	Benzene,NADH:oxygen oxidereductase (1 2-hydroxylating)
Recommended Name	Benzene. 1,2-dioxygenase
Synonyms	Benzene dioxygenase Benzene hydroxylase Oxygenase, benzene 1,2-di- More (formerly registered together with toluene dioxygenase EC 1 .14.12.11)
Reaction And Specificity	CATALYZED REACTION Benzene + NADH + H^+ + O_2 = cis –cyclohexa-3, 5-diene-1, 2-diol + NAD^+ (i.e cis-benzene glycol)
Reaction Type	Redox reaction
Natural Substrates	Benzene + NADH + O_2 (reaction in benzene catabolism, the three-component enzyme system (1--7) contains a monomeric (2) or dimeric (7) reductase which is an iron-sulfur flavoprotein (FAD), a monomeric ferredoxin and an tetrameric (7) iron-

	sulfur oxygenase [2]. all three components necessary for activity [5], the enzyme is closely related to toluene dioxygenase EC 1.14,12.11 [2], induced by benzene (1, 5, 6) [1-7]
Substrate spectrum	1. Benzene + NADH + O_2 (best substrate [2]) [1-7] 2. More (the enzyme metabolizes lower alkyl substituted (CH_3 > C_2H_5 >> C_3H_7) and halogenated (F > CI > Br) benzenes [7]. substrates of cell-free extract are toluene (50% as good as benzene) and naphthalene (2.2% as good as benzene) [2], substrates that lead to oxygen consumption: toluene, fluoro-, chloro-, brorno- and iodobenzene [5]. No substrates are benzoic acid [5, 7]: benzaldehyde, phenol [5, 7]. acetophenone, p-xylene, naphthalene [5], nitrobenzene [7]) [1-7]
Product spectrum	cis-Cydohexa-3,5-diene-1,2-diol + NAD^+ (i.e. cis-benzene glycol)
Inhibitor(s) Cofactor(s)/prostethic group(s)/activating agents	FAD (reductase (A_2): flavoprotein) [3-7] NADH (requirement) [1, 2,7]; 2.6-Dichlorophenolindophenol (activation, is reduced in vitro only in the presence of NADH and reductase [5] [3, 5]; NADPH (10% or less as effective as NADH) [5]; Cytochrome c (activation, is reduced in vitro only in the presence of reductase (A_2), ferredoxin (B) and NADH) [5]; More (none of the three enzyme protein compounds shows catalytic activity in the absence of the other. NADH and Fe^{2+}
Metal compounds/salts	Fe^{2+} (requirement, multi-component system consists of two non-here iron-sulfur proteins [5], a terminal oxygenase (A1) with two 2Fe-2S clusters (Mossbauer spectroscopy [3])[2-4, 7] per molecule of MW 215300 [1] and an electron-transport mediating ferredoxin-like protein (B) with presumably 2Fe-2S [1, 2] per molecule of MW 12300 [1], two additional Fe^{2+} -ions are required [4,7] for 10-fold increase of activity, enzyme possibly bears an additional Fe^{2+} -binding site [4], additional Fe^{2+} required for activity [5], of 5 cysteine-residues detected 4 are required to coordinate FeS-cluster [6], midpoint redox

potential at pH 7.0: —155 mV (ferredoxin), —112 mV (oxygenase) [7]) [1-7]

2.7 Enzyme Purification

Enzyme purification is a process involving purification of protein depends on its unique structure, which determines both biological function and properties in solution. This enzyme can be used industrially to catalyse just one reaction with no undesired by-products. Some industrially available enzyme has been prepared by genetic modification. It is normally necessary to undertake at least a partial purification to obtain a useful product.

There are several method involved in enzyme purification such as affinity chromatography, ion exchange chromatography and salt precipitation. Affinity chromatography is a separation of the enzyme based on specific biological interaction such as its substrate recognition (Maxwell, et al., 2014). Other method is salt precipitation. Salt precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out. The solubility of proteins varies according to the ionic strength of the solution, thus increases in the ionic strength of the solution cause a reduction in the repulsive effect between identical molecules of a protein. It also reduces the forces holding the solvation shell around the protein molecules. Hence, when these forces are sufficiently reduced, the protein will be precipitated. Ammonium sulphate is effective because of its high solubility, lack of toxicity to most enzymes and its stabilizing effect on some enzymes (Chaplin, 2014). For example from *P. putida,* can produce enzymes such as benzene reductase enzyme and cathacol 2,3-dioxygenase enzyme.

2.8 Summary

Petrochemical wastewater can bring harm to environment and living organisms. It is very vital to ensure the wastewater is treated properly. Chemicals wastes contain in it such as BTX are very dangerous because even only short term exposure to those compounds it can gave severe sickness. Benzene is colourless liquid that can dissolve in

water and evaporate into the air very quickly. Due to long-term exposure of benzene, it can cause leukaemia and cancer of the blood-forming organs. Toluene is also a colourless liquid but insoluble in water. By exposing toluene to the body it can give several symptoms such as headaches, sleepiness or even death for high level of exposure. As for xylene, its chemical properties are colourless liquid and insoluble in water. For long-term exposure of humans it can cause headache, dizziness, fatigue, tremors, and incoordination; respiratory, cardiovascular, and kidney effects. This petrochemical wastewater must undergo a treatment before been discharge. There are three type of wastewater treatment which is physical treatment, chemical treatment and biological treatment. Therefore by using enzyme produce from *P. putida*, it can help remove or reduce the amount BTX in the wastewater through biological treatment. From *P. putida* in can produce variable of enzyme, for example benzene dioxygenase, toluene oxygenase and xylene oxidase, and catechol 2, 3-dioxygenase. Furthermore, this enzyme will undergoes purification process to obtain useful product.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter presents all the chemicals and procedure of the experiments carried out in order to achieve the objectives of this work. The purpose of this experiment is to extract pure enzyme produced and to investigate the effect of concentration of BTX on the production of enzyme.

3.2 Materials

Benzene, toluene, xylene have 99% purity. Phosphate buffer solutions use were disodium hydrogen phosphate, sodium dihydrogenphosphate and sodium chloride. Lysis buffer used was tris base, 15% glycerol. Nutrient agar and broth was used as the bacteria growth medium.

3.3 Methodology

This chapter elaborates the procedures that used in the experiment of BTX treatment from petrochemical wastewater using *P. putida.* The methodology of this experiment consists of preparation culture of *P. putida*, *P. putida* growth curve and cell dry weight, preparation of BTX stock solution, extraction and dilution procedure of sample and also analysis process by UV-Vis Spectrophotometer and Gas Chromatography (GC).

3.3.1 General Process Procedure

Figure 3.1 shows the general process of BTX treatment from petrochemical wastewater using *P. putida* started from the beginning until the end of the process. Before starting any procedure, each of glassware use in this experiment should be sterilized properly in autoclave to avoid contamination. Contamination will affect the accuracy of the result obtained from the experiment. When dealing with microorganism, the process must be carried out in laminar flow hood as a safety precaution.

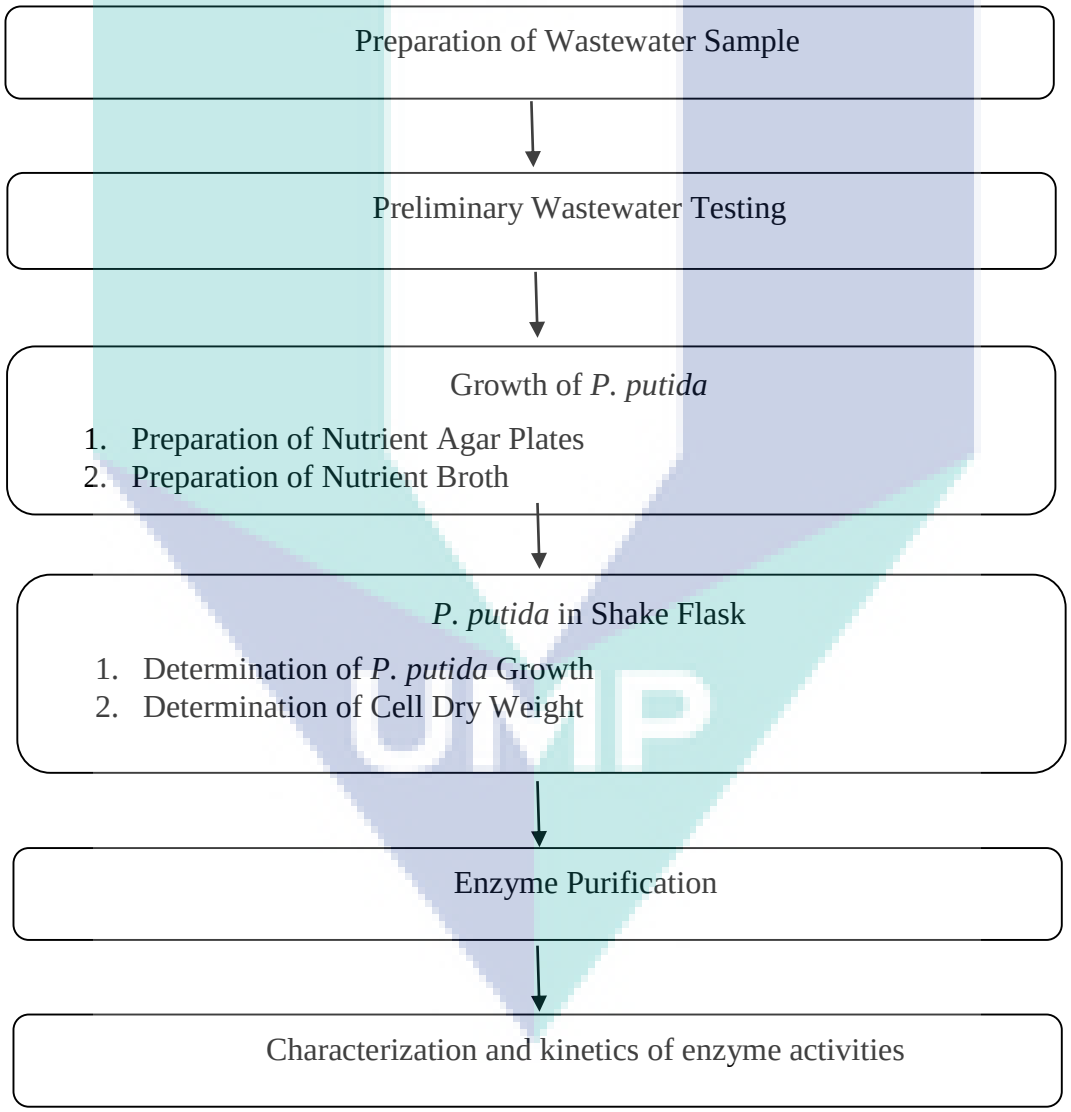

Figure 3.1: General process of BTX Treatment

3.3.2 Preparation of nutrient agar plates

24 g of nutrient agar powder was weighted and mixed with 1000mL of distillate water inside 1L of sample bottle. All the apparatus used in this experiment are sterilised to avoid contamination. After that, the agar medium was prepared by transferring the nutrient agar inside laminar flow chamber to avoid contamination and then was let to cool for a few minutes as shown in **Figure 3.2. Figure 3.3** shows preparation of *P. putida* by inoculum it into petri dish that contain agar medium inside a laminar air flow chamber by streaking method. Then, nutrient agars with inoculum of *P. putida* were put in incubator for 24 hours. (Azoddein, et al., 2015)

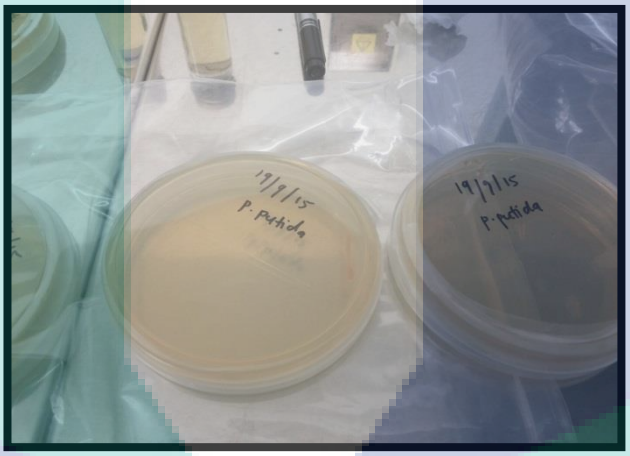

Figure 3.2: Nutrient agars were cooled for a few minutes inside laminar flow

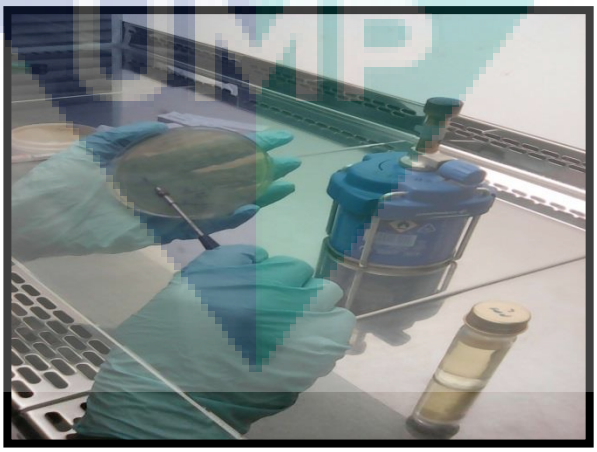

Figure 3.3: Inoculum of *P. putida* into petri dish by streaking method

3.3.3 Preparation of Nutrient Broth

Nutrient broths were prepared by weighted nutrient food for about 8g and mixed with 1000mL of distilled water inside 1L sample bottle. After 24 hours, three loops of *P. putida* colonies from nutrient agar were transferred into the sample bottle contained 20 ml of nutrient broth solution using inoculating loops. It was then placed in incubator oven at 37°C for 24 hours. **Figure 3.4 (a)** shows the appearance of bacteria solution before placing inside the incubator oven. The appearance turns into cloudy after incubation as shown in **Figure 3.4 (b)**. The nutrient food is stored inside incubator oven at temperature 37^{o}C in 24 hour to allow it to grow to form colonies.

(a) **(b)**

Figure 3.4: (a) *P. putida* colonies before 24 hours (b) *P. putida* colonies after 24 hours turn to cloudy

3.3.4 Preparation of Bacteria Growth and Cell Dry Weight

20ml of bacteria colony was added into conical flask containing 180ml of nutrient broth. Then the conical flask was placed in incubator shaker at 37^{o}C at 180 rpm. An amount of sample will be taken at certain hours and placed in the cuvette that will immediately analyse using UV-VIS with wave length 600nm. The procedure of cell dry weight is carried out simultaneously with bacteria growth. 1.5ml of sample was transferred in the microbiological centrifuge tube. The 1.5ml sample in tube was

centrifuged at 12000 rpm for 20 minutes before placed in the oven at 70^{o}C for the sample to be dry overnight. Weights of tube with samples were recorded before and after the sample were dried (mohamed khir, 2012).

3.3.5 Preparation of Stock Solution (BTX)

15 ppm of benzene stock solution was prepared by dilute 67mL of benzene with 1000mL of distilled water. The stock solution is stored inside freezer to avoid contamination. Therefore, the same step is repeated for other hydrocarbon (mohamed khir, 2012)

3.3.6 Preparation of Cell Free Extract

Harvested cell were suspended in Phosphate buffer solution (PBS) (NA_2HPO_4,$NA_2H_2PO_4$,NaCl,H_2O), at pH 7.3 and then centrifuged at 12000 × g for 10 minutes at 4^{o}C. Next, the cells were re-suspended with the same buffer and washed three times with PBS buffer. **Figure 3.5 (a)** and **(b)**, show appearance of sample before and after centrifuged (McMahon, et al., 2007).

(a) **(b)**

Figure 3.5: (a) Sample before centrifuge (b) Sample after centrifuge

3.3.7 Enzyme Purification

After that, the pallet was re-suspended with lysis buffer (Tris base, glycerol) and maintain at 0^{o}C for sonification at a peak amplitude (10 microns) for 30 second. The

unbroken cells and cell wall materials were removed by centrifugation at 12000 × g for 10 minutes at 4°C to remove cell debris and the supernatant was decanted and kept at 4°C (McMahon, et al., 2007). Further enzyme purification was conducted on at 4°C using SDS-PAGE for 1 hour (T. H. T. Abdul Hamid, 2011) as shown in **Figure 3.6** below.

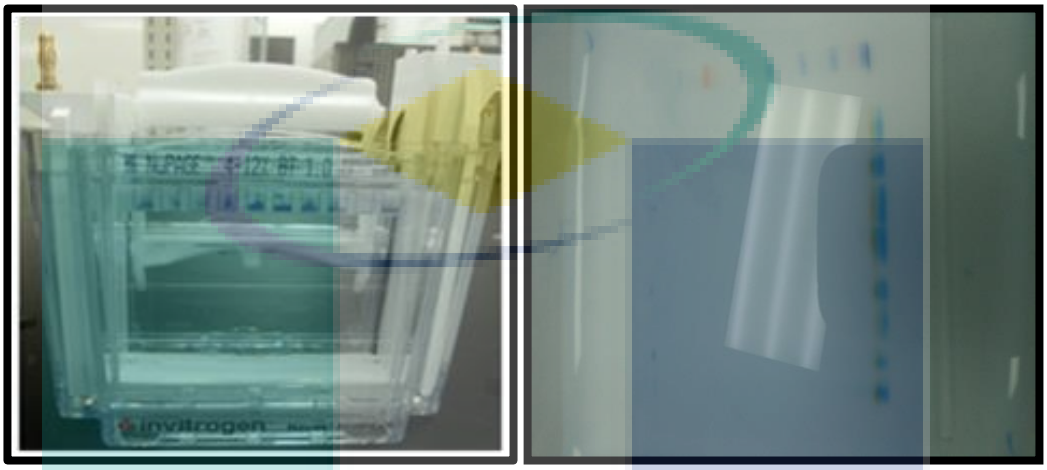

Figure 3.6: SDS-PAGE

After the SDS-PAGE was completed, the gel was stained using blue staining of SDS-PAGE. The gel was placed in orbital shaker for 1 hour at suitable speed, to ensure the gel will not damage. Next, the gel was de-stained by pouring off stain and de-staining with distilled water. Distilled water was replaced for several times. Staining and de-staining of the gel is shown in **Figure 3.7** below

(a) **(b)**

Figure 3.7: (a) Staining of SDS PAGE Gel (b) De-staining of SDS PAGE Gel

3.3.8 BTX Treatment

The procedure of BTX treatment using ratio bacteria to solvent solution effect is performed by added each stock solution of BTX in conical flask with ratio 1:1:1 to obtain 1000ml sample solution. Then the sample solution will be added with bacteria solution with variable bacteria ratio. Then, gas chromograpy (FID) is used to identify the removal BTX. At each concentration of BTX, SDS-PAGE was done, to identify whether the enzyme can be produce or not.

3.3.9 Dilution of Benzene

99% purity of benzene is diluteed, step by step to have an accurate concentration before been diluted with nutrient broth as shown in **Table 3.1**.

1,000,000 ppm → 100,000 ppm → 10, 000 ppm → 1,000 ppm

$$M_1V_1 = M_2V_2$$

Table 3.1: Dilution of benzene using nutrient broth

Concentration (ppm)	Volume required from 1000 ppm of benzene to dilute with nutrient broth (V_1)
150	(1000ppm)×V_1 = (150ppm)×(180mL) v_1= 27 mL
200	(1000ppm)×V1 = (200ppm)×(180mL) V_1= 36 mL
250	(1000ppm)×V_1 = (250ppm)×(180mL) V_1= 45 mL

3.3.10 Dilution of Toluene

99% purity of toluene is diluted, step by step to have an accurate concentration before been diluted with nutrient broth as shown in **Table 3.2**

1,000,000 ppm → 100,000 ppm → 10, 000 ppm → 1,000 ppm

$$M_1V_1 = M_2V_2$$

Table 3.2: Dilution of toluene using nutrient broth

Concentration (ppm)	Volume required from 1000 ppm of toluene to dilute with nutrient broth (V_1)
20	(1000ppm)×V_1 = (20ppm)×(180mL) V_1= 3.6 mL
25	(1000ppm)×V_1 = (25ppm)×(180mL) V_1= 4.5 mL
30	(1000ppm)×V_1 = (30ppm)×(180mL) V_1= 5.4 mL

3.3.11 Dilution of Xylene

99% purity of xylene is diluted, step by step to have an accurate concentration before been diluted with nutrient broth as shown in **Table 3.3**

1,000,000 ppm → 100,000 ppm → 10, 000 ppm → 1,000 ppm

$M_1V_1 = M_2V_2$

Table 3.3: Dilution of xylene using nutrient broth

Concentration (ppm)	Volume required from 1000 ppm of xylene to dilute with nutrient broth (V_1)
1	(1000ppm)×V_1 = (1ppm)×(180mL) V_1= 0.18mL
3	(1000ppm)×V_1 = (3ppm)×(180mL) V_1= 0.54mL
5	(1000ppm)×V_1 = (5ppm)×(180mL) V_1= 0.9mL

3.3.12 Sample Extraction and Dilution

Before going through the extraction procedure, the sample obtained was centrifuged at 10000×g for 10 minutes and filtered using the 0.25 µm of nylon filter to remove the biomass retain in the sample solution. Each sample prepared was extracted with 50ml of dichloromethane (DCM) using separator funnel to separate water from the

BTX solvent. After that the sample obtained was diluted with DCM in 10ml of volumetric flask as shown in **Figure 3.8.** Next, 1.5ml sample prepared in the volumetric flask was transferred to GC vial for the analysis procedure (mohamed khir, 2012).

Figure 3.8: Sample Extraction and Dilution

3.3.13 Gas Chromatography (GC)

The operation of the FID is based on the detection of ions formed during combustion of organic compounds in a hydrogen flame. The generation of these ions is proportional to the concentration of organic species in the sample gas stream. Hydrocarbons generally have molar response factors that are equal to number of carbon atoms in their molecule. To analyse the sample prepared using GC, the specific condition should be apply to obtain the result. The condition applied to analyse the BTX solvent is:

Table 3.4: Specification of GC

Types of Column	**BD-Wax**
Detector	FID
Carrier gas	Helium
Sample inlet	split
Temperature Detector	300^{o}C
Temperature column	250^{o}C

3.4 Summary

The growth curve of *P. putida* was studied using UV-spectrophotometer and also the cell dry weight. Besides that, the process of cell free extract was done through sonification and to remove the unbroken cells and cell wall materials were completed by centrifugation. At each concentration of BTX, SDS-PAGE will be done, to identify whether the enzyme can be produce or not.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter served to tabulate the results obtained from the experimental work and provide discussion from the result. The subchapters include *P. putida* growth, cell dry weight, effect of concentration, growth curve at different concentration, and enzymes purification.

4.2 *Pseudomonas putida* Growth Curve

The growth of *P. putida* obtained is quite similar to the standard growth of bacteria from literature (Widdel, 2007). The microorganism growth curve is generally depends on the condition of the batch culture. *P. putida* is initially suitable with state of incubator shaker of 37^{o}C and shaking velocity of 180rpm. The bacteria growth curve has four stages; lag, exponential growth, stationary and death phase. **Table 4.1** tabulated the percentage absorption of *P. putida* culture. The percentage absorption will represent the growth rate of the bacteria inside the culture. The data is further presented in growth curve as shown in **Figure 4.1** in which the growth is proportional to percentage absorption.

Table 4.1: The percentage absorption of *P. putida* culture

Time (h)	Optical Density (OD)	Time (h)	Optical Density (OD)
0	0	**26**	2.176
1	0.222	**28**	2.227
2	0.53	**30**	2.235
3	0.82	**32**	2.245
4	0.951	**34**	2.246
5	1.488	**36**	2.277
6	1.743	**38**	2.289
8	1.885	**40**	2.33
10	1.909	**44**	2.339
12	1.956	**48**	2.345
14	2.078	**52**	2.299
16	2.175	**56**	2.2865
18	2.186	**60**	1.9835
20	2.192	**64**	1.982
22	2.206	**68**	1.978
24	2.216		

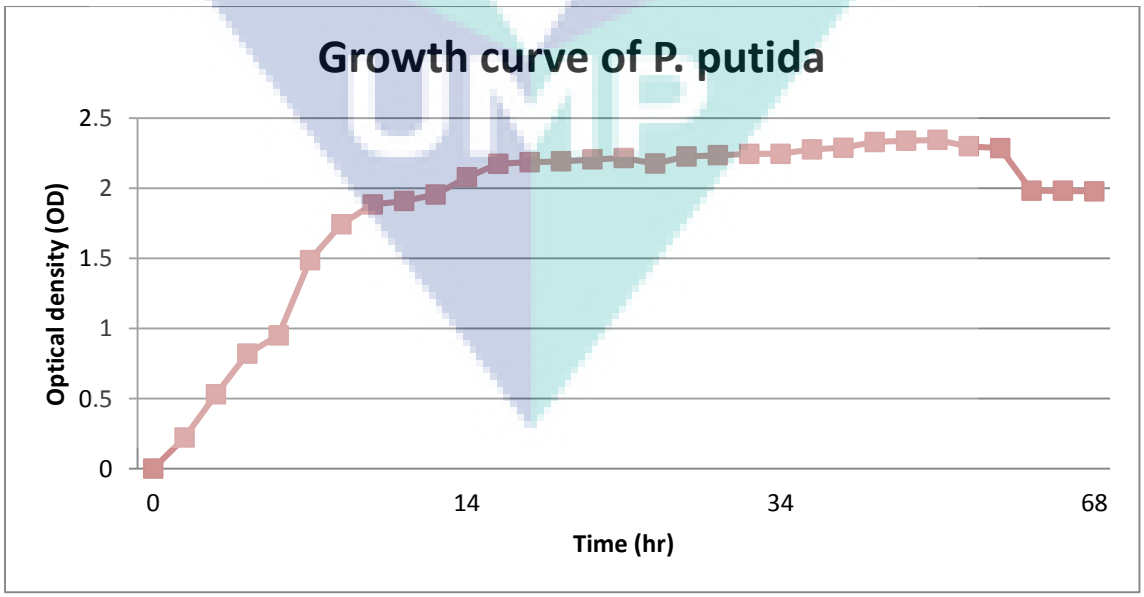

Figure 4.1: *P. putida* growth curve

Lag phase occur in the period of time from 0 to 1st hour. At this point the microorganism was introduced into fresh medium, and take time to adjust to the new environment. This is the time where metabolism is increased and the cell size increase, but does not able to replicate thus slightly or no increase in cell mass (Widdel, 2007). The second phase is exponential phase from time 1st to 7th hours. At this stage the living bacteria is rapidly increased within time. During this phase the bacteria is comfortable with the environment supply, called as optimum condition for the *P. putida* to growth up and create as much as colonial possible by degrading the substrate (Hamzah, et al., 2011).

The third phase is stationary phase that last from 7th hour to 50th hours. As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. The reproduction rate will slow down; the cells division is equal to the number of cell death before finally stops its division completely. The cell number is not increased and thus the growth rate is stabilised (Widdel, 2007)

Lastly, the death phase happened at 52nd to 68th hours. This is the stage where the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions. The death is rapid and at a uniform rate. The number of dead cells exceeds the number of living cells (Widdel, 2007).

4.3 Cell Dry Weight

Table 4.2 tabulates the cell dry weight of *P. putida* taken over time from the culture batch. The value is represented as a graph in **Figure 4.2.** The figure demonstrates that the mass of cell dry weight is proportional to the time of experiment. During exponential and stationary phase the increase of cell dry weight is due to the generation of new living bacteria through reproduction instead of the amount of waste and by product produced in the culture. Meanwhile during the death phase the growth rate decrease but the cell weight still increase due to the culture of bacteria that occur in the batch process .Thus the amount of mass will keep remaining in the batch process until the experimental end.

Table 4.2: The cell dry weight of *P. putida*

Time (h)	Net weight (g)

0	0.0002
1	0.0006
2	0.0008
3	0.0013
4	0.0018
5	0.0022
6	0.0015
8	0.0018
10	0.002
12	0.0024
14	0.0025
16	0.003
18	0.0047
20	0.0023
22	0.0102
24	0.0074
26	0.0056
28	0.0066
30	0.0089

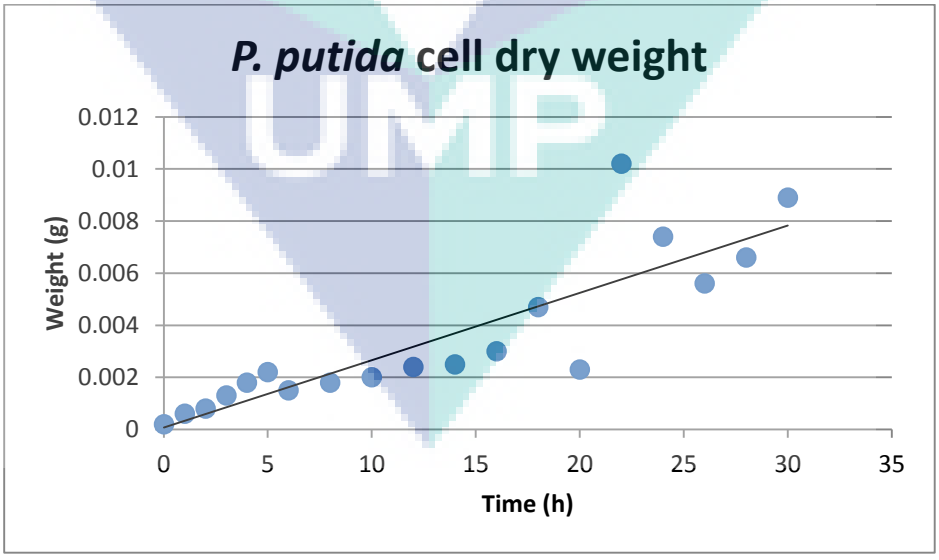

Figure 4.2: *P. putida* Cell Dry Weight

4.4 Effect at different concentrations

For the effect of variable concentration, the shaking speed is 180 rpm and the bacterium to the solvent ratio is 1:9. **Table 4.3** below shows the effect of concentration on the percentage removal.

Table 4.3: Percentage removal at different concentration

Sample	Temperature (ºC)	Hour	Concentration (ppb)	Concentration after treatment	% Removal
Benzene	37	24	1500	298.56	80.096
Benzene	37	24	2000	438.66	78.067
Benzene	37	24	2500	645.55	74.178
Toluene	37	24	0.20	0.0738	75.405
Toluene	37	24	0.25	0.0855	65.785
Toluene	37	24	0.30	0.1137	62.112
Xylene	37	24	0.01	0.0171	42.846
Xylene	37	24	0.03	0.0073	26.544
Xylene	37	24	0.05	0.0380	23.992
BTX	37	24	50		
Benzene	37	24	25	1.4813	94.075
Toluene	37	24	12.5	3.9051	68.759
Xylene	37	24	12.5	7.8866	36.907
BTX	37	24	100		
Benzene	37	24	50	3.9075	92.185
Toluene	37	24	25	8.481	66.076
Xylene	37	24	25	16.626	33.495
BTX	37	24	150		
Benzene	37	24	75	6.057	91.924
Toluene	37	24	37.5	14.046	62.543
Xylene	37	24	37.5	26.9756	28.065

The data show that benzene has the highest percentage removal from at any given concentration. Benzene has percentage removal of more than 70%. Toluene percentage removal is between 60% to 75% while Xylene percentage removal is only between 23% to 42%. Mixing the three main contaminant forming BTX mixture shows the significant reduction however the same pattern occurs in which benzene will have the highest removal percentage compare to toluene and xylene. Increasing the concentration of BTX mixture from 5 ppm to 15 ppm does not give huge percentage removal different yet there is sight reduction of the removal. The degradation of benzene, toluene and xylene suggests in the present of TOL pathway (Otenio, et al., 2005).

P. putida initiates toluene degradation at the methyl group, eventually forming benzoate, which is degraded by the meta-cleavage route. Xylene also undergoes the same oxidative reaction, giving rise to the methyl-benzyl alcohol formation. However, TOD pathway utilizes benzene as substrate (Otenio, et al., 2005).

4.5 *P. putida* at growth curve at difference concentration of BTX

Table 4.4 tabulates the result of *P.putida* growth measured using UV-Vis. The data was represented in **Figure 4.3**. From **Figure 4.3**, growth of *P. putida* at 15 ppm of BTX slightly lower compare to at 5ppm and 10 ppm. Growth at 5ppm and 10ppm does not have any significance different. This growth curve agreed with the previous result in **Table 4.3** in which increasing the concentration of BTX will reduce the percentage removal. Bacteria at high concentration of BTX might have difficulty to growth. The percentage removal is lowest at high concentration because the amount of bacteria is not compatible with the amount of substrate that provided in the solvent solution (Otenio, et al., 2005).

Table 4.4: Effect of BTX concentration on growth of *P. putida*

	Optical Density (OD)		
Time (h)	**5ppm**	**10ppm**	**15ppm**
0	0.116	0.11	0.073
1	0.123	0.114	0.083
2	0.19	0.188	0.122
3	0.234	0.225	0.22
4	0.444	0.4	0.39
5	0.615	0.505	0.476
6	0.756	0.684	0.624
8	0.987	0.943	0.82
10	1.777	1.687	1.54
12	1.987	1.807	1.63
16	2.027	2.011	1.673
20	2.046	2.035	1.725
24	2.114	2.071	1.808

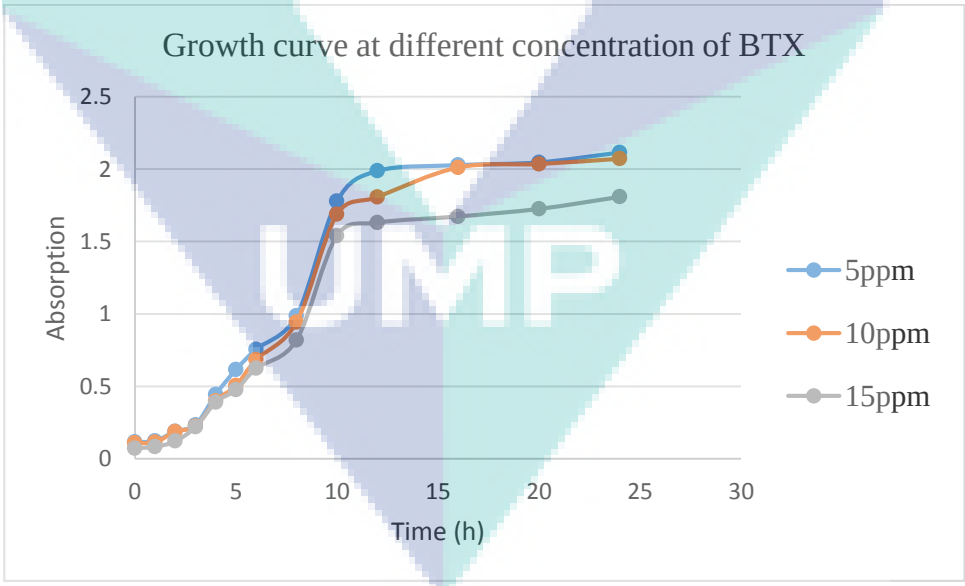

Figure 4.3: *P. putida* growth curve at difference concentration of BTX

4.6 Production and Purification of Enzyme

The purified enzyme were analysed through SDS-PAGE. After staining, the gel showed a protein band, which indicates the molecular mass of the protein.

4.6.1 Benzene, toluene and xylene

All concentration of benzene, toluene, xylene and BTX were analysed using SDS-PAGE to observe the appearance of enzyme produce by *P. putida* from the treatment of the BTX. However, only the SDS-PAGE result from benzene treatment can be read and presented in **Figure 4.4** below. Result for toluene and xylene was not readable.

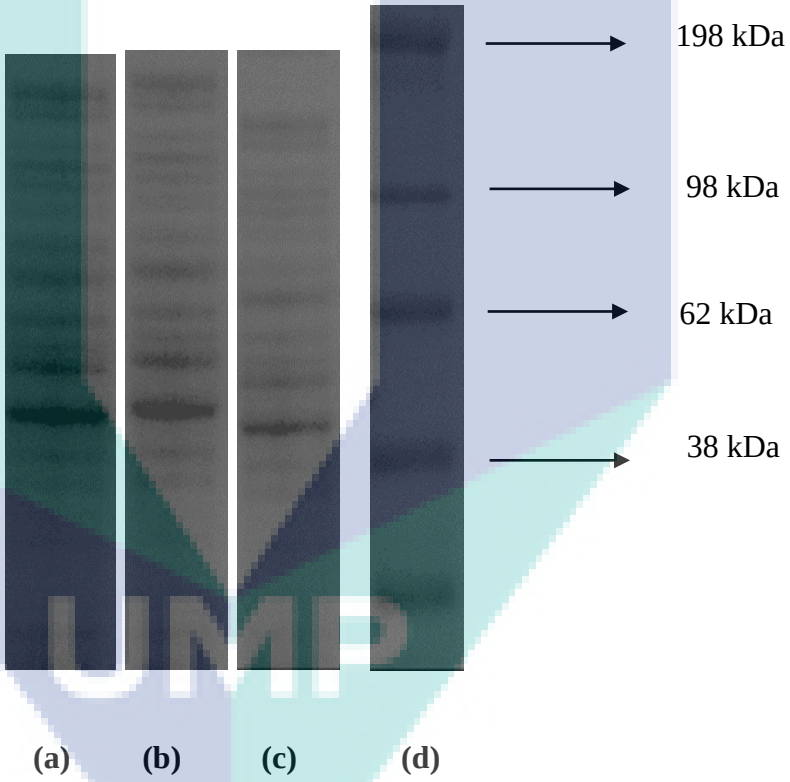

Figure 4.4: SDS-PAGE staining result at different concentration of benzene (a) 150ppm (b) 200 ppm (c) 250 ppm (d) indicator

From **Figure 4.4** above, it is estimated that the molecular mass of the protein is 39kDa which indicate the presence of benzene dioxygenase. The benzene 1,2-dioxygenase from *P. putida* catalysed the dihydroxylation of benzene to (1R,2S)-cis-

cyclohexa-3,5-diene-1,2-diol (benzene cis-dihydrodiol). The enzyme system has three components which are a flavoprotein reductase and a ferredoxin, which transfer electrons from NADH, and a catalytic iron-sulfur protein (ISPBED). ISPBED contains a Rieske-type [2Fe-2S] cluster, a mononuclear iron oxygen activation centre, and a substrate-binding site (Bagnéris, et al., 2005).

4.6.2 BTX

All three concentration of BTX was analysed using SDS-PAGE to see the presence of enzyme. **Figure 4.5** shows the SDS-PAGE result of the enzyme from the treatment of BTX at different concentration. The presence of benzene dioxygenase in this mix of BTX samples determined based on protein molecular value of 39 kDa. This might happened because the ratio of benzene is higher compare to toluene and xylene. *P. putida* react with BTX as a catalyst which will break down the bonding between carbon-carbon bonds such as benzene dioxygenase enzyme mediated the meta-cleavage of benzene-like metabolism from wide range of aromatic compounds. The specificity of an enzyme for its substrate is generally a function of the enzyme's "active site" or binding site. The improvement of the techniques in the enzyme production, purification and characterization will affect the reaction with BTX.

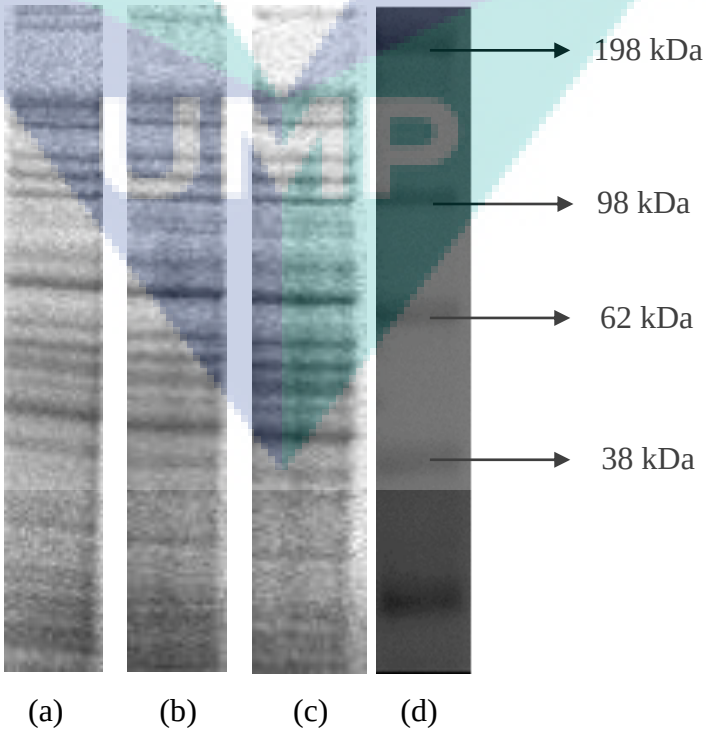

Figure 4.5: SDS-PAGE staining result for different concentration of BTX (a)5ppm (b) 10ppm (c) 15ppm (d) indicator

4.7 Summary

The growth curve obtained almost identical in pattern to the typical bacteria growth phase with lag phase, exponential phase, stationary phase and dead phase. All of the concentrations of BTX were able to be removed by *P. putida* and the enzyme produce is benzene dioxygenase.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The enzyme produce from the treatment of the BTX is benzene dioxygenase. Form the separate treatment of benzene, toluene and xylene, only enzyme produce from benzene treatment can be purified and analysed. The enzyme production from toluene and xylene are uncertain. Expected production of toluene oxygenase and xylene oxidase, and catechol 2, 3-dioxygenase from the treatment is either unsuccessful or unable to be purified. Despite that, there is significance removal of toluene and xylene from the separate treatment of mixing of those three main compounds. Increase in the concentration of benzene, toluene and xylene will reduce the percentage removal. This can be expected as bacteria will have difficulty to survive in higher concentration of toxic substances. The growth curve of *P. putida* obtained in this study is match with the typical pattern of the bacterial growth curve in batch culture. The growth curve of *P. putida* has complete the bacteria life cycle in period 64 hours. The lag phase occur in 0 to 1 hours and continuous with exponential phase at 1 until 7 hours. Then proceed to stationary phase at 7 hours until 50 hours and last phase is death phase that occur at 54 hours until 68 hours. The cell dry weight obtain is proportional to the time of experimental. The removal of benzene is at 74% to 80%. The removal of toluene is at 62% to 75%. The removal of xylene is at 23% to 42%. Increasing the concentration of contaminants will reduce the removal capabilities. It can be relate with the survival of the bacteria inside highly toxic chemical in high concentration and time taken for the degradation.

5.2 Recommendation for future works

This study was conducted using artificial petrochemical waste water based on the literature. For future study, it is recommended to conduct using the real petrochemical wastewater to test the credibility of the bacteria in a real situation. The petrochemical waste water does not only associate with BTX, but involving a lot other toxic compound and contaminants. Survival of bacteria in this kind of environment is paramount important for the application in industries.

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