CHARACTERIZATION OF ENZYME PRODUCED BY *Pseudomonas putida* IN TREATING MERCURY FORM PETROCHEMICAL WASTEWATER

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ABSTRAK

Merkuri adalah salah satu daripada unsur toksik dan sangat berbahaya. Pendedahan merkuri boleh membantutkan kesihatan dan pendedahan merkuri termasuklah dari segi dos, umur orang yang terdedah, tempoh laluan terdedah terdedah dan tempoh terdedah. Di Malaysia, terdapat beberapa tempat proses perlombongan emas yang menggunakan jumlah merkuri yang banyak untuk pengekstrakan bijih. Raksa digunakan akan dilupuskan ke tempat yang terdekat dan menyebabkan air dan tanah tercemar. Merujuk Jabatan Alam Sekitar (JAS) pelupusan merkuri yang betul haruslah mempunya kepekatan kurang daripada 0.05 ppm untuk piawai B dalam sisa air untuk keselamatan manusia dan alam sekitar. Terdapat beberapa teknologi yang digunakan dalam penyingkiran merkuri seperti rawatan kimia, pertukaran ion, penjerapan , serta bioremendasi yang digunakan untuk penyingkiran merkuri dalam kajian ini. Kertas kerja ini membentangkan aktiviti enzim dari *P. putida* yang dihasilkan dalam rawatan merkuri dari air sisa petrokimia pada keadaan optimum. Tiga kepekatan merkuri yang berbeza iaitu 3 ppm, 5 ppm dan 7 ppm telah didedahkan dengan pelbagai suhu dan kelajuan gocangan. .kajian dilakukan pada suhu 33 ˚C, 37 ˚C dan 41 ˚C manakala kelajuan goncangan adalah pada 140 rpm, 180 rpm dan 220 rpm. Pertumbuhan *P. putida* pada keadaan optimum dalam kepekatan merkuri yang berbezadiperhatikan. Kesimpulannya, keadaan optimum kepekatan merkuri dan kelajuan goncangan pada 3 ppm adalah 37 ˚C dan 140 rpm masing-masing, dengan mampu mengurangkan 86,13% kepekatan merkuri. keadaan optimum kepekatan merkuri dan kelajuan goncangan pada 5 ppm adalah 41 ˚C dan 180 rpm masing-masing, dengan mampu mengurangkan 71,47% kepekatan merkuri. Akhir sekali, keadaan optimum kepekatan merkuri dan kelajuan goncangan pada 7 ppm adalah 41 ˚C dan 180 rpm masing-masing, dengan mampu mengurangkan 92.59% kepekatan merkuri. Semasa proses penyingkiran merkuri, reductase merkurik telah dihasilkan pada nilai 56 kDa.

ABSTRACT

Mercury is one of the toxic element and very harmful. The exposure of the mercury will mainly cause health effect and the exposure can be in term of dose, age of person exposed, duration exposed route exposed and duration of exposed. In Malaysia, Mercury is one of the heavy metals of concern, found in wastewaters coming from oil refinery and petrochemical industries. Mercury and mercurial compounds are highly toxic contaminants in the aquatic systems and soils. The proper disposal of wastewater should be implement with the Department of Environment of Malaysia (DOE) which is the concentration of mercury should be 0.05 ppm for standard B in wastewater for the sake of people and environment health. There are many types of mercury removal technology to reduce the concentration of mercury such as chemical treatment, ion exchange, membrane filtration, adsorption as well as bioremediation which used for mercury removal in this study. This paper present the activity of enzyme from *P. putida* is produced in the mercury treatment from petrochemical wastewater at optimum condition. Three different concentration of mercury which were 3 ppm, 5 ppm and 7 ppm were exposed to various temperature and shaker speed. The temperature study was measured at 33 ˚C, 37 ˚C and 41 ˚C while for speed shaker were at 140 rpm, 180 rpm and 220 rpm. *P. putida* growth at optimum condition in mercury different concentration was observed. Conclusion, optimum condition concentration of mercury and shaker speed at 3 ppm is 37 ˚C and 140 rpm respectively, with able to reduce 86.13 % mercury concentration. Optimum condition concentration of mercury and shaker speed at 5 ppm is 41 ˚C and 180 rpm respectively, with able to reduce 71.47 % mercury concentration. Lastly, optimum condition concentration of mercury and shaker speed at 7 ppm is 41 ˚C and 180 rpm respectively, with able to reduce 92.59 % mercury concentration. During the processes of mercury removal, mercuric reductase was produced at value of 56 kDa.

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LIST OF SYMBOLS

Hg	Mercury
nm	Nanometre
µL	Micro litres
mL	Mililitres
µ	Micro
g	Gram
kg	Kilogram
s	Second
min	Minutes
hr	Hour
µg/g	Microgram per gram
°C	Degree celcius
µM	Micro Meter
kDa	Kilodalton
W/cmK	Watts per centimetre kelvin
g/cm^3	Gram per cubic centimeter
$cm^3/mole$	Cubic centimeter per mole
kJ/mole	Kilojoule per mole
Pa	Pascal
L	Liters
Psi	Per square inch
Rpm	Revolutions per minutes

LIST OF ABBREVIATIONS

OD	Optical Density
P. putida	*Pseudomonas putida*
PPM	Part Per Million
PPB	Part Per Billion
PTFE	Polytetrafluoroethylene
DOE	Department of Environment
ABS	Absorbance
PHA	Polyhydroxyalkanoates
PBS	Phosphate buffer solution
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

1.1   Background of the Study

Mercury is a trace component of all fossil fuels including natural gas, gas condensates, crude oil, coal, tar sands and other bitumen. The use of fossil hydrocarbons as fuels provides the main opportunity for emissions of the mercury they contain to the atmospheric environment but other avenues also exist in production, transportation and in processing systems (Wilhelm and Bloom, 2000). These other avenues may provide mercury directly to air, water or solid waste streams. In addition, the distribution and transformation of mercury in production, transportation and processing are considered relative to the determination of mercury in air emissions, wastewater, and products from oil and gas processing facilities (Rebhun and Galil, 1994). The disposal of waste water that contains mercury should follow the Department of Environment (DOE) regulation which is 50 ppb concentration (Environment Quality for Sewage and Industrial Effluent Regulations 2009 Third Schedule, 2010). The higher concentration of mercury will caused the fish and shellfish contaminated with mercury. From the past research it shows that the mercury level in fish are $0.007 - 0.914$ μg/g (Parvaneh, Jinab, Abu, & Bakar, 2010). The mercury level in the fish shows the closed relationship with concentration of mercury in river. Besides that, the previous study showed that the mercury concentration at the surface of water is >3.0 ppm (S.Ghosh et al, 1999). This subsequently effect of the human health as they used the source from the river. The exposed for long term and high concentration can be harmful to the brain, heart, kidneys, lungs and immune system of human at all ages.

1.2   Motivation and Problem Statement

Mercury is one of the heavy metals of concern, found in wastewaters coming from oil refinery, chloralkali manufacturing industry, paint, pharmaceutical, paper and battery

manufacturing industries. Mercury and mercurial compounds are highly toxic contaminants in the aquatic systems and soils. They are dangerous pollutants because they can disperse widely into environment due to their high mobility and potentially dangerous concentration throughout the food chain (Carpi, 2001).

Mercury contamination of hydrocarbon production and processing systems can be more than a mere nuisance (Li et al., 2009). Early detection and accurate quantification of mercury is necessary to assure equipment integrity, to comply with regulations and to ensure safety of workers. High concentrations of mercury are found in several regions of the world and operators have developed measures to cope with the major ramifications but all such measures benefit from early recognition of potential problems. Routine maintenance and inspection activities become non-routine when mercury is present in fluids above a few ppb and become problematic when mercury concentrations reach approximately 100 ppb.

Methods for removing mercury ion (Hg^{2+}) from aqueous solution mainly consist of physical, chemical and biological technologies. Among conventional methods suggested by researchers for removing mercury ions from aqueous solution are chemical precipitation and electrochemistry (Pascal et al., 2007), ion exchange (Chen et al., 2008), membrane technologies (Broom et al., 1994), adsorption on activated carbon and etc (Demirbas, 2008). Most of mercury remediation through common physico-chemical technologies are expensive, not environmental friendly and suitable only for the treatment of higher mercury concentration in wastewater treatment plants.

One potential process in biotechnology that can be implemented in treatment of mercury contaminated wastewater is by introducing *Pseudomonas putida* (*P. putida*) as a biosorption approach which utilizes natural materials of biological origin. This method was pioneered by Nakamura el al., (1986) for the treatment of mercury contaminated wastewater discharged near Minamata Bay. The biosorption process possesses metalsequestering properties and can be used to decrease the concentration of mercury ions in solution. It can effectively sequester dissolved mercury ions out of dilute complex solutions efficiently and quickly, thus making it an ideal candidate for treating high volume and low concentration complex wastewaters (Wang and Chen, 2006).

P. putida plays an important role in the decomposition and biodegradation of wastewater undergoing treatment. In addition, P. putida have been used in applied studies by chemists and environmental engineers for green chemistry application and bioremediation for its enzymes (Felske et al., 2003). Thus, in this research aims to study effect temperature and shaking speed on the activity of enzyme from P. putida in mercury concentration removal. Besides that, this study also hoped to provide treatment alternatives and to widen the varieties for treatment of wastewater in the petroleum industry.

1.3   Objective

The objective of this research is to study effect temperature and shaking speed on the activity of enzyme from P. putida in mercury concentration removal.

1.4   Scopes of this research

The following are the scopes of this research:

i.   To study the behaviour of P. putida and isolation of enzyme before mercury treatment.

ii.   To investigate the effect of ratio of P. putida to mercury concentration.

iii.   To analyse the effectiveness of enzyme in the removal of mercury from industrial wastewater.

iv.   To analyse the characteristic of P. putida enzyme.

1.5   Main contribution of this work

Based on the scope above, the research should be able to remove the mercury at higher percentage at optimum condition. It is also important to reduce the mercury level in the petrochemical wastewater.

1.6   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 mercury that is 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 which first part explaining about *P. putida* characteristic. This chapter covers the review of mercuric reductase and mercury which are include application, exposure risk of mercury and mercury sources in petrochemical industry. This chapter presents general method treatment for mercury removal in wastewater by using physical, chemical and biological methods. Mechanism of mercury removal also states in this chapter, which are important in understanding activity of enzyme from *P. putida*. This chapter ends with the summary of the literature review.

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

This study continous with Chapter 4 presents the results and discussions. The subchapters include *P. putida* growth, cell dry weight, effect of temperature, effect of shaker speed, effect of *P. putida* in mercury removal, and enzymes purification. Last but not least, Chapter 5 presents about the conclusion and recommendation of this study.

CHAPTER 2

LITERATURE REVIEW

2.1   Introduction

This chapter describe the characteristics of the *P. putida*, mercuric reductase, overview of mercury, mercury in petrochemical industry, and the optimal condition of the study. Besides that, this chapter also had an overview of the physical, chemical and biological methods of the mercury removal in wastewater treatment.

2.2   Characteristic of *P. putida*

The genus *pseudomonas* has been heterogeneous since Migula first named it in 1894. *Pseudomonas* a gram-negative, strictly aerobic, a polar flagellated rods (Gundala, Jayakumar, K, & Chintala, 2013). Table 2.1 shows name of order of *Pseudomonas putida*.

Table 2.1**:** Name of order of *Pseudomonas putida*

Domain	Bacteria
Phylum	Proteobacteria
Class	Gamma proteobacteria
Order	Pseudomonadales
Family	Pseudomanadaceae
Genus	Pseudomonas
Species	Pseudomonas putida
Others name	Pseudomonasovalis, Pseudomonas striata, Arthrobacter, siderocapsulatus, Bacillus putidus, etc.
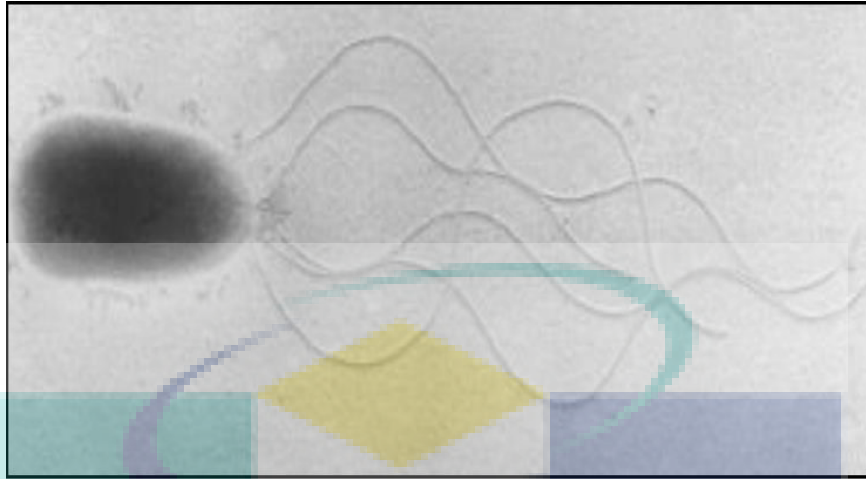
Figure 2.1: *Pseudomonas putida*, 1.0 μm.

From Figure 2.1, the *P. putida* was a rod-shape and flagellated. *P. putida* was a gram-negative bacterium that is found in most soil and water habitats where there is oxygen (Krish Hamilton, 2007). It grows optimally at $25 - 30\ ℃$ and can easily isolate. *P. putida* has several strains including the KT2440, a strain that colonizes the plant roots in which there is a mutual relationship between the plant and bacteria. The surface of the root, rhizosphere, allows the bacteria to thrive from the root nutrients. *P. putida* has a very diverse aerobic metabolism that is able to degrade organic solvents such as toluene and also to convert styrene oil to biodegradable plastics Polyhydroxyalkanoates (PHA) (Mohd Azoddein, 2013).

P. putida is unique because it has the most genes involve in breaking down aromatic and aliphatic hydocarbons which are hazardous chemicals caused by burning fuel, coal, tobacco, and other organic matter. Most pseudomonas is free-living saprophytic organisms in soil or water where they play important role decomposition, biodegradation and the carbon and nitrogen cycle. Because of this lifestyle, pseudomonas is characterized by great metabolic diversity and is able to utilize a wide range of carbon sources, including molecules which few other organisms can break down (Kris Hamilton, 2007).

Another strain of *P. putida* was SP1 that isolated from marine environment. It was found to be resistance to 280μM of $HgCl_2$. SP1 was also highly resistance to another metals, including $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$ and $ZnSO_4$ and the antibiotics

ampicillin (Ap), kanamycin (Kn), chloramphenicol (Cm) and tetracycline (Tc). *P. putida* SP1 was able to vitalized almost 100% of the total mercury it was exposed to and could potentially be used for bioremediation in marine environment (Zhang et al, 2011).

2.3 Mercuric Reductase

The flavoprotein mercuric reductase (reduced Nicotinamide adenine dinucleotide phosphate (NADP): mercuric ion oxidoreduction) is an important component of an organ mercurial detoxification system found in many bacteria (Silver et al, 1983) (Summers et al, 1977). This enzyme was function to remove or reduce amount of concentration of mercury in solution. The enzyme catalysed the cytoplasmic reduction of inorganic mercuric ions to elemental mercury which is volatile, and is thus no enzymatically removed from the growth medium. In some study, the operon is found on a transportable element (Fox and Walsh, 1981). Table 2.2 shows the characteristic of the mercuric reductase (Springer Book).

Table 2.2: Characteristic of mercuric reductase

EC number	1.16.1.1
Systematic name	Hg:NADP$^+$ oxidoreductase
Recommended name	Mercuric (II) reductase
Synonym	Mer A Mer A protein Mercurate (II) reductase Mercurate ion reductase Mercuric reductase Mercury reductase Reduced NADP: mercuric ion oxidoreductase Reductase, mercurate (II)
CAS registry number	67880-93-7

Sources Organism	<1> Pseudomonas aeruginosa (PAO9501; Tn501-encoded; enzyme is encoded by the transposon Tn501) <2> Escherichia coli (containing the cloned mercury resistance genes from plasmid NR1; W3110 laclq containing the plasmid pPSO1; Tn501 mercuric ion reductase; PWS1) <3> Thiobacillus ferrooxidants (TFI 29) <4> Pseudomonas stutzeri <5> Pseudomonas cepacia <6> Bacillus sp. (strain RC607) <7> Penicillium sp. (MR-2 strain) <8> Bacillus spaericus <9> Bacillus polymyxa [10, 18] <10> Rhodococcus sp. [10, 18]

The enzyme was purified by native gel electrophoresis. The weight of the mercury reductase was found at 54 kDa for major band and 69 kDa for minor band. This enzyme is soluble in cytosolic flavoprotein that contain both flavin adenine dinculeotide (FAD) and a redox active disulphide that catalyses the reaction (S.Ghosh et al., 1999). In the other study, the mercuric reductase also found at 59 kDa and 62 kDa (Fox and Walsh, 1981)

2.4   Mercury Overview

Mercury is known as one of the most toxic metal in the word. Mercury is a metal that exist as the liquid in room temperature. Its boiling point is 356.6℃ and freezing point is -38 ℃. Mercury has higher density which is 13.6 heavier than water. Mercury is a metal with silver colour. It has high density and high surface tension. Mercury is easily break up into small droplets. It is slightly soluble in water. Mercury has an ability to form alloy with all metals except iron and platinum. These alloys known as amalgams. The mercury can be soluble in both polar and non-polar solution (Mohd Azoddein, 2013). The summary of the physical properties of mercury was show in Table 2.3.

Table 2.3: Physical properties of the mercury

Atomic mass average	200.59 ℃
Boiling Point	357
Electrical conductivity	0.0104×10^6 /cm Ohm
Thermal conductivity	0.0834 W/cmK
Density	13.546 g/cm^3 @300 K
Melting point	-38.72 ℃
Molar volume	14.81 cm^3/mole
Vapor pressure	0.0002Pa @ -38.72 ℃
Heat of vaporization	59.229 kJ/mol

In terms of chemical properties, mercury valance state can be form of elementary Hg^0, mercurous Hg^{+1} and mercuric Hg^{+2}. The elementary mercury is the most stable state of mercury. Mercury also can be in ionic form Hg^{22+} and Hg^{2+}. Inorganic mercury mainly in form of salts and mostly less soluble in water (Mohd Azoddein, 2013). Figure 2.2 shows the mercury phase room temperature.

Figure 2.2: The mercury phase room temperature

2.4.1 Application of Mercury

There are many applications of the mercury and the major uses of the mercury are (Kumar and Tate, 1982):

i.   It is used as a liquid contact material for electrical switches. It is working fluid in the vacuum.

ii. It is used in the manufacture laboratory equipment such as mercury-vapour rectifiers, thermometers, barometers, manometers and mercury-vapour UV lamps.

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iii.   In micro gas analysis, mercury was used as a sealing liquid for the evolved gases.

iv.   In manufacture of chlorine and sodium hydroxide, mercury was used as cathode.

v.   In the calomel electrode and in the Weston standard cell, mercury is used in contact solution which contains mercury (I) salts as the solid phase.

vi.   Most of dry cells contain amalgams of zinc and cadmium to prevent impurities from shortening the life of the battery. Mercury battery contains HgO. The latter is also used in skin ointments.

vii.   For filling dental cavities, mercury is grown with Ag_3Sn. The resulting semisolid amalgam, on standing, sets to form a hard solid mixture of stable and non-toxic compounds Ag_5Hg_8 and Sn_7Hg. Silver and gold amalgams are used for filling teeth.

viii.   Merbaphen, an organomercurical, is used as a diuretic. Mercurochrome and merthiolate are most popular antiseptic.

2.4.2   Exposure Risk to Mercury

The inorganic mercury Hg (II) transforms into methylmercury by the bacteria that living in the sediments of aqueous environments, a potent neurotoxin that concentrates through the food chain in the tissue of fish and marine mammals. Then, the ingestion of methylmercury by human from seafood and other dietary and environmental sources is connected to serious sensory, motor and cognitive disorder (Darbha et al., 2007). The major effect of mercury poisoning is neurological and renal disturbance as well as impairment of pulmonary function (Green-Ruiz, 2005). Hg is a bio accumulative toxin that attacks the central nervous and endocrine systems (Oehmen, et al., 2014). Mercury can be exposed directly from breathing (evaporated mercury) or from contaminated food (fish and marine sources). The short term exposed will make someone to have nausea, diarrhoea, cough, eye irritation and etc. For long term mercury exposed will caused man to have Anorexia, loss of appetite, anxiety, fatigue and many more. The mercury are particularly sensitive to pregnant woman, infants and also young children. The exposure to high Hg levels can be harmful to the brain, heart, kidneys, lungs and immune system of humans of all age. While the excessive exposure over long periods of time can result in brain damage and in extreme cases, death (Oehmen, et al., 2014).

2.5   Mercury Sources in Petrochemical Industry

Mercury can be released directly to environment during manufacture and processing of some products. The indirect contamination may be caused by the inadvertent or accidental release of mercury and mercury containing industrial, agriculture and consumer products. For example, an undetermined amount of mercury finds its way into waterways and sewer systems as a result of indiscriminate disposal of mercury containing paints, pharmaceutical, fungicides (Kumar & Tate, 1982), fossil fuel burning, metallurgical processes (Green-Ruiz, 2005) (R. Dash & Das, 2012), mining and chor-alkaly industry (Oehmen, et al., 2014).

The main wastewater streams that derive from petroleum production and processing are produced water from both oil and gas production and refinery wastewaters. Very minor amounts of water (relative to produced water and refinery wastewater) derive from gas processing and these are mainly water from separators at gas plants (essentially produced waters) and condensed water from dehydration. No wastewater streams originate from transportation systems other than the very small amounts that come from pipeline pigging operations and tanker ballast (Wilhelm and Bloom, 2000).

Besides that, normal production operations of both crude oil and natural gas involve primary separation of water, gas and oil. Separated water (referred to as produced water when separated close to the well) is either discharged (to an ocean, lake or stream or evaporation pond) or reinjected (usually to the formation it came from). Reinjection is utilized to enhance oil recovery (EOR) or to comply with regulatory requirements stemming from environmental concerns. Produced water normally containing high (>100.00ppb) concentration of mercury dissolved (Wilhelm and Bloom, 2000).

Furthermore, the chemical compositions of refinery wastewater vary widely, as do the volumes of water (per barrel of oil processed) produced by refineries (Campanella et al., 1986). Gill and Fitzgerald (1987), reported range of mercury pollution in ocean waters is broad i.e. 0 – 1000 ppb. Major of water compositional differences stem from process configuration (products produced) and from the type of crude oil that is processed (high sulphur crude, sweet crude). The production of petroleum based industry

wastewater generally consists of polycyclic and aromatic hydrocarbons, cyanide, oil, phenols, metal derivatives, sulphides and other chemicals (Campanella et al. 1986).

The mean and range of mercury concentration in refinery wastewater cannot be stated with certainty. A little information is available in the published literature that speaks directly to this issue. Environment ProEPA study of refinery effluents from the early 80″s (Ruddy, 1982) provides a mean close to 1 ppb but the methodology to arrive at this number is poorly documented. The amount of refinery wastewater discharged to the environment (rivers, lakes and oceans) is approximately 1.50 billion barrels yearly (U.S. EPA 2001 and U.S. EPA 1997). Applying the 1982 EPA mean value of 1 ppb as maximum to this amount yields approximately 250 kg as an upper limit to the total amount of mercury discharged in refinery wastewater.

2.6 General Method of Treatment

There were existing approaches for Hg removal treatment include adsorption, ion exchange, reverse osmosis and nanofiltration, chemical precipitation and bioremendation and hollow fibre external membrane of bioreactor (Oehmen, et al., 2014) (Shafie, 2009). Mercury is present in water as a micropollutant, at level much lower than other ions that will compete for example chemical precipitation, ion exchanges, and adsorption sites, increasing the amount of material needed to achieve Hg removal (Oehmen, et al., 2014). The more advance method of mercury removal was by using the bacteria.

2.7 Mechanism of Mercury Removal

Among the various type of mercury removal method, the removal by using bacteria was use in this experiment. The microorganism choose was *P. putida* as it might be the most widely found toxic ion resistance system occurs in all bacteria division where it has been sought. Mercury resistance occur widely with Gram negative and Gram positive bacteria and it shows the right choice in choosing the *P. putida* as the micro bacteria in the gold mining wastewater treatment. During the mercury removal treatment, the mercury reductase was produced to remove the mercury contaminant (Silver & Phung, 2005).

The most common functional genes of the mercury reductase are merA and merB, which code for mercuric ion reductase and organomercurial lyase. The lyase responsible in reducing highly toxic organomercurical compound such as methylmercury and phenol mercuric acetate into almost nontoxic volatile element mercury with help of the enzyme mercury reductase (R. Dash & Das, 2012). The dissimatory metal reducing bacterium (DMRB) Shewanella oneidensis (S. oneidensis) MR-1 reduces ionic mercury (Hg [III]) to elementary mercury (Hg [0]) by an activity that not related to the MerA mercuric reductase. In S. oneidensis, this activity is constitutive and effective at Hg (II) concentration too low to induce mer operon function. The reduction of Hg (II) by MR-1 required the present of electron donor and electron acceptors (A. Wiatrowski, Ward, & Barkay, 2006).

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 mercury is very dangerous because even only long term exposure to those compounds it can gave severe sickness. Mercury is the only common metal liquid at ordinary temperatures. It rarely occurs free in nature and is found mainly in cinnabar ore (HgS) in Spain and Italy (De and Ramaiah, 2006). There are three important types of mercury which is the pure element, inorganic compounds such as mercuric nitrate and organic mercury compounds such as phenyl mercuric propionate. Furthermore, three important types of mercury are considered among the most harmful materials in the environmental because of its toxicity, high volatility and potential for bioaccumulation even at low concentration (Byun et al., 2011).

Chronic mercury poisoning may develop gradually without conspicuous warning signs because mercury accumulates in body tissues (Hussein et al., 2005). Symptoms of repeated exposure include gray skin color, gum problems, tremors, memory and concentration problems, mood changes, and visual disturbances including clouding of the eyes. In the aquatic environment under the suitable conditions, mercury is bioconverted to methylmercury, called methylation (Wood, 1974). Methylmercury accumulates in fish at levels that may harm the fish and the other animals that eat them. This petrochemical wastewater must undergo a treatment before been discharge. There are three types 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 concentration of mercury in the wastewater through biological treatment. From *P. putida* in can produce variable of enzyme which is mercuric reductase. Furthermore, this enzyme will undergoes purification process to obtain useful product.
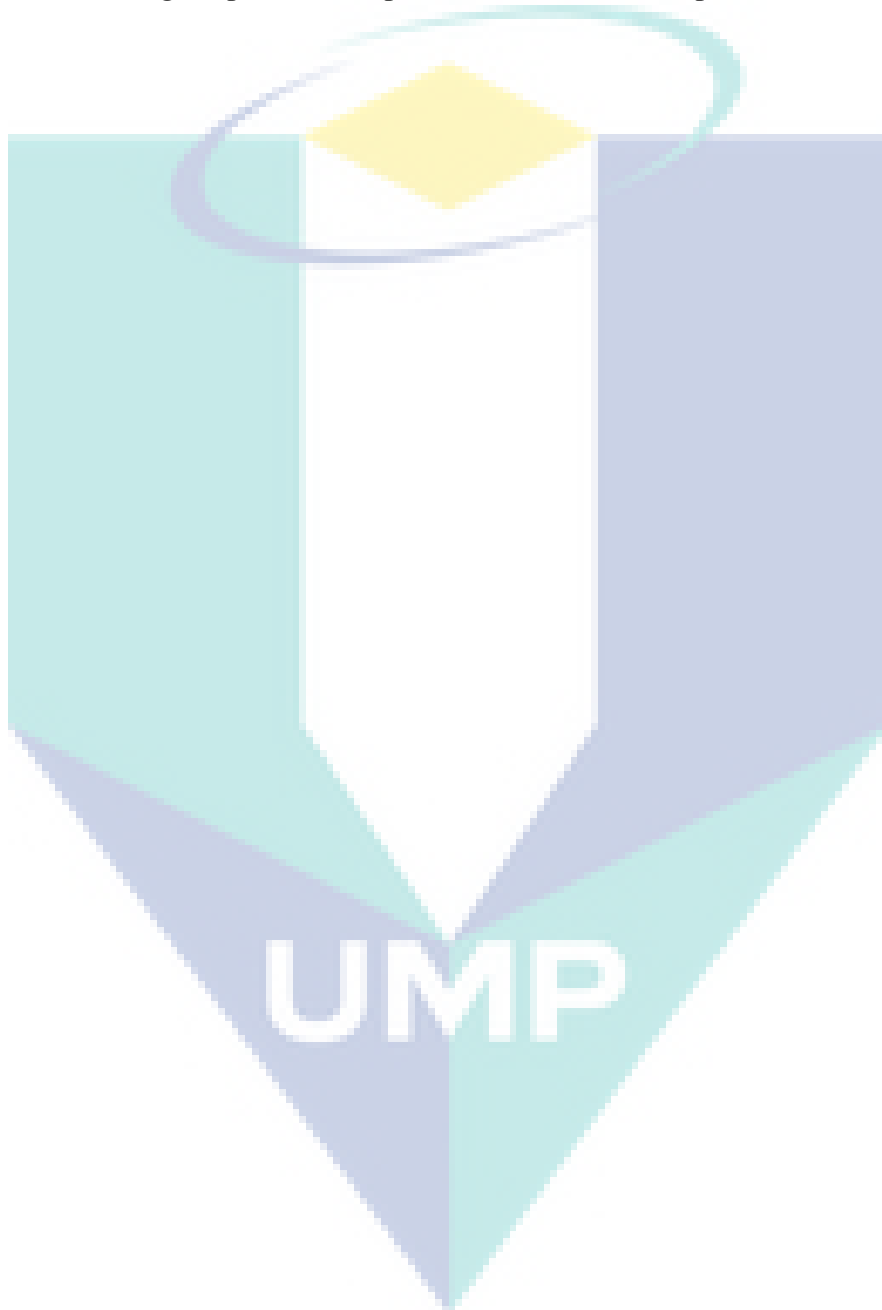
CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter describes the details of all research techniques and activities carried out in this study consisting of materials, process equipment, apparatus used for experimental analysis, sterilization techniques, culture preservation and fermentation process. The flowchart provides an overview of the research elements considered and undertaken in achieving the objectives mentioned in Chapter 1. Glassware used in this experiment underwent through autoclaving and proper sterilization to avoid contamination. Safety measures and precautions have been taken into account when designing and preparing the experiments and the procedure related to microorganism were carried out in the laminar flow hood.

3.2 Materials

The materials used in this research consisted of microorganisms and chemicals for the preservation of the microbe in slant, culture medium and for analytical procedures. Various chemicals and materials are also used for sample analysis of wastewater and also as cleaning chemical.

3.2.1 Microorganism

Bacteria *P. putida* used in this study was obtained from Merck (Malaysia) Sdn. Bhd as a local agent dealing with the bacteria. *P. putida* used is colony stock from laboratory. The growth mediums for *P. putida* were prepared by suspending 8 grams nutrient powder in 1 L of de-ionized water. The growth media was sterilized in autoclave at 121°C, at pressure of 15 psi for 20 minutes. The culture was keep below 5 °C and this culture was used for all subsequent works (Stanbury et al, 1984).

3.2.2 Chemicals

The chemical used in this experiment are divided by three parts. Firstly used in sample and standard preparation are 1000 ppm mercury (Hg (NO₃)), Stannous Chloride powder, H_2SO_4 and L-cysteine. Secondly in enzyme purification, the chemicals used are phosphate buffer solution (PBS), lysis buffer solution and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reducing agent. Lastly, in terms of apparatus cleaning, HNO_3 and 70 % Ethanol was used.

Analytical grade Ethanol used as a cleaning solvent was supply by Merck (Malaysia) Sdn. Bhd. Tap water was used for washing glassware, cleaning and as cooling water. Distilled water was used to prepare cultured medium and de-ionized water was used for analytical purpose such as in spectrometer and mercury analyser, and also in preparation of chemical standard solution to determine standard calibration curve and for sample dilution. The dilution of HNO_3 was used to immerse the apparatus that contact with mercury especially the test tubes.

3.2.3 Medium

The medium was used for inoculum, growth and waste water treatment purpose. Nutrient broth (NB) used for stock culture is 8 g of nutrient was suspended in 1L of de-ionized water. For nutrient agar, (NA), 23 g of agar was suspended in 1L of de-ionized water. Both of the solutions

were sterilized in an autoclave at temperature of 121 ˚C at 15 psi for 20 minutes before used (Mohd Azoddein, 2013).

3.2.4 Stock Culture

The purity of the stock culture must be concern so that the culture can be used for a long period without losing or lacking of nutrient. The preserved nutrient broth can only last for few weeks and after that start to decay due to nutrient depletion and accumulation of toxic. The stock culture can be stored in refrigerator up to six months (Stanbury et al, 1984).

3.3 Equipment

In this study, in general the equipment used can be divided into experimental apparatus and analysis apparatus. Both apparatus have their own function in setting up a series of experimental activities for data collection.

3.3.1 Experimental Apparatus

The experimental apparatus are the basic parts of the apparatus that are required in order to carry out the experiments. These experimental apparatus that required in the experiments are auto-clave (H+P Varioklav Stream Sterilizer ESCO), incubator shaker, microbiological incubator, laminar flow cabinet (ESCO) and oven.

3.3.2 Analysis Apparatus

The analysis apparatus was used to determine the condition of the compound or substrate as well as to identify operating parameters of processes. In this experiment, the analysis apparatus used are analytical balance (Mettler Teledo), UV Vis-spectrophotometer (U-1800, Hitachi), and mercury analyser (RA-3000 Mercury, NIC).

3.4 **Methods**

This section discussed the further details of the experimental procedure. The aims of the experiment are to achieve the objectives previously mention in Chapter 1. The calculated and graphically data was collected properly and then summarized in discussion leading to a conclusion presented.

3.4.1 **Overall Experimental Sequence of the Study**

The experiments carried out in this study divided into three main stages. The first stage is carried out to determine the growth curve of the *P. putida*. The second stage is to determine the effect of the temperature and shaker speed in mercury removal. The last stage is to determine the activity of the enzyme in mercury removal. The summary of the experimental procedure are summarize in the Figure 3.1.

RIG/EQUIPMEN	PROCESSES	MATERIALS/CHEMIC

-Autoclave

-Glassware

-Laminar flow cabinet

-Pipette

-Incubator shaker

-Weighing balance

-Centrifuge

-70% Ethanol

- *P. putida* colony

-Nutrient broth

-Nutrient agar

Experiment rig set

P. putida growth at optimum

Change Method

P. putida growth

No

Yes

-Incubator shaker (180 rpm)

-T = (33˚C, 37 ˚C, 41 ˚C)

-UV-vis spectrophotometer (600 nm)

-Electrophoresis

Study the effect of temperature

- Study of growth curve
- OD reading
- Reduction of Hg

-T = (37 ˚C)

-Incubator shaker (140, 180, 220 rpm)

- UV-Vis spectrophotometer (600 nm)

- Electrophoresis

Study the effect of shaker speed

- Study of growth curve
- OD reading
- Reduction of Hg

-*P putida*

-Growth media

-Hg in DI water

Data analysis

Conclusion

Figure 3.1: Method in conducting experiment

3.4.2 Preparation of the bacteria growth

In preparing the stock culture, the culture from slanting agar must be transfer first into the broth culture in a laminar flow cabinet. The sterility of the media and apparatus must be maintained during transfer the pure culture of *P. putida.*

First, all the apparatus is sterilized by autoclaving in autoclave. Secondly, as the needed apparatus and are ready in the biohazard laminar flow, the inoculating loop is sterilized using a flame from Bunsen burner until it is red hot. Cooled down the loop before streaking on the surface of the agar culture. The cap and lip of the bottle sample containing nutrient broth are sterilized using the flame from Bunsen burner. Then, the inoculating loop that contains a smear of culture is immersing into the bottle sample. Slightly shake the bottle sample to dipping the pure culture. Then flame again the cap and lip also the loop. For every bottle of sample (20 mL), there were three looping of the culture from bacteria colony in Figure 3.2. The one loop of culture was standardizing as one line of streaking. The bottle sample containing of the pure culture in nutrient broth is then incubated at 37 ˚C for 24 hours. It is then kept in a refrigerator to be used as a stock culture.

Figure 3.2: *P. putida* streaking colony.
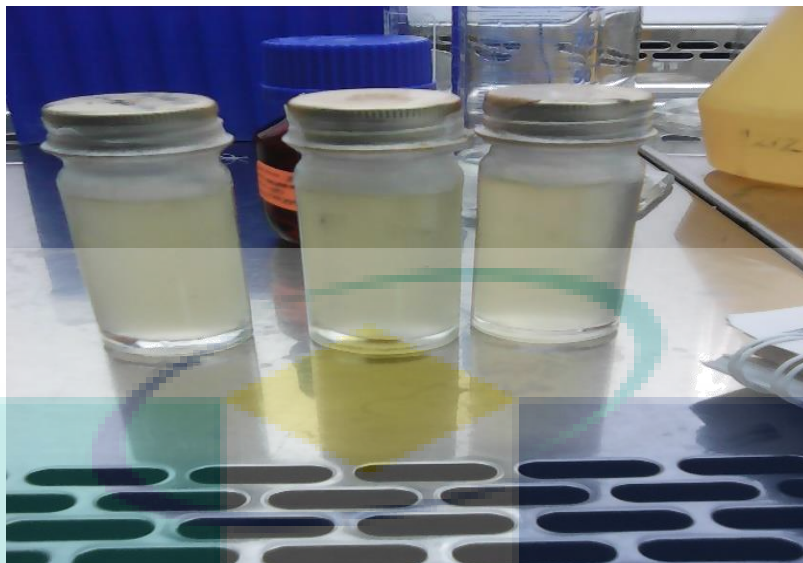
Figure 3.3: *P. putida* cultured broth

As the stock culture was ready, added 20 mL of the broth culture into 180 mL of the broth solution to determine the growth curve pattern. *P. putida* cultured broth is shows in Figure 3.3. The solution is then placed in incubator shaker at 37 ˚C with 180 rpm speed as shown in Figure 3.4. The mouth of the conical flask was covered with cotton and gauze first before wrap with the aluminium foil. This was to prevent the contaminated from the surrounding effect.
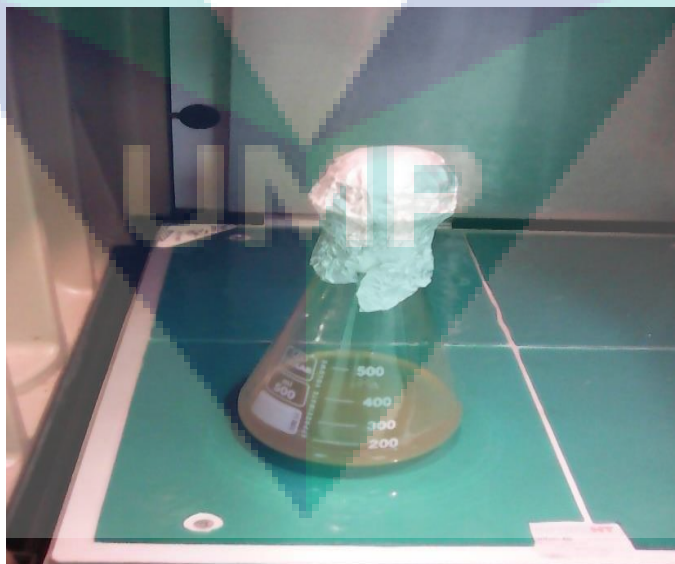
Figure 3.4: *P. putida* growth in incubator shaker

3.4.3 Analyzing the bacteria growth and cell dry weight

2.5 mL of the solution was taken and test by using the UV-vis spectrometer. 600 nm wavelength was used and broth as a blank (Cabral et al, 2014) (Mohd Azoddein, 2013). The data was record at 1 hour interval for first 6 hours, 2 hour interval (4 reading), and 4 hour interval until the bacteria death. After undergoes UV-vis spectrometer test, 1.5 mL of the solution was put into the centrifuge tube (weight the centrifuge tube before use). The solution was centrifuge with 10000 rpm for 5 minutes. The broth solution was removed and placed the centrifuge tube into the oven (37°C). The weight of the centrifuge tube with dry cell after 24 hours was record.

3.4.4 Preparation of Stock Solution (mercury solution)

In preparing the stock solution, continues dilution had done to make sure that the mercury mixed and diluted well. 10 mL of 1000 ppm mercury was diluted first in 100 mL volumetric flask to form 100 ppm stock solution. Then, 50 mL of 100 ppm stock was dilute again in 100 mL volumetric flask to form 50 ppm stock solution.

3.4.5 Study the effect of temperature

In study of the effect of temperature, the sample was prepared based on Table 3.1 below. The blank was set as only broth solution in order to analysis the growth pattern of *P. putida* at three differences concentration. At constant shaker speed of 180 rpm, various the effect of temperature of 33˚C, 37˚C and 41˚C was study.

Table 3.1: Sample preparation of mercury solution

Sample Concentration (ppm)	Broth Solution (ml)	50 ppm Mercury Standard (ml)	Brooth Culture with *P. putida* (ml)	Total sample solution (ml)
0	200	0	0	200
3	169.5	10.8	20	200
5	162.0	18.0	20	200
7	154.8	25.2	20	200

3.4.6 Study the effect of shaker speed

In study the effect of the shaker speed, the sample preparation was same as in temperature effect study in Table 3.1. Three differences shaker speed at 140 rpm, 180 rpm and 220 rpm was study at constant temperature was study.

3.4.7 Enzyme purification

The enzyme purification is to purify the type of enzyme produced during the Hg removal by *P. putida*. The purification methods used are SDS-PAGE method with electrophoresis gel. 45 mL of the sample was centrifuge in (centrifuge model) at 10,000 rpm, temperature of 4˚C for 10 minutes. As the pallet form at the bottom of the centrifuge tube is shows in Figure 3.5, remove the solution (used for mercury removal test). Then, rinse with 1 mL of the phosphate buffer solution by centrifuge at 12,000 rpm, temperature of 4˚C within 5 minutes for three times. Then, added 1 mL of lysis buffer and centrifuge again at 12,000 rpm, temperature of 4˚C for 10 minutes.

Figure 3.5: Pallet form at the bottom of the centrifuge tube

Take out 1.0 µL of the sample supernatant added with 2.5 µL of NuPAGE LDS Sample Buffer (4x), 1.0 µL of NuPAGE Reducing Agent (10x) and the rest are distilled water to make total volume 10.0 µL. The sample is centrifuge again in (centrifuge name) at 10,000 rpm for 5 minutes. Then, heat the sample at 70˚C. The sample undergoes the

electrophoresis gel and staining for 24 hours before view under the bio-imaging equipment. Then band form in the gel, was discuss in next chapter.

3.4.8   Mercury removal test

Before analyse the percentage of the mercury removal, the standard curve of the Hg should be prepared first. As the limitation of the R-1001 Mercury analyzer, it only detects the Hg concentration up to maximum concentration 15 ppb, thus the standard curve preparation should be range in 0 – 15 ppb of Hg concentration.

3.4.8.1   Preparation of the reagent and Hg standard

The standard curve of the mercury was prepared first as a reference to determine the mercury concentration of the sample. All important reagents were prepared in order to study the mercury removal. All this reagent acts as an agent that convert the ionic mercury to elementary mercury in mercury analyzer test.

Reagent preparation

The reagent consists of 50% of sulphuric acid, 10% stannous chloride and 0.001 % L-cysteine solution. 25 mL of Sulphuric acid was added slowly into 25 mL of distilled water in an ice bath with a constant stirring. The ice bath is must because of the exothermic processes occurred. In preparing 10% Tin (II) chloride, 5 g of the stannous chloride was dissolved in 50% sulphuric acid in 50 mL volumetric flask. The stannous chloride reagent must be freshly prepared for every used to avoid the reducing ability lost. Lastly, the preparation of 0.001% L-cysteine solution, the 2 mL of concentrated nitric acid was added into the 10 mg of L-cysteine powder. Then, the distilled water was added to make total of 1000 mL solution.

Hg calibration standard

Five differences of mercury concentration were prepared as standard. Table 3.2 shows the summary of the Hg standard calibration preparation.

Table 3.2: Hg Standard calibration preparation

Hg Concentration (ppm)	100 ppb of Hg (µL)	Distilled Water (ml)	Total volume (µL)	50 % Sulphuric acid	10 % Tin (II) Chloride (µL)	0.001 % L-cysteine (µL)
0	0	5000	5000	200	200	200
2	100	4900	5000	200	200	200
4	200	4800	5000	200	200	200
6	300	4700	5000	200	200	200
8	400	4600	5000	200	200	200

The stannous chloride was freshly added into the solution before analyse to prevent the reduction processes occurred.

3.4.8.2   Mercury removal

In order to analyse the percentage of mercury removal, some steps had done consist of sample preparation, sample dilution and finally sampling test.

Sample preparation

The solutions that centrifuge before (from enzyme purification) are filter again to remove the remaining microorganism using 4.5µ Polytetrafluoroethylene (PTFE) filter. Then, the filtered solution is diluted into 1 mL per 50 mL solution and undergoes second dilution 1 mL per 24 ml total to make the concentration of the Hg below the Mercury analyzer limitation test. The used of the PTFE filter because (PTFE) membranes are chemically resistant to nearly all solvents, acids and bases. The membrane has low extractable and good thermal stability. PTFE is hydrophobic and requires rewetting prior to use with aqueous solvents.

Sampling test

5 ml of the pre-digested samples was transfer into the sample tubes. 200 µL of H_2SO_4 was added into the sample tube followed by 0.001 % L-cysteine (µL) and 200 µL of Tin (II) Chloride. The impinger cap was closed start the measurement analyzer. As the sound of the buzzer heard, it signals of the end of the analysis. The impinger cap was

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removed and rinses thoroughly the bubbler with DI water. The result of the percentage mercury removal is discussed in the next chapter.

3.5   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 mercury, 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 discuss about the result and discussion that obtain from the laboratory experiment. The subtopics that will be discussed are the *P. putida* growth and cell dry weight curve. Besides, there will be about the effect of temperature and shaker speed in in mercury removal at three differences Mercury concentration and the purification of the enzyme.

4.2   Growth pattern and Cell dry weight.

The growth and cell dry weight method was study to determine the growth pattern of the *P. putida* at condition of 37 ˚C and 180 rpm. This condition known as an optimum condition in previous (Mohd Azoddein, 2013).

4.2.1 *P. putida* growth curve

The OD reading of the bacteria was record at 1 hour interval for first 6 hours, followed by 2 hours interval for 8 hours and 4 hours interval for the rest reading. At every time of OD reading taken, the continuous steps going with cell dry weight method. Both methods was use to compare the approximation and precious phase that occurs at certain time. As the reading taken for three time each, the average value was record for the data. The data of the relationship between OD reading in terms of %ABS and cell dry weight in Table 4.1 was display in graph pattern to discrete its pattern at certain time. The growth

pattern was determined to express the capability of the *P. putida* to growth and determine the growth rate at point.

Table 4.1: Relationship between OD reading (%ABS) and cell dry weight data

| Time (hr)	% ABS	Dry Shell (gram)	Dry shell + dry Bacteria (gram)	Net weight (gram)
0	0.0000	1.0983	1.0983	0.0000
1	0.0000	1.0886	1.0886	0.0000
2	0.0000	1.0895	1.0895	0.0000
3	0.1470	1.0971	1.0974	0.0003
4	0.2570	1.0899	1.0906	0.0007
5	0.3673	1.1009	1.1025	0.0016
6	0.7583	1.0850	1.0900	0.0050
8	1.5056	1.0940	1.1033	0.0093
10	1.8401	1.0891	1.0993	0.0102
12	1.9030	1.0882	1.0989	0.0107
14	1.9670	1.0952	1.1061	0.0109
18	2.0360	1.0964	1.1075	0.0111
22	2.0760	1.0949	1.1062	0.0113
26	2.1020	1.0863	1.0979	0.0116
30	1.6340	1.0863	1.0962	0.0099
34	1.3863	1.0854	1.0944	0.0090
38	1.1108	1.0912	1.0991	0.0079
42	1.0955	1.0981	1.1048	0.0067
46	1.0944	1.0936	1.0999	0.0063
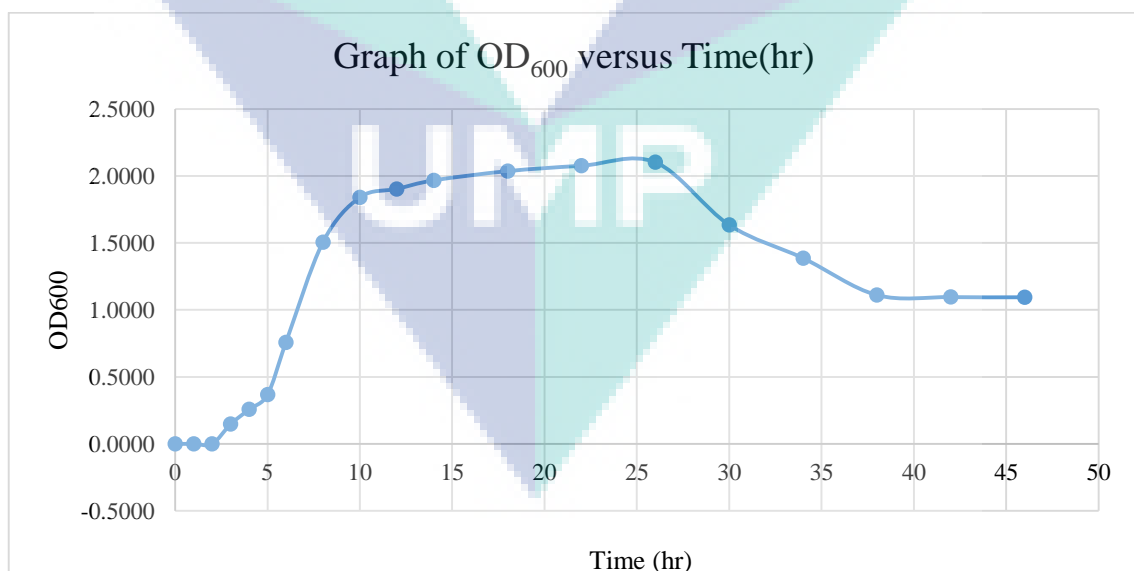
Figure 4.1: *P. putida* growth curve at 37°C and 180rpm.

The bacteria growth basically divided into four stages which are lag phase, log (exponential) page, stationary phase and death (decline) phase. From the graph in Figure 4.1, the OD reading (% ABS) shows the present or living bacteria at certain time. The growth pattern of the *P. putida* is quite difference to the standard growth of bacteria claim in Mohd Azoddein (2013).

Figure 4.1 shows the growth pattern of the *P. putida* at optimum condition. The lag phase occurs early between 0 to 3 hours. At this phase, the *P. putida* tried to adapt with the condition. The short time for the lag phase shows that the conditions are suitable for the *P. putida* growth as it can easily adapt to growth after that.

After 3 hours, the exponential phase occurs. There the activity of the *P. putida* is vigorously growth until 10 hours. At this phase, the *P. putida* adapt well by eating most of the nutrient present in the solution, multiplying its amount and known as the healthier phase of the *P. putida* in growth. Compare with Mohd Azoddein (2013), it shows the exponential growth after 7 hours.

Then, as the processes occur in bath culture, the nutrient supply reaches its limits and starts to degrade. During this stationary phase, the growth rate is equal to death rate. This phase shows the longest phase in this pattern of growth.

Finally, the death phase occurs after 25 hours. During this phase, the number of living *P. putida* start to decrease and the nutrient supply is considered to finish. Besides that, decreasing of OD reading (% ABS) shows that the *P. putida* death quite faster within the time. It may cause by the waste and toxic that produced by the living thing itself.
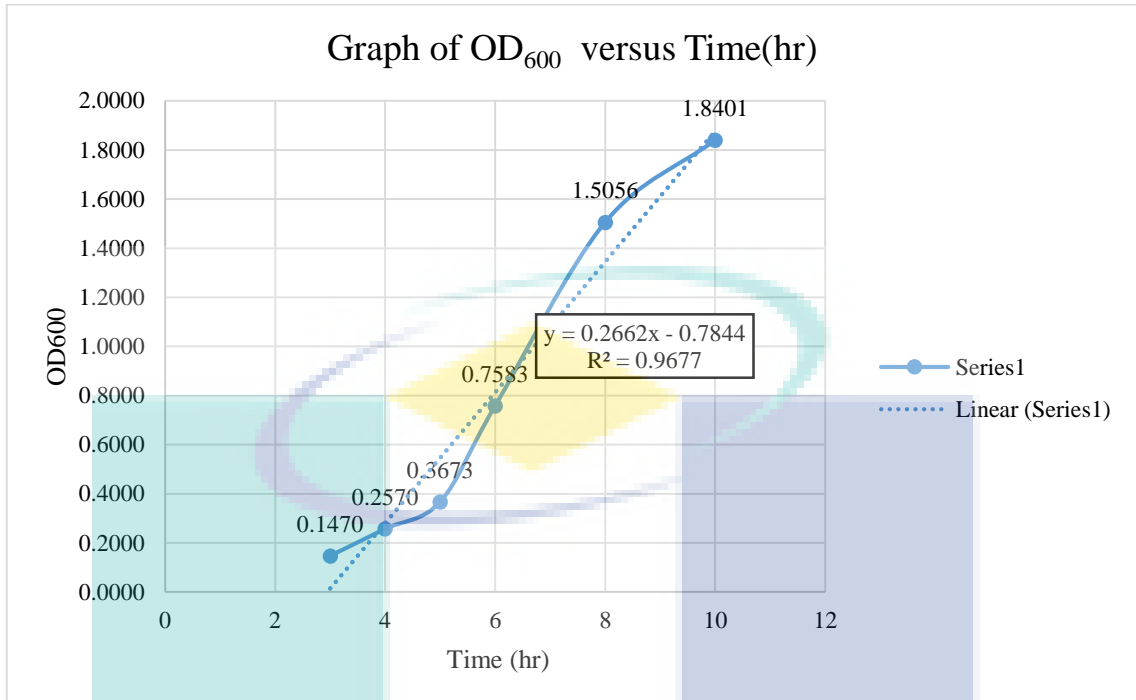
Figure 4.2: Exponantial phase of *P. putida* growth curve

Exponential phase was expanded to see the details growth of the *P. putida*. The Figure 4.2 shows the experimental phase and expected phase that the *P. putida* should live. At 3 to 6 hours, the *P. putida* live vigorously upper the exponential linear line while at 6 to 10 hours the *P. putida* live below the exponential linear line. Even though, it is consider as acceptable of growth pattern as the R-square values show strongly positive value between the actual and linear line of data.

4.2.2 Cell dry weight
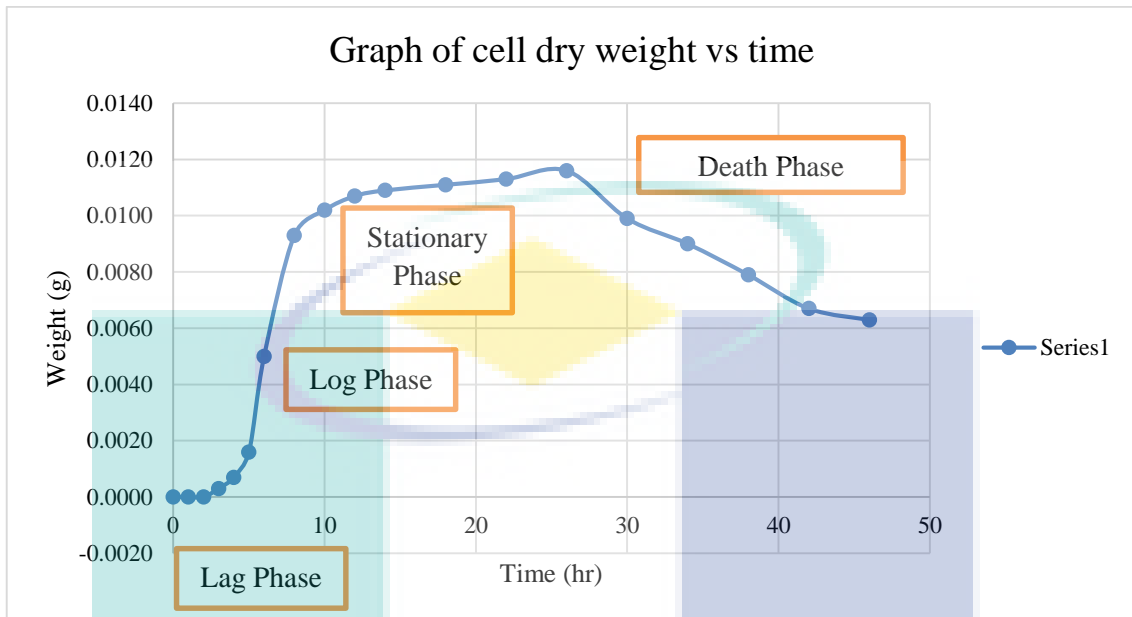
Figure 4.3: Graph of cell dry weight of the *P. putida* curve

Besides analyse the growth curve based on percentage absorbance, it is also can be define based on the cell dry weight analysis as shows in Figure 4.3. If compare with percentage absorbance, the growth pattern shows same pattern for both methods which is consist of four stages lag phase, log phase, stationary phase and death phase. The lag phase occurs at 0 to 3 hours followed by log phase until 10 hours. After that stationary phase occurs after 10 hours until 27 hours then followed by death phase. For detail result, the exponential phase is carried out again in the next graph.
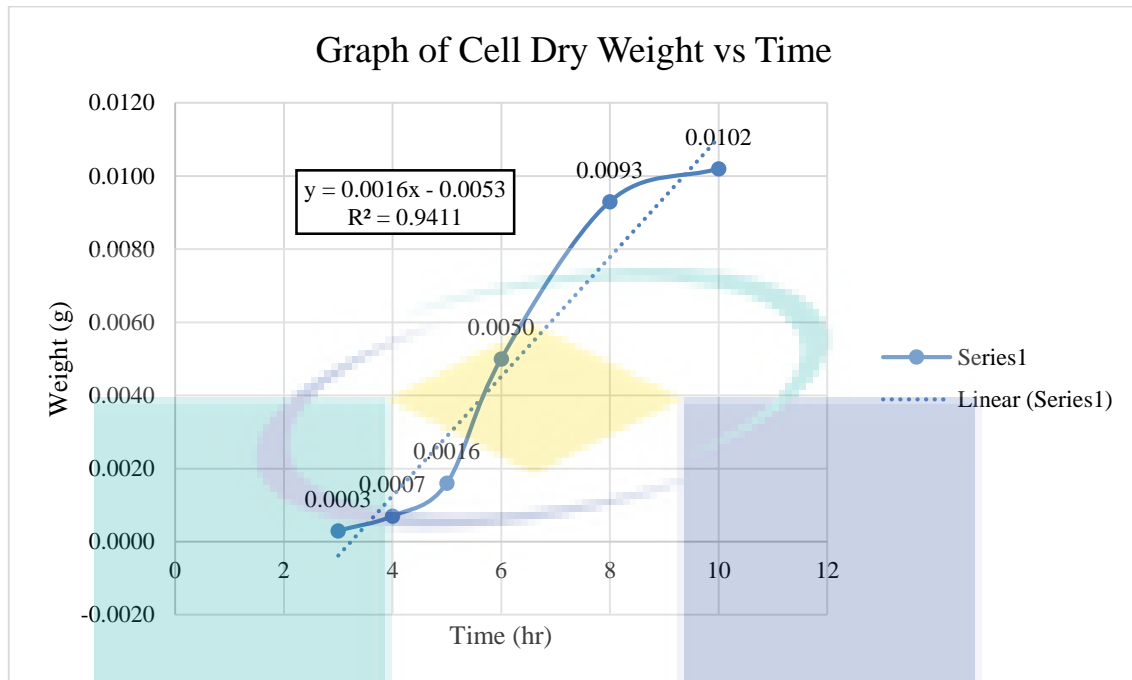
Figure 4.4: Exponential phase of the cell dry weight *P. putida* growth curve

From Figure 4.4 it shows that the mass of cell dry weight is proportional to the time of experimental. The increasing of cell dry weight is effected also by the growth that occurs during exponential and stationary phase. During the exponential and stationary phase, the weight cell keeps increase due to the generation on new living bacteria through reproduction instead of the amount of waste and by product that produce. Meanwhile during the death phase the growth rate is decrease but the cell weight still keeps 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 finish experimental.

4.3   Effect of Temperature and Shaker speed

Experiment was carried out at three differences temperature (33˚C, 37˚C, 41˚C) with differences speed of incubator shaker (140 rpm, 180 rpm, 220 rpm). Firstly, the adaptable of *P. putida* condition at 33˚C was summarized in Table 4.2.

Table 4.2: Summary of the OD reading at 33 °C

Speed (rpm)	Concentration (ppm) OD Reading (%ABS)					
	3		5		7	
	Max	Min	Max	Min	Max	Min
140	0.243	0.163	0.337	0.300	0.485	0.400
180	1.500	0.168	0.372	0.319	0.380	0.319
220	4.000	0.160	0.343	0.328	0.548	0.509

From Table 4.2, at 140 rpm, the 7ppm mercury solution has higher growth of *P. putida* with OD value of 0.485 and minimum value represent in 3 ppm mercury solution. At 180 rpm, 3 ppm mercury solution indicates maximum OD reading (4.00) and the minimum OD reading (1.68). At 220 rpm, 3ppm mercury solution shows maximum reading of OD value with 4.00 and minimum OD reading with value of 0.16. Concentration mercury at 3 ppm was expected to have the highest mercury removal at 33 ˚C for rotatory shaker speed at 140 rpm and 220 rpm compare with 5 ppm and 7 ppm mercury.

Secondly, experiment was carried out at three differences temperature (33˚C, 37˚C, 41˚C) with differences speed of incubator shaker (140 rpm, 180 rpm, 220 rpm) with the adaptable of *P. putida* condition at 37˚C was summarized in Table 4.3.

Table 4.3: Summary of the OD reading at 37 °C

Speed (rpm)	Concentration (ppm) OD Reading (%ABS)					
	3		5		7	
	Max	Min	Max	Min	Max	Min
140	0.223	0.165	0.388	0.290	0.570	0.467
180	0.215	0.141	0.283	0.142	0.345	0.223
220	0.500	0.168	0.283	0.079	0.261	0.178

From Table 4.3, at condition 37˚C, the growth of *P. putida* shows the increasing in OD reading at almost all condition compare in 33˚C condition. For 3ppm mercury solution, the highest reading with 0.5 %ABS shows at 220 rpm shaker speed. For 5ppm mercury concentration, the highest OD reading shows at 140rpm shaker speed with value of 0.388 %ABS. in 7ppm mercury concentration, 140rpm shaker speed shows the higher reading of %ABS with value of 0.570. In overall, at 37˚C, the *P. putida* shows the best growth reading in 7ppm mercury concentration with the maximum OD reading of 0.57

and minimum of 0.178. The *P. putida* shows the decrease in growth with increasing the shaker speed in 5ppm and 7ppm mercury concentration.

Lastly, experiment was carried out at three differences temperature (33˚C, 37˚C, 41˚C) with differences speed of incubator shaker (140 rpm, 180 rpm, 220 rpm) with the adaptable of *P. putida* condition at 41˚C was summarized in Table 4.4.

Table 4.4: Summary of the OD reading at 41 ºC

Speed (rpm)	Concentration (ppm) OD Reading (%ABS)					
	3		5		7	
	Max	Min	Max	Min	Max	Min
140	0.279	0.180	0.343	0.160	0.542	0.513
180	0.231	0.179	0.301	0.225	0.336	0.265
220	0.363	0.179	0.337	0.268	0.363	0.217

From Table 4.4, at 41˚C, for 3ppm and 5ppm mercury concentration, *P. putida* growth shows the quite same adaptable in all condition of 140rpm, 180rpm and 220rpm shaker speed. In other side, the *P. putida* growth shows the better growth in 7ppm mercury solution with OD reading of 5.42 %ABS. *P. putida* shows decline in growth at 180 rpm at every differences concentration of mercury solution.

In general, by comparing Table 4.2, Table 4.3 and Table 4.4, at temperature of 33˚C and 37˚C, 3ppm mercury concentration was expected to have the highest mercury removal while 7ppm should be the lowest mercury removal.

Firstly, the adaptable of the *P. putida* growth at concentration 3ppm with different effect of temperature and shaker speed was summarized in Table 4.5. This point of view shows the capability of *P. putida* growth at specific concentration literally.

Table 4.5: Effect of temperature and shaker speed in 3 ppm solution

Temperature (°C)	Shaker Speed (rpm)	OD$_{600}$	
		Max	Min
33	140	0.243	0.163
	180	1.500	0.168
	220	4.000	0.160
37	140	0.223	0.165
	180	0.215	0.141
	220	0.500	0.168
41	140	0.279	0.180
	180	0.231	0.179
	220	0.363	0.179

Based on Table 4.5, the OD$_{600}$ reading maximum was represented at 33°C for 220rpm with reading of and the minimum represent at 180rpm for 37°C. On the other side, minimum readings only shows a slightly differences between each parameter. This shows that, the capability of the *P. putida* started to adapt were same for each stages. In average, the higher temperature showed the lowest adaptable of the *P. putida* and the lowest temperature shows the higher average of the *P. putida* adaptable.

Secondly, the adaptable of the *P. putida* growth at concentration 5ppm with different effect of temperature and shaker speed was summarized in Table 4.6.

Table 4.6: Effect of temperature and shaker speed in 5 ppm solution

Temperature (°C)	Shaker Speed (rpm)	OD$_{600}$	
		Max	Min
33	140	0.337	0.300
	180	0.372	0.319
	220	0.343	0.328
37	140	0.388	0.290
	180	0.283	0.142
	220	0.283	0.079
41	140	0.343	0.160
	180	0.301	0.225
	220	0.337	0.268

The adaptable of the *P. putida* in 5ppm mercury solution shows the intermediate OD$_{600}$ reading based on Table 4.6. There was no huge differences between each parameters as the maximum OD$_{600}$ was 0.372 (33 °C, 180 rpm) and the minimum OD$_{600}$

was 0.079 (37 ˚C, 220 rpm). Thus, it can say that, the *P. putida* can be adapted at any differences of temperature and shaker speed in 5ppm solution.

Lastly, the adaptable of the *P. putida* growth at concentration 7ppm with different effect of temperature and shaker speed was summarized in Table 4.7.

Table 4.7: Effect of temperature and shaker speed in 7 ppm solution

Temperature (˚C)	Shaker Speed (rpm)	OD_{600}	
		Max	Min
33	140	0.485	0.400
	180	0.380	0.319
	220	0.548	0.509
37	140	0.570	0.467
	180	0.345	0.223
	220	0.261	0.178
41	140	0.542	0.513
	180	0.336	0.265
	220	0.363	0.217

From Table 4.7, the maximum OD_{600} reading was 0.570 (37 ˚C, 140 rpm) and the minimum reading represent was 0.178 (37˚C, 220rpm). The differences between the minimum and maximum reading at each parameters show slightly differences at 33 ˚C and 41 ˚C, while at 37 ˚C there were more differences of the OD_{600} reading. This shows that, the *P. putida* was more adaptable at 37 ˚C in 7 ppm mercury solution.

4.4   Effect of *P. putida* in mercury removal

In study the effect of the mercury removal, the standard curve of the solution was done as a reference of reading the absorbance.

4.4.1   Mercury Standard Curve

The standard curve of the mercury solution most important to analyze the absorbance of the sample and subsequently calculated the percentage of the mercury removal at differences temperature and shaker speed.
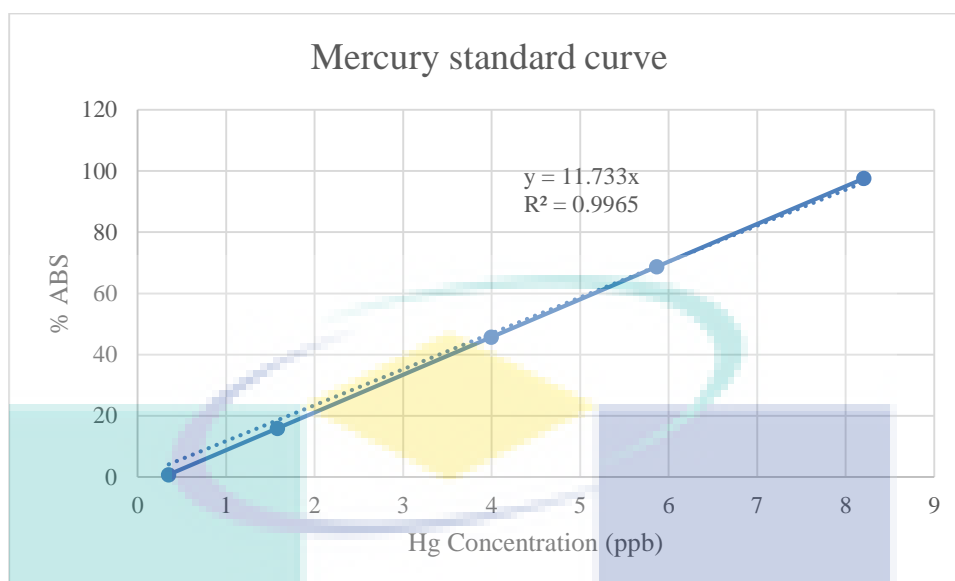
Figure 4.5: Graph of mercury standard calibration

Figure 4.5 show graph of mercury standard calibration showed the standard curve of the 5 differences concentration of mercury solution. The linear line of the standard curve plot having the value of R^2 of 0.9965. This shows that, the linear line was strongly positive value and was been acceptable as a reference. From the graph, the equation form was y = 11.733 X, that's mean as increasing 1ppb of mercury concentration, hence the absorbance value also increases by 11.733%. From the graph, we can relate that, the higher reading of %ABS the higher mercury concentration in the solution.

4.4.2   Percentage Mercury removal at differences temperature and shaker speed.

Firstly, the percentage of mercury removal in 3ppm solution is shows in Table 4.8. The percentage of the mercury removal is calculated based on the formula below:

% Removal = $(A^0 - A^1)/ A^0$ x 100

A^0 = initial concentration of Hg

A^1 = final concentration of Hg

Table 4.8: The percentage of mercury removal in 3 ppm solution

Concentration (ppm)	Shaker Speed	Temperature (˚C)	Average Removal (%)

3	140	33	75.29
		37	86.13
		41	51.25
	180	33	50.32
		37	72.54
		41	73.21
	220	33	78.00
		37	84.41
		41	80.25

From Table 4.8, the highest percentage of mercury removal in 3ppm solution showed at 140 rpm and 37 ˚C with 86 % of removal followed by 84 % at 220 rpm and 37 ˚C and 80 % at 220 rpm and 41 ˚C. The lowest percentage removal represents at 33 ˚C and 180 rpm with only 50.52 % removal.

The average of the removal was at the highest shaker speed 220 rpm, with more than 80 %. The average removal shows the lowest at 180 rpm, with less than 70 %. This shows that, the average preferred of mercury removal in 3ppm solution was at 220 rpm speed shaker, while the most preferred was at 37 ˚C and 140 rpm. Secondly, the percentage of mercury removal in 5ppm solution is shows in Table 4.9.

Table 4.9: The percentage of mercury removal in 5 ppm solution

Concentration (ppm)	Shaker Speed	Temperature (˚C)	Average Removal (%)
5	140	33	66.86
		37	71.47
		41	49.88
	180	33	53.44
		37	65.85
		41	86.40
	220	33	9.625
		37	53.83
		41	33.75

Table 4.9 shows the summary of the mercury removal at 5 ppm mercury solution with differences of the temperature and shaker speed. The highest removal represents at 41˚C and 180 rpm followed by 71.47 % at 37 ˚C and 140 rpm and 66.86 % at 33 ˚C and 140 rpm. The lowest percentage of mercury removal represent at 33˚C, 220 rpm with 9.625 %. The average removal was at 180rpm with an average of above 65 % closely

followed by 140 rpm condition. This shows that, there only a slightly difference of removal between of 140 rpm and 180 rpm shaker speed. Although that, the in specifically, the most preferred condition in removal of 5ppm mercury solution was at 41˚C and 180 rpm. Lastly, the percentage of mercury removal in 7ppm solution is shows in Table 4.10.

Table 4.10: The percentage of mercury removal in 7ppm solution

Concentration (ppm)	Shaker Speed	Temperature (˚C)	Average Removal (%)
7	140	33	54.75
		37	68.93
		41	60.13
	180	33	24.66
		37	57.94
		41	92.59
	220	33	25.68
		37	56.21
		41	12.59

Table 4.10 shows the summary of the percentage of mercury removal in 7 ppm solution. The highest removal was represented at 41 ˚C and 180 rpm with 92.59 % followed by 140 rpm with 68.93 % at 37 ˚C and 60.13 % at 41 ˚C. The lowest removal was represented at 41 ˚C and 220 rpm with 12.59 % removal. The average removal showed the condition of 140 rpm has more than 55 % percent removal followed by at 180rpm. There were only slightly differences in average, if compared in both condition, but the 140 rpm shows the closed removal at three differences temperature at constant speed compared with 180 rpm shaker speed. In specifically, the optimum condition for the 7 ppm mercury removal was at 180 rpm and 41 ˚C.

4.5   Enzyme activity and purification

For study the enzyme activity in mercury removal, the enzyme purification was done to determine the dominant types of enzyme produced.
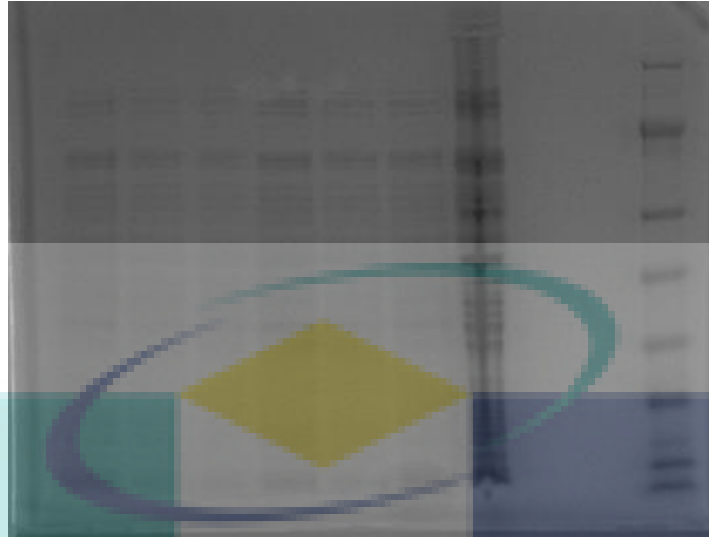
Figure 4.6: Indicator for the activity of mercuric reductase from polyacrylamide gel electrophoresis (PAGE)

Figure 4.6 shows the gel from Polyacrylamide gel electrophoresis (PAGE) which is used for separating proteins ranging in size from 5 to 120 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel. Care must be used when creating this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered forms. The samples of mercuric reductase from the same enzyme preparation which had been stored under different conditions were applied to 7.5% slab gels and electrophoresed as described under "Methods". This method does have limitations and for example, identification of a band on a protein gel is not considered positive proof of identity.

As recall in Table 3.1, the proportion of the mixture of mercury solution and the broth culture with *P. putida,* different proportion was used such as for the band labelled as 7 is the mixture with the highest volume of mercury solution in the proportion. Based on the intensity of the band, the sample 7 recorded as the most active enzyme compared to the other samples. Thus, it is proven that there are enzyme activities occur in all the samples but the samples 7 is the highest activities recorded.

Meanwhile, the addition of reducing agent helps to cleave protein disulfide bonds and ensure that no quaternary or tertiary protein structure remains. Consequently, when these samples are electrophoresed, proteins separate according to mass alone, with very little effect from compositional differences. Large ones can't get through the gel easily so they stay close to the top. All the samples have the same pattern as it digested downline through the electrophoresis gel. By the rightest side lane is the molecular weight markers (MW markers) or standards. The number 62, 49 and 3 kDa marked on the Figure 4-2 are the selected sets of reference proteins which are recorded as the estimated molecular weight of the protein. The line band form at 56kDa at line 1 to 6, where the mercuric reductase present. Mercuric reductase produced as an enzyme that react in mercury removal. S. Ghosh Et. (1999) said that the molecular weight of mercuric reductase present at 54kDa and 69kDa and Fox and Walsh (1982) the enzyme also compose at 56kDa and 62kDa. The presence of the mercury reductase shows that, there is an enzyme activity occurred in the mercury removal processed.

On the contrary, another pattern to be in the lookout is the condition of a preparation such as extent to which proteins have degraded. As we have observed in the Figure 4.2, all the samples show the fading band fragments. For an example, in Lane 6. As the band exist at the 62 kDa, as it moves downwards, the intensity of the band is reduced at it shows that the protein in this process is degraded and may giving a slight error for the reading.

Mercuric reductase is the enzyme produced, and it is a central enzyme in the organomercurial resistance system elaborated by many soil and enteric bacteria. It enables them to reduce organomercurials to volatile, elemental mercury and is thus relevant to an understanding of the biogeochemical cycles of mercury in the environment. In addition, an understanding of the structure and catalytic mechanism of this enzyme should provide molecular insights into the biochemical toxicology of mercury compounds.

4.6   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 mercury were able to be removed by *P. putida* and the enzyme produce is mercuric reductase.
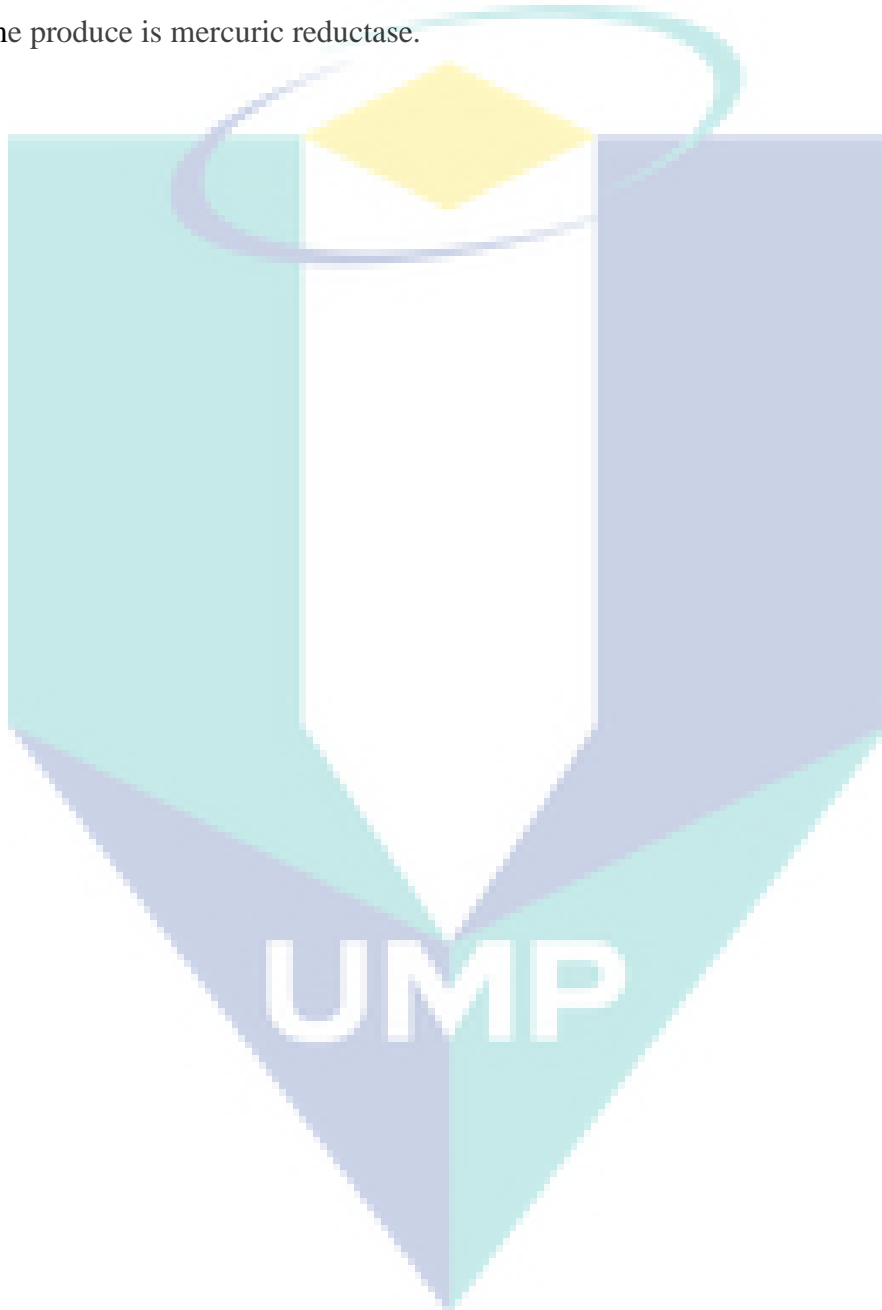
CHAPTER 5

CONCLUSION

5.1   Conclusion

In this study, the growth pattern of the *P. putida* shows four phase of growth which was lag phase, log phase, stationary phase and death phase. The log phase occurs between 3hr to 10hr.

From the study, the optimal condition of the mercury removal at differences concentration showed the difference specific condition in terms of temperature and shaker speed. For 3 ppm solution the optimum condition was represent at 37 ˚C and 140 rpm with 86.13% of mercury removal. In 5ppm solution the optimal condition represent at 41˚C and 180 rpm with 71.47% of mercury removal.

Lastly, for the 7 ppm solution, the optimal condition at 41 ˚C and 180 rpm with 92.59% removal was represented. It can be concluded that, the removal was preferred occurred at lower speed shaker as the result shows the optimal condition was at 140 rpm and 180 rpm optimal. Besides that, the higher temperature also more preferred in mercury removal as there was optimal at 37 ˚C and 41 ˚C was presented.

The mercury reductase also present in electrophoresis gel shows that there was enzyme produced during the processes. As there are an amount of mercury removal shows that there are also showed the activity of the enzyme occurred. The mercury reductase was found at 59 kDa molecular weight.

5.2 Recommendation for future works

P. putida has an ability to remove the mercury at higher temperature with the lower speed shaker. The extended research need to study in actual petrochemical wastewater to prove the capability of the mercuric reductase do its activity in mercury removal at the optimal condition.
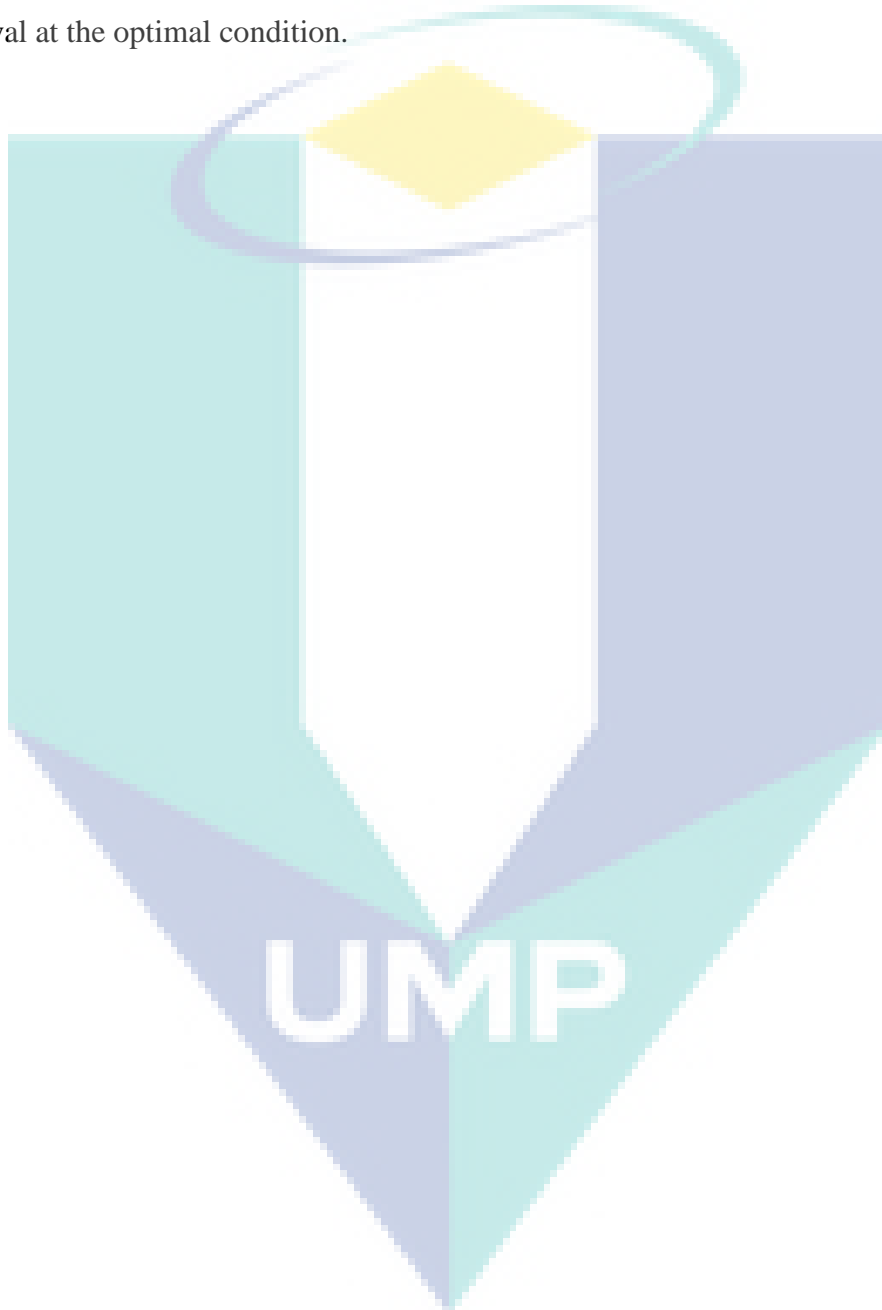
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ACHIEVEMENTS

Publications

1. Characterization of Enzyme Produced From *Pseudomonas Putida* for BTX (Benzene, Toluene & Xylene) Treatment in Petrochemical Industry Wastewater System
2. Assessing Storage of Stability and Mercury Reduction of Freeze-Dried *Pseudomonas putida* within Different Types of Lyoprotectant
3. Effect of Mercury Concentration on *P. putida* Growth in Mercury Removal
4. Study of enzyme purification method and growth pattern for *Pseudomonas putida* in mercury removal
5. Effect of Temperature on *Pseudomonas Putida* to Remove Mercury