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Exploring Thermophilic Bacilli and Their Thermostable Amylase Activity in Minahasa Hot Springs, Indonesia

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Running title: Thermophilic Bacilli from Minahasa Hot Springs

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ABSTRACT: Minahasa, Indonesia, is part of the Sangihe Islands Arc-related system within the Molluca Sea Collision Zone. The Molluca Sea Collision Zone is situated at the intersection of the Eurasian, Australian, Pacific, and Philippine plates. This region has many hot springs that span a wide temperature range from 45 to 98°C and are located at relatively high altitudes (700-900 m above sea level). This study aimed to isolate and characterize thermophilic bacteria from several hot springs in Minahasa, Indonesia. Mud and water samples were collected from three hot springs in Minahasa, Indonesia, and enriched in nutrient broth at 55°C. Thermophilic bacteria were isolated and characterized morphologically and biochemically. Isolates producing thermostable α-amylase were selected using amylose-containing media. Genomic DNA was extracted, and the 16S rDNA gene was amplified and sequenced. Phylogenetic analysis was performed using ClustalX and MEGA software, and the sequences were confirmed via BLAST and EZBioCloud databases. In this study, thirty-six thermophilic bacteria were isolated from three hot springs in Minahasa. Three distinct isolates, designated as FRM-LHD03, FRM-KNN03, and FRM-KRM06, were selected for further study. Phylogenetic analysis and a BLAST search in the EZBioCloud database revealed that FRM-LHD03 and FRM-KNN03 are *Geobacillus kaustophilus*, while FRM-KRM06 is *G. stearothermophilus*. This represents the first documented isolation of *Geobacillus* species from hot springs in Minahasa, Indonesia. The isolates demonstrated the ability to produce thermostable amylase, indicating potential for industrial applications.

KEYWORDS: thermophilic bacteria, hotspring, Minahasa, amylase, *Geobacillus*

INTRODUCTION

Bacteria are highly abundant and diverse organisms capable of thriving in a wide range of extreme environments where most other organisms cannot survive. Studies over the past three decades have revealed that 99% of the

world's bacteria remain unexplored and uncultured in laboratory settings. Consequently, their ecological functions and biotechnological applications are not yet well understood [1]. Thermophilic bacteria are microorganisms predominantly found in hot springs, capable of living and reproducing at temperatures exceeding 70°C.

The discovery of thermophilic bacteria capable of performing metabolic activities at very high temperatures in the hot springs of Yellowstone National Park, United States, has spurred industrial applications of enzymes produced by these thermophiles. Consequently, efforts to isolate thermophilic bacteria from geothermal environments have been conducted globally. These bacteria are known to inhabit various geothermal areas, including deep-sea hydrothermal vents and volcanic craters [2]. Thermophilic bacteria are also found in heat-producing fermented materials, such as compost piles and land-based waste [3].

The ability of thermophilic bacteria to reproduce at elevated temperatures is attributed to the chemical stability of their macromolecules [4]. Due to their growth at high temperatures and unique macromolecular properties, thermophilic bacteria exhibit high metabolic rates, producing larger quantities of end products despite having lower growth rates than mesophilic bacteria. Since their discovery, thermophilic bacteria have presented researchers with intriguing and challenging opportunities. However, the microbial diversity within thermal ecosystems remains largely unexplored due to the difficulties in isolating and maintaining pure cultures [5].

Thermophilic bacteria have been extensively utilized in biotechnology on a large scale [6]. These microbes exhibit potential for diverse applications, including their use in the production of thermostable Taq polymerase for polymerase chain reaction (PCR) [7], various enzymes in biofuel manufacturing [8], microbial applications in mining [9], and compounds utilized in the food and cosmetics industries [10]. Furthermore, they play roles in the production of lactose-free milk [11], antibiotics, antifungals, and anticancer drugs [12], as well as in bioelectrochemical systems for generating and storing currents [13].

Minahasa Regency, situated in North Sulawesi Province, Indonesia, lies within the Asian segment of the Pacific Ring of Fire. Geotectonically, Minahasa forms part of the Sangihe Islands Arc system, adjacent to the Molucca Sea Collision Zone [14]. This collision zone, where the Eurasian, Australian, Pacific, and Philippine plates converge, creates complex geological formations within the North Sulawesi Arc. Seismic activity along the Molucca Sea Collision Zone has resulted in numerous hot springs throughout Minahasa Regency [14], providing optimal habitats for thermophilic bacteria. While previous studies have documented thermophiles in Indonesian

hot springs, comprehensive exploration of their biotechnological potential remains limited. This study aims to establish an ongoing research initiative dedicated to discovering, isolating, and characterizing new thermophilic organisms that may prove valuable for biotechnological and environmental applications.

MATERIALS AND METHODS

Sample Collection and Characterization

Mud and water samples were collected in 50 ml Falcon tubes from Hutan Pinus Lahendong Hot Spring (71°C, pH 6.3), Bukit Kasih Kanonang Hot Spring (96°C, pH 2.2), and Karumenga Langowan Hot Spring (75°C, pH 6.4). For enrichment, samples were immediately placed in nutrient broth at 55°C. To isolate individual colonies, a 50-µl aliquot of the one-day enrichment culture was spread onto nutrient agar (NA) plates (HiMedia, Mumbai). Plastic freezing bags were utilized to prevent sample desiccation during incubation. The growing colonies were subsequently replated on nutrient agar (NA) and incubated at gradually increased temperatures up to 70°C to isolate individual thermophilic colonies. For further analysis, the colonies were transferred to nutrient broth (NB) containing 15% glycerol and stored at -20°C. Each isolate was subjected to Gram staining and examined morphologically for size, color, margin, elevation, and pigmentation. Additionally, several standard biochemical reactions were performed to characterize the isolates biochemically.

Screening of Isolates for Thermostable α-Amylase Production

To screen isolates for thermostable α-amylase production, the isolates were cultured on solid media containing amylose as the carbon source, using a modified method of Souza and Leal [15]. The medium composition was 2% agar, 2% amylose, 0.5% peptone, 0.2% yeast extract, 0.015% CaCl2, 0.05% NaCl, and 0.05% MgSO4 at pH 7.0. Isolates were grown on amylose-containing Petri dishes and incubated at 65°C for 24 hours. Plates were then flooded with 2% iodine solution for 15 minutes. Hydrolyzed amylose by bacteria appeared as clear zones against a deep purple background.

PCR Amplification and DNA Sequencing of the 16S rDNA Region

Genomic DNA from bacterial isolates was extracted using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The 16S rDNA gene segment was amplified using the primer pair 27F (5’- AGA GTT TGA

TCM TGG CTC AG-3') and 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3'). PCR conditions included an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 48°C for 45 seconds, and extension at 72°C for 90 seconds, with a final extension at 72°C for 5 minutes. Amplified PCR products were analyzed by agarose gel electrophoresis (1%). Purified PCR products were sequenced at First Base Laboratories Malaysia. Sequences were checked for chimeras using Decipher Software, and then denoised and trimmed to generate single fasta files. These fasta files were used as query sequences in BLAST searches across various databases to identify homologous sequences.

DNA Sequence and Phylogenetic Analyses

The ClustalX 1.8 software package was utilized for multiple sequence alignment using the ClustalW algorithm. Phylogenetic tree construction was performed with the neighbor-joining method [16] included in the Mega v.11.0.8 software [17]. To verify the topology of the resulting phylogenetic trees, additional phylogenetic analyses were conducted using various methods. TreeView (Win32) 1.6.0 software [18] was employed to visualize and edit the phylogenetic trees. Final confirmation of isolate identification was achieved through BLAST searches on the NCBI webpage (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EZBioCloud database [19].

RESULTS

Hot Springs Diversity in Minahasa

The three hot springs sampled in Minahasa, Indonesia, present a diverse range of chemical and physical properties, each harboring unique bacterial isolates (Table 1). Hutan Pinus Lahendong boasts a near-neutral pH of 6.3 and a moderate temperature of 71°C, fostering the growth of 11 bacterial strains (FRM-LHD01 to FRM-LHD11). In stark contrast, Bukit Kasih Kanonang, with its acidic pH of 2.2 and scorching temperature of 96°C, supports a distinct community of 13 bacterial isolates (FRM-KNN01 to FRM-KNN13). Lastly, Karumenga Langowan, with a pH of 6.4 and a temperature of 75°C, nurtures 12 unique strains (FRM-KRM01 to FRM-KRM12). This diversity in environmental conditions across the hot springs likely contributes to the unique composition of bacterial communities within each site, suggesting that specific adaptations are required for survival in these extreme environments. The discovery of thermophilic bacteria capable of amylase production in such varied conditions emphasizes the adaptability and resilience of microbial life in hot springs.

Table 1. Chemical dan physical properties of the hot springs.

Thermophilic α-Amylase-Producing Isolates

To identify isolates capable of producing heat-stable α-amylase, the isolates were cultured on a solid medium containing amylose as the sole carbon source, using a modified version of the method described by Souza and Leal [15]. Figure 1 showcases the results of an amylase activity test for three bacterial isolates: FRM-LHD03, FRM-KNN03, and FRM-KRM06. The presence of a clear zone around the bacterial colonies is a visual indicator of amylase activity. Amylase, an enzyme secreted by the bacteria, breaks down starch (amylose) in the surrounding medium. The clear zones in the figure directly demonstrate that all three isolates are capable of producing amylase, which degrades the starch in the agar, leading to the observed transparent areas. The varying sizes of the clear zones may suggest differences in the amount of amylase produced or the efficiency of the enzyme from each isolate. These isolates, therefore, represent potential sources of amylase for various biotechnological applications.

Figure 1. Isolates exhibiting positive results in the starch hydrolysis test: a) FRM-LHD03, (b) FRM-KNN03, and c) FRM-KRM06.

Phenotypic Characterization of the Isolates

Phenotypic characterization of the α-amylase-producing isolates, (FRM-LHD03, FRM-KNN03, and FRM-KRM06) that have shown positive results for amylase activity, is presented in Table 2, Table 3, and Figure 2. The characterization includes morphological observations, microscopic examination (Gram staining), and biochemical tests. All three isolates are Gram-positive, meaning they have a thick peptidoglycan layer in their cell wall. They are also rod-shaped (bacilli) and exhibit motility, indicating the presence of flagella or other means of movement. These characteristics are commonly associated with bacteria from the genus *Bacillus*. The isolates show some variations in their biochemical properties. All three are catalase-positive, indicating they can break down hydrogen peroxide. They are also negative for oxidase and Voges-Proskauer tests. However, there are differences in indole, citrate, and methyl red reactions. FRM-KRM06 shows a weakly positive methyl red test, suggesting mixed acid fermentation.

Table 2. Colony morphology of the isolates.

Figure 2. Gram staining of isolates showing positive results in the starch hydrolysis test: a) FRM-LHD03, (b) FRM-KNN03, and c) FRM-KRM06.

Table 3. Biochemical characters of the isolates.

Molecular Identification of the Isolates

By using forward and reverse primers, the PCR-amplified products were sequenced from both directions. The sequences were deposited in GenBank under accession numbers MN822929, MN822930, and MN822931. The sequences of the three isolates were then entered into a BLAST search to identify bacterial species with the highest sequence homology. BLAST analysis revealed that the three isolates had high homology with *Geobacillus* species. Sequences of the species with the highest homology were downloaded from GenBank for further analysis.

Phylogenetic analysis was conducted to identify the three isolates at the species level. The results of the phylogenetic analysis using the Neighbor-Joining method (Figure 4) showed that FRM-LHD03 and FRM-KNN03 formed a cluster with *Geobacillus kaustophilus* and *G. thermoleovorans*. The FRM-KRM06 isolate formed a cluster with *G. stearothermophilus*. Phylogenetic analyses using different algorithms, including Maximum Likelihood, UPGMA, and Parsimony methods, yielded phylogenetic trees with similar topologies (data not shown). Thus, the phylogenetic analysis of the isolates capable of producing thermostable amylase in this study can be considered valid.

Figure 3. PCR products of isolates exhibiting positive results in the starch hydrolysis test.

Figure 4. Phylogenetic analysis using Neighbor-Joining method. The tree was constructed using Mega v.11.0.8.

DISCUSSION

Hot springs represent unique and stable extreme environments. These terrestrial hotspots host diverse and unconventional life forms, genes, and metabolic products [20]. Several studies suggest that thermophilic microorganisms thrive in these high-temperature conditions due to molecular-level adaptations, both within their cells and at subcellular levels.

In this study, muddy soil samples were collected from three hot springs in Minahasa, Indonesia to isolate, characterize, and identify bacterial strains exhibiting amylase activity. Among the 36 bacterial strains isolated, three were found to exhibit significant amylase activity, as evidenced by the formation of clear zones surrounding bacterial colonies. The phenotypic characterization suggests that the three isolates are likely closely related and belong to the genus *Bacillus* or a closely related genus. The variations in biochemical tests indicate some level of diversity within these isolates, which could be due to different species or strains within the same species. The Gram-positive, rod-shaped morphology, along with the positive catalase test, aligns with the typical characteristics of Bacillus species, which are known to include many amylase-producing bacteria. The ability of these isolates to produce amylase, as evidenced by the clear zones in the starch hydrolysis test (mentioned earlier but not shown here), further supports their potential for biotechnological applications where amylase is required.

Molecular identification using 16S rRNA gene sequencing revealed high homology of the three isolates with *Geobacillus* sp. Phylogenetic analysis further confirmed that isolate FRM-KRM06 clustered closely with *G. stearothermophilus*, supported by a high bootstrap value of 98%. Therefore, based on phylogenetic analysis and BLAST search results, FRM-KRM06 is identified as *G. stearothermophilus*. This species (formerly known as *B. stearothermophilus*) [21] is a rod-shaped, Gram-positive bacterium belonging to the Firmicutes division. This thermophilic species is prevalent in soil, marine sediments, and hot springs, and is recognized for its ability to spoil food products. It thrives in temperatures ranging from 30 to 75°C. Numerous studies have documented its capacity to produce the α-amylase enzyme [22-24].

Isolates FRM-LHD03 and FRM-KNN03 clustered together with two species, *Geobacillus kaustophilus* and *Geobacillus thermoleovorans*. These two *Geobacillus* species are closely related, forming clusters known as

"kaustophilus clades" according to molecular phylogenetic analysis [25]. To confirm the identification of these isolates, a search was conducted in the EzBioCloud database. EzBioCloud provides a quality-controlled similarity-based search for a 16S rRNA sequence database [19]. The top-hit information from EzBioCloud identification for FRM-LHD03 and FRM-KNN03 confirmed that both isolates belong to *G. kaustophilus*, with 99.86% identity for FRM-LHD03 and 99.93% for FRM-KNN03. In the past, scientists used a 16S rRNA gene sequence similarity threshold of less than 97% to differentiate between species and less than 95% to differentiate between genera [26]. However, this threshold has been revised to 98.2–99.0% depending on the studied taxa [27]. A recent large-scale study also supports this revised threshold, demonstrating a similar threshold of 98.65% when comparing 16S rRNA gene sequence similarities with genome-based average nucleotide identity values [28].

In this study, *G. kaustophilus* was found in two hot springs with distinctly different physicochemical properties: Hutan Pinus Lahendong, characterized by an average temperature of 71°C and pH 6.3, and Bukit Kasih Kanonang, characterized by an average temperature of 96°C and pH 2.2. This bacterial species is known to tolerate pH ranges from 2 to 12 [25 Previous studies have indicated that its upper temperature limit is 74°C [29]. Therefore, it is noted here that *G. kaustophilus* can inhabit an environment with an average temperature of 96°C.

In our previous study, conducted at a different location, a different thermophilic bacterium designated as *Anoxybacillus thermarum* FRM-RBK02 was isolated and characterized [30]. This species was shown to possess the capability to produce thermostable α-amylase. This finding, along with the results obtained from the present study, indicates that hot springs in Minahasa harbor thermophilic bacteria that have the potential to be exploited as enzyme sources for industrial applications.

CONCLUSIONS

This study reinforces the notion that hot springs are reservoirs of diverse thermophilic bacteria with significant biotechnological potential. The isolation of *Geobacillus stearothermophilus* and *Geobacillus kaustophilus*, both known producers of α-amylase, from hot springs in Minahasa, Indonesia, underscores this potential. However, the identification of *G. kaustophilus* in an environment with an average temperature of 96°C challenges the previously reported upper temperature limit for this species,

suggesting the need for further investigation into its thermal tolerance and adaptability. These findings, combined with our previous research identifying *Anoxybacillus thermarum* with thermostable α-amylase production capabilities, highlight the rich diversity of thermophiles in Minahasa and their potential as sources of industrially relevant enzymes. Further exploration of these hot springs may unveil additional novel microorganisms and biomolecules with valuable applications.

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TABLES LEGENDS

Table 1. Chemical dan physical properties of the hot springs.

Table 2. Colony morphology of the isolates.

Table 3. Biochemical characters of the isolates

FIGURES LEGENDS

Figure 1. Isolates that showed positive results with the starch hydrolysis test. A) FRM-LHD03, (b) FRM-KNN03, and c) FRM-KRM06.

Figure 2 Gram stain of the isolates that showed positive results with the starch hydrolysis test. A) FRM-LHD03, (b) FRM-KNN03, and c) FRM-KRM06.

Figure 3. PCR products of the Isolates that showed positive results with the starch hydrolysis test.

Table 1. Chemical and physical properties of the hot springs.

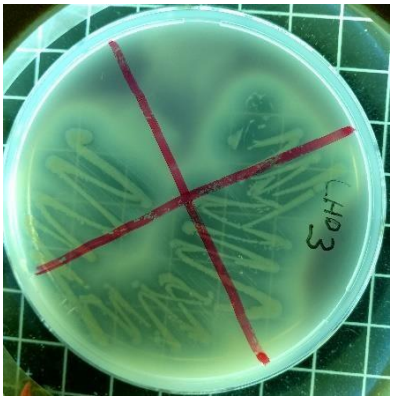
Location	pH	Temperatures (°C)	Isolates
Hutan Pinus Lahendong	6.3	71	FRM-LHD01 – FRM-LHD11
Bukit Kasih Kanonang	2.2	96	FRM-KNN01 – FRM-KNN13
Karumenga Langowan	6.4	75	FRM-KRM01 – FRM-KRM12

Table 2. Colony morphology of the isolates.

Isolate	Form	Elevation	Margin	Opacity
FRM-LHD03	circular	raised, convex	entire	transparent
FRM-KNN03	circular	raised, convex	entire	transparent
FRM-KRM06	circular	raised, convex	entire	opaque

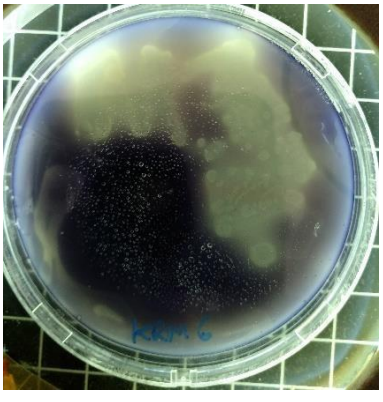
Table 3. Biochemical characters of the isolates.

Biochemical Test	Isolate		
	FRM-LHD03	FRM-KNN03	FRM-KRM06
Indole	-	-	+
Citrate	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Methyl red	+	+	+
Vogues-Proskauer	Weak +	-	-



A)

B)



C)

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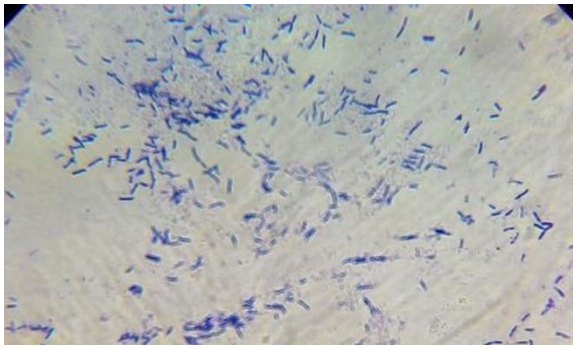
a)



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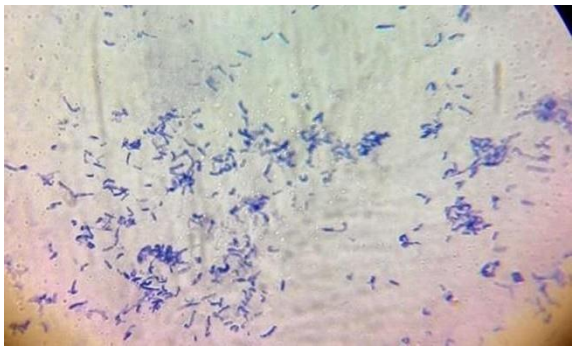
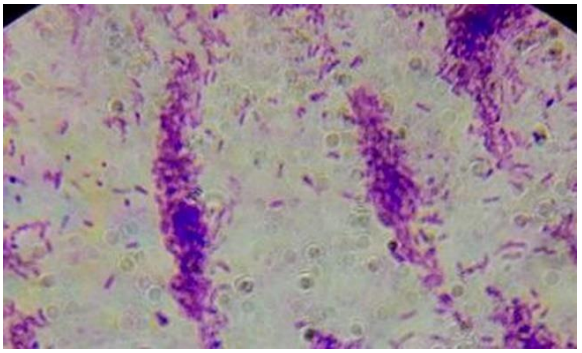


c)



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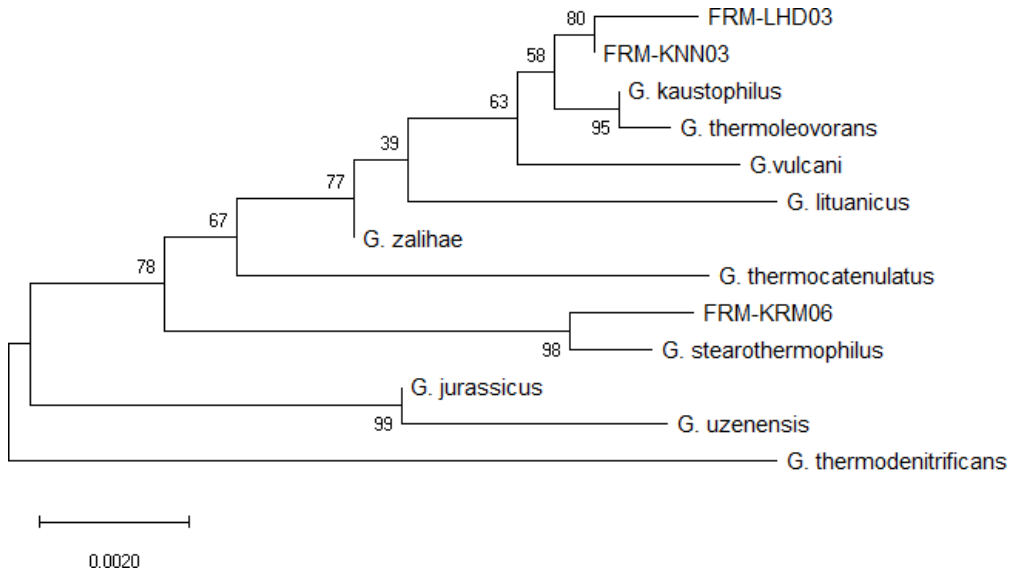

Figure 4. Phylogenetic analysis using Neighbor-Joining method. The tree was constructed using Mega v.11.0.8.