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Selection of Bacteria from Mamuju's NORM as Uranium and Thorium Bioleaching Agents

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ABSTRACT

Natural materials that can cause increased radiation exposure to the surrounding environment are called Naturally Occurring Radioactive Materials (NORM). NORM contains uranium and thorium, critical elements with strategic and economic value. Conventional separation methods include chemical leaching and partial precipitation with strong acids and bases. These methods require large costs and produce waste harmful to the environment. This study explores bioleaching as an efficient and eco-friendly alternative to address these limitations. The indigenous bacteria used in bioleaching were isolated directly from NORM in Mamuju. This study aims to isolate, select, and evaluate bacteria from NORM as potential bioleaching agents. The methodology of this study includes NORM characterization, bacterial isolation and selection, molecular identification, and resistance testing of selected bacteria. The study successfully isolated eight bacterial strains from NORM, among which isolate L0A demonstrated the highest bioleaching potential. After five days of incubation, L0A achieved uranium and thorium concentrations of 2.508 mg/L and 10.5946 mg/L, respectively. Molecular identification revealed that L0A belongs to *Bacillus* sp. These findings demonstrate the potential of *Bacillus* sp. L0A is a bioleaching agent, paving the way for developing efficient, sustainable, and environmentally friendly methods for extracting valuable radioactive elements from NORM.

INTRODUCTION[*]

Naturally Occurring Radioactive Materials (NORM) are natural materials that can cause increased radiation exposure to the surrounding environment [1]. Natural radionuclides such as ^{40}K, ^{232}Th, ^{235}U, and ^{238}U are mostly contained in natural materials [2]. One of the natural materials that contain high concentrations of ^{238}U and ^{232}Th is britholite ore from Mamuju, West Sulawesi, Indonesia [3]. The elements of uranium and thorium have strategic and economic values, especially in their use as a core element of nuclear fuel [4]. Their applications are widely used in various sectors. Uranium is the most well-known and commonly used actinide element, mainly because of its importance to the nuclear fuel cycle. In addition, uranium can be utilized in broader fields, such as organometallic synthesis, catalysis, nuclear medicine, industrial X-rays, radiometric dating, and material science. Like uranium, thorium is a natural radioactive element identified as a potential alternative fuel to nuclear energy [5]. In order to maximize its potential, NORM should be processed to extract radioactive elements such as uranium and thorium.

Currently, the technology of separating radioactive elements of uranium and thorium in minerals uses chemical leaching and partial

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precipitation methods using strong acids and bases [6]. The process requires large quantities of strong acids such as sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), and bases such as ammonia (NH_3), sodium hydroxide (NaOH), and sodium carbonate (Na_2CO_3) [7] so it requires high costs. Also, the process produces toxic and hazardous liquid chemical waste that has the potential to pollute the environment. Based on these considerations, it is necessary to develop methods for separating radioactive elements using more effective and environmentally friendly methods, such as bioleaching [8].

Bioleaching is a 'clean technology' that is an alternative method of metal extraction using microorganisms and their metabolites. It is environmentally friendly and effective compared to hydrometallurgical and pyrometallurgical processes [9]. Research related to metal extraction using bioleaching techniques has been developed using bacteria or fungi [10]. The microorganisms used in the bioleaching process usually produce metabolic organic acids or oxidizers of iron and sulfur [11]. The bacteria used in the bioleaching process should be indigenous, so they need to be isolated from the environment of the extracted sample. Therefore, this study aims to isolate and select the bacteria from NORM.

METHODOLOGY

Materials

The NORM sample used in this research was collected from Mamuju, Sulawesi Selatan, Indonesia. All chemicals and solvents used were analytical grade with high purity. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were purchased from Himmedia. Standard solutions of uranium and thorium were purchased from Inorganic Ventures.

Physicochemical characterization of NORM

Physicochemical characterization of NORM, including levels of proximate moisture content, ash content, dry matter content, and nitrogen were carried out according to the Association of Agricultural Chemists method [12]. Carbon content was analyzed by UV-vis spectrophotometry [13]. In addition, the elemental content of uranium and thorium was analyzed using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) Thermo Scientific, radioactivity was measured using a Gamma Spectrometer, and NORM pH was measured using a Metrohm pH meter.

Isolation of bacteria from NORM

One gram of the NORM sample was dissolved in 0.85 % NaCl solution and subjected to multistage dilution. NaCl diluted the sample into 10 levels of dilution, and then each level of dilution was taken to as much as 0.1 mL and put into a Petri dish which contained Nutrient Agar (NA). The sample was grown on medium for 24 hours at room temperature. The different colonies were isolated and grown on Petri dishes containing NA to characterize morphology and cells using Gram staining. Purified isolates were grown on agar slants and stored at 4 °C as stock cultures [14].

Bacteria selection

The isolated bacteria were rejuvenated on an agar slant. The isolates were incubated at 37 °C for 24 hour. Two loopfuls of bacteria isolates were taken and inoculated into a Nutrient Broth (NB). They were then incubated for one day at 30 ^{o}C with 100 rpm. 10 % bacterial culture (A 660 nm = 1) was inoculated into NB containing 1 % NORM and incubated for 5 days at 30 ^{o}C and 100 rpm agitation. Then, the culture was analyzed for the total viable count of bacteria, pH, and metal leaching. The bacterial culture supernatant was analyzed using ICP-OES to obtain metal leaching. The selected isolates were those with the highest leaching ability.

Molecular identification

DNA extraction was performed using the Quick-DNA Magbead Plus Kit (Zymo Research, D4082), followed by DNA amplification using the MyTaq HS Red Mix, 2X (Bioline, BIO-25048) with universal primers 27F and 1492R. The amplified 16S gene products were analyzed using electrophoresis, followed by bidirectional sequencing using the Sanger DNA Sequencing using the Capillary Electrophoresis method with the same universal primers (27F and 1492R). The sequencing results were then analyzed bioinformatically, where the nucleotide base sequence was tested for similarity using the Basic Local Alignment Search Tool (BLAST) feature on the NCBI website. The similar results were subsequently used to construct a phylogenetic tree.

Resistance test of selected bacteria to uranium and thorium

Selected bacteria were used in the uranium and thorium resistance test. The bacteria were first cultivated in 40 mL of TSB. Then, the bacterial culture was diluted to reach an OD$_{660}$ of 1. Next, a drop test was carried out on the TSA, which had been adjusted to pH 8, with uranium and thorium at concentrations of 0, 50, 100, 200, 300, 500, 700, 1000, and 1500 ppm. The TSA was then incubated for 24 hours at room temperature. Bacterial growth was observed, and colony diameters were measured.

RESULTS AND DISCUSSION

The Mamuju's NORM characterization results show that although the conditions in the britholite ore are extreme, this environment can still support bacterial growth (Table 1). NORM mineral characterization results show that this sample has a pH of 8.89, which indicates alkaline properties. The low C-organic content of 0.32 % wt and nitrogen of 0.02 % wt shows a lack of organic matter and biological activity. The ash content of 8.11 % wt indicates the dominance of inorganic material, while the low % water content of 0.33 % wt confirms dry environmental conditions. The radionuclide activity in this sample is quite high, with ^{238}U reaching up to 32,450 Bq/kg and ^{232}Th at 205,049.28 Bq/kg. This condition indicates the presence of high concentrations of radioactive elements. The uranium and thorium concentrations at NORM reached 10,574.25 mg/L and 35,819.30 mg/L, respectively, indicating considerable potential radiological hazard for microorganism life. However, despite this extreme environment, the number of bacterial cells detected in the sample reached 1.55x10^{5} CFU/g, which means that these extreme physicochemical conditions can still support bacterial growth. Although these conditions are not ideal, bacteria can live in alkaline conditions (alkaliphile) [15]. In addition, bacteria can live in conditions with low water content and low nutrients (C and N content) by forming endospore structures, so bacteria have thick, heat-resistant walls usually produced by Gram-positive bacteria in the form of *Bacillus* [16].

Observations of the colony and cell morphologies of the eight bacterial isolates identified from the NORM samples showed a consistent pattern with the observed characteristics (Table 2). All isolates generally have a circular colony shape with entire edges and raised elevation. Colony size varied from small to large, with a generally shiny appearance, although two isolates, L1B and L1D, appeared dull. Colony pigment color varies between white and cream, while the optical properties are dominantly opaque, with some isolates being translucent. At the cell morphology level, the isolates showed uniform shapes, called *Bacillus* in six and *Streptobacillus* in two isolates. All isolates showed positive results on Gram staining. Gram-positive bacteria have thicker peptidoglycan cell walls (20 – 80 nm), whilst Gram-negative is only 2-3 nm. Gram-positive *Bacillus* can form spores and survive in extreme environments for years [17].

Table 1. Physicochemical Characterization of NORM.

Parameter	Value
pH	8.89
C-Organic Content (% wt)	0.32
Nitrogen Content (% wt)	0.02
Ash Content (% wt)	8.11
Water Content (% wt)	0.33
Radionuclide Activity (Bq/kg)	
U-238	32,450.00
Th-232	205,049.28
Concentration (mg/l)	
Uranium	10,574.25
Thorium	35,819.30
Cell Amount (CFU/g)	1.55 x 10^{5}

Table 2. Colony, Cell Morphologies Observation, and Isolated Cell Bacteria.

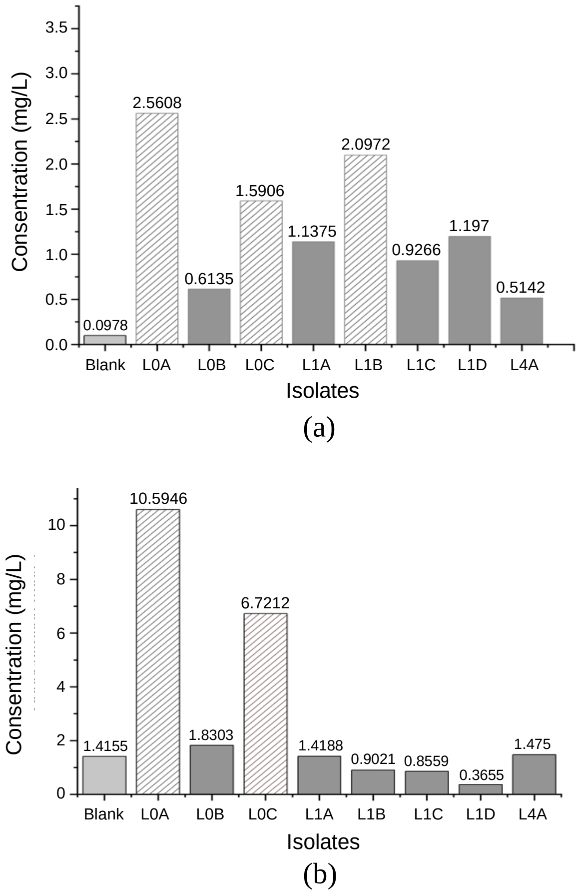
No.	Isolates	Colony morphologies							Cell morphologies		
		Shape	Margin	Elevation	Size	Appearance	Pigmentation	Optical	Shape	Gram straining	Cell amount (CFU/mL)
1	L0A	Circular	Entire	Rised	Small	Shinny	White	Opaque	Bacillus	Positive	4,80,E+07
2	L0B	Circular	Entire	Rised	Small	Shinny	Cream	Opaque	Strepto-bacillus	Positive	5,26,E+08
3	L0C	Circular	Entire	Rised	Moderate	Shinny	Cream	Opaque	Bacillus	Positive	2,19,E+08
4	L1A	Circular	Entire	Rised	Small	Shinny	Cream	Opaque	Bacillus	Positive	8,15,E+09
5	L1B	Circular	Entire	Rised	Small	Dull	White	Trans-lucent	Bacillus	Positive	3,70,E+08
6	L1C	Circular	Entire	Rised	Moderate	Shinny	White	Opaque	Bacillus	Positive	3,71,E+10
7	L1D	Circular	Entire	Rised	Moderate	Dull	Cream	Trans-lucent	Bacillus	Positive	9,45,E+07
8	L4A	Circular	Entire	Rised	Large	Shinny	Cream	Opaque	Streptobacillus	Positive	4,90,E+07

The selected bacteria showed the ability to leach thorium (L0A and L0C) and uranium (L0A, L0C, and L1B) after 5 days of incubation (Figs. 1a-b). This leaching ability is characterized by increasing concentrations of uranium and thorium in the supernatant, which indicates that the two isolates can extract uranium and thorium from NORM. This increase in uranium and thorium concentrations in the supernatant strongly indicates the potential of bacteria as bioleaching agents. The increase in uranium and thorium concentrations can occur due to the desorption mechanism by bacterial exudates through producing ammonia to dissolve metals from ores [18].

The other studies mention that desorption of uranium and thorium can occur due to the increase in biological weathering of minerals, which impacts the reduction of mineral particle size [19]. However, the CFU count of L0A bacteria in the supernatant showed no significant variation between day 0 and day 5. As shown in Fig. 2, the colony-forming unit (CFU) count of L0A bacteria in the supernatant was recorded as 4.80 × 10^7 CFU/mL on day 0, which decreased to 2.40 × 10^6 CFU/mL by day 5. This slight decrease in bacterial counts could be due to bacterial mortality resulting from the failure to adapt during the bioleaching period.

The increase in pH of the isolate solution (Fig. 3) and the increase in extracted uranium and thorium concentration are also essential characteristics of the bioleaching process. According to prior studies, the bacteria that can extract uranium are *Acidithiobacillus ferroxidans* bacteria. The bacteria extract uranium indirectly by oxidizing pyrite (FeS_2), a mineral associated with uranium, and Fe^{2+} into Fe^{3+}. Fe^{3+} then attacks uranium minerals by oxidizing U^{4+} to U^{6+}, which is soluble in H_2SO_4 [20].

The bioleaching process can occur at a strong alkaline pH. Therefore, alkaliphilic bacteria with a pH tolerance can carry out the bioleaching process. Alkaliphilic microorganisms, which require a pH of around 9 for optimal growth, appear to be involved in this process, while a lower pH (around 6.5) would inhibit their growth [21]. Studies have shown that heterotrophic bacteria, such as *Bacillus* and *Pseudomonas*, can thrive in alkaline environments, producing ammonia and organic acids that facilitate metal dissolution from ores [18]. Although previous research focused on other metals, this strongly suggests that uranium and thorium bioleaching can also be achieved using similar mechanisms. While further research is needed, the findings of this study provide a promising basis for developing bioleaching processes for uranium and thorium in alkaline conditions.



(a)

(b)

Fig. 1. Relationship between Isolates and (a) Uranium and (b) Thorium Concentrations in Supernatant.

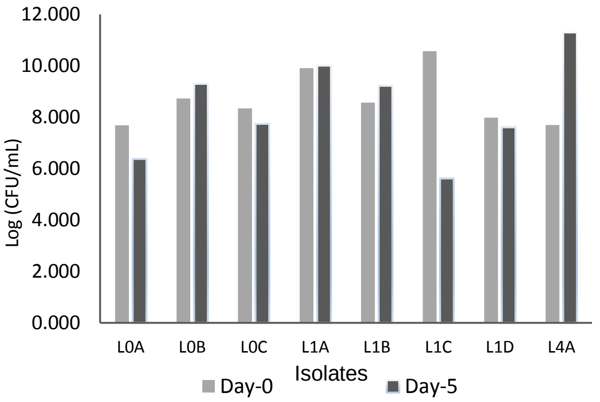


Fig. 2. Relationship between Isolates and Cell Count of Bacterial Isolates.

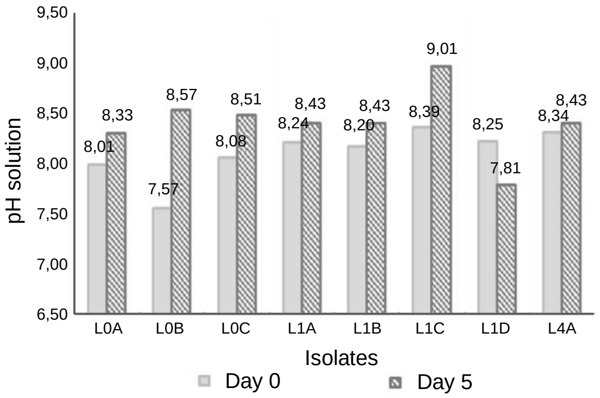


Fig. 3. Relationship between Isolates and pH Changes in Supernatant.

Based on the bioleaching potential test, the L0A isolate demonstrated the highest capability as a bioleaching agent for uranium and thorium, extracting these elements more efficiently than other isolates. Phylogenetic analysis using 16S rRNA gene markers (Fig. 4) revealed that L0A is closely related to *Bacillus* sp. PS06. The phylogenetic tree confirmed the isolate's classification within the *Bacillus* group, which is well-known for its bioleaching abilities, particularly in extracting heavy metals from mineral-rich environments. For instance, a study by [14] showed that bacteria from the *Bacillus* group, such as *Bacillus licheniformis* JAJ3, possess significant bioleaching potential, although not specifically for uranium or thorium.

The tolerance of *Bacillus* sp. L0A to uranium and thorium was tested by culturing the bacteria for 24 hours on Tryptic Soy Agar (TSA) containing various concentrations of uranium or thorium. The results showed that *Bacillus* sp. L0A could survive and grow at concentrations up to 1,500 ppm (Fig. 5), demonstrating its potential as a bioleaching agent for minerals with high uranium and thorium content. Several studies have frequently reported bacteria from the *Bacillus* group that are resistant to high uranium and thorium levels [22]**.** This ability suggests that the bacteria have developed an adaptation mechanisms to counter the toxic effects of uranium and thorium, which typically damage cells. However, the drop test results showed a decrease in bacterial colony diameter (to 0.8 cm for uranium and 0.9 cm for thorium) as concentrations increased, suggesting that while the bacteria can tolerate high metal concentrations, extreme conditions may still negatively affect their growth. This decline in growth could be attributed to the cumulative toxic effects of uranium and thorium, which may cause oxidative stress or disrupt cellular integrity at higher concentrations. Such findings emphasize the need for further exploration into the specific mechanisms of metal resistance and how they could be enhanced for more efficient bioleaching. Additionally, understanding how these bacteria manage the metal ions at high concentrations could have significant implications for developing sustainable bioleaching strategies, particularly extraction of uranium and thorium from mineral ores and contaminated sites. Although indications of bioleaching are already visible from these preliminary results, further research is needed. The research that requires a more in-depth characterization of the biochemical mechanisms involved and long-term observations of leaching behaviour under various environmental conditions will be essential for further confirmation.

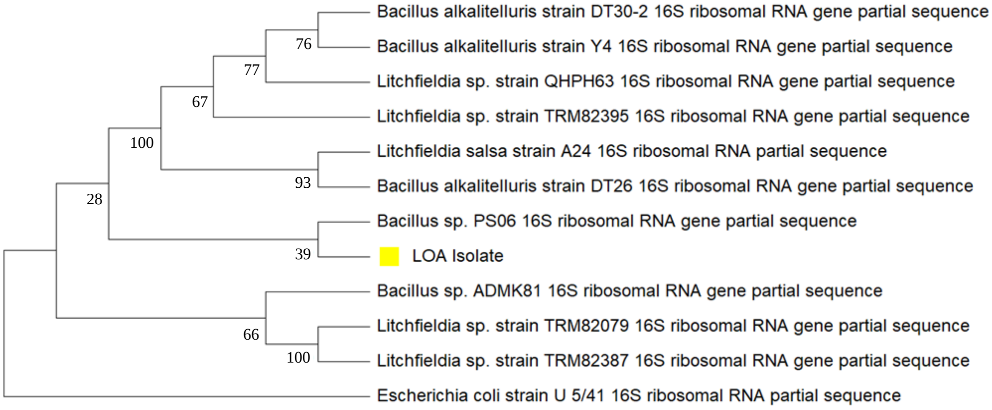
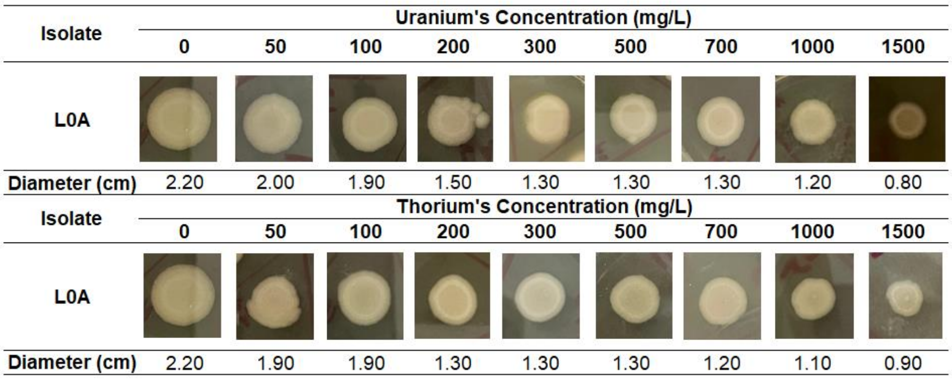


Fig. 4. Phylogenetic Tree of Isolates Tree of L0A.



Isolate	**Uranium's Concentration (mg/L)**								
	0	**50**	**100**	**200**	**300**	**500**	**700**	**1000**	**1500**
L0A									
Diameter (cm)	2.20	2.00	1.90	1.50	1.30	1.30	1.30	1.20	0.80
Isolate	**Thorium's Concentration (mg/L)**								
	0	**50**	**100**	**200**	**300**	**500**	**700**	**1000**	**1500**
L0A									
Diameter (cm)	2.20	1.90	1.90	1.30	1.30	1.30	1.20	1.10	0.90

Fig. 5. Resistance of L0A to Uranium and Thorium Concentration.

CONCLUSION

The results of this study indicate that the L0A bacterial isolate has the best ability to desorb uranium and thorium from NORM. The L0A isolate is a Gram-positive bacteria with the closest relationship to *Bacillus* sp. PS06. L0A can survive at uranium and thorium concentrations of 1,500 mg/L. However, further research is still needed regarding the application of bioleaching under optimum conditions, the mechanism of bioleaching, and the concentration limits of isolate resistance to uranium and thorium.

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AUTHOR CONTRIBUTION

S. Indryati, A. Mujiyanto, I. Sugoro: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – original draft, and Writing – review and editing. I. G. Sukadana: Data Collection. K. S. Widana: Formal Analysis and Writing – review and editing. A. E. Hidayat, F. J. Rahma, C. A. Shabirah, R. I. Laksmana, A. A. Pratama, K. Trinopiawan, R. Prasanti, T. Purwanti, A. W. Putra: Investigation. All authors read and approved the final version of the paper.

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