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Study of Enzyme Purification Method and Growth Pattern for *Pseudomonas putida* in Mercury Removal

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Abstract: Mercury is one of the toxic elements and very harmful. The mercury exposure will mainly cause health effect, and the exposure can be in terms of dose, the age of person exposed, duration exposed route exposed, and duration of exposure. Mercury is one of the heavy metals of concern in Malaysia, 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 implemented with the Department of Environment of Malaysia (DOE), which is the mercury concentration should be 0.05 ppm for standard B in wastewater for people and environmental health. There are many types of mercury removal technology to reduce the concentration of mercury, such as chemical treatment, ion exchange, membrane filtration, adsorption, and bioremediation used for mercury removal in this study. This paper present the activity of enzyme from *Pseudomonas putida* (*P. putida*) is produced in the mercury treatment from petrochemical wastewater at optimum condition. *P. putida* growth at the optimum condition in the different mercury concentrations was observed. The conclusion of optimum condition concentration of mercury and shaker speed at 7 ppm is 41°C and 180 rpm, respectively, with the ability to reduce 92.59% mercury concentration. During the processes of mercury removal, mercuric reductase was produced at a value of 56 kDa.

Keywords: *Enzyme purification, growth pattern, P. putida, mercury removal*

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I. INTRODUCTION

Mercury is a trace component of all fossil fuels including natural gas, gas condensates, crude oil, coal, tar sands and other bitumen [1]. These other paths may provide mercury directly to air, water or solid waste streams in an oil refinery and petrochemical industries. Furthermore, the dispersion and transformation of mercury in production, processing, and transportation are considered relative to the determination of mercury in air emissions, wastewater, and products from oil and gas processing facilities [2]. The disposal of wastewater that contains

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mercury should follow the DOE regulation which is 50 ppb concentration [3]. The higher concentration of mercury will cause the fish and shellfish to contaminate with mercury. The past research shows that the mercury level in fish is 0.0070.914 μg/g [4]. The mercury level in the fish shows the closed relationship with the concentration of the mercury in the river. Besides that, the previous study showed that the mercury concentration at the surface of the water is >3.0 ppm [5, 6, 7].

The genus Pseudomonas has been heterogeneous since Migula first named it in 1894. Pseudomonas a gram-negative, strictly aerobic, a polar flagellated rods [8, 9]. *P. putida* was a rod-shape, flagellated and a gram-negative bacterium that is found in most soil and water habitats. *P. putida* has the most genes involved in breaking down aromatic and aliphatic hydrocarbons which are hazardous chemicals produced by burning fuel, coal, tobacco, and other organic matter. Most pseudomonas is free-living saprophytic organisms in soil or water where they play an important role for 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 [7]. The flavoprotein mercuric reductase (reduced NADP: mercuric ion oxidoreduction) is an important component of an organ mercurial detoxification system found in many bacteria [10]. This enzyme was functioning to remove or reduce the 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 [11]. The enzyme was purified by native gel electrophoresis. The weight of the mercury reductase was found at 54 kDa for the major band and 69 kDa for the minor band. This enzyme is soluble in cytosolic flavoprotein that contains both flavin adenine dinucleotide (FAD) and an active redox disulfide that catalyses the reaction [10]. In the other study, the mercuric reductase also found at 59 kDa and 62 kDa [11].

II. MATERIAL AND METHODS

A. Microorganism

Bacteria *P. putida* used in this study was acquired from Merck (Malaysia) Sdn. Bhd as a local agent dealing with the bacteria. *P. putida* used is colony stock from the laboratory.

B. Chemicals

The chemical used in this experiment is divided into three parts. Firstly used in sample and standard preparation are 1000 ppm mercury (Hg (NO_3)), 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 SDS page reducing agent. Lastly, in terms of apparatus cleaning, the HNO_3 and 70% of Ethanol was used.

C. Methods

The experiments carried out in this study divided into three main stages. The first stage is carried out to determine the growth pattern and dry cell weight of the *P. putida*. The second stage is to determine purification and activity enzyme from *P. putida* produced. The last stage is to determine mercury removal at 7 ppm.

D. Preparation of the Bacteria Growth

First, all the apparatus is sterilized by autoclaving in an autoclave. Secondly, as the needed apparatus and are ready in the biohazard laminar flow, the inoculating loop is sterilized using a flame from a Bunsen burner until it is red hot. Cooled down the loop before streaking on the surface of the agar culture. Then, the inoculating loop that contains a smear of culture is immersing into the bottle sample. The bottle sample containing the pure culture in nutrient broth is then incubated at 37°C for 24 hours. After 24 hours, added 20 mL of the broth culture into 180 mL of the broth solution to determine the growth curve pattern. The solution is then placed in an incubator shaker at 37°C with 180 rpm speed.

E. Determine the Bacteria Growth and Dry Cell 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 [12, 13]. The data was recorded at 1-hour interval for first 6 hours, 2-hour interval (4 reading), and 4-hour interval until the bacteria death. After it undergoes UV-Vis spectrometer test, 1.5 mL of the solution was put into the centrifuge tube (weigh the centrifuge tube before use). The solution was centrifuged 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 recorded.

F. 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 centrifuged

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 shown in Figure 3, remove the solution (used for mercury removal test). Then, rinse with 1 mL of the phosphate buffer solution by centrifuge at 12,000 rpm, the 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 the 4°C for 10 minutes. Take out 1.0 μL of the sample supernatant added with 2.5 μL of NuPAGE LDS Sample Buffer (4×), 1.0 μL of NuPAGE Reducing Agent (10×) and the rest are distilled water to make total volume 10.0 μL. The sample is centrifuged 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 stained for 24 hours before view under the bio-imaging equipment.

G. *Mercury Removal*

The samples centrifuged before (from enzyme purification), are filtered again to remove the remaining microorganism using 4.5μ Polytetrafluoroethylene (PTFE) filter. 5 ml of the pre-digested samples were transferred into the sample tubes. 200 ţL 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 starting the measurement analyzer. As the sound of the buzzer heard, it signals of the end of the analysis. The impinger cap was removed and rinses the bubbler thoroughly with DI water.

III. RESULTS AND DISCUSSION

A. *Growth Pattern and Dry Cell Weight of P. putida*

The growth and cell dry weight method were studied to determine the growth pattern of the *P. putida* at the condition of 37°C and 180 rpm. At 37°C *P. putida* were adaptable with new environment as well as 180 rpm was chosen due to the highest optical density value. These conditions were known as the optimum condition [13].

B. *P. putida Growth Curve*

The growth pattern was determined to express the capability of the *P. putida* to growth and determine the growth rate at the point. The bacteria growth basically divided into four stages which are lag phase, log (exponential) page, stationary phase and death (decline) phase as shown in Figure 1. % ABS shows the present or living bacteria at certain time. The growth pattern of the *P. putida* is quite different from the standard growth of bacteria [13].

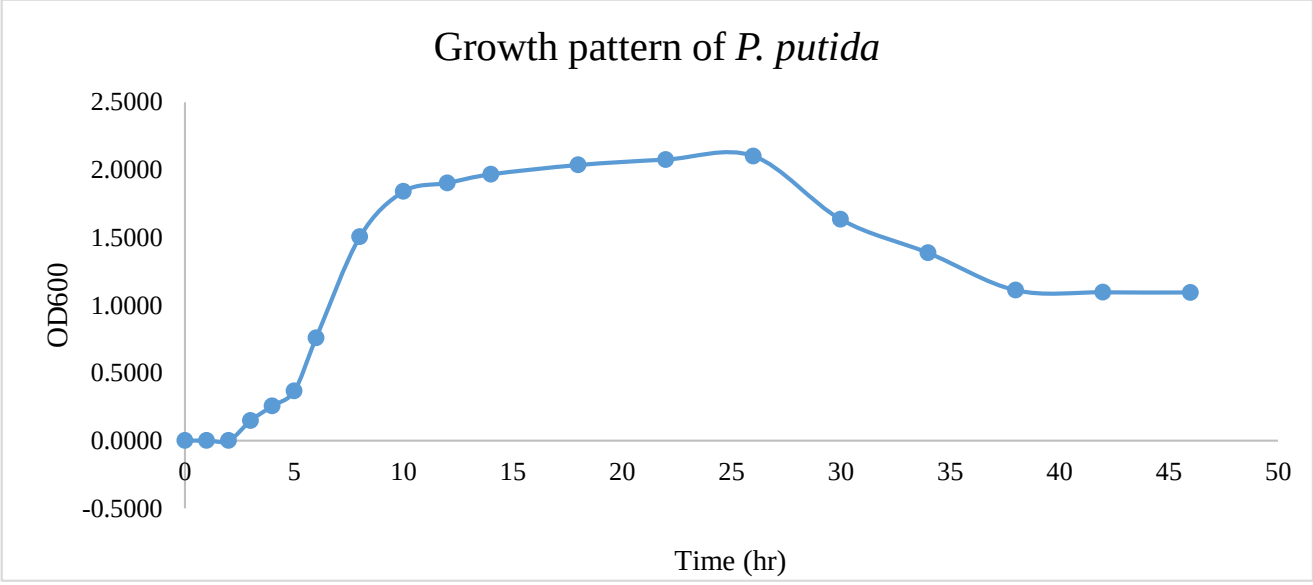

Fig. 1. *P. putida* growth curve at 37°C and 180rpm

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 condition is 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. Then, as the processes oc-

curs in bath culture, the nutrient supply reaches its limits and starts to degrade. During this stationary phase, the growth rate is equal to the 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, the decreasing of the % ABS reading show 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.

C. *Cell Dry Weight*

Figure 2 shows that the mass of cell dry weight is proportional to the time of the experimental. The increasing of cell dry weight is also affected by the growth that occurs during the exponential and stationary phase. During the exponential and stationary phases the cell weight keeps increasing due to the generation on new living bacteria through reproduction instead of the amount of waste and by-product that is produced. Meanwhile, during the death phase, the growth rate is decreasing but the cell weight still keeps increasing due to the culture of bacteria that occur in the batch process. Thus the amount of mass will keep remaining in the batch process until the experimental end.

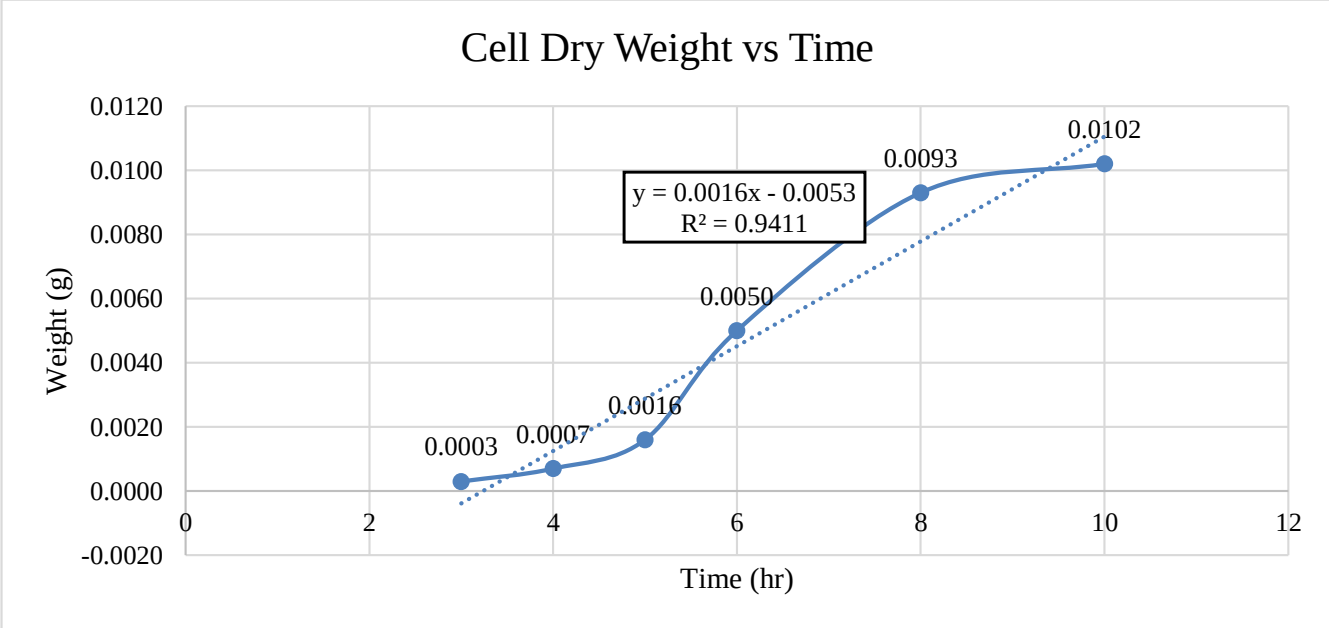

Fig. 2. Cell dry weight of *P. putida*

D. *Mercury Removal by P. putida*

Table 1 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 slight 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. In the previous study, mercury concentration was used in the range of 3 ppm and 5 ppm [13]. However, the average percentage mercury removal is lower than 7 ppm. Thus, the mercury concentration at 7 ppm was chosen in this study, which shows the highest percentage of removal, i.e. 92.59%.

E. *Enzyme Activity and Purification*

For this study, the enzyme activity in mercury removal, the enzyme purification was done to determine the dominant types of enzyme produced. Figure 3 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 applied while

making this sort 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.

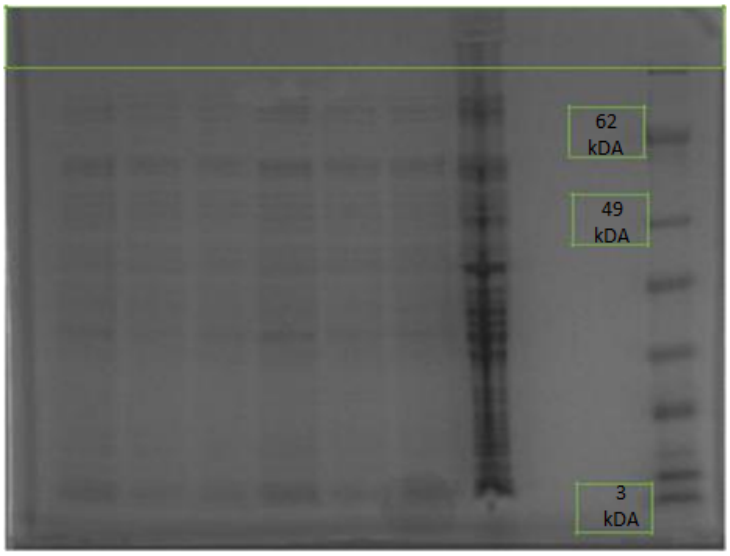

Fig. 3. Indicator of the activity of mercuric reductase from Polyacrylamide Gel Electrophoresis (PAGE)

F. Mercury Removal by P. putida

From Table 1, the proportion of the mixture of mercury solution and the broth culture with *P. putida*, the different proportion was used such as for the band labeled as 7 is the mixture with the highest volume of mercury solution in the proportion. Based on the intensity of the band, 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.

TABLE 1
REMOVAL OF MERCURY AT CONCENTRATION 7 PPM

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

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 Figure 3 is the selected sets of reference proteins which are recorded as the estimated molecular weight of the protein. The line band is formed at 56kDa at line 1 to 6, where the

mercuric reductase present. Mercuric reductase produced as an enzyme that reacts in mercury removal. Felske et. al. (2003) said that the molecular weight of mercuric reductase present at 54kDa and 69kDa and the enzyme also compose at 56kDa and 62kDa [10]. 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 Figure 3, all the samples show the fading band fragments. For example, in Lane 6. As the band exists 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 give 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 allows them to reduce organomercurials to volatile, elemental mercury and is thus relevant to an understanding of the biogeochemical cycles of mercury in the environment. Moreover, an understanding of the structure and catalytic mechanism of this enzyme should provide molecular insights into the biochemical toxicology of mercury compounds.

IV. 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. The mercury reductase also present in electrophoresis gel shows that there was enzyme produced during the processes. As there is 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. 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 a 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.

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