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Bioremediation Potential of Heavy Metal-Resistant Bacteria in Koto Panjang Reservoir, Riau, Indonesia

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Abstract: Koto Panjang Reservoir serves as a vital water resource supporting the social well-being of the local community in the Riau Province. The flow of the Kampar River, acting as the riverine zone and water inlet to the reservoir, provides environmental support for the construction of hydroelectric power plants (PLTA) and the aquaculture practice of Floating Net Cages (FNC). Over the past three years, there has been a decline in environmental quality due to heavy metal pollution, exceeding the threshold limits for Cd, Pb, and Zn. This study aims to explore the presence of heavy metal-resistant bacteria in the waters of Koto Panjang Reservoir, which will be evaluated and developed as in situ bioremediation agents through bioaugmentation techniques. The study was initiated into the following stages: Isolation of heavy metal-resistant bacteria, determination of their maximum tolerance limits, and molecular identification of superior isolates or strains. The physico-chemical conditions of the water indicate that Cd, Pb, and Zn pollution in the FNC area has surpassed the minimum threshold for river water and similar categories, as per Regulation of the Government of the Republic of Indonesia No. 22 of 2021, Appendix VI. Heavy metal-resistant bacterial isolates were obtained through enrichment techniques using water samples and Nutrient Broth (NB) media enriched with 1 mM Cd, Pb, and Zn. Eight bacterial isolates were successfully purified from water samples in the FNC area and the upstream area (control). The tolerance capacity to heavy metals expressed in MIC_{50} values shows that all isolates have a tolerance range ranging from 8-43 mM or equivalent to 1,500-12,000 ppm against heavy metal stress. Our findings highlighted the occurrence of indigenous bacterial strains with the potential for use in bioaugmentation to mitigate pollution in the Koto Panjang Reservoir in the future.

Keywords: Bioremediation, Cd, *Comamonas,* 16S-rRNA, MIC_{50}, Pb, Zn

Introduction

Kampar River, with a length of approximately ±413 km, is one of the largest rivers in Riau Province that has undergone environmental engineering to create a new ecosystem, namely Koto Panjang Reservoir (Indonesian Institute of Sciences, 2019). Koto Panjang Reservoir is the second-largest reservoir in Southeast Asia (±12,400 ha) and the largest national reservoir in operation since 1986. It holds a functional water capacity of ±1,040,000,000 m^3, integrated as a source for a 114 MW hydropower plant (Budijono *et al*., 2021).

The reservoir has provided functional ecosystem services to the local community, meeting their needs for clean water and providing a location for fisheries cultivation. The reservoir is home to 29 fish species, with annual catch productivity reaching 223.32 kg/ha/year (Sumiarsih, 2021). Aquaculture activities, specifically using floating net cages (*Keramba Jaring Apung/* FNC in *Bahasa*), have been ongoing since 2001 and continue to function, focusing on key commodities such as common carp (*Cyprinus carpio*) of the Rajadanu variety, gourami (*Osphronemus gouramy*) and tilapia (*Oreochromis niloticus*) (Sumiarsih, 2021). The reservoir ecosystem plays a crucial role in human life, serving as a source of clean water, a fishery resource, and a hub of local to regional biodiversity (Jones *et al*., 2019).

However, the concerning condition of reservoirs requires serious attention to reduce pollution and restore the sustainability of these ecosystems (Reid *et al*., 2019). Since the construction of Koto Panjang Reservoir until now, there has been no formal policy by the local government regarding sustainable water management and reservoir utilization. The lack of such policies has the potential to become a significant issue in the future (Ivey *et al*., 2006). One of the main challenges in the development of fisheries in reservoir waters is the decline in water quality caused by an increase in pollution from various anthropogenic activities in the upstream (riverine zone) and the reservoir (littoral zone) itself, including plantation, agriculture, settlement and tourism activities (Hasibuan *et al*., 2017; Budijono *et al*., 2021).

Budijono and Hasbi (2021) reported the bioaccumulation of heavy metals (Cd, Pb, Zn) in the bodies of cultured fish, with high concentrations of zinc (Zn) in 2019. In 2020, Budijono and Hasbi (2021) also stated that the levels of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) exceeded environmental tolerance limits, indicating pollution from organic pollutants originating from the riverine zone. A study on ecotoxicology in Koto Panjang Reservoir in 2021 further confirmed that heavy metal pollution still occurs in the reservoir waters, particularly cadmium (Cd) and lead (Pb) (Harjoyudanto *et al*., 2022). Periodical absorption of heavy metals by water bodies and contaminated organisms over an extended period can lead to health problems, such as food poisoning, liver damage, cardiovascular disorders, and even death in humans and cultured fish (Balali-Mood *et al*., 2021).

Surface water pollution in most reservoirs in Riau and globally generally originates from soluble organic compounds (primarily from municipal or industrial waste discharge), nutrients (from surface runoff of agricultural land or point sources), and suspended solids from various mineral origins and organic fractions (Mazurkiewicz *et al*., 2020). Microbiological bioremediation is one of the most promising practices in the restoration of polluted surface water and other aspects of environmental engineering. In freshwater ecosystems, microbes play a crucial role in the global energy flow and various biogeochemical cycle pathways (C, N, P, S, and other elements) in both deep reservoirs and shallow lakes (Savvichev *et al*., 2018).

Compared to oligotrophic and stable saline ecosystems (oceans), the ecological conditions of freshwater habitats serve as primary containers for distinct microbial communities between water bodies and sediments (Zhang *et al*., 2020). Environmental conditions temporally influence the species and local bacterial communities, indirectly indicating that each freshwater ecosystem, including reservoirs, possesses its own biodiversity to be explored. One benefit of this biodiversity is the potential of specific isolates or strains that can be utilized as bioremediation agents for the habitat if it becomes contaminated in the future (Muter, 2023).

As of now, it is unclear whether the indigenous bacterial communities in Koto Panjang Reservoir, which have been exposed to pollution for an extended period, demonstrate tolerance to high concentrations of heavy metals (Cd, Pb, Zn). If these communities exhibit tolerance, specific strains with the potential for bioaugmentation could be screened and identified. This approach aims to reduce heavy metal levels and mitigate the risk of further catastrophic pollution in the reservoir.

Materials and Methods

Collection of Samples

The water sampling was conducted within the Koto Panjang reservoir of the Kampar River, Riau Province, Indonesia, which serves as a location for aquaculture in the form of floating net cages (Figs. 1-2). The water sampling was purposively carried out at 10:00 using 100 mL sterile polystyrene bottles collected randomly at each point and composited into 1000 mL. Metal concentrations of Cd, Pb, and Zn were measured using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) in mg/L. Water samples from each point were utilized as a source for bacterial isolation and stored under cold conditions (4°C) for further use. Environmental parameters, serving as supportive data and providing an overview of the current conditions in Koto Panjang Reservoir, were also determined based on standard procedures.

Isolation of Heavy Metal Tolerant Bacteria

The enrichment technique was employed to isolate heavy metal-resistant bacteria. A total of 100 mL water samples from each point were mixed with 100 mL of Muller Hinton Broth (MHB) medium (pH 7.3) supplemented each with heavy metals using 1 mM of $CdCl_2$, $Pb(NO_3)_2$ and $ZnSO_4$ for Cd, Pb and Zn tolerance, respectively. The mixture was then incubated at 30°C for 72 h with agitation at 150 rpm. The enriched culture, appearing turbid, was serially diluted to 10^6 CFU/mL and cultured on solid Muller-Hinton media supplemented with 1 mM of each metal (Thompson *et al*., 2005). Bacterial isolates with distinct morphologies were purified on Nutrient Agar (NA) media and stored as stocks for further processing.

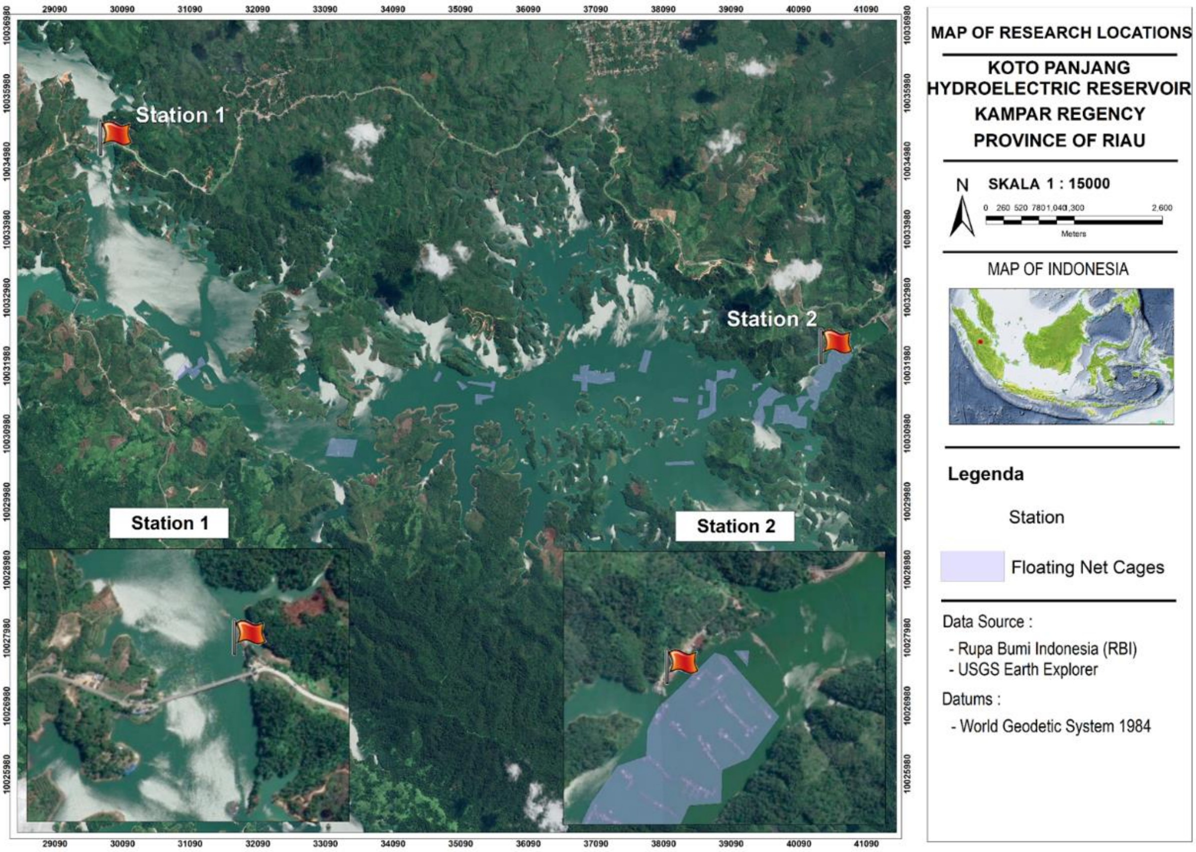

Fig. 1: Map of sampling sites showing Station 1 as a control site and Station 2 as polluted area or floating net cages

Fig. 2: Overview of (A) Station 1, control site, and (B) Station 2, floating net cages near Koto Panjang Reservoir

Minimum Inhibitory Concentration (MIC) Assay

The toxicity of metal ions in bacterial liquid cultures was evaluated to assess the potential of these isolates to tolerate metal ions from liquid media. In brief, metal tolerance in liquid media was determined for all isolates by calculating the MIC_{50} values, which represent the concentration at which bacterial growth was half-maximally inhibited by heavy metal ions using an online tool, AAT Bioquest (https://www.aatbio.com/tools/ic50-calculator). The procedure involved diluting overnight cultures by a factor of 1:100 in fresh Mueller-Hinton Broth (MHB) media supplemented with increasing concentrations of each metal (3.125, 6.25, 12.5, 25, 50, and 100 mM). The cultures were then incubated at 32°C for 24 h. Following incubation, the optical density was determined spectrophotometrically in triplicate at 600 nm (OD_{600}). The MHB media with the corresponding concentrations of lead were used as a blank to eliminate absorption attributed to metal precipitates formed by the media components.

Molecular Identification

Bacterial species identification for each potential heavy metal-resistant bacterial isolate/strain was carried out through molecular analysis of the 16S-rRNA region, outsourced to Macrogen, Inc. (Singapore). Sequencing results from both the forward and reverse sites were combined and aligned with the electronic database in GenBank using the BLASTn feature. The construction of a phylogenetic tree was performed to validate the BLAST results, utilizing the MEGA-XI program.

Results and Discussion

Based on the current environmental conditions, the concentrations of Cd, Pb, and Zn exceed the threshold limits set by the water quality standards for rivers (Classes 1–3) and similar classifications stipulated in the Government Regulation of the Republic of Indonesia in 2021, Appendix VI (Table 1). The concentration values obtained represent a composite from several points around the sampling location. The current levels of heavy metal concentrations in Koto Panjang Reservoir are considerably higher compared to previous findings. Hendrik *et al*. (2021) reported heavy metal concentrations ranging from 0.144-0.170 ppm for Cd and 0.008-0.075 for Pb in 2022. Budijono and Hasbi (2021) also reported that in 2021, concentrations of Cd, Pb, and Zn were still within normal conditions for water bodies, with the highest concentrations in the order of Zn > Pb > Cd.

Hashmi *et al*. (2002) stated that anthropogenic sources of heavy metal pollution in freshwater arise from diesel-fueled transportation modes and diagenetic exchanges between water and sediment. Additionally, domestic waste, agriculture, such as inorganic fertilizers, and industrial activities can also serve as sources of heavy metal pollution, posing threats to the existence of aquatic organisms (Nriagu, 1978; Praveena *et al*., 2008). However, Hendrik *et al.* (2021) confirmed that the presence of Cd and Pb in the reservoir originates from the accumulation of river water flow, runoff, or groundwater flow and was not attributed to the activities of hydroelectric plants in the surrounding area. The total bacterial density from each enrichment exhibited varying results, with the highest log CFU/mL obtained in the following order: Zn(FNC) > Pb(FNC) > Cd(FNC) > Zn(Control) > Cd(Control) > Pb(Control) as depicted in Fig. (3).

In general, the water conditions in the aquaculture site (FNC) indicated the presence of heavy metal-resistant bacteria, suggesting that the area was inhabited by indigenous strains that are tolerant. In the Pb enrichment using water from the upstream (control), no bacterial isolates were obtained to grow on the NA medium. The bacterial enrichment technique using pure heavy metals is intended to select bacterial species or strains that are less tolerant while maintaining the genetic expression (phenotypic) associated with tolerance in the face of heavy metal stress. Orji *et al*. (2021) added 200 mg/L (ppm) of each heavy metal, including Cd, Cu, Hg, Ni, Pb, and Zn, to select for heavy metal-resistant bacteria from the waters of Uburu Lake, Nigeria. Rodríguez-Sánchez *et al*. (2017) also used varying concentrations of $Pb(NO_3)_2$, ranging from 1.0-7.0 mM, to cultivate bacterial isolates resistant to heavy metals from Pb-contaminated soil in Mexico.

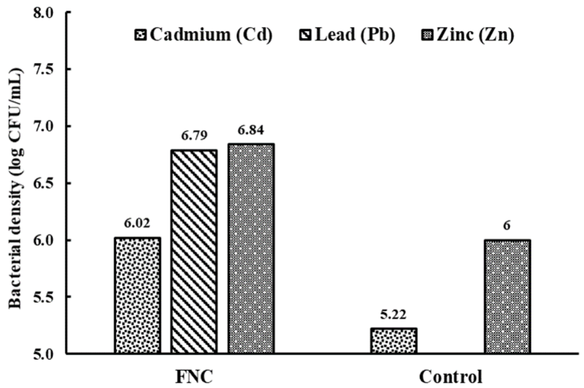

Fig. 3: Total plate count of bacterial density in each enriched sample with heavy metals (Cd, Pb, Zn) among stations

Table 1: Water physicochemical parameters in study sites. FNC = Floating net cages, SNI = Indonesian National Standard or Standard Nasional Indonesia in Bahasa, USEPA = U. S. Environmental Protection Agency, LOD = Limit of detection, Regulatory standard was based on the Indonesian Governmental Regulation No. 22 of 2021, Appendix VI

No.	Parameter	Units	Method	Value		Regulatory Standard (Status)	
A	Physical			FNC	Control	FNC	Control
1	Total Suspended Solids (TSS)	mg/L	SNI 6989.3:2019	7	4	Normal	Normal
2	Total Dissolved Solids (TDS)	mg/L	SNI 6989.27:2019	28	44	Normal	Normal
3	Cadmium (Cd)	mg/L	ICP-MS	0.28	<LOD	>0.01	Normal
4	Lead (Pb)	mg/L	ICP-MS	0.15	<LOD	>0.03	Normal
5	Zinc (Zn)	mg/L	ICP-MS	0.32	<LOD	>0.05	Normal
B	Chemical						
1	Acidity (pH)	-	SNI 06-6989.11-2019	6.8	6.7	Normal	Normal
2	Dissolved oxygen (DO)	mg/L	SNI 06-6989.14-2004	6.7	6.5	Normal	Normal
3	Total nitrogen (N)	mg/L	USEPA	<1.5	<1.5	Normal	Normal
4	Total phosphate (P)	mg/L	SNI 06-6989.31-2005	0.023	0.003	Normal	Normal
5	Detergent content	mg/L	SNI 06-6989.51-2005	0.31	0.069	>0.2	Normal

A total of eight tolerant bacterial isolates (Cd: 3 isolates, Pb: 1 isolate, Zn: 4 isolates) were collected. The tolerance profile of heavy metal from each isolate was modeled (Fig. 4) and expressed in MIC_{50} value (Table 2). The results demonstrate variations in tolerance among heavy metal-resistant bacterial strains obtained from Koto Panjang Reservoir. In sequence, the highest tolerance levels were exhibited by ZnWa01 > ZnWa02 > ZnSu01 > ZnSu02 > PbWa01 > CdSu02 > CdWa01 > CdSu01.

Table 2: Tolerance level (MIC50) of tolerant bacterial isolates

No.	Isolate(s)	MIC_{50} (mM)	Parts per million (ppm)
1.	PbWa01	30.55	10,118
2.	ZnSu01	34.42	9,896
3.	ZnSu02	33.91	9,749
4.	ZnWa01	43.93	12,630
5.	ZnWa02	37.41	10,755
6.	CdWa01	10.48	1,921
7.	CdSu01	8.27	1,516
8.	CdSu02	11.76	2,156

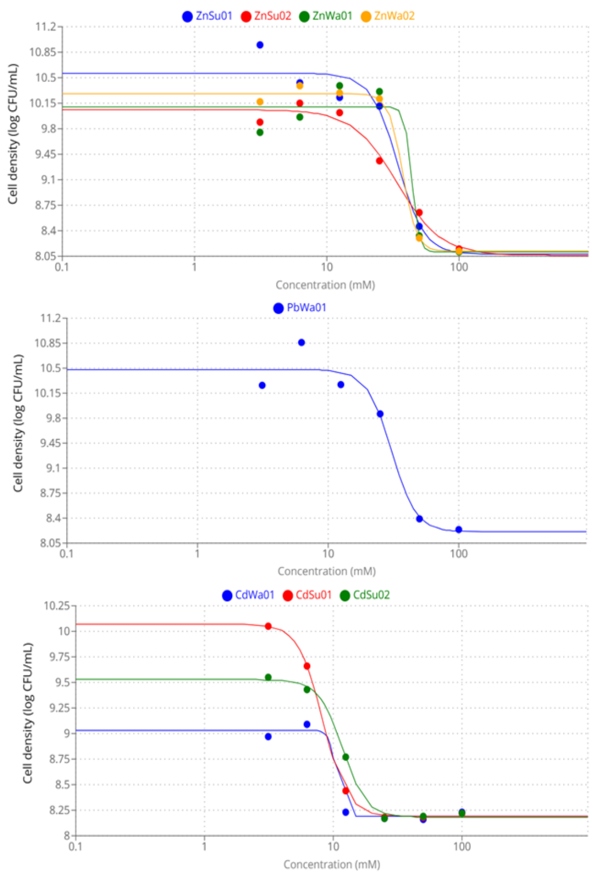

Fig. 4: Heavy metals (Cd, Pb, Zn) tolerance profile of each bacterial isolate from Koto Panjang Reservoir using four parameter logistic (4PL) regression model

The differential toxicity of zinc (Zn) compared to cadmium (Cd) and lead (Pb) towards bacteria can be elucidated through several factors. Firstly, the chemical speciation of metal groups plays a crucial role, where Zn tends to form less toxic or less compatible speciation compared to Cd and Pb (Choudhury and Srivastava, 2001). Secondly, the biochemical role of Zn as a microessential element for many organisms, including bacteria, leads to the evolution of regulatory and control mechanisms in the uptake, storage, and utilization of Zn, which is relatively stable within bacterial cells (Hussain *et al*., 2022; Panina *et al*., 2003). Zinc element also shares similarities with other essential metals, such as magnesium and manganese, allowing bacterial cells to uptake zinc through transportation systems designed for these essential metals (McDevitt *et al*., 2011). Furthermore, the binding affinity of Cd and Pb to biological molecules is often higher than that of Zn, resulting in stronger interactions with cellular components and, consequently, more significant impacts.

Lastly, the toxicity mechanisms of Cd and Pb, involving substitution for essential metals in enzymes and proteins, can disrupt cellular functions, whereas Zn, as an essential metal, does not interfere with cellular processes in the same way because bacterial cells have mechanisms to distinguish between essential and non-essential metals (Bayle *et al*., 2011). It is crucial to note that the toxicity of heavy metals can vary among different bacterial species and strains and environmental conditions also play a significant role in determining the impact of these metals on bacterial cells.

All bacterial isolates were identified molecularly and submitted to GenBank as accessions (OR921364-OR921371). The results indicate that the heavy metal-resistant bacterial communities in the waters of Koto Panjang Reservoir and the upstream area exhibit differences in terms of genus and species. Phylogenetic tree construction was performed to confirm the species identity through BLASTn searches (Fig. 5). *Comamonas* is the genus with the most abundant species and strains, uniquely obtained from both floating net cages (FNC) and the upstream area. Several strains of *Comamonas testosteroni* are environmental bacteria typically found in samples from polluted environments. This group utilizes steroids and aromatic compounds but infrequently employs carbohydrates as a carbon source, demonstrating resistance to various heavy metals and antibiotics (Liu *et al*., 2015). Meanwhile, the understanding of the presence of other species may warrant further investigation through literature review, as some species categorized as potentially pathogenic to humans may not exhibit such traits since these isolates were collected from an environment that tends to be lesser in pathogenicity.

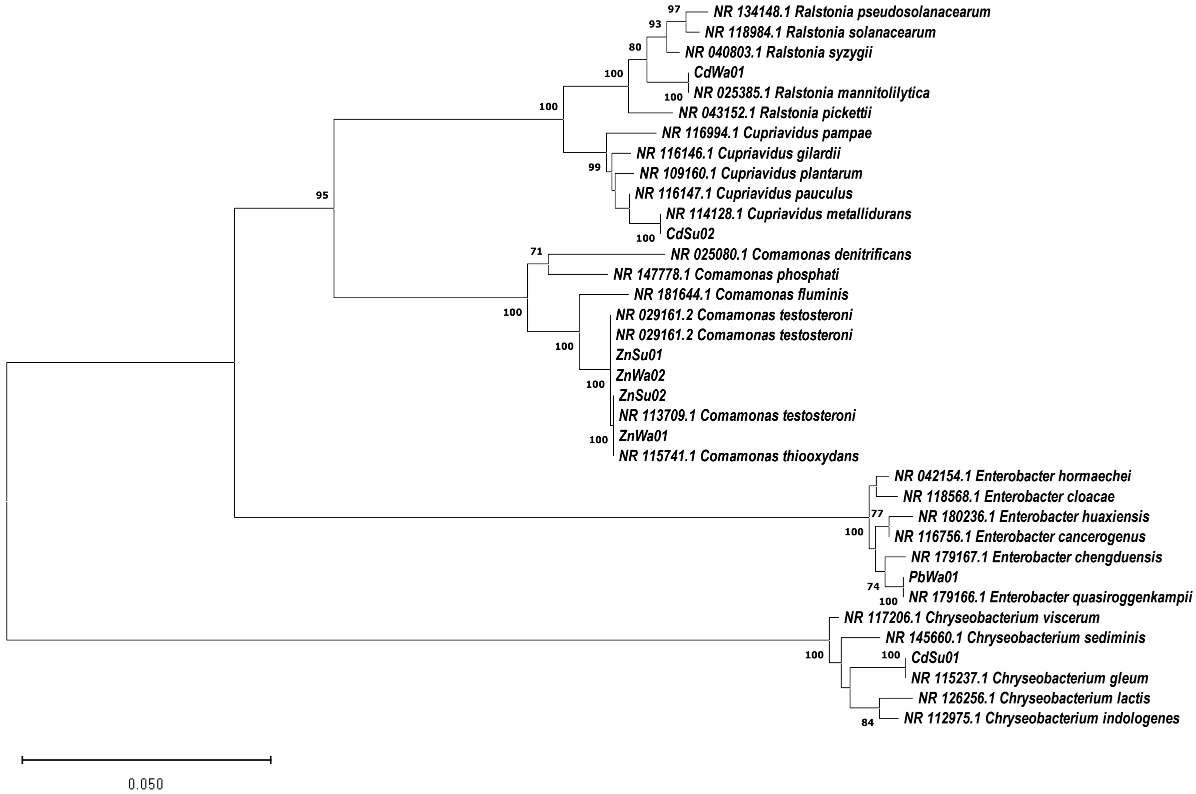

Fig. 5: Dendrogram showing the species identity of each heavy metal-tolerant bacterium based on online database (accessions) in GenBank. Phylogenetic construction was modelled using neighbor-joining using Kimura-2 parameter with bootstrapping (1000×)

Conclusion

The Koto Panjang Reservoir continues to face challenges from heavy metal pollution, serving as a biological reservoir for metal-tolerant bacteria. In our study, we have collected eight bacterial isolates, each exhibiting a distinct tolerance level under Cd, Pb, and Zn stresses in toxicity assays. Further investigations will delve into elucidating the unique mechanisms employed by each isolate to survive and counteract the adverse effects induced by the presence of metal ions.

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Author’s Contributions

Irda Sayuti: Conceptualization, funding acquisition, methodology, writing original draft preparation.

Darmadi: Funding acquisition, investigation, project administration.

Adrian Hartanto: Visualization, writing original draft preparation, writing review, and editing.

Darmawati: Formal analysis, validation.

Imam Mahadi: Formal analysis, investigation.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issue is involved.

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