

Biochemical Properties of Mercuric Reductase from Local Isolate of *Bacillus sp* for Bioremediation Agent

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ABSTRACT

Mercuric reductase is the important enzyme which catalyzes a reduction of a toxic Hg^{2+} to non-toxic Hg^0 . The enzyme which has been potentially used as mercury bioremediation agent is produced by mercury resistant bacteria. These research aims are to determinate the resistance level of a local *Bacillus sp* to $HgCl_2$ in media, to determine the mercuric reductase activity from the bacteria, and to determine the biochemical properties of the mercuric reductase. The *Bacillus sp* was grown in the *Nutrient Broth* media with various of 0; 20; 40; 60; 120; and 160 μM $HgCl_2$ to know the response of the bacteria against mercury, The cell growth of *Bacillus sp* was measured by *optical density* (OD) method of λ 600 nm. The mercuric reductase activity was assayed in the solution of MRA (*Mercury Reductase Assay*), then the oxidized NADPH was observed by the spectrophotometry method at λ 340 nm. The result showed that the *Bacillus sp* has been resistant to media containing mercury at 120 μM , but the microbial growth was decreased by 50% in media containing mercury 80 μM . The *Bacillus sp* could produce highly the mercuric reductase enzyme at 16 hours of growth time with enzyme activity as 0.574 Unit/ μg . The mercuric reductase from the bacteria has an optimum activity at pH 6 and temperature 37 °C

Key words: *Bacillus sp.*, mercuric reductase, bioremediation

INTRODUCTION

The development of industry in Indonesia is so rapid, then it followed by the addition of the amount of waste in the environment. Waste on environmental pollution contains toxic chemicals, one of which is heavy metal mercury (Hg). The Government of Indonesia gives the mercury contamination threshold through Government Regulation of Republic of Indonesia number 82 the year 2001 for group I equal to 0.001 mg/L, group II is 0.002 mg/L, group III is 0.002 mg/L, while group IV is 0.005 mg/L. Various methods are used to reduce the mercury contamination from nature. One of them with the method of bioremediation which represent as a technique using microorganisms to reduce pollutants from environments. The mercury resistant bacteria could growth well on the medium containing mercury more than 5 mg/L (De Jaysankar, Ramaiah, Mesquita, and Verlekar, 2003; Adriana, Michele, Michele, Josino, and Ana, 2008). The potency of the bacteria is important to be used as a microbial bioremediation agent to clean mercury contaminant in environments.

The ability of mercury-resistant bacteria can produce mercuric reductase which is an enzyme with activity in the reducing of Hg^{2+} to Hg^0 . This case facilitates the bacteria can survive to a toxic of ionic mercury (Hg^{2+}) in the (Nascimento, and Charton-Souza, 2003). Mercury-resistant bacteria can reduce mercury ions by involving mercuric reductase enzymes and NADPH via the redox reaction equation (**Figure 1**).

Some bacteria have been reported to produce mercuric reductase enzymes are *E. coli*, *Acinetobacter*, *Enterobacteriaceae*, *Pseudomonas sp.*, *Xanthomonas sp.*, *Bacillus sp.*, and *Staphylococcus sp.* (Zeroual, Moutaouakkil, Dzairi, Talbi, and Chung, 2003). The *Bacillus sp.* has been isolated from Malang agricultural, but its potency has been unknown to produce the mercuric reductase enzyme. The research was developed to observe the mercury resistance level of the bacteria. In addition to detect the properties of mercuric reductase enzyme which is produced by the bacteria as a bioremediation agent to reduce mercury waste.

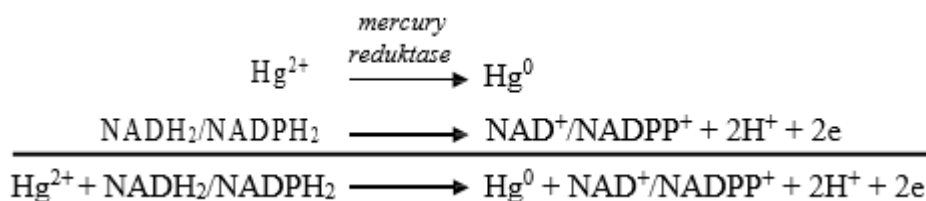


Figure 1. Redox reaction mercury ions by involving mercuric reductase enzymes and NADP

According to the classification of enzymes internationally, mercuric reductase is an enzyme in the class of oxidoreductase that is an enzyme that catalyzes the electron transfer reaction (Lehninger, 2004). There are four types of enzymatic mechanisms associated with mercuric reductase, namely: (1) reducing Hg^{2+} to Hg^0 ; (2) break down the organomercury compound (including MeHg^+), which results in the form Hg^0 ; (3) methylation of Hg^{2+} , and oxidation of Hg^0 to Hg^{2+} . The process of reduction of Hg^{2+} by mercuric reductase is done by coenzyme NADPH and NADH (Barkay, Miller, and Summer, 2003; Møller, Barkay, Hansen, Norman, and Hansen, 2014). The mechanism of action of mercuric reductase is carried out by the transfer of electrons from NADPH via FAD to reduce the active part of the cysteine. The active cysteine is converted into two shaped cysteine forms of SH function. One of the forms of cysteine forms a complex transfer charge with FAD, and the active part of cysteine reduces Hg^{2+} to C-terminal cysteine, forming Hg^0 (Lian, Guo, Riccardi, Dong, and Parks, 2014); Freedman, Chengsheng, and Barkay, 2012).

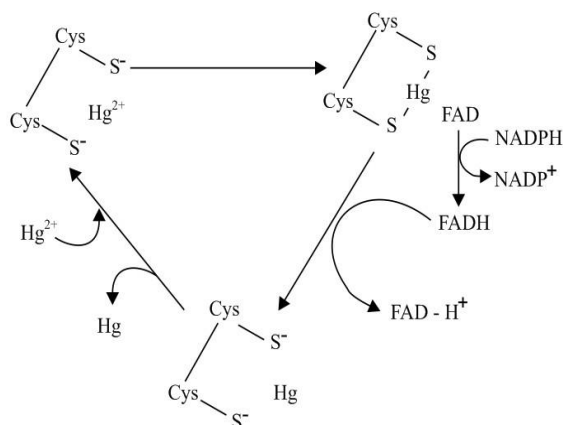


Figure 2. Mechanism of mercuric reductase action (Lian, et. al.,2014)

EXPERIMENTAL SECTION

Chemicals and Instruments

Bacillus sp. used is obtained from the agricultural land of Malang. Nutrient Agar (NA) and Nutrient Broth (NB), HgCl_2 , EDTA,

NADPH, β -Mercaptoetanol, MgSO_4 , CH_3COONa , CH_3COOH , Na_2HPO_4 and NaH_2PO_4 growth medium. The instrument used in this research are autoclave, micropipette, pH meter, thermometer, laboratory commonly used glass equipment, UV-Vis spectrophotometer Shimadzu UV-1800, batch ultrasonicator, shaker, incubator, Laminar Air Flow Kottermann 8580, centrifuge (Beckman 201).

Procedures

Culturing of Bacteria

The *Bacillus sp.* was grown on Nutrient Agar (NA) media composed by 0.3% (b/v) Beef extract and 0.5% (b/v) peptone, then incubated for 24 hours at 37 °C to achieve optical density (OD) 0.1 at λ 600 nm and then used as a starter.

The HgCl_2 Resistance Assay

One percent (v/v) of starter of *Bacillus sp.* cultured in 20 mL Nutrient Broth (NB) medium containing HgCl_2 in various concentration of 0; 20; 40; 60; 120; and 160 μM . The cultures then incubated at 37 °C with shaking at 150 rpm for various time of 0, 8, 16, 24, and 48 hours. The grown of bacterial cells were calculated by the method of turbidimetry at λ 600 nm.

Preparation of Growth Curve

One percent (v/v) of *Bacillus sp.* was cultured in 50 mL of Nutrient Broth (NB) medium without HgCl_2 and with HgCl_2 . Subsequently the culture was incubated at 37 °C at a rate of 150 rpm with shaking. Every four hours of cell suspension was measured OD at λ 600 nm, then plot the absorbance value to be a graphic

Isolation of Mercury Reductase Enzymes

The cell suspension obtained during cells harvesting was centrifuged at 9000 rpm, 4 °C for 20 min. The supernatant was discarded, then the cell was resuspended in 30 mL buffer Na-phosphate pH 7. The cell suspension was then sonicated for 60 seconds with an ultrasonicator batch (2% power, 50Hz frequency). After that, it was centrifuged at a

rate of 9000 rpm for 30 min at 4 °C. The supernatant is transferred into a clean and sterile bottle carefully. The resulting supernatant is a crude extract of the mercuric reductase enzyme (Lusiana, 2014; Kannan, and Krishnamoorthy, 2006).

Protein Content Assay

Determination of protein content is done by using Bradford method. The test was performed by adding 80 µL crude extract enzyme with 500 µL of Bradford reagent into ten mL measuring flask and then adding with distilled water to the boundary marker, incubated at room temperature for 10 minutes. The protein content can be determined by the wavelength of λ 595 nm (Bradford, 1976). The protein content can be determined by calculating based on the absorbance value of the sample entered into the BSA standard curve regression equation.

Mercury Reductase Enzyme Activity Test

Three mL of MRA (Mercury Reduction Assay) solution which composed by 50 mM of Na_3PO_4 buffer (pH 7), 0.5 mM EDTA, 0.2 mM MgSO_4 , 0.1% (v/v) β -mercaptoethanol, 0.1 mM NADPH was reacted with 1 mL 80µM HgCl_2 and 0.1 mL of crude enzyme, incubated at 37 °C for 15 min, then residual of NADPH was measured at λ 340 nm. A unit of mercuric reductase is defined as the number of enzymes that oxidize µM NADPH per minute in experimental condition (Zeroual et al. 2003; Purkan, Nurmalyya, and Hadi, 2016)

Determination of pH and temperature optimum of enzyme activity

The mercuric reductase enzyme extract was assayed its activity at different pH and temperature. The 50 mM sodium phosphate buffer was used to adjust pH 6-8. Whereas the pH five was adjusted with acetate buffer. The range temperature which used to optimize in

the enzyme activity assay is 30, 37 , 40, and 45 °C.

RESULTS AND DISCUSSION

Resistance of *Bacillus sp* toward HgCl_2

The growth response of *Bacillus sp* to mercury was measured by the turbidity test of bacterial culture in media containing HgCl_2 at various concentration per time. Results of the growth response of *Bacillus sp.* without and in the presence of HgCl_2 is presented in **Figure 3**. The growth response on mercury resistance test showed that the *Bacillus sp.* has survived in mercury media until concentration of 80µM. For the incubation time of 32 h, the bacteria *Bacillus sp* is still able to survive well in media containing 80µM HgCl_2 compared with its growth in media without HgCl_2 .

Growth Curve *Bacillus sp*

The growth curve of mercury-resistant bacterial was observed in media without and with HgCl_2 in concentration of 80 µM. The growth showed 4 phases which are the exponential phase or logarithmic phase, stationary phase and death phase (**Figure 4**).The lag phase or adaptation phase is shown at an incubation time of 0 to 8 hours. At an incubation time 8 hours to 24 hours growth begins to increase rapidly so that this phase is called logarithmic phase or exponential phase. Making the inoculum is very good if done in logarithmic phase. The stationary phase is shown at incubation time of 32 hours to 40 hours. In this phase the number of cell populations remains due to the number of cells growing equal to the number of dead cells (Pelczar, and Chan, 2010); Giovanella, Cabral, Bento, Gianello, & Camargo, 2016; Waluyo, 2008). At incubation time of 44 hours, there is a decrease of OD (Optical Density) value so that at 44 hours incubation time is called death phase.

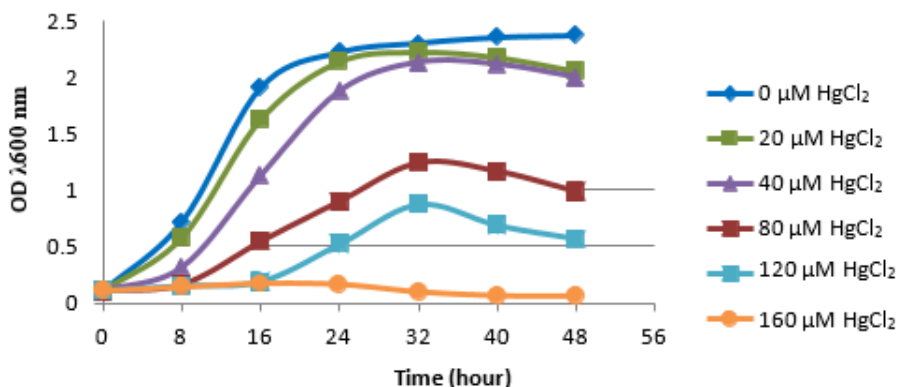


Figure 3. Growth response of *Bacillus sp.* toward HgCl_2 at various concentration

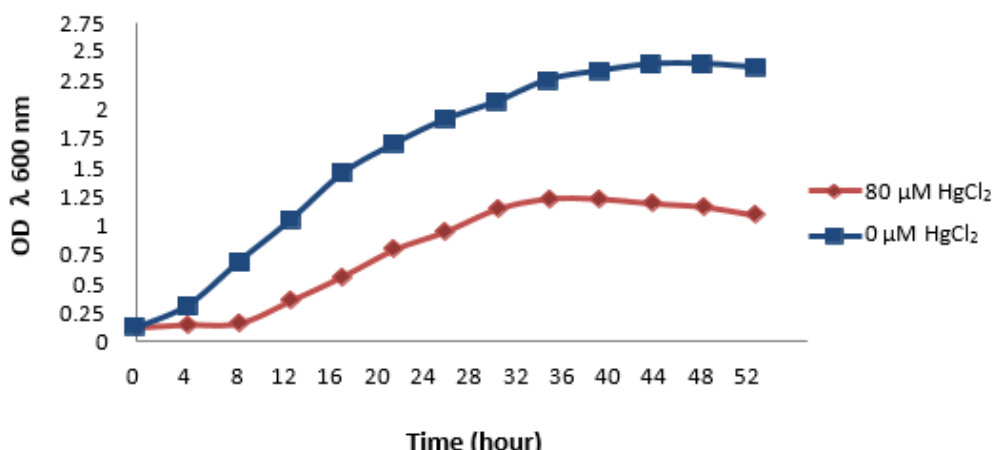


Figure 4. The growth curve of *Bacillus sp* in media without HgCl₂ and 80µM HgCl₂

Enzyme Activity and Protein Content

The isolation of enzyme and activity test were conducted to determine the ability of *Bacillus sp* in producing mercuric reductase enzyme. The result of isolation of mercuric reductase enzyme then determined its protein level and used for enzyme activity test. Measurement of protein content was done by Bradford method. The Bradford test is used to measure the total protein concentration by colorimetry in a solution. The mercuric reductase activity test from *Bacillus sp* shown in **Figure 5**.

The specific activity of an enzyme is obtained by dividing the activity of the enzyme with protein content. Based on the **Figure 5** was known that the mercuric reductase enzyme from *Bacillus sp* local isolate had optimum activity at 16 hours harvesting time with a total activity of 0.5714 Units μ g and Hg reduced by 16.8 μ M. According to Marthiana (2010), the determination of optimum incubation time is intended to obtain the right time when enzyme activity is highest so that the best possible harvesting of enzymes can be done.

Table 1. Protein content in crude enzyme of mercuric reductase

The time of harvesting of enzyme (hours)	OD λ 595nm	Protein content(μ g/mL)
0	0.150	19.60
8	0.151	20.38
16	0.150	19.60
24	0.151	20.38
32	0.150	19.60
40	0.151	20.38
48	0.151	20.38

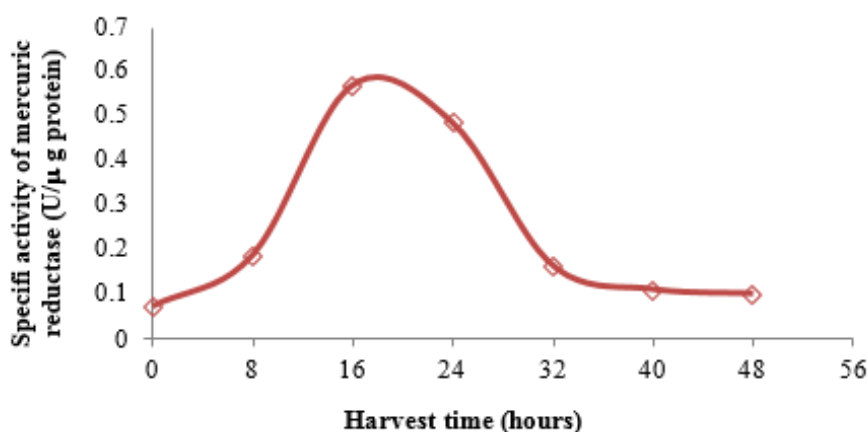


Figure 5. Effect of harvest time on specific activity of mercuric reductase enzyme

Characteristics of Mercury Reductase Enzymes

The optimum pH

The stability of the enzyme is influenced by many factors such as temperature, pH, solvents, cofactors and the presence of surfactants (Kannan, and Krishnamoorthy, 2006). The enzyme shows maximum activity in the optimum pH range of the enzyme with high stability (Sulastri, 2002). Determination of pH of the enzyme mercuric reductase was determined by enzyme activity with a variation of pH (4, 5, 6, 7 and 8). The result data of pH effect on enzyme activity is shown that activity of mercuric reductase is optimum at pH 6 (Figure 6).

At acidic pH, the amount of enzyme mercuric reductase *Bacillus sp.* is low. The condition does not support for mercuric reductase enzyme to do a catalytic activity. While at a slightly acidic in the pH 6, the enzyme activity is optimum with mercuric reductase activity of 12 Units / mL, and there was 18 μ M reduced Hg. High enzyme activity

can occur due to the high specificity between the enzyme and the substrate, i.e., the functional group on the catalytic side is at the appropriate ionization. At pH seven enzyme activity also can be said good because the result of mercuric reductase activity equal to 11.2 Units / mL, and there was obtained 16.8 μ M reduced Hg. At base pH that is pH 8 activity of enzyme mercuric reductase decreasing that is equal to 8.5 Unit / mL. This condition may cause a denaturation of the enzyme activity (Podjadi and Supriyanti, 2005).

Optimum temperature

The effect of temperature on enzyme is related to the energy factor which needs to catalyze a reaction and also related to denaturation effect (Sulastri, 2002). The optimum temperature of mercuric reductase enzyme was determined in various temperature of 30, 37, 40, and 45 °C. In the Figure 7 is shown that optimum activity of mercuric reductase is took placed at 37 °C.

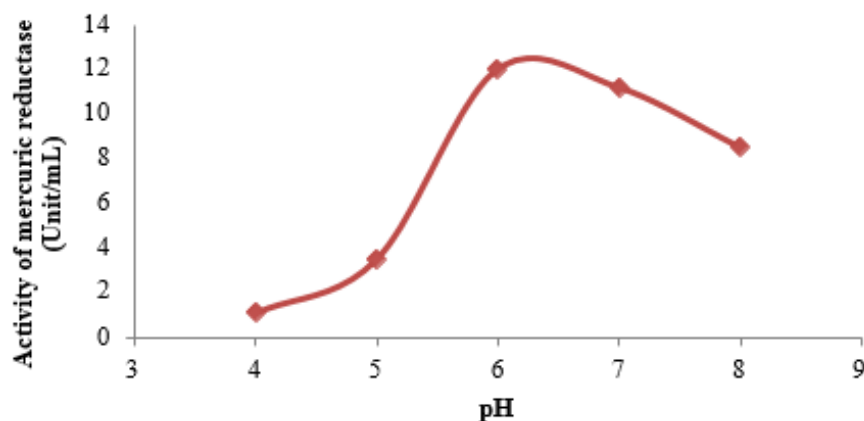


Figure 6. Effect of pH on the activity of mercuric reductase enzyme

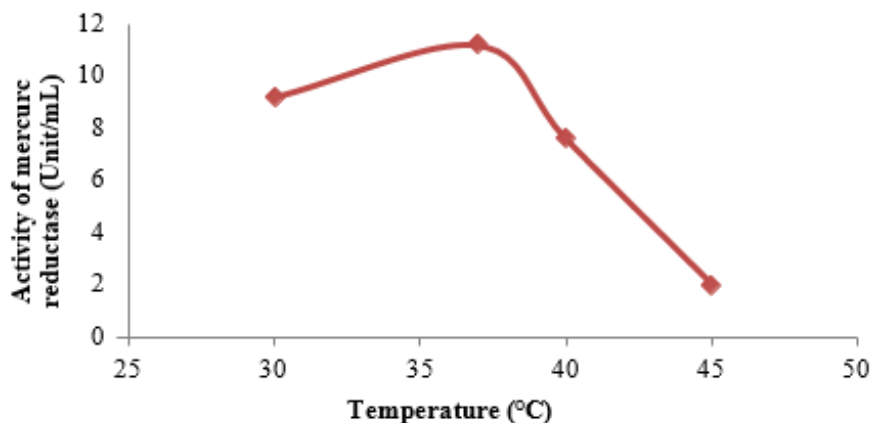


Figure 7. Effect of temperature on the activity of mercuric reductase enzyme

The activity of mercuric reductase enzyme at 30 °C was low, which was 9.2 Units/mL corresponding to reduced Hg form 13.8 µM. The energy in this condition might be insufficient to make reaction running, so then cause less optimal enzyme activity. The enzyme has optimum activity at 37 °C with 11.2 Units/mL and followed by 16.8 µM reduced Hg. At the optimum temperature the enzyme's energy is equal to the energy required to initiate the reaction between the enzyme and the substrate so that it proceeds well. At a temperature of 40 °C the activity of the enzyme decreased slightly by 7.6 Units/mL. This condition might create a denaturation effect, so the speed of enzyme reaction decreases up to four times. Based on the result, it is suggested to apply the local isolate of *Bacillus sp* for bioremediation agent in the environment which contaminated with mercury in the setted temperature of 37 °C.

CONCLUSION

The Local isolate of *Bacillus sp*.exhibited a potential bioremediation agent and showed resistance level in HgCl₂ up to 80 µM in growth media. The bacteria could produce the mercuric reductase enzyme when it was grown until 16 hours of fermentation time, and exhibited a specific enzyme activity of 11.2 Units/mL. The mercuric reductase enzyme of *Bacillus sp* had an optimum activity at pH 6 and 37 °C respectively.

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