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Mercury Removal Using *Pseudomonas putida* (ATTC 49128): Effect of Acclimatization Time, Speed and Temperature of Incubator Shaker

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***Abstract*—**Microbes have been used to solve environmental problems for many years. The role of microorganism to sequester, precipitate or alter the oxidation state of various heavy metals has been extensively studied. Treatment using microorganism interacts with toxic metal are very diverse. The purpose of this research is to remove the mercury using *Pseudomonas putida* (*P. putida*), pure culture ATTC 49128 at optimum growth parameters such as techniques of culture, acclimatization time and speed of incubator shaker. Thus, in this study, the optimum growth parameters of *P. putida* were obtained to achieve the maximum of mercury removal. Based on the optimum parameters of *P. putida* for specific growth rate, the removal of two different mercury concentration, 1 ppm and 4 ppm were studied. From mercury nitrate solution, a mercury-resistant bacterial strain which is able to reduce from ionic mercury to metallic mercury was used to reduce ionic mercury. The overall levels of mercury removal in this study were between 80% and 89%. The information obtained in this study is of fundamental for understanding of the survival of *P. putida* ATTC 49128 in mercury solution. Thus, microbial mercury removal is a potential bioremediation for wastewater especially in petrochemical industries in Malaysia.

Keywords*—Pseudomonas putida*, growth kinetic, biosorption, mercury, petrochemical wastewater.

I. Introduction

MERCURY pollution of the environment by mining activities and industrial petrochemical wastewater has resulted in worldwide contamination of large areas of soils and sediments [1]-[4], and let to elevated atmospheric mercury levels [5]. Because of lack of suitable cleanup technologies, efforts to deal with polluted sites are directed toward the mechanical removal of contaminated material and its deposition elsewhere [6], [7]. Such processes are costly and often result in remobilization of toxic mercury compounds during the dredging process.

Mercury is one of the most toxic elements. It binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions [8]. After discharge into the environment, mercury enters the sediments where it persists for many decades. It is taken up by aquatic organisms in the form of highly toxic methylmercury and is subsequently biomagnified through the food chain. The health of top predators, e.g. birds, fish, seals, and man, is thereby threatened [9], [10]. At high concentrations, mercury vapor inhalation produces acute necrotizing bronchitis and pneumonitis, which is lead to death from respiratory failure. Long term exposure to mercury vapor primarily affects the central nervous system. Mercury also accumulates in kidney tissues, directly causing renal toxicity, including proteinuria or nephritic syndrome [11]. Increasing of mercury concentration can cause impairment of pulmonary function and kidney, chest pain and dyspnousea [12]. Therefore, the discharge of mercury into the environment needs to be prevented by efficient and cost-effective end-of-pipe treatment technologies for mercury emitting industries.

Purification of areas polluted by heavy metals such as mercury is difficult, because the metals cannot be transformed into harmless elements. Over a few decades, community is devoting concentrated efforts for the treatment and removal of heavy metals in order to face this problem [13]. Various types of technology is available for removing of mercury in water and wastewater including chemical precipitation, conventional coagulation, reverse osmosis, ultrafiltration, magnetic filtration, ion exchange and activated carbon adsorption and chemical reduction [14].

Biological systems have been thought to be adapted for removal of toxic heavy metals from petrochemical wastewater [15]. Bioremoval is biological systems for removal of metals ion from polluted water has the potential to achieve greater performance at lower cost than nonbiological wastewater treatment [16]. Developments in the field of environment biotechnology indicate the bacteria, fungi, yeasts and algae can remove heavy metals from aqueous solution by adsorption [17].

In bacteria resistance to mercury is related to enzymatic reduction of Hg^{2+} to volatile Hg^0 [18]. Mercury detoxification process originated from *mer* operon located on either plasmids or transposable elements in the mercury resistant microorganisms. Specific transport of bulk mercury across the cell membrane is achieved by two *mer* operon genes *mer*P and *mer*T, which express cystein-rich protein to deliver ambient mercuric toward intracellular mercuric reductase for subsequent reduction of mercuric ions to volatile Hg^0 [19]. In the present investigation after isolation bacteria from

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petrochemical wastewater the ability of isolated bacterium, *P. putida,* has been assessed for removal, biosorption and up take, mercury ion.

II. Mathematical Modeling of the Kinetics Description

Cell culture systems are extremely complex. There many inputs and many outputs. Unlike most chemical systems, the catalyst themselves are self-propagating. To assist in both understanding quantifying cell culture systems, biotechnologists often use mathematical models [20]. A mathematical model is a mathematical description of physical system. A good mathematical model will focus on the important aspects of particular process to yield useful results. Chemical reaction can be simplified. For example, a first order chemical reaction in which mole of reactant (*S*) is converted to a product (*P*)

$$S = nP \tag{1}$$

Can be expressed as a differential equation of the form:

$$\frac{d[S]}{dt} = -k[S] \tag{2}$$

where [*S*] is the concentration of the reactant and *k* is a rate constant.

A differential equation describing product formation is:

$$\frac{d[P]}{dt} = -n\frac{d[S]}{dt} \tag{3}$$

or

$$\frac{d[P]}{dt} = -nk[S] \tag{4}$$

where [*P*] is the concentration of the product and n is the stoichiometric yield constant describing the relationship between the removal of *S* and formation of *P*. Note that as the concentration of *S* decrease, the concentration of *P* increases and hence the negative sign in (3) and (4). By solving these two equations, it is possible to predict the values of *S* and *P* at any time [20].

A. The Exponential Growth Curve

The rate increase cell (or biomass) is depending on the concentration of cells present in the reactor can be described as:

$$\frac{dX}{dt} \alpha X \tag{5}$$

where *X* is the concentration of biomass in flask. Biomass concentrations are typically expressed in g.L-1 of dry weight. The proportionality expression in (5) can be replaced with a constant, known as specific growth rate (μ). Equation (5) thus can be re-written as:

$$\frac{dX}{dt} = \mu X \tag{6}$$

Hence,

$$\mu = \frac{1dX}{Xdt} \tag{7}$$

where μ is the specific growth rate. This model of microbial growth referred to as the exponential growth model. The specific growth rate (μ) describes how fast the cells are reproducing. The higher the value of specific growth rate, then the faster the cells are growing. When cells are not growing, then their specific growth rate is zero. During exponential phase, the specific growth rate is relatively constant [20].

III. Materials and Methods

A. Materials and Equipment

P. putida (ATTC 49128), Mercuric nitrate (Hg $(NO3)_2$), Acid nitric (HNO_3), Nutrient broth (Merck: BD 234000) and Nutrient agar (Merck: BD 213020) were supplied by the Merck (Malaysia) Sdn. Bhd. The equipments required in this study are autoclave (H+P Varioklav Stream Sterillizer ESCO), Shaker (B. Braun, German, Microbiological Incubator, Mermmert-Germany/BE 600), Laminar Flow Cabinet (ESCO), Oven (Haeraeus), Mercury Analyzer (RA-3000 Spectrophotometer), UV-VIS (U-1800, Hitachi) and pH Meter.

B. Growth Media for the Microorganism

Bacteria *P. putida* used in this study was obtained from Merck (Malaysia) Sdn. Bhd as local agent dealing with the bacteria. The *P. putida* used is ATCC 49128 (freeze dried) sourced from Microbiologics, 217 Osseo Ave. North, St. Cloud, USA. The ingredients of *P. putida* were 5% of pepton meat and 3 % of extract meat. The media growth for bacteria was prepared by suspending 8g in 1 liter of demineralized water, if required dispense into smaller container. The media growth should be sterilized in an autoclave about 15 minutes at 121˚C.

C. Agar Preparation

Preparation for nutrient agar to the stock culture in test tubes is almost the same as the growth media preparation. The ingredient of agar was 5% of pepton from 3% of meat extract and 12% of agar. The nutrient agar was taken from Merck Sdn. Bhd. In preparing the nutrient for the stock culture, suspend 20g in 1 liter of demineralized water by heating in a boiling water bath or in a current of steam and autoclave about 15 minutes at 121˚C.

D. Method of Culturing Freeze-Dried P. putida ATTC 49128

P. putida (freeze dried) were cultured in nutrient broth (BD 234000), which was prepared by suspending 8 g nutrient broth into 500 ml deionized water in schott bottle and autoclave at 121^{o}C for 15 minutes. Freeze dried culture was removed from a thin skin of cellulose by soaking in water for a few minutes. Disinfect the ampule with alcohol after scoring with a sharp file and pouring to 0.5 ml nutrient broth with mixed well for forming a cell concentration [10]. Then all of these were

transferred to 5 ml nutrient broth to form a cell suspension and incubated at 30°C overnight. Few drops of this suspension was introduced to agar slant, nutrient broth and plate, and incubated at 30°C for 1 – 3 days to check the growth.

E. Stock Culture

Stock culture is very important to make sure a pure culture can be used for a long time period without losing or lacking any nutrient. A nutrient broth can only last for a few weeks and after that the culture start to decay based on nutrient depletion and accumulation toxic by product. A stock culture can be up to six month in the refrigerator [21].

Actually, there are several ways to transfer a culture from broth culture to an agar plants. The best technique is aseptic technique. It requires transferring the pure culture and maintaining the sterility of the media. First, the inoculating loop was flamed with the Bunsen burner until red hot to sterilize it. It was cooled down before entering the culture broth. Using free hand, the test tube that contains the pure culture is shaken to disperse the cells. The tube cap was removing using free fingers to hold sterile inoculating loop and the lip of the test tubes is also flamed using a Bunsen burner. The culture tube was held at a slanted position while the inoculating loop is inserting to the culture broth.

After that, the culture tube lip was flamed again and closed with its caps. After leaving the culture tube aside, using the free hand, a test tubes containing nutrient agar is opened and the lip was flamed. The inoculating loop, which contains a smear of culture, is inserted to the test tube and slide gently on the agar surface in a continuous streaking motion. The loop is also flamed after finishing using it. The test tubes containing pure culture on nutrient agar was incubated at 30°C in one day. It is then kept in the refrigerator to be used as stock culture.

F. Cultivation of Inoculum

To prepare the inoculum, a loop full of colony *P. putida* ATTC 49128 was picked from nutrient agar freshly grown culture was transferred 10 ml nutrient broth then were incubated at 30°C for 1-3 day. After 1-3 day, it was transferred to 250 ml inoculum flask containing 90 ml nutrient broth. Cells were grown at 30°C with vigorous shaking at 180 rpm. Samples of 10 ml were withdrawn an aseptically at regular time interval, 30 minutes for analysis using syringe. The cells were then centrifuged at 5000 rpm for 5 minutes, washed once with normal saline solution to give optical density (OD) at 600nm for *P. putida* ATTC 49128 growth monitoring.

G. Experimental Work

In this research, the parameters that will be going to observe are the effect of shaker speed on the bacteria growth, the effect of temperature shaker, the effect of time growth of *P. putida* in microbiological incubator and the effect of mercury on *P. putida* growth. Each set of experiment will be carried out in shake flask. The experiment will used 20ml of *P. putida* that growth one day before running the experiment. The bacteria will mix with 180ml of nutrient broth in conical flask.

1. Effect of Shaker Speed on the Bacteria Growth

In this experiment, three speeds of shaker will be used to identify the best condition for *P. putida* growth. The speeds are used at 140 rpm, 180rpm and 200 rpm. The operating temperature of this experiment is at 37°C and shake flask were shaken for 2 days.

2. Effect of Acclimatization Time of *P. putida* in Microbiological Incubator

In this experiment, the *P. putida* were grown in microbiological incubator for one day, two days and three days. The operating temperature of shaker that used was 37 °C and the shaker speed at 180 rpm for 2 days.

3. Effect of Temperature Shaker

In this experiment, effect of temperature shaker was studied. The shake flask were shaken at different temperatures which are 25°C, 30°C,33°C, 37°C and 42°C. The shake flask speed set at 180 rpm for 2 days. The chosen of different temperature is due to actual application in Malaysia climate.

4. Mercury Concentration Reduction by *P. putida*

In this experiment, stock of 1 ppm and 4 ppm of mercury nitrate were prepared. The stock solution were prepared about 1 liter. Then 180 ml of stock solution was added to shake flask that containing 20 ml of *P. putida*. After that, the samples were shaken at 180 rpm. The operating temperature of shaker that used is 37°C.

H. Mercury Measurement

In this experiment, the effect of *P. putida* to mercury was determined using mercury analyzer. Before the sample was measured by mercury analyzer there are several steps that must be taken carefully due to sensitivity of instrument. The maximum concentration of mercury that can be measured was 15 ppb. So the sample that used must be diluted to avoid the damage of mercury analyzer.

A solution containing hydrogen sulphate (97%) and ultrapure water was prepared with 1:1. 40 ml hydrogen sulphate (97%) was measured and mixes with 40 ml ultrapure water using measurement cylinder. The solution was poured into cleaned glass bottle. A mixture of stanum chloride ($SnCl_2$) and hydrogen sulphate (H_2SO_4) was prepared. After that, 2g of stanum chloride ($SnCl_2$) is weighted in beaker using electronic weight measure. 19 ml ultrapure water and 1ml hydrogen sulphate (97%) is poured into the beaker. The mixture was stirred till the solid stanum chloride dissolved using glass rod. 10ml of sample was poured into test tube and using micropipette, 250 microlitre of stanum chloride mixture and hydrogen sulphate (1:1) was measured. Both measured solutions were added into sample.

The test tube was plugged into the Mercury Analyzer test tube's socket. Samples are labelled and the start button within the software is clicked. Finally after 180 seconds, the concentration of sample result is recorded in ppb unit.

IV. Results and Discussion

A. The Effect of Acclimatization Time of P. putida in Microbiological Incubator

In this experiment, time growth of *P. putida* in microbiological incubator was investigated. Growth and culture of *P. putida* in microbiological incubator were taken for one day, two day and three day. Fig. 1 shows the typical growth curve for *P. putida* population after transferring inoculums process in Microbiological Incubator for one day.

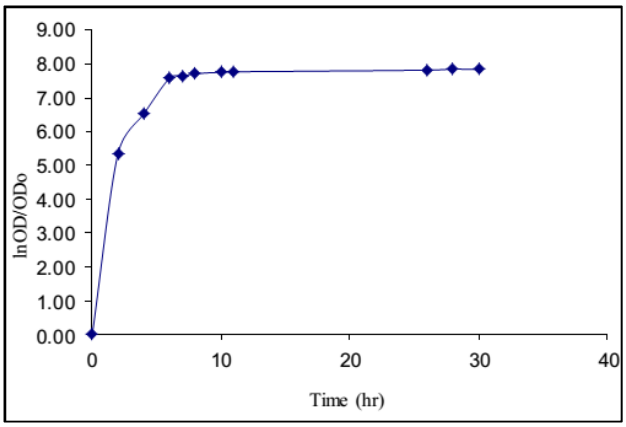

Fig. 1 Growth of *P. putida* in microbiological incubator in one day

Fig. 1 shows that *P. putida* can adapted to new environment in only one day after inoculums process. In lag phase, *P. putida* growth immediately after inoculation and is a period of adaption of cells into a new environment. *P. putida* organizes their molecular constituents when they are transferred to a new medium. During this period, cells increased slightly. In five hours, most of the bacteria showed and increased their cell density. This phase was called exponential phase where the *P. putida* has adjusted to their environment and multiply rapidly. The comparison of effect of time growth of *P. putida* in microbiological incubator is shown in Fig. 2.

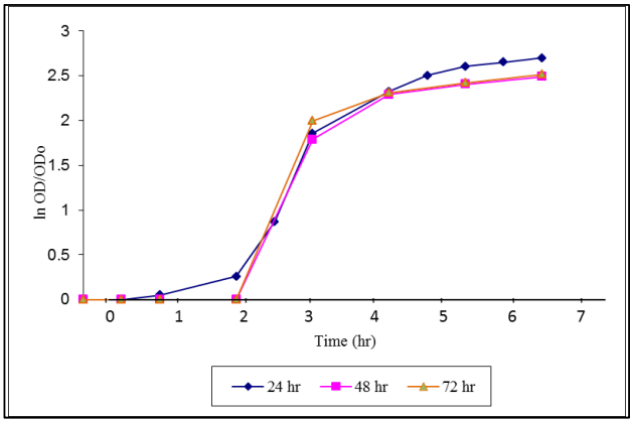

Fig. 2 Effect of acclimatization time on *P. putida* growth

In this experiment, the specific growth rate, μ, for the different acclimatization times were determined. The specific growth rate, μ represents the average growth rate of all cells present in a culture, but not necessary the maximum specific growth rate of individual cells, as most microbial cultures are divided asynchronously [22]. In this case the specific growth rate, μ of *P. putida* obtained from the experiments are 0.58 hr^{-1} for 24 hours acclimatization, 0.56 hr^{-1} for 48 hours acclimatization and 0.56 hr^{-1} for 72 hours. For the different acclimatization times of 24 hours, 48 hours and 72 hours, as shown in Fig. 2. Based on analysis of the effect of acclimatization times on the growth of *P. putida* and the behavior during the different growth phase as well as the growth related parameters, it can be concluded that *P. putida* requires 24 hours only to acclimatize to a new environment in order to achieve optimum growth and will minimize the time required to grow.

B. The Effect of Shaker Speed

Investigating the effect of shaker speed to the growth rate at different shaker speed, the shake flask was shaken horizontally in the incubator shaker at 37°C at 140, 180 and 200 rpm. The shaker speed between 140 and 200 rpm was chosen because the best conditions of *P. putida* to growth were in that range. Fig. 3 illustrated the effect of shaker speed on *P. putida* growth measured by optical density (OD). At high shaker speed the growth of bacteria increased more in exponential phase. The growth of *P. putida* is important in this phase because *P. putida* has potential to remove mercury and *P. putida* will achieve maximum rates of reproduction as well.

The effect of shaker speed is increasing the interfacial area between the media and the microbe in the aqueous phase [23]. Shaker speed is actually related to the mass transfer area for the bacteria; higher shaker speed has resulted higher area of mass transfer, and as a consequence, higher rate of microorganism growth is obtained. Mass transfer area actually provides a medium for the bacteria to react, grow and metabolize [24].

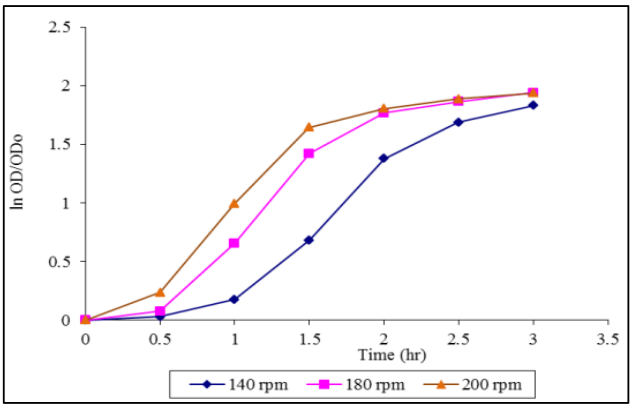

Fig. 3 Effect of shaker speed on *P. putida* growth measured by optical density (OD)

For shaker speeds of 180 and 200 rpm, the growth of P. *putida* increased more rapidly. At high shaker speeds, the growth of bacteria increased slightly higher in exponential

phase. The specific growth rate, μ of *P. putida* for 140 rpm shaker speed is 1.04 hr-1, 1.17 hr-1for 180 rpm and 1.07 hr-1 for 200 rpm. From the specific growth rate and parameters related to growth of *P. putida* results, it can be concluded that the shaker speed at 180 rpm is the optimum speed for *P. putida* growth [25].

C. Effect of Temperature Shaker

In this experiment, the effect of temperature shaker was investigated. The shaker speed that used was 180 rpm. Shake flask were shaken at 25°C, 30°C, 33°C, 37°C and 42°C. Fig. 4 shows the effect of temperature shaker on *P. putida* growth measured by optical density (OD).

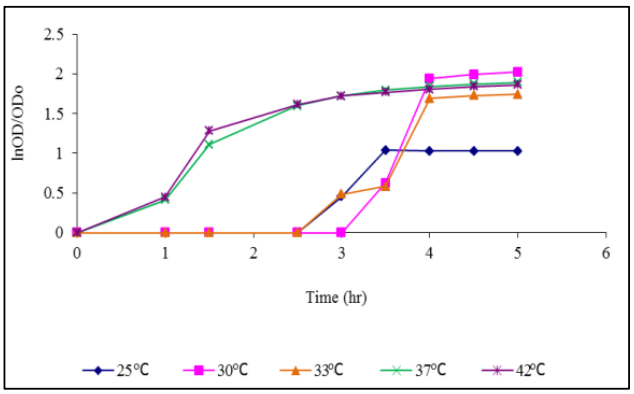

Fig. 4 Effect of different temperature shaker on *P. putida* growth measured by optical density (OD)

Fig. 4 shows that higher growth of *P. putida* was achieved at 37°C. At the lag phase, both temperatures 37°C and 42°C, give the value of optical density (OD) and show that *P. putida* growth faster in this temperature. This phase occurs immediately and it is a period of adaption of cells into a new environment.

To better understand the growth rate in this experiment, the specific growth rate, μ for the different temperatures were determined. The highest specific growth rate, μ of 0.25hr^{-1} is observed when the temperature is 37^{o}C. In addition, it is found that the durations required for *P. putida* to adapt to the environment which is at different temperature are as follows: where at, 2.50 hours (25^{o}C), 3.00 hours (30^{o}C), 2.50 hours (33^{o}C), 1 hour (37^{o}C and 42^{o}C). Thus, 37°C is a suitable temperature to be applied for petroleum based industries wastewater treatment as this temperature is more economical to use and is the same as the temperature of the surrounding environment in countries with tropical climate like Malaysia. In addition, *P. putida* has the potential to be commercialized at petroleum based industries wastewater treatment plant without needing additional cost for heating or cooling system [26], [27].

D. Removal Mercury Using P. putida

In this experiment, the effect of *P. putida* on mercury was investigated. Fig. 5 shows the effect of *P. putida* to mercury in 1 ppm. The experiment was compared with bacteria growth and the reduction of mercury using mercury analyzer. The graph shows that the mercury was removed and the percentage of reduction is about 89% for two days. From this result it shows that mercury Hg 2+ volatile to Hg0. The final value of mercury concentration was about 0.001 ppm. The safety limit in wastewater is about 0.005 ppm. So, it proof that the mercury can remove with high efficiency using *P. putida*.

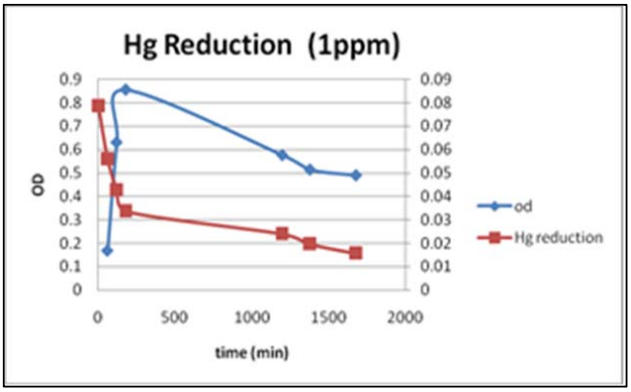

Fig. 5 Effect of *P. putida* to mercury in 1 ppm

Fig. 6 shows that mercury concentration at 4 ppm also reduced effectively using *P. putida*. The mercury increased when *P. putida* growth in exponential phases. In this phases, *P. putida* produce highly flavoprotien that contains mercury reductase. In this condition, the Hg $^{2+}$ will volatile to Hg ^{0} because of this flavoprotein. From this experiment the percentage of mercury removal was about 80%. Therefore, *P. putida* required more time to remove mercury in higher concentration, 4 ppm.

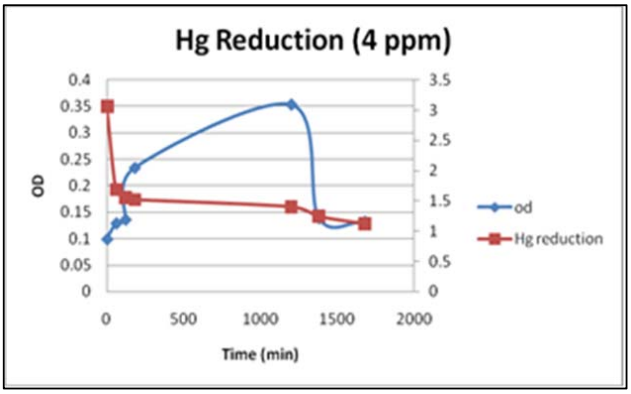

Fig. 6 Effect of *P. putida* to mercury in 4 ppm

From both results obtained, the optimum time required to remove mercury was approximately 1100 minutes and less than 500 minutes for 1ppm.

V. Conclusion and Recommendations

In this study, the growth of *P. putida* was obtained to achieve the maximum of mercury removal. In this research the growth of *P. putida* should be observed correctly because the

maximum growth of *P. putida* will increased at the optimum condition such as acclimatization time is 24 hr, shaker speed is 180 rpm, and temperature of shaker at 37^{o}C.

Removal of mercury using *P. putida* was successfully obtained. It has been shown that high-efficiency removal of mercury by mercury-resistant bacteria, *P. putida* in laboratory study. The overall levels of mercury removal in this study were between 80% and 89%, which indicated that the microbial detoxification system for mercury was highly effective under these conditions.

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