Isolation and Morphological Characterization of Lead Tolerant Bacteria Associated with *Perna viridis*

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Abstract

Lead is a heavy metal contamination that is released into sea waters and cannot be decomposed so it experiences accumulation and magnification along the food chain. Efforts to explore bacteria that have tolerance and have the potential to become lead reduction agents are a strategic step in remediating lead contamination. The aim of the research was to isolate and characterize the morphology of lead tolerant bacteria from the green mussel *Perna viridis*. The research method was carried out by isolating bacteria associated with green mussels by grinding their organelles. Next, a tolerance test was carried out for bacterial isolates using Luria Bertani media which was added with $Pb(NO_3)_2$ at a concentration of 100 ppm and its multiples, then compared with the 0 ppm control. The bacterial isolates obtained were characterized based on colony and cell morphology. The results of the research obtained 11 bacterial isolates that were able to grow at a concentration of 10 ppm. A total of 3 bacterial isolates were tolerant to lead with a lead concentration of 400 ppm. Bacterial isolates that are tolerant to lead show changes in cell morphology to reduce the impact of exposure to lead which has a lethal effect because the decrease in cell surface area that occurs can reduce lead absorption.

Keywords : isolation, characterization, lead, tolerance, mussel

INTRODUCTION

Indonesia is an archipelagic country with abundant marine wealth and high-value commodities, but along with increasing human activities, marine waters become polluted due to pollutants released. One of the pollutants released in marine waters is heavy metals that occur worldwide, including most of Indonesia's coastal waters (Putra *et al*., 2022). Some of the factors causing the presence of heavy metals with high concentrations in a body of water are industrial activities, mining activities, and agricultural activities. Some heavy metals often found in aquatic ecosystems include lead, copper, chromium, and zinc. Zinc and Copper are metals that are often found as pollutants because they are essential metals. Lead is a heavy metal with high toxic levels that can disrupt various physiological processes in organisms. Lead does not have any biological function unlike other metals, such as zinc, copper and manganese. Lead metal will act as toxic in living cells by following ionic mechanism and that of oxidative stress (Jaishankar *et al*., 2014). Over time, heavy metals in the aquatic environment will accumulate and magnify in biotic or abiotic components (Harmesa *et al.*, 2020). The accumulated heavy metal compounds will be passed through the food chain and poison every organism that consumes them. Lead (Pb) is one of the heavy metals that accumulate in aquatic living things.

Lead is heavy metal pollutes marine aquatic ecosystems that can accumulate in green mussels (*Perna viridis*). Green mussel is a marine life settled on objects in marine waters. Green mussel obtains nutrients from food consumption by filtering food sources without a selection process in waters, which makes them often used by humans as bioindicator agents (Yaqin *et al*., 2020). Lead contaminant compounds that detect and accumulate in the body of green mussel can illustrate pollution in marine aquatic ecosystems.

*Corresponding author http://ejournal.undip.ac.id/index.php/buloma Diterima/Received : 15-08-2023
DOI:10.14710/buloma.v12i3.59350 Disetujui/Accepted : 27-09-2023

The gill tissues of the mussels body showed the highest lead concentration. The circulation of green mussel consumption in a food chain can cause the transfer of heavy metal compounds Pb into the human body after consumption. Lead heavy metals entering the human body do not provide biological benefits and can cause fatal organ damage (Botte *et al*., 2022; Ashkan, 2023).

Leads were contaminated green mussel found on the north coast of Java Island, based on previous identification. High concentrations of lead due to industrial activities such as the ceramics, aluminum, and textile industries have polluted the green mussels state at the coast of Semarang Harbor (Ihsan *et al*., 2019). Green mussels exposed to lead make associated bacteria tolerant to lead. Green mussels associated bacteria tolerant to lead are a strategy to reduce lead contamination in the environment. One effective mechanism for metal binding by bacteria is bioaccumulation using metallothioneins proteins (Tiquia-Arashiro, 2018; Chatterjee *et al*., 2020). Metallothionein is a ubiquitous, cysteine-rich protein that is involved in homeostatic metal response for the essential metals (Wong *et al*., 2017). Identification of green mussel associated bacteria that can synthesize metallothionein will be a strategic first step to explore bacteria that have the potential to become lead bioremediation agents. This research aims to isolate and identify lead tolerance bacteria that are associated with green mussels so that they have the potential to be used as lead bioremediation agents.

MATERIAL AND METHOD

The research was conducted at the Biotechnology Laboratory, Diponegoro University, for four months in July-October 2023. The tools and materials used in the research are samples to Luria Bertani agar media (LBA), Luria Bertani broth (LBB) media, sterile aquadest, $Pb(NO_3)_2$, G dye (violet crystal, iodine, 96% alcohol, safranin), centrifuge, oven, atomic absorption spectrophotometer (AAS), water bath, micropipette, spreader, ice box, and shaker incubator.

Green Mussel Sampling

Green mussel sampling will be carried out in July 2023 in Semarang Harbor in three location Tambak Lorok (TL), Demak (DMK), and Banjir Kanal Timur (BKT). Green mussel samples were brought using ice boxes to maintain the freshness of green mussels during the trip to the Biotechnology laboratory of Diponegoro University.

Lead Grade Test on Green Mussels

Lead test preparation found in green mussel samples starts with separating and drying the green mussel organs using an oven with a temperature of 80°C for 12 hours. The dried green mussel organs are then mashed using a mortar and pestle. The fine sample was then weighed with a mass of 5 gs (Aznardi *et al.*, 2022). Lead levels in the prepared green mussel samples were measured using an atomic absorption spectrophotometer. Lead content test results in the form of heavy metal content points in ppm units.

Isolation and Screening of Green Mussel Symbion Bacteria

The organs of the green mussel are separated from the shell, weighed by 2 gs, and crushed using a mortar and pestle. The delicate green mussel organs are put into Luria Bertani Broth (LBB) media made with sterile seawater. Green mussel organelles that have been inserted into LBB media are then diluted in stages 7 times. Bacterial cultures at dilution levels 5, 6, and 7 were then grown on Luria Bertani Agar (LBA) media. Bacteria that grew at the beginning of isolation were then screened using Luria Bertani media by adding 10 ppm $Pb(NO_3)_2$. The bacterial isolate was incubated for 24 hours with a temperature of 37°C (Aznardi *et al.*, 2022).

Morphological Characterization of Green Mussel Symbion Bacterial Isolate

The morphology of green mussel associated bacterial isolates were characterized using two observation parameters, morphological observations (macroscopic) and cell observations (microscopic). Observations of colony morphology are based on the shape, margins, and elevation of the colony. Meanwhile, microscopic cell observations are based on G staining, cell shape, and cell size (Marzan *et al.*, 2017).

Tolerance Test of Green Mussel Symbiont Bacteria to Lead

The bacterial isolates obtained inoculated on liquid media Luria Bertani Agar (LBA) that was given $Pb(NO_3)_2$ with various concentrations, 0 ppm, 100 ppm, 200 ppm, 300 ppm, and multiples until bacterial isolates were unable to grow (Aznardi *et* *al*., 2022). Phase contrast microscopy was used to observe isolates capable of growing on lead-containing media cell morphology.

RESULT AND DISSCUSION

Lead Metal Analysis in Green Mussel Samples

The measurement of lead heavy metal levels in green mussel samples has taken to determine the environmental conditions. The condition of lead heavy metal contamination found in green mussels in Semarang Harbor has various contamination conditions (Figure 1). Several factors can cause the condition of contamination accumulated in Semarang Harbor. The opinion expressed by Ihsan *et al*. (2019) stated that the high lead contamination in Semarang Harbor caused by solid and liquid waste discharges released by Baboon River industrial activities and community waste, which included ceramic, aluminum, and textile production waste.

Heavy metal contamination released in the waters of Semarang Harbor due to anthropogenic activities accumulates in green mussels because green mussels are marine living things that live sedentary on hard objects submerged in seawater and have a mechanism of taking food without a selection process/filter feeder non-selection. This way of life makes green mussels that settle in Semarang Harbor make green mussels accumulate heavy metals in soft tissues. The statement revealed by Melinda *et al*. (2020) states that the absorption of heavy metals by green mussels exposed to heavy metals causes excess mucus production, which causes a reduction in filtration capacity even at higher levels of contamination and can cause deformation of green mussels. Green mussels taken at 3 locations (BKT, TL, and DMK) in Semarang Harbor were detected to accumulate lead, even though the measured lead levels in green mussels were below the maximum limit of heavy metal contamination in processed foods set by the Food and Drug Supervisory Agency (BPOM), which is 0.20 mg/kg for products derived from mollusks. The accumulation of lead that occurs in green mussels will be passed on in the food chain, which can eventually reach and poison humans as the top of the food chain. The statement expressed by Ashkan (2023) states that lead can have damaging effects on the human body such as neurodevelopmental disorders even though it enters with very low levels. There is no maximum limit regarding lead contamination that is good for consumption.

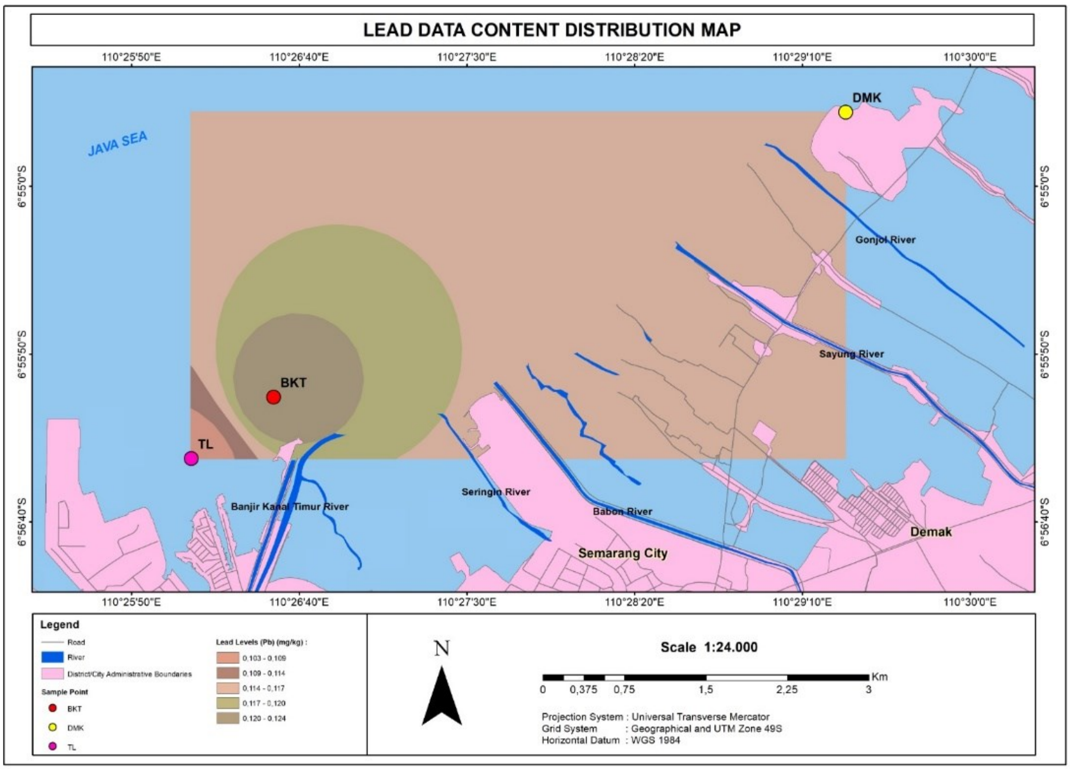

Figure 1. Map of data distribution of lead levels at Semarang Harbor (BKT, TL, and DMK)

Isolation and Screening of Green Mussel Symbion Bacterial Isolate

The results of isolation and screening of green mussel associated bacteria using leaded LBA media with a concentration of 10 ppm resulted in 11 bacterial isolates. The bacterial isolates obtained consisted of five bacterial isolates taken from the TL station, three bacterial isolates from the DMK station, and four bacterial isolates from the BKT station (Figure 2). The morphological characteristics of cells and colonies resulting from green mussel associated bacteria can be seen in Table 1. Bacterial growth in the screening media using lead with a concentration of 10 ppm indicates that bacterial isolates can grow on lead contamination of 10 mg/L, which means that bacterial isolates are able to grow with lead concentrations 100 times higher than the average lead concentration measured at the sampling site.

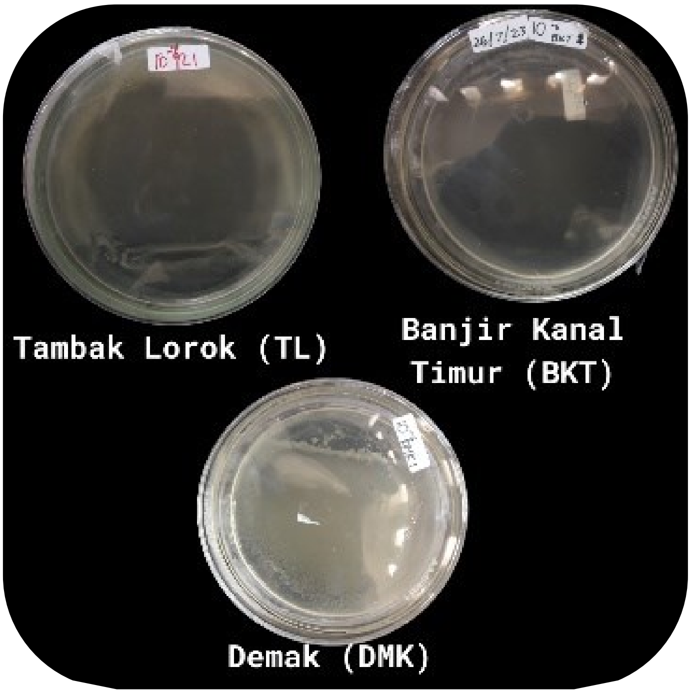

Figure 2. Results of isolation of bacteria associated with green mussels with codes TL, DMK, and BKT

Table 1. Morphological characters of cells and colonies of green mussel associated bacterial isolates from using lead ($Pb(NO_3)_2$) with a concentration of 10 ppm

Bacteria Isolate	Morphological Characteristics					
	Colony Color	Colony Shape	Colony Size	Elevation	G	Cell Shape
TL1	Non-pigmented	Circular	Punctiform	Flat	negative	Bacilli
TL2	Non-pigmented	Irreguler	Small	Flat	positive	Bacilli
TL3	Non-pigmented	Irreguler	Small	Raised	positive	Bacilli
TL5	Non-Pigmented	Circular	Punctiform	Raised	positive	Bacilli
DMK1	Non-Pigmented	Circular	Punctiform	Flat	positive	Bacilli
DMK2	Non-Pigmented	Circular	Punctiform	Flat	positive	Bacilli
DMK3	Non-Pigmented	Circular	Small	Flat	positive	Bacilli
BKT1	Non-Pigmented	Circular	Punctiform	Flat	positive	Bacilli
BKT2	Non-Pigmented	Circular	Punctiform	Flat	positive	Bacilli
BKT3	Non-Pigmented	Circular	Punctiform	Flat	positive	Bacilli
BKT4	Non-pigmented	Circular	Punctiform	Flat	positive	Bacilli

Tolerance of green mussel bacterial isolate to lead

The tolerance test of green mussel associated bacterial isolates was carried out to determine the ability of the tolerance capacity of green mussel associated bacteria isolates to lead. Isolates of green mussel associated bacteria with isolate codes TL3, DMK1, and BKT3 are known to have a tolerance capacity to lead reaching 400 ppm (400 mg/L). Aznardi *et al.* (2022) stated that lead is a non-essential metal for living things but can be tolerated by bacteria at high concentrations. The ability of bacteria to live at high concentrations is supported by several mechanisms that are able to support bacterial life, including using absorption with extracellular polymeric substance (EPS), lead bioaccumulation using metallothionein, lead precipitation, and binding using siderophores. Tiquia-Arashiro (2018) said bacterial tolerance to lead can be an alternative in lead remediation.

Isolates of green mussel associated bacteria with isolate codes TL3, DMK1, and BKT3 that have a high lead tolerance capacity in lead-added media with a concentration of 400 ppm were observed using phase contrast microscopy with the aim of determining the morphological adaptation of bacterial cells to high lead concentrations. The results of observations can be seen in Figure 3.

Bacterial isolates that grow and have tolerance to lead with high concentrations have morphological change adaptations caused by exposure to lead with concentrations of 400 ppm. Changes that occur are shown through changes in the size of elongated bacterial cells, changes in bacterial size that occur in BKT3 bacterial isolates increase by 2 times, TL3 bacterial isolates increase

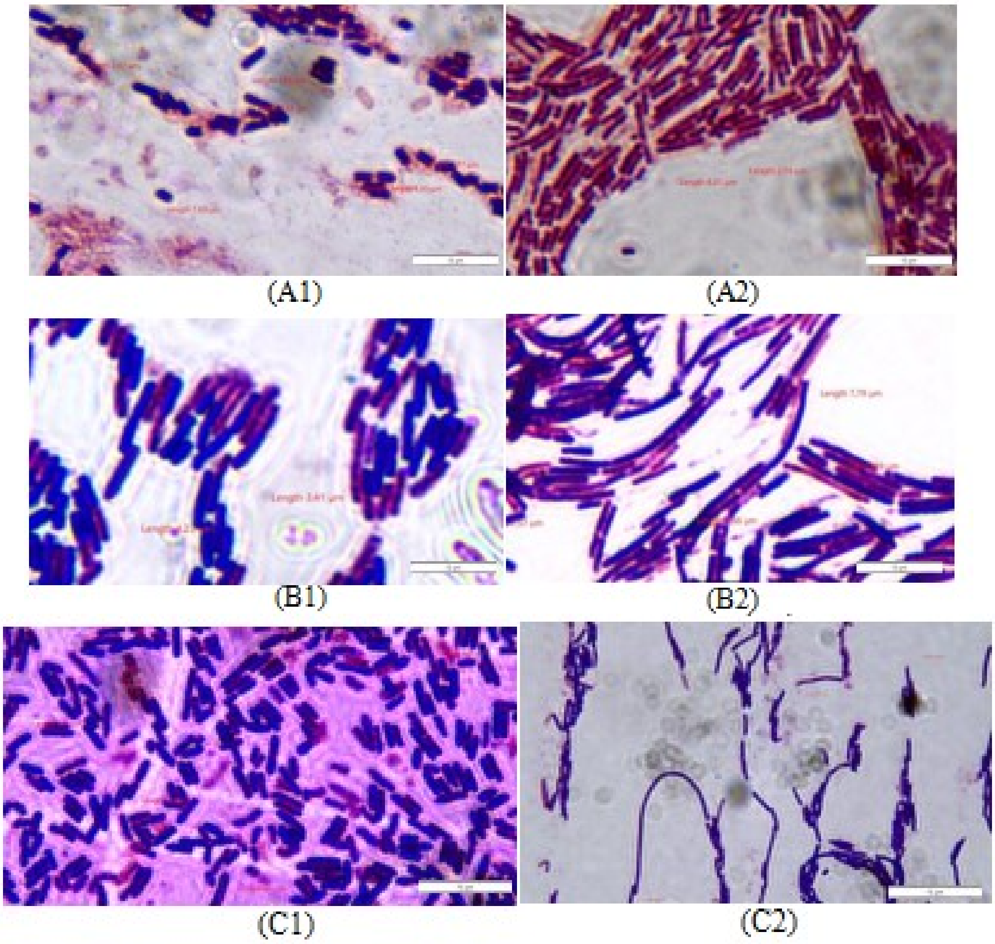

Figure 3. (A1) BKT3 bacterial isolate on lead media with a concentration of 10 ppm, (A2) BKT3 bacterial isolate on lead media with a concentration of 400 ppm, (B1) TL3 bacterial isolate on lead media with a concentration of 10 ppm, (B2) TL3 bacterial isolate on lead media with a concentration of 400 ppm, (C1) DMK1 bacterial isolate on lead media with a concentration of 10 ppm, and (C2) DMK1 bacterial isolate on lead media with a concentration of 400 ppm.

Table 2. Lead tolerance capacity to lead at concentrations of 100 ppm, 200 ppm, 300 ppm, and 400 ppm

Isolate Code	100 ppm	200 ppm	300 ppm	400 ppm
TL1	+++	++	-	-
TL2	+++	-	-	-
TL3	+++	+++	+++	+++
TL5	+++	+++	+++	+++
DMK1	+++	+++	+++	+++
DMK2	+++	++	-	-
DMK3	+++	+++	+++	+++
BKT1	+++	+++	+++	+++
BKT2	+++	+++	+++	+
BKT3	+++	+++	+++	+++
BKT4	+++	+	-	-

Note: -= non-tolerant; += weak; ++= medium; +++=strong

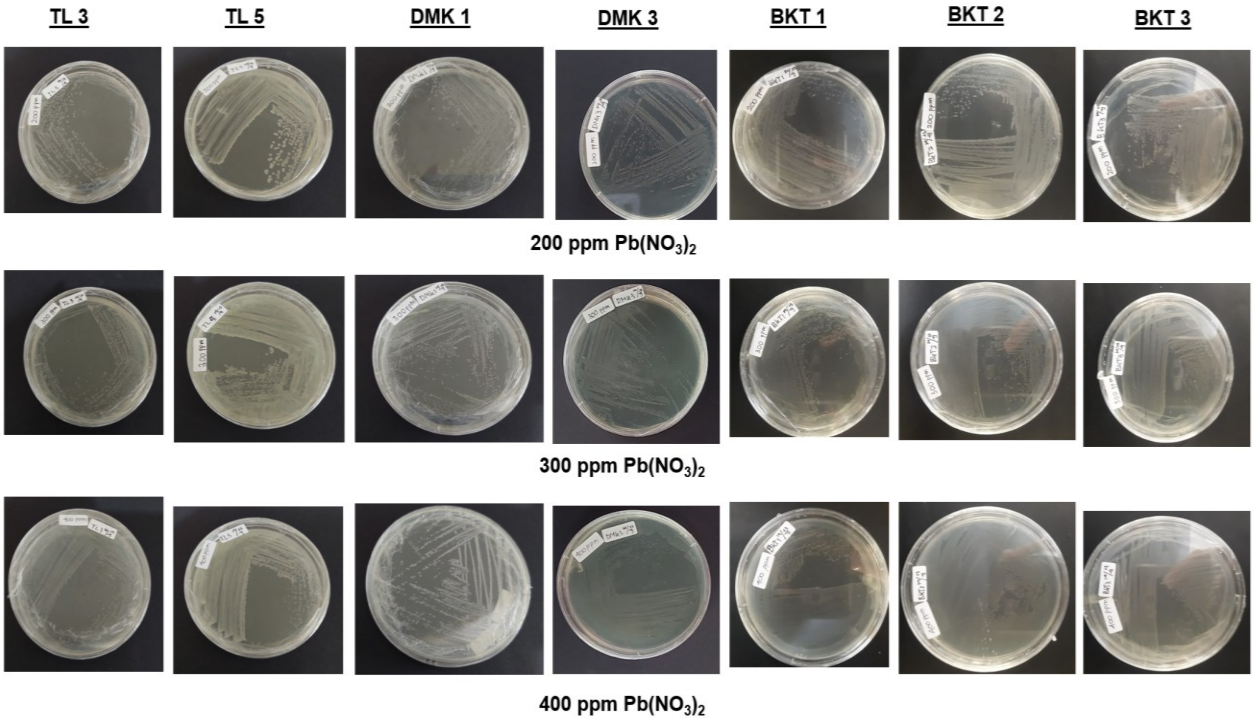

Figure 4. Tolerance bacteria that grow on Luria Bertani Agar media with lead concentrations of 100 ppm, 200 ppm, 300 ppm, and 400 ppm

by 2-3 times, and DMK1 bacterial isolates increase by 3-8 times. Sharma *et al*. (2017) exhibited that the adaptation possessed by bacteria by making structural changes is an adaptation made to reduce the impact of exposure to lead which has a lethal effect, because the decrease in cell surface area that occurs can reduce lead absorption. The lead tolerance capacity possessed by bacterial isolates with codes TL1, TL2, TL3, TL5, DMK1, DMK2, DMK3, BKT1, BKT2, BKT3, and BKT4 can be seen in Table 2.

The tolerance capacity shown by TL3, TL5, DMK1, DMK3, BKT1, BKT2, and BKT3 isolates showed strong results up to lead heavy metal concentrations of 400 ppm compared to other isolate codes (Figure 4). The ability of an isolate to grow at high lead concentrations is a sign that the isolate is tolerant to lead. This is because the ionic mechanism of lead toxicity occurs due to the ability of lead metal ions to replace other bivalent cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations such as Na^{+}. In the end, this will disrupt the biological metabolism of cells where changes occur in various biological processes such as enzyme regulation, cell adhesion, signals within cells and between cells, protein folding, cell maturation, apoptosis, ionic transport, enzyme regulation, and neurotransmitter release. In concentrations as small as picomolar, lead can replace calcium thereby affecting protein kinase C, which regulates nerve excitation and memory storage (Jaishankar *et al*., 2014).

Among the five isolates obtained, there were three isolates that showed higher growth at a lead concentration of 400 ppm, namely isolate codes TL3, DMK1, and BKT3. Bacterial defense strategies in dealing with the effects of lead are generally carried out through mechanisms of biosorption, efflux, production of metal chelators such as siderophores and metallothionein. Other mechanisms include the production of extracellular sequestration and bioaccumulation of lead within cells (Mitra *et al*., 2021). The research result showed the three isolates of tolerance bacteria that are associated with green mussels have the potential to be improved further in the next research for lead bioremediation agents.

CONCLUSION

Bacterial isolates obtained from green mussels taken from Semarang Harbor amounted to 11 bacterial isolates. There were 3 bacterial isolates of green mussel associated bacteria that had high tolerance with isolate codes TL3, DMK1, and BKT3. These three isolates showed changes in cell morphology aimed at reducing the lethal effect.

ACKNOWLEDGMENT

This research was funded by PKM-RE Year 2023 which is gratefully acknowledged

REFERENCES

Ashkan, M.F. 2023. Lead: Natural Occurrence, Toxicity to Organisms and Bioremediation by Lead-degrading Bacteria: A Comprehensive Review. *Journal of Pure and Applied Microbiology,* 17(3): 1298-1319.

Aznardi, S., Madduppa, H., & Ismet, M.S. 2022. Concentration of heavy metals in green mussels (*Perna viridis*) of Lampung Harbor and their symbione bacterial resistance. *Jurnal Ilmu dan Teknologi Kelautan Tropis*, 14(3): 395-404.

Botte, A., Seguin, C., Nahrgang, J., Zaidi, M., Guery, J., & Leignel, V. 2022. Lead in the marine environment: concentrations and effects on invertebrates. *Exotoxicology*, 31: 194-207.

Chatterjee, S., Kumari, S., Rath, S., Priyadarshanee, M., & Das, S. 2020. Diversity, Structure and Regulation of Microbial Metallothionein: Metal Resistance and Possible Applications in Sequestration of Toxic Metals. *Metallomics*, 12: 1637-1655.

Harmesa, H., Lestari, L. & Budiyanto, F., 2020. Distribusi logam berat dalam air laut dan sedimen di perairan Cimanuk, Jawa Barat, Indonesia. *Oseanologi dan Limnologi di Indonesia*, *5*(1): 19-32.

Ihsan, Y.N., Silitonga, P.N.A.Z., Fellatami, K., Syamsuddin, M.L., Zallesa, S. 2022. The distribution of heavy metals in Jakarta and Semarang Bay during the period of 2016-2019. *Jurnal Ilmu-Ilmu Perairan, Pesisir, dan Perikanan*. 11(1): 55-67

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B.B. & Beeregowda, K.N., 2014. Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary toxicology*, *7*(2): 60-72. doi: 10.2478/intox-2014-0009.

Marzan, L.W., Hossain, M., Nina, S.A., Akter, Y., & Chowdhaury, A.M.M.A. 2017. Isolation and Biochemical Characterization of Heavy-Metal Resistant Bacteria from Tannery Effluent in Chittagong City, Bangladesh: Bioremediation Viewpoint. *Egyptian Journal of Aquatic Research*. 43: 56-74.

Mitra, A., Chatterjee, S., Kataki, S., Rastogi, R.P. & Gupta, D.K., 2021. Bacterial tolerance strategies against lead toxicity and their relevance in bioremediation application. *Environmental Science and Pollution Research*, 28: 14271-14284. doi: 10.1007/s11 356-021-12583-9

Melinda, T., Samosir, A.M., Sulistiono., & Simanjutak, C.P.H. 2020. Bioaccumulation of lead (Pb) and mercury (Hg) in green mussel *Perna viridis* (Linnaeus, 1758) in Cengkok Coastal Waters, Banten Harbor, Indonesia. *International Conference on Sustainable Utilization of Natural Resources*, 800: 1-9.

Putra, M.D.N., Widada, S. and Atmodjo, W., 2022. Studi Kandungan Logam Berat Timbal (Pb) pada Sedimen Dasar di Perairan Banjir Kanal Timur Semarang. *Indonesian Journal of Oceanography*, 4(3): pp.13-21.

Sharma, J., Shamim, K., Dubey, S.K., & Meena, R.M. 2017. Metallothionein Assisted Periplasmic Lead Sequestration as Lead Sulfite by P*rovidencia vermicola* Strain SJ2A. *Science of The Total Environment*, 579: 359-365.

Tiquia-Arashiro, S.M. 2018. Lead absorption mechanism in bacteria as strategies for lead bioremediation. *Applied Microbiology and Biotechnology*, 102: 5437- 5444.

Wong, D.L., Merrifield-MacRae, M.E. & Stillman, M.J., 2017. Lead (ii) binding in metallothioneins. *Metal Ions in Life Sciences,* doi: 10.1515/9783110434330-009.

Yaqin, K., Nirwana., & Rahim, S.W. 2020. Konsentrasi Mikroplastik pada Kerang Hijau (*Perna viridis*) di perairan Mandalle Pangkajene Kepulauan, Sulawesi Selatan. *Jurnal Akuatiklestari*, 5(2): 52-57.