

Indigenous *Streptomyces* spp. isolated from *Cyperus rotundus* rhizosphere indicate high mercuric reductase activity as a potential bioremediation agent

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Abstract. Rahayu HM, Putri WA, Khasanah AU, Sembiring L, Purwestri YA. 2021. Indigenous *Streptomyces* spp. isolated from *Cyperus rotundus* rhizosphere indicate high mercuric reductase activity as a potential bioremediation agent. *Biodiversitas* 22: 1519-1526. The purification and characterization of mercuric reductase of four indigenous *Streptomyces* spp. from *Cyperus rotundus* L. rhizosphere in mercury-contaminated area have been investigated. Cell-free extract was obtained by disrupting cells using sea sand at 4 °C followed by centrifugation. Mercuric reductase was purified by ammonium sulfate precipitation, dialysis, and chromatography column (DEAE Sepharose anion column chromatography). The determination of optimum pH and temperature of mercuric reductase activity was measured based on the number of NADPH₂ oxidized to NADP per mg protein per minute using a spectrophotometer. The molecular weight of mercuric reductase was determined using SDS-PAGE. Result showed that the highest specific activity of mercuric reductase was recorded from *Streptomyces* spp. BR28. The optimum pH and temperature of cell-free extract enzyme mercuric reductase were 7.5 and 80 °C, respectively. The enzyme was purified to 431.87-fold with specific activity 21918.95 U/mg protein. SDS PAGE showed that the molecular weight of mercuric reductase in *Streptomyces* spp. BR 28 ranged from 50 kDa to 75 kDa. It can be concluded that *Streptomyces* isolates contain mercuric reductase and have potential as mercury bioremediation agent to overcome mercury contamination in the environment.

Keywords: *Cyperus rotundus*, mercuric reductase, mercury, *Streptomyces*

INTRODUCTION

MRB or Mercury resistant bacteria have a broad spectrum of gram-positive and gram-negative bacteria. They have a peculiar mechanism of encounter and eventually convert Hg from highly toxic to non-toxic form (Dash and Das 2012; Kumari et al. 2020). MRB population is proportional to the scale of Hg pollution at the site (Dash and Das 2012; Winardi et al. 2019). Many bacteria are found to be resistant to mercury and classified as MRB including *Shigella flexneri*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *P. fluorescens*, *P. aeruginosa* (Fatimawali et al. 2019; Imron et al. 2019), *Firmicutes*, *Planctomycetes* (Fatimawali et al. 2020), *Klebsiella pneumoniae*, *Morganella morgani*, *Xanthomonas*, *Achromobacter* spp., *Sphingobium* spp., *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Mycobacterium marinum*, *Bacillus* spp., *Enterobacter*, *Staphylococcus aureus*, *Sphingopyxis* spp., *Psychrobacter* spp., *Brevibacillus* spp., *Anoxybacillus* spp., *Luteimonas* spp.,

and *Geobacillus kaustophilus* (Mahbub et al. 2017), *Brevundimonas* spp. (Irawati et al. 2012), *Actinomycetes* and *Streptomyces* (Undabarrena et al. 2017).

Streptomyces spp. is one of MRB which belongs to the genera Actinomycetes. It is aerobic, gram-positive bacteria (Deepika and Kannabiran 2010; Popa and Bahrim 2011), and has G-C content > 50% (guanine and cytosine) (Rintala 2003). Morphologically, this bacteria is different from other bacteria because it has a hyphae structure that forms the mycelium (Popa and Bahrim 2011) and undergoes morphological changes during its life cycle (Popa and Bahrim 2011). *Streptomyces* is a group of saprophytic bacteria (Deepika and Kannabiran 2010), most of which can be found in soil habitats (Deepika and Kannabiran 2010; Popa and Bahrim 2011; Madigan et al. 2012) no exception to soils contaminated with mercury.

Streptomyces spp. can be used as a solution to the problem of biological mercury pollution through a detoxification process. Detoxification mainly converts toxic heavy metal ions to non-toxic ions (Madigan et al.

2012). The mechanism of mercury detoxification by bacteria occurs with various systems, one of which is the reduction of Hg^{2+} to Hg^0 (volatile) using mercury reductase enzyme (Hughes and Poole 1989; Madigan et al. 2012; Kumari et al. 2020) which is produced by *merA* (Kumari et al. 2020). Bacteria that can reduce Hg^{2+} to Hg^0 are generally mercury-resistant bacteria (Weiss et al. 1977; Gopinath et al. 1989; Huang et al. 2010) and can be used as a bioremediation agent to reduce mercury waste in the environment (Purkan et al. 2017).

Indonesian artisanal and small-scale gold mining activities (ASGM) have been found in the islands of Borneo (Kalimantan), Buru, Sulawesi, Java and operations extending to the islands of Lombok and Sumbawa (Krisnayanti et al. 2012; Male et al. 2013; Tomiyasu et al. 2016). In the ASGM, gold is recovered by miners through a two-stage process of whole-ore amalgamation and cyanidation. The primer mercury concentration of the amalgamation tailings is about 3000 mg kg⁻¹ and greater than 1600 mg kg⁻¹ for cyanidation tailings (Krisnayanti et al. 2012). Several studies have been conducted focusing on the distribution of heavy metals near-final disposal areas have already been conducted (Tomiyasu et al. 2019). It is a challenge for researchers to provide problem-solving technology for treating mercury-contaminated areas. Bioremediation includes technologies that accelerate natural processes for degrading or reducing toxic effects of harmful chemicals and thus provide a good cleaning strategy, especially in Indonesia.

The use of microorganisms has provided a safer and more economical alternative to conventional physicochemical practices. Metabolic processes of organisms mostly use contaminants as energy sources resulting in nontoxic or less toxic by-products (Winardi et al. 2019) Maziyah (2011) has been successful in obtaining four isolates of the genus *Streptomyces* spp. from the rhizosphere of the turfgrass (*Cyperus rotundus* L.) in mercury-contaminated areas in the traditional gold processing area of Selodong Hamlet, Buwun Mas Village, West Lombok, Indonesia. They are *Streptomyces* spp. AS1, *Streptomyces* spp. AS2, *Streptomyces* spp. AS6, and *Streptomyces* spp. BR28 with the best mercury resistance capability is at a concentration of 1 mM (*Streptomyces* spp. AS6 and *Streptomyces* spp. BR28) and a concentration of 2 mM (*Streptomyces* spp. AS1 and *Streptomyces* spp. AS2). The ability of mercury resistance in these isolates due to the ability of these isolates to bind mercury to the cytoplasm, membranes, and cell walls.

However, mercury reductase activity of four *Streptomyces* spp. in the process of detoxification of mercury into non-toxic or less toxic forms is not yet known, so further research is needed. This research is aimed to purify and determine the activity of mercuric reductase enzyme from *Streptomyces* spp. which can be used in future for mercury bioremediation in Indonesia.

MATERIALS AND METHODS

Isolation and cultivation of *Streptomyces* spp.

Four isolates belonging to the genus *Streptomyces* (strains AS1, AS2, AS6, and BR28) were isolated from the

Cyperus rotundus L. plant-soil samples in the traditional gold processing area of Selodong Hamlet, West Lombok (Maziyah 2011). Each isolate was taken as much as 0.1 mL from glycerol stock and inoculated in 5 mL medium Yeast Malt Extract (YM) broth. Incubation was carried out for three days at room temperature. Then as much as 0.1 mL of culture were inoculated into the SNA medium by the spread plate method and incubated at room temperature. Observations were carried out for four days to three weeks until the colonies grew, then streaked and stored in a sloping SNA medium as stock.

Induction of mercury reductase

The induction method of mercury reductase isolates belonging to the genus *Streptomyces* was modified from Amroso et al. (1998) and Maziyah (2011). One inoculating loop isolate from the SNA skew medium was grown in 5 mL YM broth medium and incubated for three days at room temperature. Then 10% (v/v) of the liquid culture isolate was grown in 20 mL of YM broth liquid medium containing 0.05 mM HgCl_2 . The culture was then incubated at 30 °C at 125 rpm incubator shaker for 5 days.

Cell-free extract preparation

Cell-free extract preparation was adapted from Ghos et al. (1998). The liquid culture was centrifuged at 1646 g for 30 minutes, then washed twice with 50 mM buffer sodium phosphate pH 7.5 containing 0.5 mM EDTA and 14 mM β -mercaptoethanol. The obtained pellets were broken down using sea sand and centrifuged 1646 g at 4°C for 30 minutes. The supernatant obtained was called cell-free extract of crude enzyme.

Determination of mercury reductase activity

Mercury reductase activity was measured by oxidation of NADPH_2 at λ 340 nm. The method of measuring mercury reductase activity was performed according to the Ogunseitan (1998) and Zeroual et al. (2003) by adding 0.5 mL of enzyme extract to 3 mL of Mercury Reductase Assay (MRA) solution containing 50 mM sodium phosphate buffer solution (pH 7.5), 100 μM NADPH_2 , 0.2 mM MgCl_2 , 0.5 mM EDTA, 0.1% (vol/vol) β -mercaptoethanol, and 200 μM HgCl_2 . Incubation was carried out for 60 minutes at 37 °C in dark. Measurements by spectrophotometer at λ 340 nm were carried out when the enzyme was added and after incubation 60 minutes to determine the initial NADPH_2 concentration and the final NADPH_2 concentration so that the amount of oxidized NADPH_2 can be determined. The NADPH_2 level was determined by the equation of the regression line $y = ax + b$ obtained from the standard NADPH_2 curve. One unit of activity was defined as the amount of oxidized NADPH_2 per mg of protein per minute (μM NADPH_2/mg protein/minute).

Determination of protein concentration (Bradford Assay)

The determination of protein concentration was carried out under the Coomassie Blue (*Bradford Assay*) method (Bradford 1976). 60 μL crude enzymes were added with 3 mL of reagent and incubated for 2 minutes at room

temperature. Furthermore, absorbance was measured at λ 595 nm. Protein concentration (mg/mL) was calculated from the equation of the standard curve for protein curves.

Enzyme purification

Streptomyces spp. Mercuric reductase purification was carried out through several stages, starting from deposition with ammonium sulfate, dialysis, and column chromatography (DEAE Sepharose anion column chromatography). At each purification stage, specific enzyme activity and protein concentration were measured.

Characterization of mercury reductase

Effect of pH

The effect of pH on mercury reductase was carried out to determine the optimum pH of mercury reductase. Measurement of the effect of pH on the activity of mercury reductase was based on the method of Zeroual et al. (2003) with modification that measures the activity of mercury reductase at various pH variations, namely pH 4 and 5 (Sodium acetate), 6 and 7.5 (Sodium phosphate), 8 and 9 (Buffer Tris-HCl) with 10-minute of incubation time.

Effect of temperature

Measurement of the effect of temperature on the activity of mercury reductase is carried out to determine the optimum temperature of mercury reductase. Measurements were made by measuring the enzyme activity at various temperature variations (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C) in the optimum pH solution from previous measurements and incubated for 10 minutes.

Measurement of enzyme molecular weight

Crude protein enzymes, the results of the deposition of ammonium sulfate, dialysis, and columns were analyzed by SDS PAGE.

Data analysis

Protein concentration was calculated from the equation of the regression line $y = ax + b$ obtained from the standard curve of protein. Data on mercury reductase activity was calculated by measuring the levels of oxidized NADPH₂ per mg of protein per minute ($\mu\text{M NADPH}_2/\text{mg protein/minute}$) obtained from the regression line equation of $y = ax + b$ for the standard NADPH₂ curve.

RESULTS AND DISCUSSION

Mercury resistance and mercury reductase activity of *Streptomyces* spp.

Results exhibited that *Streptomyces* spp. AS1, *Streptomyces* spp. AS2, and *Streptomyces* spp. AS6 showed decreased resistance to HgCl₂ at 1 mM concentration. The growth inhibition diameters of the three isolates were 10.3 mm, 9.6 mm, and 20 mm (Figure 1). The results of previous studies (Maziyah 2011) mention *Streptomyces* spp. AS1, *Streptomyces* spp. AS2, and *Streptomyces* spp. AS6 is resistant to HgCl₂ at a concentration of 1 mM with a diameter of growth inhibition successively 6.53 mm, 7.47 mm, 8.77 mm. The resilience of *Streptomyces* spp. BR 28 has decreased slightly, from 9.23 mm to 9.3 mm. So it can be concluded that the resistance ability of *Streptomyces* spp. BR 28 against HgCl₂ remains the same.

Measurement of the specific activity of mercury reductase on four isolates showed that *Streptomyces* spp. BR28 has the highest specific activity of 56.94 U/mg compared to *Streptomyces* spp. AS1 17.49 U/mg, *Streptomyces* spp. AS2 15.43 U/mg, and *Streptomyces* spp. AS6 30.66 U/mg (Figure 2).

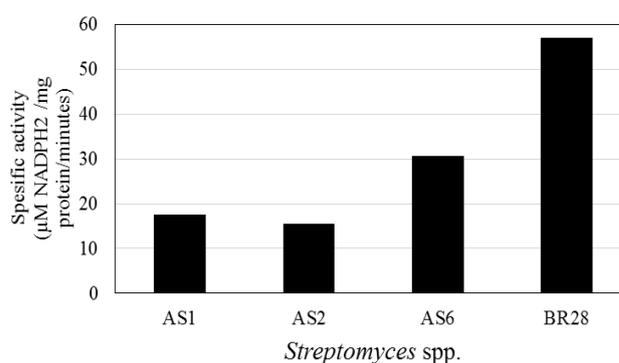


Figure 2. The specific activity of mercury reductase on four isolates of *Streptomyces* spp.

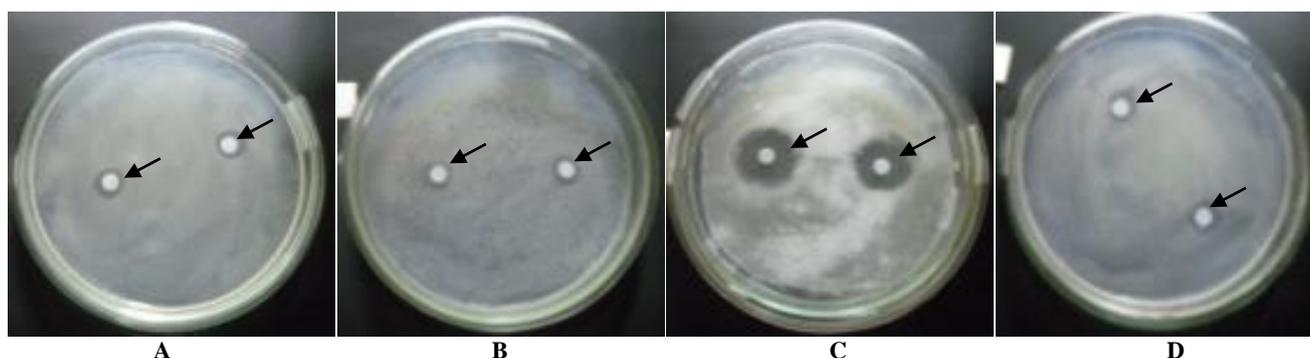


Figure 1. Mercury resistance activity of *Streptomyces* spp. A. *Streptomyces* spp. AS1 with clear zone 10.3 mm; B. *Streptomyces* spp. AS2 with clear zone 9.6 mm; C. *Streptomyces* spp. AS6 with clear zone 20 mm; D. *Streptomyces* spp. BR28 with clear zone 9.3 mm

Mercuric reductase is an enzyme located in the cytoplasm (Furukawa and Tonomura 1972; Schottel 1978; Fox and Walsh 1982; Meissner 1983; Robinson and Tuovinen 1984; Summers 1986) and is inducible (Weiss et al. 1977; Robinson and Tuovinen 1984; Nakahara et al. 1985; Summers 1986; Bogdanova et al. 1988; Petrus et al. 2015) so that mercury reductase will only be produced by organisms as a response if there are mercury compounds in the growth environment (Robinson and Tuovinen 1984). Nakahara et al. (1985) explain that there is a correlation between mercury resistance and the synthesis of mercury reductase. The ability to produce enzymes will be lost if the organism is grown in a medium that does not contain mercury compounds it eventually will lose its resistance to mercury (Olson et al. 1982; Robinson and Tuovinen 1984; Nakahara et al. 1985). So it can be concluded, the members of the genus *Streptomyces* showed a reduced ability of mercury resistance because the isolates were stored for a long time in a medium that does not contain mercury. The mechanism of transformation and mercury resistance starts from the binding process of Hg^{2+} (Nies 2009) that occurs in the periplasm (for gram-negative bacteria) and *periplasmic binding protein (merP)* (for gram-positive bacteria) (Nies 2009; Silver and Hobman 2007; Silver and Phung 2005). *merP* will bring Hg^{2+} to *merT*, then Hg^{2+} will be released by *merP* quickly through 2 of the 4 cysteines found in *merT* (Silver and Phung 2005). *merT* functions to transfer Hg^{2+} from *merP* to mercury reductase (*merA*) and Hg^{2+} will be reduced to Hg^0 (Hughes and Poole 1989; Gadd 1990; Hobman and Brown 1997; Silver and Phung 2005; Silver and Hobman 2007).

Silver and Hobman (2007) describe the reduction process of Hg^{2+} + to Hg^0 starting with the binding of Hg^{2+} to the mercury reductase subunit of the carboxyl-terminal part of Cys 557-Cys 558. Then it is moved quickly through the thiol-thiol exchange to the Cys 135-Cys 140 monomer pair. Then Hg^{2+} will be on the active site of the Cys 135-Cys 140 pair and reduced to Hg^0 by electron transport from the FAD cofactor. Eventually, the volatile Hg^0 will be removed from the microorganism cell.

Enzyme purification

The results indicate an increase in specific activities at each stage of purification. The specific activity of ammonium sulphate precipitation was 1703.477 U/mg

protein. The specific activity of cell-free enzyme extracts increased the activity 33.56 times (Table 1).

The specific activity of mercury reductase resulting from dialysis has increased. This is indicated by an increase in the specific activity value of 50.23 times the specific activity of the free cell extract. The highest specific activity was found by column chromatography fraction which was 431.87 times cell-free extract. Enzyme-specific activity was higher in line with the increasing stages of purification. The specific activity of column chromatography fraction was higher than dialysis fraction, ammonium sulfate precipitation, and cell-free enzyme extract. So it can be said that the enzymes resulting from column chromatography fractions are purer compared to cell-free enzyme extracts.

Mercury reductase activity at various pH

Testing the effect of pH on the activity of mercury reductase revealed that the enzyme activity was lowest at pH 4 and optimum at pH 7.5, then decreased (Figure 3). Increasing or decreasing the optimum pH will change the ionization group on the active site of the enzyme and the substrate which will further slow-down or prevent the formation of the enzyme-substrate complex (Byrne 2013; Giovanella et al. 2015). According to Copeland (2000), enzymes have tertiary structures that are sensitive to pH and will generally be denatured at pH values that are too low or too high.

Streptomyces spp. BR 28 has an optimal pH of 7.5 with an activity value of 47.5 U. This is in line with Copeland (2000) which states that most enzymes work optimally at neutral pH. This statement was confirmed by Bafana et al. (2017), Zeroual et al. (2003), Olson et al. (1982), Nakahara et al. (1985) which states that the optimum mercury reductase at pH approaches neutral pH.

Mercury reductase activity at various temperature

Mercury reductase showed the lowest activity at 20 °C and optimum at 80 °C and then decreased. An increase in temperature will cause an increase in the kinetic speed of the enzyme and will effectively increase the amount of fusion between the substrate and the enzyme so that the substrate-enzyme complex is formed (Byrne 2013). Each enzyme has an optimum temperature (Byrne 2013).

Table 1. Purification of mercury reductase *Streptomyces* spp. BR28

Stage	Protein			Enzyme Activity			Purification	
	Volume (mL)	Protein concentration (mg/mL)	Total of protein (mg)	Enzyme activity (Unit)	Specific activity (Unit/mg of protein)	Total of activity (Unit)	Purification factor	Yield (%)
Cell-free extract	100	0.051	5.1	0.854	50.753	258.840	1	100
Ammoniumsulphate precipitation	4	0.399	1.596	13.590	1703.477	2718.749	33.56	1050.36
Dialysis	4	0.238	0.952	12.140	2549.457	2427.083	50.23	937.68
Column chromatography	1	0.001	0.001	1.25	21918.95	21.919	431.87	8.47

The test results (Figure 4) showed that activity of mercury reductase *Streptomyces* spp. BR 28 was optimum at a 80 °C temperature of which was 199.16 U, four times higher than at a temperature of 50 °C. So it can be concluded that the mercuric reductase from *Streptomyces* was a thermophilic enzyme. Several previous studies have shown that mercuric reductase is an enzyme that is resistant to high temperatures. Including research, Sayed et al. (2014) explained that the novel mercuric reductase from the unique deep brine environment of Atlantis II in the Red sea is stable at high temperatures. Maged et al. (2019) also found that mercuric reductase from the red sea has thermal tolerance at 70 °C. Which was followed by Freedman et al. (2012) research, chemotrophic thermophilic *Aquificae* possessed thermophilic mercuric reductase.

The molecular weight of mercury reductase

The molecular weight of mercury reductase *Streptomyces* spp. BR28 has been checked by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) technique. Protein bands were parallel to the markers ranging from 50 kDa to 75 kDa (Figure 5).

The molecular size of mercury reductase of several gram-positive and gram-negative bacteria that has been identified was ranged from 56 kDa and 62 kDa in *Pseudomonas aeruginosa* PAO 9501 (Fox and Walsh 1981), 60 kDa in *Lysinibacillus sphaericus* strain G1 (Bafana et al. 2017), 69 kDa in *Bacillus* sp (Moore et al. 1989), 54 kDa and 69 kDa in *Azotobacter chroococcum* (Gosh et al. 1998), 64 kDa and 55.5 kDa in *Escherichia coli* J531 (R831) (Schottel 1977), and 62 kDa in *Klebsiella pneumoniae* (Zeroual et al. 2003). Thus, it can be estimated the molecular weight of mercury reductase *Streptomyces* spp. BR28 ranged from 50 kDa to 75 kDa. The exact molecular weight of mercury reductase was not known with certainty because the SDS PAGE fraction of column chromatography that has the highest activity did not show the presence of a single protein band to ensure the molecular weight of mercury reductase (Figure 5).

The results of the SDS page column chromatography showed three-column fractions that showed protein bands (fractions four, five, and six). Fraction four showed seven protein bands, fraction five has six protein bands, and fraction six has two protein bands (Figure 6).

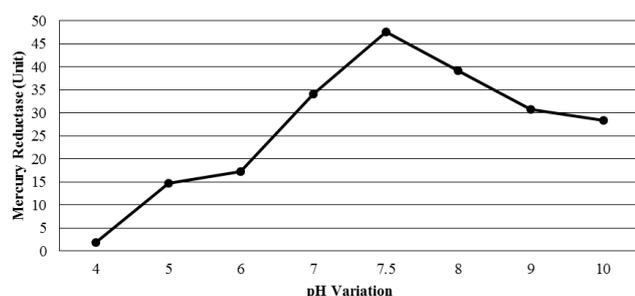


Figure 3. The activity of crude enzyme from *Streptomyces* spp. BR28 at various pH values

A large number of protein bands were visible in fractions four, five, and six suggest that the enzymes obtained from the DEAE sepharose anion column chromatography were not yet pure. Thus, purification using DEAE sepharose anion column chromatography was not able to separate mercury reductase from other proteins. Mercuric reductase is a central enzyme in the organomercurial resistance system elaborated by many soils and enteric bacteria (Fox and Walsh 1981).

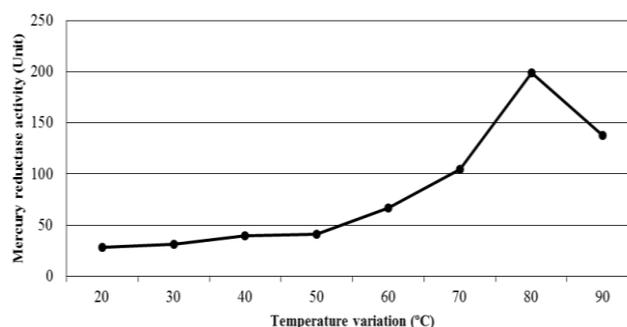


Figure 4. The activity of crude enzyme *Streptomyces* spp. BR28 at various temperatures

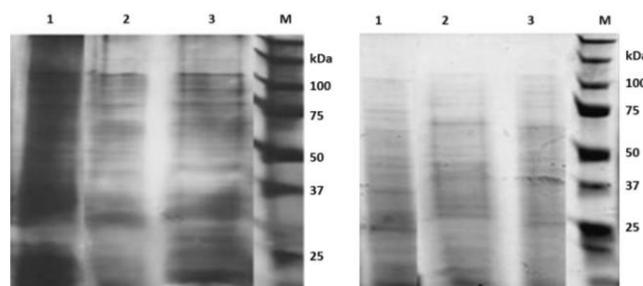


Figure 5. SDS PAGE results of mercury reductase *Streptomyces* spp. BR28 with (A) silver staining; (M) protein marker; (Lane 1) crude enzyme; (Lane 2) result of dialysis; (Lane 3) ammonium sulfate precipitation yield 30-60%; (B) coomassie blue coloring; (M) protein marker; (Lane 1) crude enzyme; (Lane 2) result of dialysis; (Lane 3) ammonium sulfate precipitation yield 30-60%

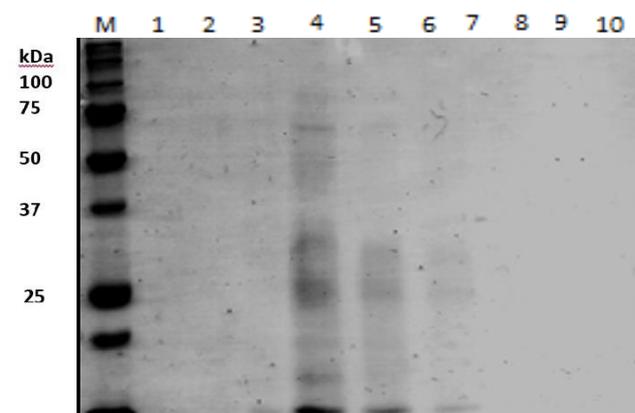


Figure 6. SDS PAGE results of mercury reductase column chromatography *Streptomyces* spp. BR 28, (M) Protein marker, (Lane 1-10) Fraction 1-10

Mercury resistant bacteria (MRB) consists of a broad spectrum of gram-positive and gram-negative bacteria that have a peculiar mechanism of countering and eventually converting Hg from highly toxic form to non-toxic form using that enzyme. Previous studies have never measured mercuric reductase activity specifically on *Streptomyces* spp. isolated from the *Cyperus rotundus* rhizosphere in mercury-contaminated areas. Therefore, in this study, several methods were applied to measure the optimum conditions for mercury reductase activity from *Streptomyces* spp. that has never been done before in Indonesia.

From the result, it is known that the total activity of the cell-free extract mercuric reductase *Streptomyces* spp. was 258.840 units of the complete protein 5.1 mg. This enzyme activity is greater than the enzyme activity in *Azotobacter chroococcum*, which is only 25.187 units of 201.5 mg of total protein (Ghosh et al. 1999). Other studies have also shown that the specific activity of the mercuric reductase *Streptomyces* spp. is superior with a value of 50,753 units/mg of protein compared to mercuric reductase of *Klebsiella pneumoniae* with a value of 49.24 units/mg of protein (Zeroual et al. 2003). Recently, mercuric reductase activity has also been measured from *Lysinibacillus sphaericus* with a value of 2.4 and 0.13 units/mg (Bafana et al. 2013). These findings in this study provided information that mercuric reductase from *Streptomyces* spp. has promising capabilities for the bioremediation process against mercury contamination.

In this study, it can be concluded that *Streptomyces* spp. BR28 can be considered as a mercury bioremediation agent because it has mercury reductase with a specific activity of 21918.95 U/mg protein with a purity level of 431.87 times cell-free extract. The optimum pH and temperature of the mercury reductase isolates were 7.5 and 80 °C, respectively. The isolate also has mercury reductase with sizes ranging from 50 kDa to 75 kDa. So, this study provided important results and gave new insight for next research, especially in the bioremediation field.

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