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Molecular Characterization of Zinc (Zn) Resistant Bacteria in Banger River, Pekalongan, Indonesia

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Abstract

Indigenous bacteria are able to remove the metals contamination in environment. This study aimed to assess the resistance of bacterial species to Zinc (Zn) in Banger River, Pekalongan City. The bacteria from three different parts of Banger River were isolated and inoculated in Zn-selective medium. Then, molecular identification to determine the bacteria species was conducted using polymerase chain reaction (PCR) by applying forward-reverse *16SrRNA* gene primers. The sequences analysis was conducted using MUSCLE and MEGA6. There were seven dominant species that possibly resistant to Zn. Approximately, every isolate could reach more than 95 % from 2000 ppm of Zn in the medium. The higher absorption of Zn was found in Z5 isolate. The seven bacteria species were clustered into nine genera i.e. *Klebsiela, Xenorhabdus, Cronobacter, Enterobacter, Escherichia, Shigella* and *Sporomusa* known as Gram Negative bacteria and *Clostridium* and *Bacillus* as Gram Positive bacteria. In Gram Positive bacteria, especially *Bacillus* sp, carboxyl group in peptidoglycan play a role as metal binder. In Gram-negative bacteria, lipopolysaccharide (LPS) which is highly anionic component on the outer membrane, able to catch the Zn. Besides that, *Enterobacter* activates endogen antioxidants such as glutathione peroxidase (GSHPx), glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD). The research found there was possible seven novel indigenous bacteria species in Banger that able to remove Zn from the sediment extremely. This finding can be developed as an eco-friendly approach to reduce metals pollution using local microorganisms.

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INTRODUCTION

Banger River is one of the major rivers in Pekalongan City. The river is located in northern part of Central Java Province, which covers an area between 6 ° 51'0 "-6 ° 55'27" S and 2 ° 50'26 "-2 ° 53'44" E. The river is functioned as a flood controller and irrigation, but it has been contaminating by domestic/ household and agricultural chemical wastes. The river is also contaminated by wastewater from industrials such as metals and textile industries. Wastewater and pollutions produced by textile factory are from coloring process using synthetic dyes. The textile dyes are composed by unsaturated organic compounds, chromospheres and auxochrome. They contain dangerous heavy metals as an activator, such as zinc (Zn), chromium (Cr), lead (Pb), nickel (Ni), copper (Cu) and manganese (Mn) (Madhav *et al.*, 2018). Due to the dyes are applicable, easy to use and inexpensive, synthetic dyes are favorable and used widely.

Heavy metals composition trigger DNA mutation in organism (Viti *et al.*, 2014), decreases water quality and reduce aquatic ecological carrying capacity for organisms that live in. Based on the investigation, one of the heavy metals that exceed the limit in the Banger river is Zn. The various forms of Zn pollution in excessive concentration are dangerous contaminant in the environment (Colin *et al.*, 2012). Actually, Zn is an essential cofactor for numerous enzymes in organisms, but it can be toxic when it presents in excess.

Microorganism especially bacteria, is an adaptable organism. It presents in the natural ecosystem and possibly able to survive in the Zn-contaminated environment. They can adapt in environment containing high Zn concentration by enduring the biotransformation and bioaccumulation ((Colin *et al.*, 2012, Edwards & Kjellerup, 2013, Hansda & Kumar, 2016). This study was determined indigenous bacteria species using molecular characterization, which is possible to be used as bioremediator. The information obtained from research can be used as base line data to develop new eco-friendly strategy for clearing environment from metals contamination.

METHOD

This research applied observational exploratory method to determine Zn-resistant bacteria abundance in Banger River, Pekalongan. All data obtained was rendered to genotype of river's sediment bacteria that growth in Zn selective media. The bacteria was identified using *16S rRNA* gene and aligned to existing-bacteria species on online based data.

Sample collection

Two-centimeter top soil of river's sediment from three-different spot; upstream area S, 6.91602°-E,109.67361°, middle area S, 6.87730°-E, 109.67740° and downstream area S, 6.85875°-E, 109.69386° were collected. The samples were collected for three repetition using Petersen grab, then the sediment samples were placed in sterile Smith Mcintyre grab plastic bags 0.1 m^2 (Hampshire, UK) per each area, separately. Then, the soil samples from different spots were mixed and homogenized manually. After that, 250 gr of soil was moved from plastic bag into polyethylene plastic zipper then store at 4 °C.

Metal detection

Every collected soil sediment was placed in the teflon beaker separately and oven-dried for 8 hours at 105 °C. After dry, the sediment was rinsed using metal-free distillated water for 3 times and re-dried at the same temperature. About 5 grams dried sediment was destroyed and destructed using HNO_3 : HCl, (ratio 3 : 1) at ±100 °C for 8 hours. The level of Zn was measured using Atomic Absorption Spectrometer (AAS) Spectra A-20 Varian plus (The Netherlands). The AAS procedure was conducted in Chemical Instrumentation Laboratory, Universitas Negeri Semarang (Unnes)

Media preparation

All preparations included bacteria culture were conducted in Microbiology Laboratory, Universitas Negeri Semarang. Before isolation, a lactose broth (LB) media was prepared from 1 gr of Tryptone, 0.5 gr of *yeast extract*, and 0.5 gr of NaCl, added to 100 mL distillated water, pH was settled at 7-7.2. All composition was mixed and autoclaved at 121°C and 2 atm for 15 minute. After that, 1 L of *Nutrient Agar* (NA) was prepared from 0.1 gr of NH_4Cl, 0.001 gr of $CaCl_2.H_2O$, 0.2 gr of $MgSO_4.7H_2O$, 0.001 gr of $FeSO_4.7H2O$, 5 gr of Na acetate, 0.5 gr of *yeast extract* and 0.5 gr of K_2HPO_4, then pH was confirmed at 7. Before sterilization, NA media was added with 10 gr of $ZnCl_2$ per litter media to make Zn NA (Zn-NA) selective medium. In preliminary experiment, the bacterial colonies, which growth in Zn-NA were uncountable, so, to get specific bacteria, $ZnCl_2$ concentration was increased until 2,000 mg per Litter.

Bacteria inoculation, isolation and DNA purification

One gram of fresh or freeze sediment samples was diluted in 9 mL sterile LB in series until concentration reached 10^{-6} gr/ mL. Then, the diluted-bacteria inoculant was spread out on the Zn-NA (selective medium) and incubated at 37 °C for 24 hours (Rehman *et al.*, 2010). To imitate the real condition of bacteria's habitat, the liquid medium was not treated with aeration (Alexandrino *et al.*, 2014).

Before genetic identification conducted, the bacteria samples were identified based on phenotype characterization (Ifandi & Alwi, 2018). The numbers of bacteria were identified by their colony form. The colony forms were separated onto different Zn-NA media. In low Zn concentration, the bacteria on the media was abundance and the identification process was complicated. The Zn concentration was increased up to 2000 ppm of $ZnCl_2$. There were seven dominant isolate bacteria popped up in the media and continued to DNA isolation. DNA from isolated bacteria were purified using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific: Jakarta, Indonesia) following the protocol from the manufacture. Polymerase chain reaction (PCR) amplification was done using primers 16S rRNA, 27 F: 5'-AGATTGATCTGGCTAGGGA-3' and 1492 R: 5'-TACGGTACCTTGTTACGCTT-3' (1500 bp product) (Vasas *et al.*, 2013)

Amplicons from PCR procedure was sequenced and analyzed using Sanger Sequencing and Fragment Analysis Software. The *16SrRNA* gene sequences were alignment to the basic local alignment search tool (BLAST) features from NCBI (https://www.ncbi.nlm.nih.gov/). The *16SrRNA* gene sequences fasta from fifteen species of nine genera were downloaded for species references. All sequences were aligned using MUSCLE software, the phylogenetic tree was developed using Mega 6 (Tamura *et al.*, 2013).

RESULTS AND DISCUSSION

High Zn contamination in Banger Rivers provides extreme environment for organisms including bacteria. Based on AAS, Zn content in river's sediment reached 128.31 ppm in upstream, 151.26 ppm in middle stream and 120.68 ppm in downstream. It is higher than WHO's standard of water pollution for Zn contamination in aquatic ecosystem .that is 1.5 ppm.

Our research was investigated several bacteria, that able to survive from high Zn concentration. This research only focused on molecular identification of bacteria and its capability on Zn absorption. The mechanism developed by bacteria to survive in metals contaminated environment needs to be more explored. From our study, at least seven species were found in the Banger River's sediment that compared with nine genera reported in NCBI.

The results of molecular identification sho-

Table 1. The reference species used in this research

Species name	NCBI Accession number	Publication
Escherichia coli strain 202	MH671481.1	(Lin, 2018)
Bacillus anthracis strain WY2	KF641920.1	(Chun *et al.*, 2012)
Bacillus aryabhattai strain APBSWPTB141	MG733614.1	(Krishnamoorthy & Gunthe, 2017)
Bacillus cereus strain SWFU2816	JN935015.1	(Wang & Zhang, 2011)
Clostridium botulinum strain CIFT_Mfb_julcb8	MG793684.1	(Athira *et al.*, 2017)
Clostridium sporogenes strain LAG1	MH760799.1	(Awotula *et al.*, 2018)
Cronobacter sakazakii strain 1	KY524290.1	(Gabra, 2017)
Enterobacter cloacae strain PB-S2	GU459209.1	(Beneduzi *et al.*, 2008)
Enterobacter tabaci strain BC2505	MF682952.1	(Bae *et al.*, 2018)
Escherichia vulneris strain NEW-ERY-1	MF079358.1	(Han, 2018)
Klebsiella pneumoniae strain KP7	MH819550.1	(Baqer, 2018)
Shigella sonnei strain E10	MH174662.1	(Hajoori & Jain, 2018)
Sporomusa malonica strain DSM 5090	NR_117652.1	(Csotonyi *et al.*, 2015)
Sporomusa rhizae strain RS	NR_042457.1	(Gößner *et al.*, 2006)
Xenorhabdus sp. PDBC SCX7 1	DQ026512.1	(Nagesh *et al.*, 2005)

wed that there were seven Zn-resistant species of bacteria that related to nine genera of existed bacteria known to be growing on high Zn concentration (Table 2). Based on preliminary biochemical identification, it was known that the seven species of isolated bacteria consisted of 4 Gram Negative bacteria (Z1, Z3, Z4 and Z6) and 3 Gram Positive bacteria (Z2, Z5 and Z7) (Table 2).

Identification process was conducted using multiple sequence alignment, MUSCLE. In order to represent the clades that related to the isolated bacteria, every sequence was aligned in BLAST online, then the closely similar sequence was chosen as a species reference. There were 15 species from nine genera accessed from NCBI were chosen due to its identical sequence with isolated bacteria (Table 1). After processing with BLAST alignment, all sequences were aligned to create phylogenetic tree (Figure 1)

The species from BLAST NCBI and Zn-resistant bacteria candidates (22 species) were aligned to build a phylogenic three. Genetic relationship between species was determined from the phylogenetic tree with the bootstrap that showed similarity among species. The Z1 bacteria was more closely related to *Klebsiella pneumoniae* strain KP7 than *Xenorhabdus sp.* PDBC SCX7 1. In the same node, bootstrap value among Z1 and *Klebsiella*, *Xenorhabdus* and several species such as *Escherichia vulneris* and *Enterobacter cloacae* was around 83%. It means there are 83 iterations that cover same nucleoside base in the sequences. Z1 bacteria was also related to the Z3 isolate that was placed in the same node with the bootstrap value reaching up to 83%. However, in spite of the closest species to Z3 bacteria was *Escherichia vulneris* strain NEW-ERY-1, the bootstrap value or similarity was only 51% and. Whereas, minimum accepted bootstrap score for similarity was 70%, so it was likely that Z3 was totally different from others reference species in the nodes.

The phylogenetic tree also showed that most of the Zn resistant bacteria were placed out of the main groups. It explained that Z1 and Z6 bacteria were out of group members of Clade 1, Z5 and Z2 were out group members of Clade II, then Z2 and Z4 were out group members of Clade III. But, the phylogenetic tree also showed that Z5 bacteria was closest to *Bacillus* genera, then Z7 was closest to *Bacillus cereus* strain SWFU2816, specifically. In the separated part, Z4 and Z2 bacteria were defined as far relative of all species, both of them were placed far from the main clades.

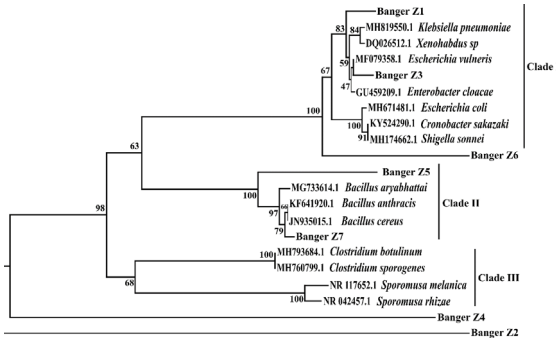

Figure 1. Phylogenetic tree of the Zn resistant bacteria isolated from Banger River. The Zn-resistant bacteria are represented by the codes Banger Z1-Z7.

Result of this study showed that the most abundance bacteria was Z2 with total 2.8 x 10^8 cells/ mL medium and the least was Z7 bacteria with concentration only reached 0.6 x x 10^8 cells/ mL medium. However, small amount of bacteria concentration in Z7 could absorb 95.57% Zn in medium. The highest absorption of Zn was occurred in Z5 isolate that reached 95.65% (Table 2).

Anaerobic environment in the sediment possibly influences bacteria's growth and develops different mechanisms in each bacteria species. Bacteria is able to adapt in the environment with high heavy metal contamination (Yin *et al.*, 2015, Fashola *et al.*, 2016). Most of species has

Table 2. Preliminary identification of isolated bacteria and Zn absorption

Isolates	Gram Type	bacteria count (cell/ mL)	Zn_{-0} (ppm) in medium	Zn_{-final} (ppm) in medium	Zn absorption (%)
Z1	Negative	0.8 x 10^8	2000	90.13	95.49
Z2	Positive	2.8 x 10^8	2000	91.44	95.43
Z3	Negative	0.7 x 10^8	2000	94.56	95.27
Z4	Negative	0.9 x 10^8	2000	99.86	95.00
Z5	Positive	2.6 x 10^8	2000	86.98	95.65
Z6	Negative	1.7 x 10^8	2000	95.15	95.24
Z7	Positive	0.6 x 10^8	2000	88.56	95.57

developing variety mechanisms to increase metals absorption and utilization, such as by metalloproteinase (Waldron & Robinson, 2009) or transport protein through cell membrane (Shin & Helmann, 2016), metal transport protein production (protein that fastening the metal transport) (Guo *et al.*, 2010), and accumulation in compartments especially inside the cell (Kambe *et al.*, 2015). This ability is important to help bacteria survive in excess heavy metals condition (Esmailzadeh *et al.*, 2016).

Physiologically, bacteria's cell wall pH provides negative charge or anionic surfactant. Environmental-exposed amino acid is hydrophilic, it provides opposite charge that interact with ions or charged molecules from environment. As a result, the metal anions or cation electrostatically will bind to the cell surface and push other same-charged ions away (Yang *et al.*, 2011). The ability of bacteria to produce extracellular polysaccharide such as endospores, which develops by *Clostridium* may protects their cell from toxic effect of heavy metals (Alexandrino *et al.*, 2014).

The study of interaction of metal ions and the cell wall of Gram-positive bacteria, especially *Bacillus sp*. showed the role of the carboxyl group of peptidoglycan or phosphoryl group in secondary polymer in metal binding (Shin & Helmann, 2016). Bacillithiol is also one of main protein that produced by *Bacillus* to reduce damage impact of heavy metal excess (Gaballa *et al.*, 2010, Gaballa *et al.*, 2014). In Gram-negative bacteria, the ability to bind metal is estimated due to the layer of lipopolysaccharide (LPS), which is highly anionic on the outer membrane and increases the regulation of endogenous antioxidant (Pandey *et al.*, 2013). In *Enterobacter*, a mechanism by activating endogen antioxidants such as glutathione peroxidase (GSHPx), glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD) has increase after the exposure to heavy metals (Banerjee *et al.*, 2015). In principle, this condition presumably because Gram-positive and Gram-negative has difference mechanism in metals interaction (Rojas *et al.*, 2014). Bacteria's cells wall also contain cell surface area in a negative charge, such as areas with the cluster of Phosphoryl (PO_4^{3-}), carboxyl (COO), sulfhydryl (SH) and hydroxyl (OH^-), resulted in metal ion interaction with the negative charge. However, our research finding showed that two species from Gram positive bacteria, more tolerant to Zn, greater than all Gram-negative bacteria.

Most of the bacteria are able to produce internal carriage protein to localize Zn such as zntR regulated by *zntA* gene (Wang *et al.*, 2012). Bacteria is also able to conduct Zn metabolism through intracellular sequestration or bound it to polymer products such as metallothionein. It is a metal-binding protein functioned to metals inactivation process or metal belay on network in every living organism (Bakiu *et al.*, 2013). The metallothionein contains thiol' (-SH) molecule group in large numbers (Hernandez & Fernandez-Lafuente, 2011) that able to bind to specific heavy metals, such as Zn (Antelmann & Helmann, 2011).

In this research, all of the cultured bacteria were reducing Zn ion more than 95 % from the media. It can be assumed that the bacteria were used absorption and localization mechanism to survive in Zn contaminated habitat. It is also a new record from Banger River about indigenous bacteria that are able to reduce Zn contaminant up to 95 %.

This research is important to help researchers to reveal Banger River's indigenous bacteria species that can be developed as an eco-friendly approach to reduce metals pollution using local microorganisms. The research is also completing new documentation of local microorganism gene sequence and its capability in Zn elimination.

CONCLUSION

There were seven indigenous species of bacteria in Banger River that able to remove the Zn from river sediment effectively. Based on the molecular identification, there were seven indigenous bacteria species that growth successfully in the excesses ZnNA. The Z1 Z3 and Z6 bacteria are closely related to Gram negative bacteria species, than Z5 and Z7 are closely related to *Gram Positive bacteria*. Meanwhile, Z2 and Z4 could not be determined because they were separated from main clades. All bacteria were able to reduce Zn more than 98% from the media

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