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Exploring the diversity and bioremediation potential of thermohalophilic bacteria from Wawolesea geothermal spring, southeast Sulawesi, Indonesia

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ABSTRACT

The Wawolesea hot springs in southeast Sulawesi, Indonesia, offer an extreme environment that presents a significant opportunity for the diversity of extremophilic microorganisms, especially thermohalophilic bacteria. These bacteria have a knack for enduring surroundings featuring both high temperatures and significant salt levels, enabling them to generate a range of enzymes that sustain their stability even in harsh conditions. This research aims to explore the diversity of thermohalophilic bacteria found in the Wawolesea hot springs and evaluate their potential to aid in bioremediation, particularly for reducing environmental pollution caused by heavy metals. The isolation of bacteria was performed using both the pour plate and streak methods on nutrient agar (NA) medium, and afterward, the bacterial cultures were kept in an incubator at 48 °C for a duration of 24 hours. By employing 16S rRNA PCR amplification, genetic identification revealed a 1300 bp target band pointing to the existence of various Enterobacter species: *Enterobacter* sp., *Enterobacter ludwigii*, *Enterobacter roggenkampii*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter mori*, *Enterobacter quasiroggenkampii*, *Enterobacter vonholyi*, *Enterobacter pseudoroggenkampii*, in addition to Cedecea davisae. Current research indicates that these bacteria have impressive bioremediation properties because they can accumulate heavy metals, such as lead (Pb), cadmium (Cd), and nickel (Ni), while also having the ability to decompose hazardous organic substances. The study's results revealed that the isolated bacteria possess considerable bioremediation potential, with a number of species showing the ability to lower heavy metal levels and accumulate toxic substances without displaying evident signs of toxicity. These findings indicate that thermohalophilic bacteria from the Wawolesea hot springs could be incredibly useful in biotechnology, especially for creating eco-friendly approaches to tackling heavy metal contamination and other detrimental compounds.

Keywords: Thermohalophilic bacteria, bioremediation, heavy metals, 16S rRNA, *Enterobacter* sp.

INTRODUCTION

Extreme environments have long been a fascinating topic for microbiologists, as they explore the diverse range of extremophilic microorganisms that thrive in such conditions. The ability to grow in extreme conditions makes the microorganisms unique. Extremophiles are a unique group of microorganisms capable of living and prospering in harsh environmental conditions. These conditions include high temperatures ranging from 60 to 110 °C, as seen in thermophiles and hyperthermophiles, and low temperatures from -2 to 15 °C, found in psychrophiles. They can also thrive in environments with high salinity levels of 2–5 M NaCl, known as halophiles, or in highly acidic conditions with a pH below 4, known as acidophiles. Additionally, they survive in highly alkaline settings with a pH above 9 called alkaliphiles, withstand high pressure as piezophiles, endure high radiation as radiophiles, and tolerate environments with

high metal concentrations known as metalophiles (Bonc et al., 2015). Extremophiles inhabit a wide range of natural habitats, including hydrothermal vents, geothermal areas, hot springs, and compost (Dalmaso et al., 2015).

Thermohalophilic bacteria are a type of bacteria that thrive in conditions of extreme heat and salinity. Reports by Straub et al. (2018) and Muzuni et al. (2022) indicate that these bacteria flourish at temperature ranges from 45 to 110 °C and salinity levels of 2 to 30%. These bacteria are often found in geothermal marine sediments and saline hot springs. In the village of Wawolesea, located in the Lasolo Subdistrict of the North Konawe Regency in Southeast Sulawesi Province, one of the saline hot springs, namely the Wawolesea hot spring, can be found. This hot spring features multiple wells, each exhibiting different temperatures and levels of salinity. According to Jamaluddin et al. (2018), the temperatures at the Wawolesea hot spring range from 48 to 70 °C. Furthermore, Jamaluddin and Umar (2017) noted that the salinity levels vary between 15% and 20%. Thermohalophilic bacteria found here could serve as potential agents for bioremediation purposes.

Bioremediation is employed to mitigate the impact of industrial and agricultural pollutants found in contaminated soil, which subsequently aids in eliminating and lessening the harmfulness of heavy metals that contaminate the soil (Hamid and Tarkhan, 2024). Therefore, the Wawolesea hot spring can serve as a source of thermohalophilic bacteria with high activity at elevated temperatures and salinity levels. These thermohalophilic bacteria are also valuable for bioremediation purposes, especially for tackling pollution in harsh environments like high-salinity or high-temperature soils and waters, where traditional microorganisms are ineffective.

Microbes are all around us, particularly when essential elements for their growth are present, with pollutants often serving as co-factors for bacterial growth within certain limits. In this regard, industrial waste estuaries serve as ideal environments for the flourishing of various microorganisms, albeit within specific boundaries. For example, nickel, iron, cobalt, and zinc are predominant in industrial waste and are essential growth factors for many bacterial communities, which have developed the ability to adapt, absorb, and convert these elements into beneficial resources (Figueira et al., 2005). Recently, scientists have sought to harness bacteria for the removal or reduction of heavy metals in water and soil. Notably, the Enterobacteriaceae family is used for this purpose; for example, Enterobacter species such as Enterobacter cloacae and Enterobacter asburiae are employed in the bioremediation of Cu^{+2}, Cr^{+2}, Pb^{+2}, Cd^{+2}, and Ni^{+2} across various polluted locations (Banerjee et al., 2015; Bestawy et al., 2013; Paul and Mukherjee, 2016; Rahman et al., 2015).

Typically, sequences amplified by PCR for the 16S ribosomal RNA gene have been grouped together based on their similarity to form operational taxonomic units (OTUs). The representative sequences from these OTUs are then compared to reference databases to predict probable taxonomy. While this method is both convenient and powerful, it has required making specific assumptions. Historically, it has been assumed that sequences with more than 95% identity indicate the same genus, while sequences with over 97% identity suggest the same species (Johnson et al., 2019).

METHODS

Sampling site and sample collection

The sampling location for obtaining thermohalophilic bacterial isolates in this study was the Wawolesea hot spring. The Wawolesea hot spring is located in Wawolesea Village, Lasolo Subdistrict, North Konawe Regency, Southeast Sulawesi. The Wawolesea hot spring features travertine deposits or limestone deposits that are yellowish-brown and white in color. The deposits formed in the Wawolesea hot spring are classified as travertine deposits, which contain a high concentration of mineral elements. The presence of these mineral elements can serve as a source of nutrients for bacterial life.

Cultivation of halophilic bacteria in nb medium

1 mL sample from the Wawolesea hot spring, with a temperature of 45–52 °C, was taken and introduced into a growth medium (NB). The sample was incubated for 2 × 24 hours. The resulting bacterial growth was then observed and subjected to serial dilution. Subsequently, the grown bacteria were isolated on to solid medium (NA) using the streak plate method (Ginting et al., 2019).

Dna extraction

A colony derived from the cultured sample on a petri dish was collected and placed into a tube with 1 mL of sterile phosphate-buffered saline (PBS). The contents were vortexed to fully dissolve the colony. Afterward, the sample underwent centrifugation for 5 minutes at 300 µg. The liquid above the sediment (supernatant) was removed, and 200 µL of PBS was introduced. Following this, 20 µL of Proteinase K was added, and the mixture was thoroughly homogenized by vortexing.

PCR Mix for Bacterial 16S rRNA

Combine the following components: 25 µL of MyTaq HS Red Mix, 1 µL of Forward primer 63f (5'–CAG GCC TAA CAC ATG CAA GTC-3'), 1 µL of Reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3'), 10 µL of DNA sample, and 13 µL of Nuclease-free water, achieving a final volume of 50 µL.

Running PCR

The initial stage, Cycle 1, involved a single process at 95°C for a duration of 5 minutes for the purpose of pre-denaturation. Following this, Cycle 2 encompassed a series of 35 repeated cycles: Step 1 at 95 °C for half a minute for denaturation, Step 2 at 55 °C for half a minute for annealing, and Step 3 at 72 °C for a full minute for extension. Lastly, Cycle 3 was carried out once at 72 °C for a duration of 5 minutes for the final extension process.

Electrophoresis

A total of 1 gram of agarose was carefully measured and dissolved in 50 mL of 0.5x TBE buffer solution. This mixture was heated over a heat source until the agarose was completely dissolved. Once the solution turned clear and uniform, it was set aside to cool down for several minutes until it reached a comfortably warm temperature (without becoming hot). At this point, 3 µL of Ethidium Bromide was introduced into the agarose solution and gently stirred to ensure even distribution. Finally, the prepared mixture was poured into a casting tray fitted with a comb and allowed to solidify fully at room temperature.

16S rRNA gene sequences

The 16S rRNA gene PCR amplification outcomes were sequenced at the Hasanuddin University Medical Research Center (HUMRC). Following this, these sequences were subjected to homology analysis using the BLAST database and subsequently submitted to GeneBank (NCBI) to acquire their accession numbers.

Analysis of phylogeny

The construction of a Phylogenetic tree was accomplished using MEGA 12 software.

RESULTS

In Figure 1, the sampling zones for thermohalophilic bacterial isolates reveal some variations in appearance. Points I and II feature yellowish deposits, while points III, IV, and V display white deposits. Points IV and V are situated further away from the hot spring source, whereas point III is at the boiling spring source. The study involves assessing temperature, salinity, and pH parameters of the Wawolesea hot spring surroundings. Sampling occurred at five distinct locations, specifically at points I, II, III, IV, and V, each having different environmental conditions.

Bacterial growth temperatures are categorized into minimum, optimum, and maximum levels. According to Pelczar et al. (1988), bacteria are grouped based on their growth temperature ranges: psychrophilic bacteria thrive between 0

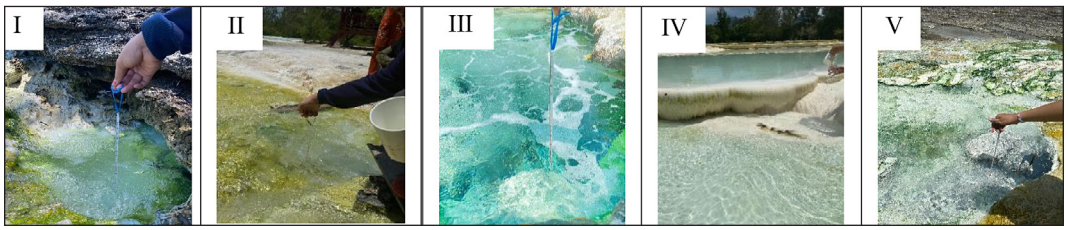

Figure 1. Locations where samples were taken from five distinct points for isolate sources

and 20 °C, mesophilic bacteria flourish between 21 and 40 °C, and thermophilic bacteria prosper at temperatures of 40 °C and above. Table 1 displays water temperature readings from points I–V that fall between 45 and 53 °C. These temperature readings demonstrate that the bacteria flourishing in the Wawolesea hot spring environment (Points I –V) are of the thermophilic variety.

Another environmental parameter measured in this study was salinity. Salinity can affect the osmotic pressure within bacterial cells, thereby influencing their survival. The salinity measurements in Table 1 show that points I, II, IV, and V had water salinity levels of 15%, equivalent to 1.5%, while point III had a salinity level of 16%, or 1.6%. These salinity data indicate that the bacteria living in the Wawolesea hot spring (Points I–V) are tolerant to salinity and can be classified as halophilic bacteria. Therefore, based on the temperature and salinity parameters, it can be concluded that the bacteria living in the Wawolesea hot spring belong to the thermohalophilic group.

One of the environmental parameters analyzed in this research was pH. The hydrogen potential (pH) is described as the activity of dissolved hydrogen ions (H^+). The environmental pH significantly affects the viability of bacteria in a given environment. The pH values presented in Table 1 reveal that the water at locations I–V had pH levels of 5.78, 5.73, 5.91, 5.84, and 5.60. These pH measurements suggest that bacteria can grow or persist in the Wawolesea hot spring. The pH range for bacterial growth spans from a minimum of 4 to a maximum of 9, with the optimal pH typically between 6.5 and 7.5 (Fajar et al., 2022).

Biochemical characterization

This Figure 2a shows the successful isolation of pure bacteria from the initial sample. This means that the selected or isolated bacteria have grown well on a specific medium without contamination from other microorganisms. This process is carried out to ensure that only a single type of bacteria is obtained, which is essential for further research. This Figure 2b shows bacteria observed under the microscope with rod-shaped morphological characteristics and a dominant pink color, indicating that they are Gram-negative bacteria. And Figure 2c represents the result of the Gram staining of a Gram-negative bacteria.

Results of bacterial electrophoresis

Amplification of the 16S-rRNA gene was carried out effectively with primers specifically aimed at bacterial DNA, resulting in a product approximately 1300 bp in size. Agarose gel electrophoresis was used to examine the PCR products, as depicted in Figure 3. The positive control (K^+) showed a clear and strong band at 1300 bp, confirming the PCR reaction's reliability. Likewise, the sample (E) presented a distinct band at the identical position (1300 bp), signifying the successful amplification of 16S-rRNA genes from bacterial isolates in the environment. The negative control (K^-) showed no band, indicating no contamination during the PCR process. A molecular marker (M, 100 bp ladder) was utilized to estimate the amplicons' size. These findings affirm the presence of bacterial DNA in the sample and demonstrate the quality and specificity of the PCR amplification.

Table 1. Results of physicochemical parameters measurement of the Wawolesea hot spring

Environmental parameters					
No	Point	Sample	Temperature (°C)	Salinity (%)	pH
1	I	I_1	50	15	5.78
		I_2	50	15	5.78
2	II	II_1	49	15	5.73
		II_2	49	15	5.73
3	III	III_1	53	16	5.91
		III_2	53	16	5.91
4	IV	IV_1	49	15	5.84
		IV_2	49	15	5.84
5	V	V_1	45	15	5.60
		V_2	45	15	5.60

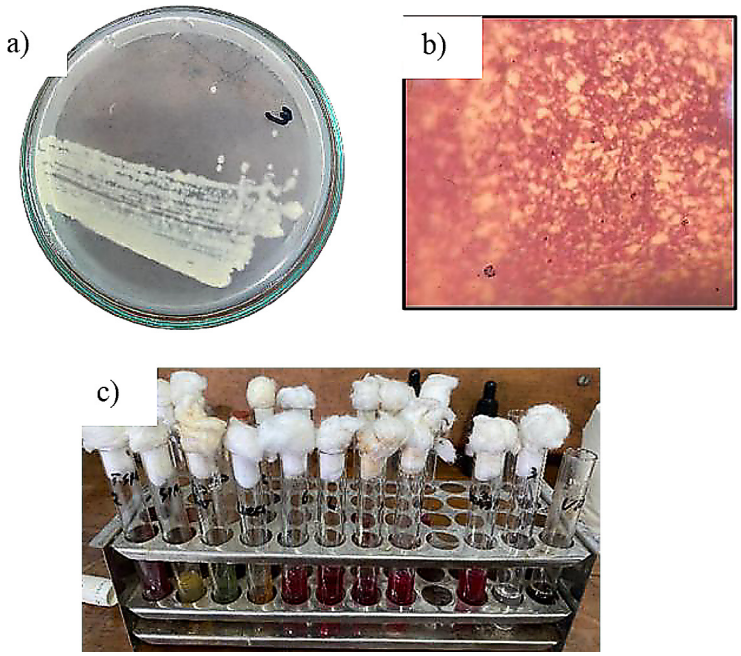

Figure 2. (a) The success of pure bacterial isolation from the initial sample, (b) observation of bacteria under the microscope and (c) bacterial staining

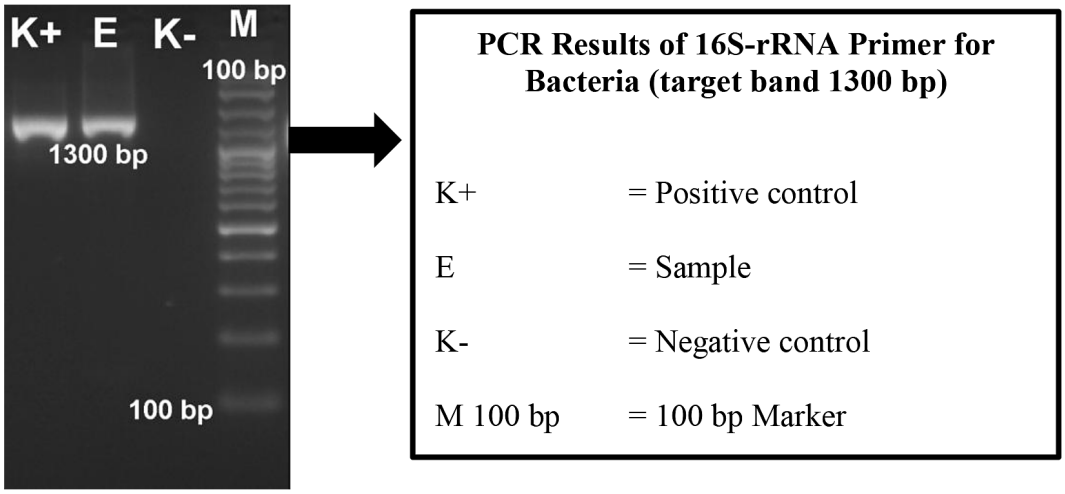

Figure 3. Electrophoresis results of bacteria

Discovering bacterial species 16S rRNA gene similarity analysis is a molecular approach employed to identify and categorize bacteria by examining the sequence of their 16S ribosomal RNA (rRNA) gene. This gene is notably conserved across various bacterial species, making it a prime candidate for phylogenetic analysis and examining evolutionary connections among different bacterial species. Table 2, labeled as 16S rRNA gene similarity analysis, presents the results of sequence comparison. The table displays the similarity percentages between the sample sequences and those of known bacterial species, aiding in the determination of bacterial species through their 16S rRNA gene.

Based on data obtained from the NCBI GenBank database, the bacteria with the highest similarity > 97% all belong to the *Enterobacter* genus and species. The similarity values ranging from 97.46 – 97.62% indicate how closely related the isolate is to other known species. A similarity of around 97.5% suggests that the isolate is classified as *Enterobacter* sp. The three species with the highest similarity are *Enterobacter* sp., *Enterobacter ludwigii*, and *Enterobacter roggenkampii*. A phylogenetic tree is a diagram that illustrates the

Table 2. 16S rRNA gene similarity analysis

Sample	Description	Scientific Name	Accession	Similarity (%)
Isolat $EAsIII_1$	Partial sequence of the 16S ribosomal RNA gene from Enterobacter sp., specifically strain Guang Xi tomato isolate K7.	*Enterobacter sp.*	MW785893.1	97.62%
	Enterobacter ludwigii strain PRFR10 16S ribosomal RNA gene, partial sequence	*Enterobacter ludwigii*	KF724149.1	97.54%
	Enterobacter roggenkampii strain 18Y001733 chromosome, full genome sequence	*Enterobacter roggenkampii*	CP138222.1	97.54%
	A partial sequence of the 16S ribosomal RNA gene from Enterobacter asburiae voucher ST56.	*Enterobacter asburiae*	KT287073.1	97.54%
	Partial sequence of the 16S ribosomal RNA gene from Enterobacter cloacae strain LT324.	*Enterobacter cloacae*	PQ782534.1	97.54%
	Partial sequence of the 16S ribosomal RNA gene from Enterobacter mori strain AS5.	*Enterobacter mori*	MT613379.1	97.46%
	Partial sequence of the 16S ribosomal RNA gene for Enterobacter quasiroggenkampii strain AC15. Enterobacter	*Enterobacter quasiroggenkampii*	OQ255853.1	97.46%
	Enterobacter vonholyi strain STK80-C chromosome, complete genome	*Enterobacter vonholyi*	CP162152.1	97.46%
	Enterobacter pseudoroggenkampii GVv1 DNA, complete genome	*Enterobacter pseudoroggenkampii*	AP038752.1	97.38%
	Cedecea davisae 16S ribosomal RNA gene, partial sequence	*Cedecea davisae*	JQ396389.1	97.46%

evolutionary connections among different biological species or entities, based on genetic likenesses and differences. Typically, this tree is built using molecular data, like the 16S rRNA gene sequence, which demonstrates how species have evolved divergently. For this particular phylogenetic tree, the 16S rRNA gene sequences of a thermohalophilic bacterial isolate from the Wawolesea hot spring were analyzed and compared with several species listed in GenBank, including *Enterobacter* sp., *Enterobacter ludwigii*, *Enterobacter roggenkampii*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter mori*, *Enterobacter quasiroggenkampii*, *Enterobacter pseudoroggenkampii*, *Enterobacter vonholyi*, and *cadeceae davisae*. The findings revealed that the isolate has a close genetic relationship with all species within the *Enterobacter* genus, displaying sequence similarities ranging from 97.46% to 97.62%.

The phylogenetic analysis of thermohalophilic bacteria revealed that Enterobacter sp. is the closest relative with a similarity score of 2161 (Figure 4). Following closely are Enterobacter ludwigii and Enterobacter roggenkampii, each with a score of 2156. These outcomes suggest that the isolate has a genetic relationship with the Enterobacter genus. However, given this similarity score and a 16S rRNA gene similarity value of less than 98.7%, there could be a potential for a new strain. Typically, the 98.7% threshold is used

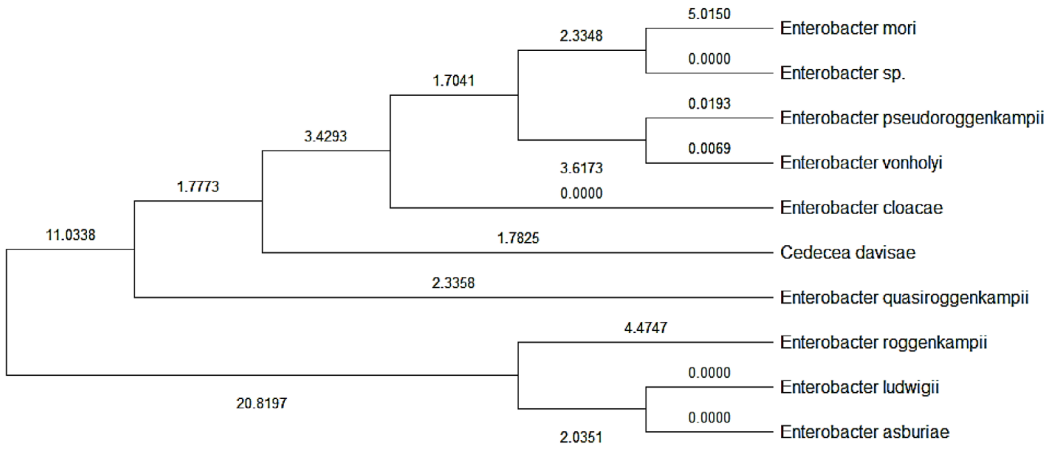

Figure 4. A phylogenetic tree

to distinguish between recognized species and those that might be novel.

CONCLUSION

After isolating bacteria, it was found that there were 15 unique isolates, each exhibiting a rod-like shape and showing Gram-negative characteristics. Sequencing analysis revealed a dominance of the Enterobacter genus, with species including *Enterobacter sp*., *Enterobacter ludwigii*, and *Enterobacter roggenkampii*. The potential for bioremediation by these isolates seems promising, as thermohalophilic bacteria are generally adept at enduring extreme conditions, such as high temperatures and salinity, and can break down a range of pollutants or other contaminants. Consequently, these isolates might play a pivotal role in bioremediation efforts aimed at combating environmental pollution, especially under challenging conditions.

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