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Molecular Identification and Characterization of Lipase-Producing Bacteria Isolated from Wawolesea Hot Spring, Southeast Sulawesi

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Abstract

The Wawolesea hot spring in Southeast Sulawesi represents an extreme ecosystem characterized by high temperature and salinity, providing a potential habitat for thermo-halophilic bacteria that produce industrial enzymes, particularly lipase. Here, we carried out molecular identification of lipase-producing bacteria isolated from the Wawolesea hot spring using 16S rRNA gene analysis. The sequence comparison showed that isolate K.111 was the most closely related to *Bacillus thuringiensis*. This supports the hypothesis that the Wawolesea hot spring harbors thermo-halophilic bacterial communities with potential applications in industrial biocatalysis.

Keywords: lipase; thermo-halophilic bacteria; Wawolesea.

Abbreviations: DNA: deoxyribonucleic acid; RNA: ribonucleic acid; PCR: polymerase chain reaction; NCBI: National Center for Biotechnology Information; Basic Local Alignment Search Tool for Nucleotides.

INTRODUCTION

Lipases constitute one of the most widely utilized groups of biocatalysts due to their ability to catalyze hydrolysis as well as various ester-related reactions under diverse physicochemical conditions. Because of this functional flexibility, lipases are extensively applied in industries ranging from food processing to renewable energy production and bioremediation (Jaeger & Eggert, 2002; Hasan et al., 2006; Sharma et al., 2001; Singh & Kumar, 2019). Their superiority over other enzyme classes arises from their catalytic versatility, enabling hydrolysis, esterification, interesterification, and transesterification reactions under a wide range of physicochemical conditions, including high salinity and extreme temperatures (Gupta et al., 2004; Panda et al., 2018). Consequently, extensive efforts have been directed toward identifying microbial sources capable of producing lipases with enhanced activity and stability under such harsh environments.

Indonesia represents a promising region for the discovery of extremophilic microorganisms due to its geological location along the Pacific Ring of Fire. Approximately 40% of global geothermal reserves are located within the country, distributed across more than 200 geothermal sites (Ifandi & Alwi, 2018; Nafian et al., 2022). One of these sites, the Wawolesea hot spring in Southeast Sulawesi, is characterized by temperatures ranging from 30 to 65 °C, elevated salinity, and near-neutral pH. These conditions are not only favorable for the proliferation of thermophilic and thermo-halophilic bacteria (Aulia et al., 2022), but they also stand out among other geothermal sites. In comparison to other Indonesian hot springs, Wawolesea ranks in the upper 10% for temperature and salinity, making it an exceptional site for the study of extremophiles. This distinctiveness underscores its appeal for extremophile hunting and potential biotechnology applications.

Thermo-halophilic bacteria thrive at temperatures between 45 and 70 °C and tolerate salinity levels of 2–30%, making them potential sources of thermostable enzymes and metabolites (Jamaluddin et al., 2018; Gurumurthy et al., 2020). Members of the genera *Bacillus*, *Geobacillus*, and *Brevibacillus* have been reported to produce highly stable lipases, underscoring their potential as industrial biocatalysts (Norashirene et al., 2013; Lee et al., 2022; Corneles et al., 2023).

The exploration of lipases from extremophilic microorganisms typically involves three main stages: (i) isolation and preliminary characterization of lipase-producing strains, (ii) enzyme purification to enhance specific activity and stability, and (iii) molecular identification based on 16S rRNA gene sequencing to elucidate phylogenetic relationships (Muharni & Anggraini, 2015; Khalid & Abdulrahman, 2024; Sendo et

al., 2022). Molecular identification provides a more reliable classification framework, as phenotypic characteristics often vary in response to environmental fluctuations.

Preliminary studies conducted at the Wawolesea hot spring have successfully isolated thermo-halophilic bacteria capable of lipase production (Jamaluddin et al., 2018; Muzuni et al., 2024). However, limited information is available regarding the purification and molecular characterization of lipase enzymes from this site. Therefore, the present study aimed to isolate, characterize, and identify thermo-halophilic lipolytic bacteria from the Wawolesea hot spring, Southeast Sulawesi. The outcomes are expected to enrich current understanding of extremophilic microbial diversity in Indonesia and provide valuable insights for the development of robust lipases for industrial applications.

MATERIALS AND METHODS

Materials

The bacterial isolates used in this study were obtained from the Wawolesea hot spring in Southeast Sulawesi. The materials included Nutrient Agar (NA), Nutrient Broth (NB), 70% ethanol, and reagents from the Wizard® Genomic DNA Purification Kit (Promega), such as nuclei lysis solution, RNase solution, protein precipitation solution, and DNA rehydration solution. Additional materials included loading dye, ethidium bromide (EtBr), 1× Tris-Acetate-EDTA (TAE) buffer, DNA ladder, primers 63F and 1387R, GoTaq® Green Master Mix (Promega), nuclease-free water (NFW), and general laboratory consumables.

Procedure

Rejuvenation of Bacterial Isolates

Bacterial isolates were rejuvenated by streaking them onto NA plates followed by incubation at 50 °C for 24 h. The NA medium was prepared by dissolving 3 g agar and 1.6 g NB in 200 mL distilled water, heating the mixture to homogenize, and sterilizing it at 121 °C for 15 min. Distinct colonies were used to inoculate NB prepared from 0.8 g NB in 100 mL distilled water. Cultures were incubated statically at 50 °C for 24 h until turbidity indicated active bacterial growth.

DNA Extraction, Amplification, and Sequencing

Genomic DNA Extraction

Genomic DNA was extracted following the Wizard® Genomic DNA Purification Kit protocol. A 1 mL aliquot of bacterial culture was centrifuged at 16,000 × g for 2 min. The resulting pellet was resuspended in 600 µL Nuclei Lysis Solution and incubated at 80 °C for 5 min. After cooling, 3 µL RNase Solution was added and the mixture incubated at 37 °C for 1 h. Protein impurities were removed using 200 µL Protein Precipitation Solution, followed by vigorous vortexing, chilling on ice, and centrifugation. The supernatant was transferred to a tube containing 600 µL isopropanol, mixed gently, and centrifuged to pellet the DNA. The pellet was washed in 70% ethanol, air-dried, and resuspended in 40 µL DNA Rehydration Solution before storage at 4 °C.

Agarose Gel Electrophoresis

DNA quality was examined through electrophoresis using a 1% agarose gel prepared in 1× TAE buffer. Approximately 4 µL DNA sample mixed with loading dye was loaded into the wells and electrophoresed at 100 V for 45 min. Gels were stained with EtBr, destained in distilled water, and visualized under UV illumination. Clear and distinct DNA bands indicated successful genomic DNA extraction (Mahadi et al., 2024; Parista et al., 2024).

PCR Amplification of the 16S rRNA Gene

The 16S rRNA gene was amplified using the universal primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387R (5′-GGGCGGAWGTGTACAAGGC-3′) (Eling et al., 2014). PCR reactions (20 µL) consisted of genomic DNA, GoTaq® Green Master Mix, primers, and nuclease-free water. Amplification conditions were: Initial denaturation: 95 °C for 5 min, 35 cycles of: Denaturation: 95 °C for 45 s, Annealing: 55 °C for 30 s, Extension: 72 °C for 1 min, and Final extension: 72 °C for 7 min. Amplicons were separated on 1% agarose gel and visualized under UV light. Smearing or faint bands in undiluted samples indicated excessive template concentration or contaminants (Muhamad et al., 2024; Arslan et al., 2021).

Nucleotide Sequencing and BLAST Analysis

PCR products were sequenced bidirectionally using Sanger sequencing at PT Genetika Science Indonesia. Forward and reverse chromatograms were assembled and edited using BioEdit 7.2, yielding a high-quality 1,274 bp sequence. Identification was performed by comparing the sequence to entries in the NCBI database using BLASTN. Sequences with ≥97% identity were considered to belong to the same genus, while ≥99% indicated species-level similarity (Khairunnisah et al., 2022). Genetic distances were calculated using the p-distance model, and phylogenetic trees were constructed using the Neighbor-Joining method with 1,000 bootstrap replications in MEGA 11 (Saleky & Amir, 2023; Vertiana et al., 2023).

RESULTS AND DISCUSSION

Genomic DNA Extraction and Quality Assessment

Genomic DNA extraction is a critical step in molecular identification, as DNA quality directly influences the accuracy of amplification and sequencing. Electrophoresis of the genomic DNA from isolate K.111 (Figure 1) displayed thick, distinct DNA bands without

smear formation. The absence of smearing indicates minimal degradation and suggests that the extracted DNA was intact and suitable for downstream PCR (Mahadi et al., 2024). Strong band intensity further reflects high DNA concentration, consistent with the observations of Buchori et al. (2023), who noted that bright, well-defined DNA bands correspond to successful extraction with minimal fragmentation.

This result aligns with Parista et al. (2024), who emphasized that faint or smeared DNA bands often indicate the presence of degraded nucleic acids or contaminants such as proteins and salts. Therefore, the visual profile of K.111 genomic DNA confirms that the extraction procedure effectively yielded high-quality DNA appropriate for PCR amplification.

Figure 1. Visualization of genomic DNA extraction results of bacterial isolate K.111

PCR Amplification of the 16S rRNA Gene

Amplification using universal primers 63F and 1387R produced an amplicon of approximately 1,300 bp, consistent with expected fragment sizes for this primer pair (Eling et al., 2014; Muhamad et al., 2024). As shown in Figure 2a, the undiluted DNA template produced a faint and smeared band pattern. This smearing likely resulted from excessive DNA concentration or from residual impurities such as proteins or organic salts that interfered with polymerase activity (Arslan et al., 2021; Muhamad et al., 2024).

In contrast, amplification using the 50× diluted genomic DNA template (Figure 2b) yielded a sharper and more distinct band with reduced smearing. This improvement demonstrates that template dilution successfully minimized inhibitory contaminants, resulting in cleaner amplification. The obtained amplicon length (~1,300 bp) is in accordance with expected amplification products for the 16S rRNA gene region targeted by primers 63F and 1387R (Muhamad et al., 2024).

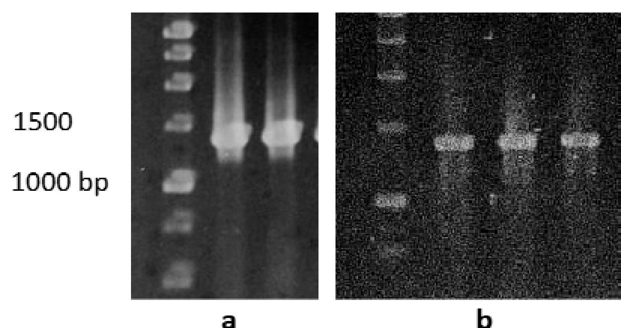

Figure 2. Visualization of the 16S rRNA Gene PCR Products on a 1% Agarose Gel. (a) Undiluted genomic DNA template, (b) Genomic DNA template diluted 50×.

PCR optimization is essential for ensuring reliable amplification. Annealing temperature significantly influences primer-template hybridization; excessively high temperatures reduce primer binding, while lower temperatures can promote non-specific amplification (Setyawati & Zubaidah, 2021). The results obtained in this study reflect optimal annealing temperature and proper template concentration, enabling successful amplification of the target gene.

DNA Sequencing

The PCR product was sequenced bidirectionally, yielding forward and reverse reads of 1330 bp and 1282 bp, respectively. After trimming low-quality ends and assembling the chromatograms, a 1,274 bp high-quality sequence was obtained. This sequence length is typical for partial 16S rRNA fragments generated by the primer pair used, as previously reported by Muhamad et al. (2024).

The sequence was considered suitable for similarity search and phylogenetic analysis. A clear chromatogram profile and the absence of ambiguous bases ensured the accuracy of downstream BLAST analysis.

```
>K.111
TATGAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAAC
CGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATG
GATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGT
TAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
TGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGT
GCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTA
AGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGA
TAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGG
ATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCG
```

Figure 3. Nucleotide sequence of the 16S rRNA gene from bacterial isolate K.111.

BLAST Similarity Analysis

BLASTN comparison of the 16S rRNA sequence of isolate K.111 with entries in the NCBI database revealed the highest similarity (100%) to *Bacillus thuringiensis*. Additionally, isolate K.111 showed 99.92% similarity with *Bacillus cereus* and *Bacillus wiedmannii*.

Table 1. Similarity analysis of the 16S rRNA gene sequence of bacterial isolate K.111 based on BLAST results.

Sampel Referensi	Max Score	Total Score	Query Cover	E Value	Per-Ident	Acc. Len	Accession
Bacillus thuringiensis JK0716S	2353	2353	100%	0,0	100%	1398	KF135459. 1
Bacillus cereus TY24	2348	2348	100%	0,0	99,92%	1518	ON506254. 1
Bacillus wiedmanni JBRI-MO-2019-0043	2348	2348	100%	0,0	99,92%	1424	MN865955. 1

According to Khairunnisah et al. (2022), ≥99% sequence identity indicates species-level similarity, while ≥97% suggests genus-level relatedness. The E-value of 0.0 obtained for all three matches signifies strong sequence homology, confirming close evolutionary relationships (Friliana et al., 2025). The 100% query coverage observed for all matches further underscores the reliability of these alignments.

Although *B. thuringiensis*, *B. cereus*, and *B. wiedmannii* are distinct species, they belong to a well-known clade of closely related *Bacillus* species, and high similarity among their 16S rRNA genes is common (Norashirene et al., 2013; Lee et al., 2022). Therefore, isolate K.111 can be placed confidently within this *Bacillus* species complex, with the highest affinity toward *Bacillus thuringiensis*.

Genetic distance analysis

Genetic distance analysis using MEGA XI revealed a value of 0.0000 between isolate K.111 and all three reference species (*B. thuringiensis*, *B. cereus*, and *B. wiedmannii*). Based on identity values (99.92%), *B. cereus* and *B. wiedmannii* were expected to show slight differentiation (~0.0008). The observed identical distances may be attributed to:Low variability within the sequenced region, Small sequence length after trimming, or The p-distance model's limited sensitivity to minimal base differences (Firda et al., 2025). The results of the genetic distance matrix analysis are presented in Table 2.

Table 2. Genetic distance matrix of 16S rRNA gene sequences between isolate K.111 and reference *Bacillus* species using the pairwise distance model.

Sample	1	2	3	4	5	6	7	8	9
K.111									
Bacillus thuringiensis KF135459.1	0.0000								
Bacillus cereus ON506254.1	0.0000	0.0000							
Bacillus wiedmanni MN865955.1	0.0000	0.0000	0.0000						
Bacilus subtilis OQ152611.1	0.0627	0.0627	0.0627	0.0627					
Geobacillus stearothermophilus FN428673.1	0.0926	0.0926	0.0926	0.0926	0.0965				
Geobacillus lituanicus NR025657.1	0.0981	0.0981	0.0981	0.0981	0.1028	0.1334			
Geobacillus kaustophilus NR115285.2	0.0926	0.0926	0.0926	0.0926	0.0981	0.0094	0.0054		
Bacillus amyloliquefaciens OQ152607.1	0.0659	0.0659	0.0659	0.0659	0,0062	0.0957	0.1020	0.0973	

Phylogenetic Tree Construction and Analysis

The Neighbor-Joining phylogenetic tree (Figure 4) formed three distinct clades with strong bootstrap support (100%). Clades 1 and 2 contained *Bacillus* species, while Clade 3 comprised *Geobacillus* species, consistent with established taxonomic relationships within the family Bacillaceae (Sahadeva & Pertiwi, 2024).

Isolate K.111 clustered tightly with *B. thuringiensis*, reflecting the 100% sequence identity obtained from BLAST analysis. Meanwhile, *B. cereus* and *B. wiedmannii* formed neighboring branches within the same clade, further demonstrating their close genetic relationship to K.111. High bootstrap support (>90%) indicates robust phylogenetic inference (Saleky & Amir, 2023; Vertiana et al., 2023).

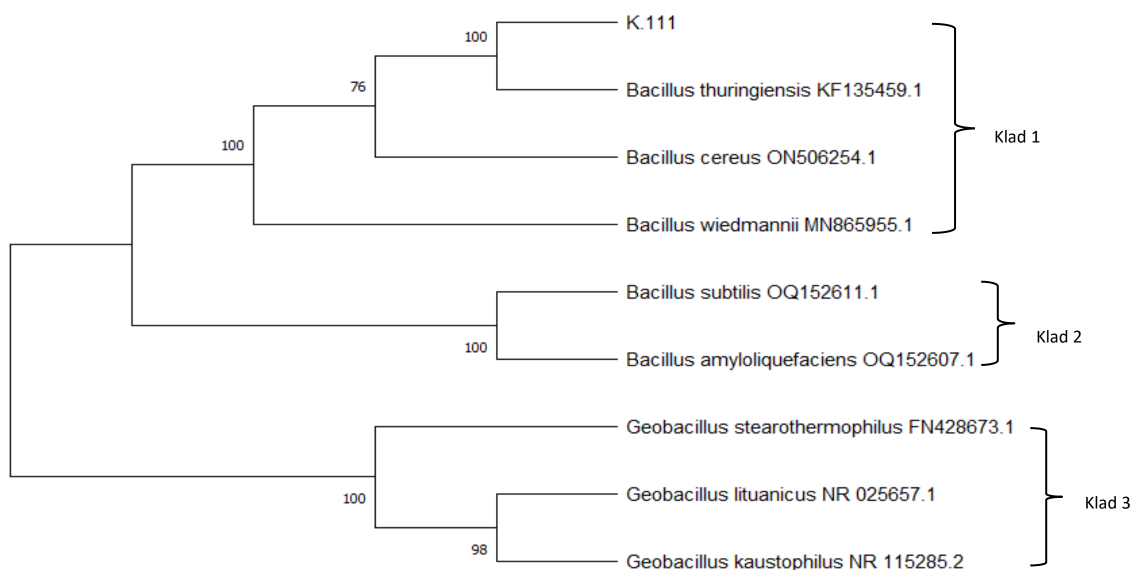

Figure 4. Phylogenetic tree of isolate K.111 and related reference species constructed using the Neighbor-Joining method based on 16S rRNA gene sequences with 1000x bootstraps.

These results are also in agreement with previous studies reporting frequent isolation of lipase-producing *Bacillus* species from extreme geothermal environments (Norashirene et al., 2013; Lee et al., 2022; Khalid & Abdulrahman, 2024).

CONCLUSION

The 16S rRNA gene of strain K.111 was molecularly characterized and had a length of 1274 bp, indicating a partial gene. Sequence analysis showed 100% similarity with *Bacillus thuringiensis an*d 99.92% similarity with *Bacillus cereus* and *Bacillus wiedmannii*. The genetic distance among these *Bacillus* species was 0.0. Phylogenetic analysis showed a bootstrap support value of 100%, confirming a strong evolutionary relationship.

Collectively, sequence similarity, genetic distance, and phylogenetic analyses consistently positioned isolate K.111 within the *bacillus* group, with the closest affiliation to *Bacillus thuringiensis*, *Bacillus cereus*, and *Bacillus wiedmannii*. This indicates that isolate K.111 belongs to the *Bacillus* group. It might be a thermotolerant variant adapted to the hot spring environment.

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Competing Interests: The authors declare that there are no competing interests.

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