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Research Article
Isolation and Identification of Arsenic-resistant Bacteria for Possible Application in Arsenic Bioremediation

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Abstract

Background and Objective: North Sulawesi is rich in minerals, among them gold is also present. The gold mining in the Buyat area produces heavy metal waste which can pollute the environment, among others is arsenic. Arsenic is a heavy metal that is very toxic to humans, so an agent is needed for the remediation process. The aim of this study was to isolate and identify arsenic-resistant bacteria from the Buyat estuary and beach to analyze the isolates' ability to detoxify arsenic. **Materials and Methods:** Soil sediment samples were obtained from Buyat estuary and beach in North Sulawesi. Isolation of arsenic-resistant bacteria was carried out by growing the samples in LB broth media containing 100, 500 and 1000 ppm arsenite. Indentification of arsenic-resistant bacteria was carried out by microbiological, biochemical and biomolecular analysis. The ability to detoxify arsenite was analyzed by CVAFS. **Results:** The study showed that there were 4 isolates of arsenic-resistant bacteria isolated from the soil samples. All isolates are rod-shaped, Gram-negative and non-motile bacteria. BLAST results showed that isolates A was *Stenotrophomonas* sp., isolate B was *Stenotrophomonas maltophilia*, isolate C was *Pseudomonas* sp. and isolate D was *Pseudomonas putida*. All isolates reduced the levels of arsenic in media by almost 100% within 72 h. **Conclusion:** The study suggested that *Stenotrophomonas* sp., *S. maltophilia*, *Pseudomonas* sp. and *P. putida* had the potentials to be used in the bioremediation of arsenic.

Key words: Arsenic remediation, arsenic-resistant bacteria, arsenic detoxification, Buyat Bay, Buyat estuary

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Arsenic, a natural element that is widespread in the earth's crust, is chemically classified as metalloid, having both metal and nonmetal properties. It exists is in the form of arsine/arsane (-3), elemental arsenic (0), arsenite (+3) and arsenate (+5). According to the Agency for Toxic Substances and Disease Registry[1], arsenic which is usually found in a combination in the environment with other elements such as oxygen, chlorine and sulfur is called inorganic arsenic, while when combined with carbon and hydrogen is called organic arsenic. Inorganic arsenic is naturally found in soils and in various types of rock, especially in minerals and ores that contain copper or lead. When this ore is heated in a smelter, most of the arsenic builds up and enters the air as fine dust. Smelters can collect this dust and form arsenic compounds called arsenic trioxide (As_2O_3)[2].

In rock or soil, arsenic is distributed as a mineral. The highest arsenic level is in the form of arsenide from copper, lead, silver and sulfide forms from gold. Human involvement in causing or exacerbating arsenic pollution can be traced to mining related activities[3,4]. Sediment samples from Buyat and Ratatotok bays were studied in 2002 and 2004 for the analysis of minerals and trace elements[5]. The sediment samples at this location contained heavy metal wastes generated by gold mining activities[3]. Their study found high concentrations of arsenic (590-660 ppm) in shallow marine sediments both from Buyat Bay and Ratatotok. Arsenic concentration in the interior of Buyat Bay ranged from 0-25 ppm. Furthermore, a study on characterization of the metal composition of river mine tailings and marine sediments in Buyat and Ratatotok was conducted[5]. The study showed that sediments affected by tailings had an arsenic concentration 30 times than uncontaminated sediments. This was confirmed again in a report conducted by an integrated team of the Ministry of Environment in 2004, which stated that illegal mining resulting from the amalgamation process waste had been dumped into a tributary upstream of the Ratatotok river[6]. Research conducted in 2010 showed that the highest arsenic levels at 6 sample points in Buyat and Ratatotok bays were found in the Ratatotok coastal area (127.8 ppm). The highest arsenic level at this location was associated with the accumulation of arsenic waste originating from the Ratatotok river estuary which had arsenic levels of 4-130 ppm. This shows that the disposal of waste through rivers into the sea caused by illegal mining has contributed to polluting the Ratatotok bay[7,8].

Arsenic is a pollutant for the environment, the search for arsenic remediation agents is urgently needed. Arsenic resistant-bacteria are the target of many studies in order to use them as bioremediation agents. Arsenic resistance in bacteria is mediated by the presence of the ars operon gene. The ars operon consists of arsC operon which encodes arsenic reductase, arsB and arsA which encodes the arsenic efflux pump, arsR which encodes the transcription regulator and arsD which encodes for the transfer of arsenite from the glutathione complex to the arsA subunit of the arsAB complex and activates it (Satyapal *et al*.[9] and Kruger *et al*.[10]).

Various studies have identified arsenic-resistant bacteria from water and soil. In addition, research on livestock meat was also carried out. The types of bacteria found in water and in the soil included *Klebsiella pneumoniae*, *Enterobacter* sp., *Bacillus* sp., *Aneurinibacillus aneurinilyticus*, *Enterobacter asburiae* and *E. cloacae*[11-13]. While *Campylobacter* spp. was found in the meat of cattle[14]. These studies showed that the bacterial colonies that exist in a community can adapt to pollutants such as arsenic, through resistance mechanisms or what is known as pollution-induced community tolerance (PICT)[15].

Research on the use of arsenic-resistant bacteria for bioremediation is still very limited, therefore the purpose of this research was to isolate and identify arsenic resistant-bacteria that were resistant to high concentration of arsenite obtained from sediment soils of Buyat estuary and beach which reduced arsenite levels in the media.

MATERIALS AND METHODS

Sampling sites: Sediment samples were obtained from Buyat estuary and beach which are located on the southern coast of the Minahasa Peninsula, North Sulawesi, Indonesia. The study was conducted from March-August, 2019. In each location, three sampling sites were chosen. In each sampling site, the pH was measured and the sediment samples were put into sterile polyethylene plastics and brought to the laboratory using a cool box for further analysis.

Isolation of arsenic-resistant bacteria: Each of soil sediment was added with sterile 10 mL of 0.9% NaCl and stirred evenly. Into each of 10 mL of sterile Luria Bethani (LB) medium, 100 µL of NaCl solution containing sediment samples and mixed evenly. From each sample, 100 µL was poured onto Nutrient Agar (NA) media, spread evenly and grown at 37°C for 2 days. Several single colonies with different morphological appearance were picked from the agar and purified by the streak method to obtain pure single colonies. Each pure colony was grown on Nutrient Broth (NB) supplemented with

500 ppm and 1000 ppm of arsenic (As_2O_3) and incubated for 24 h at 37°C. Cultures grown on arsenic media were transferred onto NA containing 500 and 1000 ppm arsenic. Each different colony was grown by the streak method to obtain pure colony. Colonies with the different morphological appearance which survived on arsenic NA media were identified. The identification procedure is described in the section 16S rRNA-based identification.

Bioremediation simulation test for arsenic-resistant bacterial isolates: The bacterial isolates that were survived in 500 and 100 ppm of arsenite were inoculated in LB broth containing 100 ppm arsenite and incubated at 30°C. After incubating for 72 h, the solution was centrifuged at 10,000 rpm for 10 min to separate bacteria from the media, then the concentration of arsenic in the media was measured using the Cold Vapor Atomic Absorption Spectroscopy (CVAAS).

Morphological-, physiological- and biochemical-based identification of arsenic-resistance bacterial isolates: Morphological- and physiological-based identification were carried out using Gram staining, bacterial cell shape and motility test. Biochemical-based identification was performed based on IMViC (Indol, Methyl red, Voges-Proskauer and Citrate) tests.

16S rRNA-based identification: The identification of the isolates based on 16S rRNA followed the previous protocol[16]. The extraction and purification of bacterial DNA were performed using Genomic DNA Mini Kit (Geneaid). The pure DNA was stored at 2-8°C for further analysis. DNA amplification using universal BactF1 and UniB1 primer pairs were done in PCR combi block machine (Whatman Biometra Germany). The composition of the PCR reaction is ddH_2O 20 µL, UniB1 primer 30 pmol μL^{-1} 1.5 µL, BactFl primer 30 pmol μL^{-1} 1.5 µL, 2x MyTaq HS Red Mix 25 µL and DNA template 2 µL. The PCR reaction condition is as follow: An initial denaturation 95°C for 180 sec, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 72°C for 30 sec and extention at 72°C for 90 sec. The final extention was done at 72°C for 60 sec. The PCR products were sent to 1 Base Malaysia for sequencing.

Analysis of sequencing result of 16S rRNA fragments: Sequencing analysis followed the protocol provided by Suresh *et al.*[17]. Briefly, chromatograms were edited using Geneious R10 software. The sequences were trimmed approximately 50 nucleotides at both ends. The edited sequences were subjected to Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to query for highly similar sequences. The highest identity results determined the expected species or genera.

RESULTS AND DISCUSSION

In this study, 4 isolates were able to be isolated and purified based on different morphological appearance on LB agar media with each concentration of arsenic. Isolates A and B were purified from agar containing 500 ppm arsenic while isolates C and D were purified from agar containing 1000 ppm arsenic. All purified isolates were subsequently inoculated on the agar slant media for further identification. The identification results based on morphological and physiological tests are presented in Table 1. All isolates were rod-shaped, Gram negative and nonmotile bacteria. Based on the test, isolates A and B gave similar results, while C and D gave different results.

The molecular identification based on 16S rRNA gene is presented in Table 2. Isolate A had 93% identity with *Stenotrophomonas maltophilia* strain C-1, isolate B had 99.8% with *S. maltophilia* strain C-1, isolate C had 94.63% identity with *Pseudomonas entomophilia* strain AS90 and isolate D had 99.73% identity with *P. putida* strain ZJB-LLJ. The lower identity results for isolates A and C raise doubts about the results of identification to state that they are the species in question. Some researchers have proposed different thresholds for bacterial species delineation using 16S rRNA gene including[18-20] 98.65, 95-96 and 97%. This indicates that the cut off is set to minimum 95% for 16S rRNA. Using this threshold, it implies that isolate A is not *Stenotrophomonas maltophilia* but *Stenotrophomonas* sp., even though using the morphological, physiological and biochemical tests showed the similar morphology and characteristics. Using the same principle, then isolate C is not *Pseudomonas entomophilia* but *Pseudomonas* sp.

Several bacteria belonging to the genera *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium* and *Pseudomonas* have been reported to be resistant to arsenic[21,22]. Resistance to arsenic species in both Gram-positive and Gram-negative bacteria is resulted from energy-dependent efflux of either arsenate or arsenite from the cell mediated via the ars operon[23]. In *E. coli*, an arsA-arsB complex functions as a primary arsenite pump[24]. In *S. aureus*, arsB alone is sufficient to act as a chemiosmotic secondary transport system for arsenite resistance without the presence of an arsA ATPase[25].

Table 1: Identification results based on morphological and physiological tests

	Code of isolates			
Tests	A	B	C	D
Morphology	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped
Gram staining	-	-	-	-
Motility	-	-	-	-
Glucose	+	+	+	+
Indole	-	-	+	+
Citrate	+	+	+	+
Lysine	-	-	-	+
H_2S	-	-	-	-
H_2O_2	+	+	+	+

+: Present, -: Absent

Table 2: Results of BLAST analysis of 16Sr RNA sequence

Isolates	Species description	Max. identity (%)	Query cover (%)
A	*Stenotrophomonas maltophilia* strain C-1	93.00	99
B	*Stenotrophomonas maltophilia* strain C-1	99.80	99
C	*Pseudomonas entomophilia* strain AS90	94.63	99
D	*Pseudomonas putida* strain ZJB-LLJ	99.73	99

Stenotrophomonas maltophilia is a commensal and an emerging pathogen earlier noted in broad-spectrum life threatening infections among the vulnerable, but more recently as a pathogen in immune-competent individual. This bacterium is also an environmental bacteria occurring in water, rhizospheres, as part of the animals' microflora, in foods and several other microbiota[26]. *Pseudomonas putida* and *Pseudomonas mendosina*, also found in arsenic-contaminated environments in Bulgaria, which were of particular interest, because they appeared to gain metabolic energy during the arsenic transformation, were characterised[27]. Bacteria adapt to arsenic toxicity mainly by the development of resistance mechanisms conferred by chromosomal or plasmid-encoded arsenical resistance (ars) operons. Once the trivalent form of the metalloid accumulates in the cell, resistance is produced by their removal from the cytosol[28]. The results of the arsenite absorption of isolates that analyzed by CVAFS showed that all isolates can reduce the levels of arsenic in media by almost 100% within 72 h. This indicates that all isolates have the ability to be further investigated as bioremediation agents in the arsenic-contaminated sites.

CONCLUSION

The present study revealed that *Stenotrophomonas maltophilia*, *Stenotrophomonas* sp., *Pseudomonas* sp. and *P. putida* isolated from Buyat estuary and beach were able to reduce the arsenite concentration quickly. These bacteria can be investigated further for their potential to remediate the environment with arsenic contamination.

SIGNIFICANCE STATEMENT

This study discovered that *Stenotrophomonas maltophilia, Stenotrophomonas* sp., *Pseudomonas* sp. and *Pseudomonas putida* showed the high ability to detoxify arsenite and can be used in arsenic-contamination site for bioremediation of arsenic. However, it is advisable to develop a safe method of arsenic detoxification by using enzymatic methods which can be carried out in further studies.

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