THE DISCOVERY OF MERCURY-RESISTANT BACTERIA KLEBSIELLA PNEUMONIAE ISOLATED FROM SARIO RIVER ESTUARY THAT CAN BE USED TO DETOXIFY INORGANIK MERCURY WASTES

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ABSTRACT

Mercury is well known for its high toxicity and strong affinity toward the thiol group of proteins. Mercury-resistant bacteria can be used for detoxify mercury wastes due to the ability of these bacteria to reduce toxic inorganic mercury into mercury metal which is volatile and less toxic. The aim of this study was the selection of bacterial strains resistant to inorganic mercury, as well as to show their capacity to reduce mercury in pure culture media Nutrient Broth. Six strains bacteria was isolated and one of them, A1.1.1. isolate was selected for its capacity to reduce mercury $HgCl_2$ in culture media nutrient broth. The one bacterial strains belong to the spesies *Klebsiella pneumoniae*. Inoculated in pure culture, these strain showed a mercury reduction of 75% in 1 hour, 92% in 12 hours, and 99,4% in 24 hours. Therefore, this bacterium could be useful in detoxification of inorganic mercury wastes**.**

Keywords : Bacteria, *Klebsiella pneumoniae,* Hg-resistant, detoxification

ABSTRAK

Merkuri dikenal karena sifat toksik dan afinitas yang kuat terhadap gugus tiol dari protein. Bakteri resisten merkuri dapat digunakan untuk mendetoksifikasi limbah merkuri karena kemampuannya untuk mereduksi merkuri anorganik menjadi logam merkuri yang mudah menguap dan sedikit toksik. Tujuan penelitian ini yaitu menentukan strain bakteri yang resisten merkuri anorganik, yang ditunjukkan oleh kemampuannya untuk mereduksi merkuri dalam kultur *nutrient broth*. Enam strain bakteri telah diisolasi. Isolat A1.1.1. dipilih karena kemampuannya untuk mereduksi $HgCl_2$ dalam kultur nutrient broth. Salah satu strain bakteri merupakan milik spesis *Klebsiella pneumoniae*. Strain tersebut menunjukkan pengurangan merkuri sebesar 75% selama 1 jam dan 92% selama 12 jam, dan 99,4% selama 24 jam. Karena itu, bakteri tersebut dapat digunakan untuk mendetoksifikasi merkuri anorganik dalam air limbah**.**

Kata Kunci : bakteri, *Klebsiella pneumoniae*, resisten merkuri, detoksifikasi

INTRODUCTION

The release of heavy metals into the environment can harm the ecosystems and cause serious harm to human health. Mercury is one of the most dangerous heavy metals and appears in the environment in various forms. Mercury compounds in the form of Hg (II) can be bound to the cysteine residues of human protein and lead to lose of its activity. In addition to Hg (II), mercury compounds are most harmful to human health is an organic mercury compounds, especially methyl mercury, and phenyl mercury. These compounds are highly reactive and have high mobility compared to Hg (0) and Hg (II), can also attack the human nervous system through the bloodstream (Rasmussen et al., 2008).

Source of mercury pollution can be derived from geological and biological processes, but not comparable to the mercury pollution caused by human activities such as burning coal, petroleum products, usage of fungicides, mercury catalyst and gold mining using mercury as a gold extraction.

One attempt to detoxify mercury can be done by using mercury-resistant microorganisms such as mercury resistant bacteria. Detoxifications of mercury by mercury resistant bacteria occur because mercury resistant bacteria have mercury resistant genes, operon mer (Silver and Phung, 1998). Structure of operon mer is different for each type of bacteria. The structure of operon mer generally consists of metaloregulator gene (MerR), mercury transformation gene (merT, merP, merC), mercury reductase gene (merA) and organo mercury lyase (merB). Bacteria possess only mercury reductase gene (merA) is called a narrow spectrum mercury-resistant bacteria. Some bacteria instead of having merA gene, also have merB gene, then the bacteria are called broad spectrum mercury-resistant bacteria. MerA protein has the function to reduce toxic mercury ions into metallic mercury Hg (O) which is less toxic and easily evaporate at room temperature, whereas merB protein has the function to catalyze of breaking mercury-carbon bond to produce organic compounds and ionic Hg (II) (Tamar Barkay 2003).

Several studies have revealed that the microorganisms in mercury polluted areas play a major role in the detoxification of mercury, therefore microorganisms in the mercury polluted area is a source for the isolation of mercury-resistant bacteria. One of the problems in North Sulawesi is contamination of land or water by mercury from gold mining waste disposal that use mercury as an ingredient to extract gold. In water and soil, metallic mercury will change into mercury ion that are soluble in water and pollute the waters. Therefore in this study were isolated narrow spectrum mercury resistant bacteria from mercury polluted areas named Sario River estuary located in Manado North Sulawesi.

Materials and Methods

Materials

Spiritus lamp, sterile plastic, incubator, microscope, glass slides, öse needle, CV-AAS, Laminar Air Flow, glasses tools, Sartorius Scales, Freezer, Kit Extraction Wizard Genomic DNA (Promega): EDTA, Lytic enzyme, nuclei lysis solution, RNAase solution, protein precipitation solution, isopropanol, 70% ethanol, DNA rehydration solution. Primers Bact-FI .5 'AGAGTTTGATCMTGGCTCAG3' / Uni-B1.5 'GGTTACSTTGTTACGACTT3' (Eurogentic AIT), agarose (Vivantis), Etidiumbromide, loading dye (Vivantis), marker (Vivantis), $HgCl_2$.

Bacteria Resistance Level Test

Bacteria grown in 0.9% NaCl solution. Subsequently inoculated in nutrient broth medium with $HgCl_2$ with concentrations of 10, 20, and 40 mg / l. Incubated at 30^{o}C

for 24 hours. Culture that grow on the medium with the highest mercury concentrations, grown on an slant agar medium and are used for identification and testing of reduction power against $HgCl_2$.

Mercury Reduction Test of Mercury Resistant Bacteria

1 Öse Bacterial isolate A1.1.1 taken from slant agar medium. Grown in nutrient broth medium containing 2 ppm $HgCl_2$. Incubated at 37 ° C for 1 hour, 12 hours and 24 hours. At the end of incubation, add 2 drops of concentrated H_2SO_4 to kill bacteria. Mercury levels analyzed by the CVAAS method and analysis of blank.

16S rRNA gene amplification by PCR Technique

Amplification was performed using PCR machine Combi Block (Whatman Biometra Germany). Primers used for the PCR process is universal primer of bact F1 (forward) pair and Uni B1 (reverse). Molds used for amplification of the 16S rRNA gene is a isolated genomic bacteria DNA. Amplification by PCR technique performed with the variation of the composition of PCR reagents and PCR reaction conditions as below.
Composition of reagents for PCR of 16S rRNA : 1 Tube for PCR: ddH_20 15.8 mL, 10 x 2.5 µl PCR Buffer, $MgCl_2$ 25 mM 3.0 µL, 10 mM 0.5 µL dNTP, 30pmol/µl 1.0 µl UniBI Primer, Primer BactFI 30 pmol / µL 1.0 mcL, Taq DNA Pol (5U/µl) 0.2 µL, Template (10x dilution) 1.0 µL, 25.0 µL total.
PCR condition : Initial denaturation 94^o , denaturation 94^o1' 30 cycles, Annealing 62^o 1 '30 ", Polymerization 72^o 1' 30", Stabilization 72^o 10 '.

Electrophoresis and Visualization of 16S rRNA gene

DNA that had been amplified, separated with 1% agarose of electrophoresis gel, visualization is then performed using the ethidium bromide dye solution and detected by UV light in the UV-transiluminator. Detection results are documented.

16S rRNA gene sequencing

Sequencing was performed to determine the nucleotide sequence of the DNA fragment detected from the visualization of DNA that are amplified in the PCR process using automated DNA sequencing machines. Sequencing process is done in Macrogen Korea. DNA sequencing results were analyzed using the method of BLAST via online media NCBI, to seek common nucleotide sequences of 16S rRNA gene in determining the species of inorganic mercury-resistant bacteria isolated from mercury contaminated locations.

RESULTS AND DISCUSSIONS

Mercury Resistance Level Testing Results

The growth medium nutrient broth (NB) with $HgCl_2$ concentration of 5 mg / l to 5 isolates can grow but only 1 isolate that can be grown in NB medium with $HgCl_2$ levels 10 and 20 mg / l, and no growth in NB medium with high levels of $HgCl_2$ 40 mg / l. Zeng *et al*., 2010, also found that Pseudomonas aeruginosa was isolated from soil that can live on solid medium containing 60 mg / l Hg^{2+}, but when Hg^{2+} was added in the liquid medium as much as 60 mg / l, then the bacteria will not grow. $HgCl_2$ is most frequently used for experimental studies because it is soluble and toxic (Schelert, 2003). Bacterial resistance level of A1.1.1 isolates is higher than that found by Nofriani and Gusrizal, 2004, which found that two species of bacteria *Enterobacter hafniae* and *Enterobacter cloacae* from the former

mining areas Mandor, West Kalimantan, both types of these bacteria can only live in LB medium with $HgCl_2$ levels of 10 mg / l, instead of that Zeyaullah *et al.*, 2010, that isolated the bacterium Escherichia coli from mercury contaminated Locations in India , which can be grown in LB medium with $HgCl_2$ levels between 25-55 mg / l. The presence of mercury-resistant bacteria often associated with levels of mercury contamination in the environment, although also found in non-mercury contaminated environments.

Molecular Identification of Isolates A1.1.1 with analysis of 16S rRNA gene Results

Based on the Blast results of 16S rRNA gene sequence, A1.1.1 isolates have 99% similarity with *Klebsiella pneumoniae, Klebsiella variicola, Klebsiella oxytoca* and *Klebsiella singaporensis*, but after aligning it online, it appears that A1.1.1 closely related isolates with *Klebsiella pneumoniae*. The results of nucleotide sequence alignment of 16S rRNA gene sequencing results of A1.1.1 isolate is *Klebsiella pneumoniae*. *Klebsiella pneumoniae* resistant to mercury has been widely reported. Zeroual *et al.*, 2003, *Klebsiella pneumoniae* isolated from industrial waste in Casablanka, and to purify and characterize mercury reductase. Dzairi et al., 2004, isolated *Klebsiella pneumoniae* strains that can be grown in 1200 μM $HgCl_2$ that can vaporize $HgCl_2$ into a volatile Hg0, isolated from hydrocarbon-contaminated river in Morocco.

Mercury Reduction Test Results of Mercury Resistant Bacteria A1.1.1 Isolate

Isolates A1.1.1 quickly respond to the presence of mercury in the media, in 1, 12, and 24 hours, respectively decreased 75%, 92% and 99.4%. This reduction exceeds the capability of reducing ability of the bacteria *Klebsiella pneumoniae* isolated from industrial waste in Casablanca Morocco, has a high resistance to mercury with minimal inhibitory concentrations of 2400 μM in solid and liquid media Muller Hinton, higher than that reported by (Spangler et al., 1973, Blaghen et al., 1983, Devincent et al., 1990, Filali *et al*., 2000) (Zeroual *et al.*, 2001). *Klebsiella pneumoniae* were found to reduce levels of mercury continuously up to 100% within 10 days, without loss of activity (Zeroual *et al*., 2001). Mercury reductase of *Klebsiella pneumoniae* has been purified and characterized, in which the results of the analysis of mercury reductase protein, has a molecular weight of 62kDa (Zeroual *et al.*, 2003.

CONCLUSIONS

In the waters of North Sulawesi, at the Sario River estuary , there are mercury-resistant bacteria *Klebsiella pneumoniae* that can reduce inorganic mercury HgCl2 75% in 1 hour, 92% in 12 hours, and 99.4% in 24 hours so it can be used to detoxify inorganic mercury waste waters.

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