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Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia

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Abstract. *Ginting EL, Wantania LL, Moko EM, Tumbol RA, Siby MS, Wulur S. 2021. Isolation and identification of thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Biodiversitas 22: 3326-3332.* The aims of the research were to isolate and identify the amylase-producing thermophilic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia. The bacteria were characterized based on the colony and cell morphology and subsequently screened for their amylase activities. The bacterial isolates were identified based on 16S rRNA gene sequences. There were 12 thermophilic bacteria isolates from Likupang Marine Hydrothermal that were able to produce amylase. Two selected isolates (L3 and L9) had an amylolytic index value in the range of 3.04-3.52 at 55°C. The colonies of L3 and L9 are circular, and they are Gram positive, rod-shaped, and motile bacteria. Based on the 16S rRNA gene sequences and phylogenetic tree analysis showed that L3 was closely related to *Bacillus caldotenax* with 93% similarity while L9 had 99% similarity to *B. caldotenax*. Hence, we identified L3 isolate as *Bacillus sp.* strain L3 and L9 isolate as *B. caldotenax* strain L9. Both of these bacteria were grouped as *Geobacillus thermoleovorans*.

Keywords: Amylase, *Bacillus*, identification, Likupang, thermophilic

Abbreviations: BLAST: Basic local alignment search tool, DDH: DNA-DNA hybridization, DNA: deoxyribonucleic acid, PCR: polymerase chain reaction, TMMB: thermus medium modified broth, TMMA: thermos medium modified agar, 16S rRNA: 16 Svedberg ribosome-ribonucleic acid, VP: Voges-Proskauer

INTRODUCTION

Bacterial populations are widespread in various environmental conditions, including many places with extreme conditions, such as pH, salinity, and temperature. Bacteria that can survive in high temperatures are known as thermophilic bacteria. One of the notable abilities of thermophilic bacteria is to produce enzymes that are stable at high temperatures, and the enzymes catalyze their chemical reactions at high speed at high temperatures (Singh et al. 2011; Mohammad et al. 2017). Moreover, thermophilic bacteria are the ideal choice for producing thermostable enzymes, which makes them interesting research subjects. In particular, the thermostability of their cell components in highly elevated temperatures has led to their extensive use in various biotechnological and industrial applications (Raddadi 2015; Charbonneau et al. 2012). They were very useful in industrial application because they have several advantages, i.e., high growth rate, accelerate fermentation processes two to three times over those with mesophilic producers, reduced risk of microbial contamination, higher diffusion rate, and mass turnover, reduced the viscosity of culture liquids, and improved solubility of polymeric substrates and fats that are usually insoluble compounds (Kambourova 2018). The thermostability of cell components in high and elevated temperatures is the reason for their extensive use in industrial applications and multiple biotechnological (Raddadi 2015; Charbonneau et al. 2012).

In high-temperature bioprocessing, the enzymes produced by thermophilic bacteria can reduce risk of contamination, improve solubility of substrates (i.e., lignocellulosic biomass), lower chances of phage infection, and reduce cooling costs. The cooling cost specifically are reduced due to greater temperature differential between the fermenter and the ambient air, which is the ultimate heat acceptor with the addition of continuous recovery of volatile chemical products directly from fermentation broth. (Frock and Kelly 2012; Keller et al. 2014). One of the enzymes produced by thermophilic bacteria is amylase which has an important role in biotechnological studies and the world enzyme market (25%-33%) (Nguyen et al. 2002). Amylase hydrolyze starch to produce maltose, glucose, malto-oligosaccharides, and various α-limit dextrin-containing α (1-6) bonds. They have a spacious range of uses in pharmaceutical, cosmetic, agricultural industries, food, and nutritional processes (Satrimafitrah et al. 2019; Gazali and Suwastika 2018; Luang-In et al. 2019; De Souza and Magalhaes 2010).

Some hot springs in Indonesia are sources of thermophilic bacteria that could produce amylase (Gazali et al. 2018; and Ardhi et al. 2020). Thermophilic bacteria can also be found in shallow water hydrothermal and seabed hot springs. Marine hydrothermal spots can be discovered in many places throughout Indonesia including North Sulawesi. One of the marine hydrothermal in North Sulawesi is in Likupang. A study by Myu[42]Ji et al. (2012) revealed microbial diversity in Likupang based on the 16S rRNA gene sequence. One hundred and five representative clones from this thermal vent showed that the highest proportion was γ-Proteobacteria (33.3%), followed by Bacteroidetes (27.6%) and unclassified bacteria group (20%). The minor groups were affiliated to Firmicutes (7.6%) and α-Proteobacteria (4.8%). On the other hand, the research on amylase-producing thermophilic ba[45]ia from this area has not been reported yet. Therefore, the present study was carried out to isolate and identify the thermophilic amylolytic bacteria from Likupang Marine Hydrothermal, North Sulawesi, Indonesia.

MATERIALS AND METHODS

Sampling

Marine water samples were collected from 3 points on the hot spring location at East Likupang beach, North Minahasa District, North Sulawesi Province, Indonesia (1°39'54.87"N, 125°06'19.41"E) (Figure 1). They were taken directly using sterile bottles and placed in cool box. Samples were carried to the laboratory for bacteria isolation and further testing. The temperatures during the sampling were measured on site using glass thermometer.

Culture and bacteria isolation

Twenty µL of marine water sample was inserted into 5 mL of Thermus Medium Modified Broth (TMMB-0.01% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, 0.35% $(NH_4)_2SO_4$, 0.1% NaCl, 0.05% Yeast extract, 0.05% peptone) and incubated at 55°C for 24-48 hours. As much as 0.1 mL of cultured broth was spread onto TMMA (TMMB + 2% Agar) using L-glass followed by incubation at 55°C for 24-48 hours. Single growing colonies with different morphological characteristics (e.g., color, colony, growth shape, and edges) were individually isolated, which were then developed on new TMMA, and observed more carefully for their morphology characteristics.

Screening the thermopiles bacteria producing amylase

The single colony of the bacterium was spotted onto the surface of TMMA containing 2% of amylum on a plate, followed by incubation at 55°C for 24[28] hours. The ability of the bacteria to produce amylase was observed by the [17]earance of a clear zone around the colony by flooding it with Lugol's iodine solution (w/v) (1% iodine in 2% potassium iodide). The clear zone and colony diameters [6]re estimated using a caliper rule (Poletto et al. 2018). The amylolytic index value of each isolate was determined based on the ratio of clear zone diameter and diameter of a colony (Soy et al. 2019).

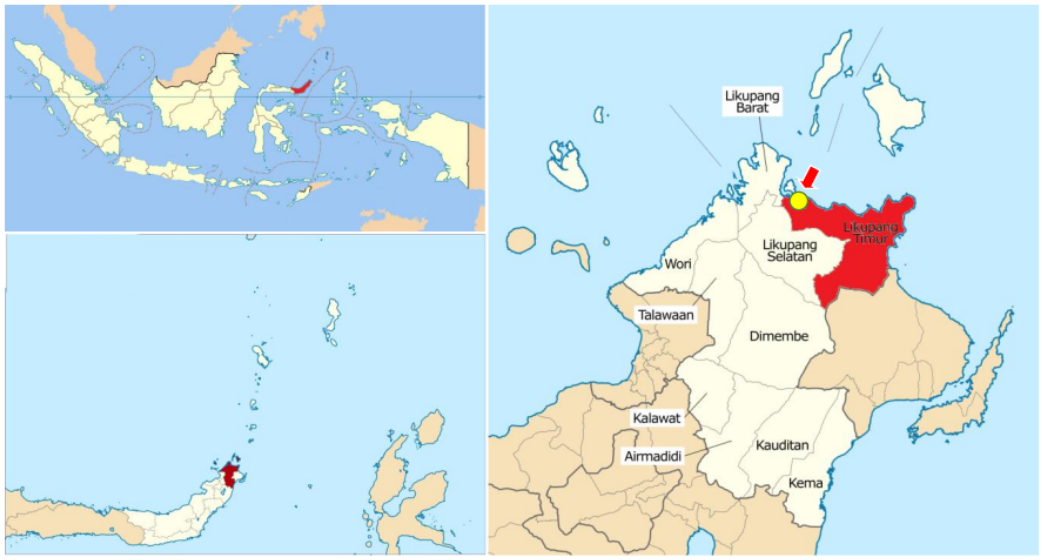

Figure 1. The map of North Minahasa District, North Sulawesi Province, Indonesia. The sampling location is in the Likupang Marine Hydrothermal, East Likupang Sub-district as indicated by an arrow

Identification of the active amylolytic thermophilic bacteria

Morphological and physio-biochemical characterization

Preliminary identification of the bacterial isolates with amylolytic activity was carried out based on morphological and physio-biochemical characteristics. Morphological characteristics observed were Gram-stain (Thairu et al. 2014), motility of bacteria, and biochemical properties. Bacterial motility was determined using semi-solid media, while biochemical properties included parameters of carbohydrate utilization, gas production, sugar fermentation (glucose, lactose, sucrose, maltose), indole production, methyl red, Voges-Proskauer (VP), citrate utilization, and H_2S production.

Molecular identification and phylogeny

Identification of bacterial isolates was confirmed by 16S rRNA gene analysis. Genomic DNA from each sample was prepared based on the protocol in the QIAprep Miniprep kit (Qiagen Hilden, Germany). The genomic DNA was used as the template for PCR amplification of 16S rRNA gene region with the universal primer pair (Integrated DNA Technologies-IDT, Singapore) 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACT-3′) (Zhang et al. 2019). Amplifications were carried out with 4 µl master mix (5× HOT FIREPol Blend Master Mix, Solis BioDyne, Estonia), 12 µl dH_2O, 1 µl primer mix, and 3 µl bacterial DNA (total volume 20 µl). The PCR steps consist of pre-denaturation at 95°C, for 30 seconds; annealing at 52°C, for 30 seconds; and elongating at 72°C, for 30 seconds. The PCR process consists of 35 cycles. After that, the phase post-PCR starts at 72°C for 10 minutes, and the stop PCR at 4°C. Nucleotide bases of the PCR products were then separated using 1% agarose gel in electrophoresis at 30-40 V and 28-29 mA, with a 10.000 bp DNA ladder as a marker (Solis BioDyne, Estonia). The PCR products were subsequently sent for nucleotide sequence to a DNA gene analysis service at First-Base Co., Selangor, Malaysia.

DNA sequences of the 16S rRNA gene quality were assessed using Sequence Scanner version 2.0 Software (Applied Biosystem) and the sequence traces were trimmed, assembled, and edited using MEGA Version 7. The sequences were subjected to BLAST using rRNA type strains/prokaryotic16S_ribosomal RNA (bacteria and archaea) database setting in The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Selected sequences from the NCBI database were aligned using CLUSTX (http://www.clustal.org/). Gaps and the 5′ and 3′ ends of the alignment were edited using BioEdit sequence alignment editor software version 7.2.5 (http://www.mbio.ncsu.edu/bioedit/page2.html). The phylogenetic tree was developed by neighbour-joining in MEGA Version 7 (Kumar et al. 2016).

RESULTS AND DISCUSSION

In total, 12 thermophilic bacteria were successfully isolated from Likupang Marine Hydrothermal, which were able to grow at 55°C. All of these isolates had an amylolytic index at TMMA containing 2% of amylum. There were two isolates (L3 and L9) that have the highest amylolytic indexes were further identified.

L3 and L9 had different morphological and physio-biochemical characteristics (Table 1). The ability of these bacteria to degrade amylum is indicated by the appearance of a clear zone around the colonies on the media containing 2% amylum at 55°C (Figure 2). The average amylolytic index of L3 and L9 were 3.04 and 3.52, respectively. The more amylase, the wider the clear zone due to the degrading of amylum on the medium which renders enhancement the amylolytic index.

The 16S rRNA gene of the two thermophilic amylolytic bacteria was successfully amplified and showed a DNA band at around 1500 bp (Figure 3). Amplification 16S rRNA sequences of bacteria were established on ~1500 bp amplicon (Akihary et al. 2020; Wullur et al. 2020; Ginting et al. 2019; Wantania et al. 2019). Furthermore, after sequence 16S rRNA was trimmed, assembled, and edited, the 16S rRNA gene sequence of L3 was 936 bp and L9 was 1120 bp.

Based on 16S rRNA gene sequence data of the bacterial isolates which were analyzed using rRNA typestrains/prokaryotic16S_ribosomal RNA database in NCBI showed that L3 was closely related to *Bacillus caldotenax* (93% similarity), and L9 to *B. caldotenax* (99% similarity) (Table 2). Both 16S rRNA genes had the same as the top five hits against Nucleotide BLASTed, which were *B. caldotenax, Geobacillus kaustophilus,* and *Geobacillus thermoleovorans* species. According to Nazina et al. (2001) *B. thermoleovorans*, *B. kaustophilus,* and *Bacillus thermocatenulatus*, which also included *Bacillus caldolyticus*, *Bacillus caldovelox,* and *B. caldotenax* should be combined into one species, namely *G. thermoleovorans* as shown in Table 2. This grouping was based on the level of DNA-DNA reassociation values. It was also consistent compared to the phylogenetic data of Nazina et al. (2005) and Spiegler (2005). Moreover, Aliyu et al. (2016) proposed for the strains of *G. kaustophilus* and *G. thermoleovorans* to be referred to as *G. thermoleovorans* species.

Table 1. Physio-biochemical characteristics of the L3 and L9 thermophilic bacteria

Characteristics	L3	L9
Cell shape	Rods	Rods
Colony shape	Circular	Circular
Colony color	Cream/Yellow	Cream/Yellow
Gram stain	+	+
Motility	+	+
Carbohydrate utilization	-	-
Gas production	-	-
Glucose	-	-
Lactose	+	+
Sucrose	+	+
Maltose	+	+
Indole Production	+	-
Methyl red	-	-
Voges-Proskauer (VP)	-	+
Citrate Utilization	-	-
H_2S Production	-	-

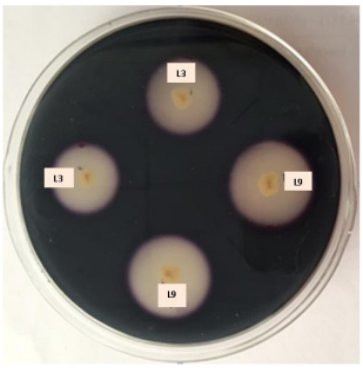

Figure 2. Bacterial isolates were spotted on TMMA containing 2% amylum. The clear zone around the bacteria indicated the amylum degradation by amylase that produced the bacteria.

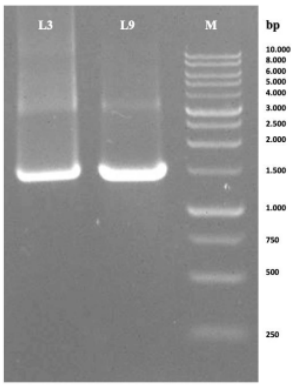

Figure 3. The amplified 16S rRNA gene of the two isolates amylase-producing thermophilic bacteria in the 1% agarose gel electrophoresis, using 10.000 bp DNA ladder as a marker.

Table 2. Top five nucleotide blast hits of 16S-rRNA gene of the bacterial using rRNA typestrains/prokaryotic16S_ribosomal RNA (bacteria and archaea) database setting in the NCBI GenBank

Isolates	Taxonomy	Species & accession number	Score	Query cover	E-value	Identity
L3	Bacillales					
	Bacillaceae					
	Geobacillus					
	G. thermoleovorans group					
		Bacillus caldotenax, NR 126321.1	898	98%	0.0	93.48%
		Geobacillus thermoleovorans, NR 115286.2	896	84%	0.0	93.32%
		Geobacillus kaustophilus, NR115285.2	893	90%	0.0	93.32%
		Geobacillus kaustophilus, NR 114089.1	893	90%	0.0	93.16%
		Geobacillus thermoleovorans, NR 036985.1	893	90%	0.0	93.32%
L9	Bacillales					
	Bacillaceae					
	Geobacillus					
	G. thermoleovorans group					
		Bacillus caldotenax, NR 126321.1	1336	99%	0.0	99.32%
		Geobacillus thermoleovorans, NR 036985.1	1336	99%	0.0	99.32%
		Geobacillus kaustophilus, NR 115285.2	1336	99%	0.0	99.32%
		Bacillus caldotenax, NR 126322.1	1334	99%	0.0	99.19%
		Geobacillus thermoleovorans, NR 115286.2	1328	99%	0.0	99.05%

The phylogenetic analysis of L3 and L9 were presented in Figure 4. The phylogenetic analysis showed the relation between L3 and L9 with their close homologs. All the closed related homologs of the rRNA typestrains/ prokaryotic 33 _ribosomal RNA (bacteria and archaea) registered in The National Center for Biotechnology Information (NCBI) were used in the construction of the phylogenetic tree to obtain their evolutionary origin. The phylogenic analysis result showed that L3 and L9 were at different branching. It indicated that L3 and L9 were different bacterial strains. The L3 isolate has 93% similarity with *B. caldotenax* which revealed that there was a different strain of L3 with *B caldotenax*. However, in determining an isolate as a new strain, a certain analysis should be done along with 16S rRNA analy 2 s. Therefore, the result of this research indicated that L3 is 3 genus *Bacillus*.

The taxonomy of the genus *Bacillus* showed that the bacteria species were members of *Bacillus* rRNA Group 5 (Cihan 2012). 2 veral members of Group 5 were reclassified into the new genus *Geoba* 19 *s* based on a combination of 16S ribosomal RNA (rRNA) sequence analysis, fatty acid composition, and DNA-DNA hybridization (DDH) (Dinsdale et al. 2011). The morphological characteristics of L3 and L9 showed the characteristics of *Bacillus*/*Geobacillus* thermophile. L3 and L9 were Gram-positive, rod-shaped and motile. According to Nazina et al. 2001; Cho and Chung 2020, *Bacillus*/ *Geobacillus* has the characteristics of Gram-positive, rod-

shaped and motile, heat resistance and the optimum growth temperatures at 55-65°C.

The temperatures at the sampling location in Likupang Marine Hydrothermal, North Sulawesi, Indonesia were 65-70°C which strongly supports the growth of the *Geobacillus*. *G. thermoleovorans* from the volcanic geothermal environment have morphological characters as a cream colony, Gram-positive motile, and rod-shaped. These characteristics of *G. thermoleovorans* were similar to L3 and L9 characteristics. Therefore, we conclude that L9 is *B. caldotenax* and L3 is *Bacillus* sp. Both of these strains were members of the *G. thermoleovorans* group. Hence, we identified L3 as *Bacillus sp.* strain L3 and L9 as *B. caldotenax* strain L9. *B. caldotenax* were also successfully isolated from Sumber Air Panas Gunungsongo, Indonesia (Nihaya et al. 2013) and shallow hydrothermal vents of Panarea Island, Italy (Gugliandolo et al. 2012).

G. thermoleovorans group were classified on the phylum Firmicutes, class Bacilli, ordo Bacillalea and genus *Geobacillus*. According to Myung-Ji et al. (2012), Firmicutes were the minor groups of microbial diversity in Likupang Marine Hydrothermal, North Sulawesi, Indonesia. Only 7.6% of microbial diversity in Likupang Marine Hydrothermal was Firmicutes. It indicated that the population of *G. thermoleovorans* was low so that it was difficult to isolate from Likupang Marine Hydrothermal. Therefore, L3 and L9 would be re-cultured and stored as potential bacterial stock.

Amylase-producing *Geobacillus* sp. was also successfully isolated from sub-seafloor sediments (Jiang 2015). *G. kaustrophilus*, *G. thermoleovorans,* and *G. toebii* were the amylase-producing thermophilic bacteria with the optimal amylase activities at 90°C and pH 7.0. The amylase activity was not detected at a temperature less than 40°C which pointed to its thermophilic nature. Sharma et al. (2015) reported that pre-incubation of the enzyme at 100°C increased amylase activity. Ardhi et al. (2020) reported that amylase-producing thermophilic *Bacillus licheniformis* isolated from Bukit Gadang Hot Spring, West Sumatra, Indonesia was able to produce 231.33 U amylase/mL, with the enzyme-specific activity of 101.79 U/mg protein at the incubation time of 36 h. The molecular weight of α-amylase from *G. thermoleovorans* was estimated to be 58 kDa by SDS-PAGE and very active in a wide range of pH from 4.0-10.0. The temperature of 70°C was considered as the optimum temperature of the enzyme and distinct thermostability in the presence of Ca^{2+}, retaining 50% of its initial activity after 90 h at 70°C (Finore et al. 2011).

Thermostable enzymes have provided considerable benefits in the industry because the enzymes have several advantages (Frock and Kelly 2012; Keller et al. 2014). *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, and *B. amyloliquefaciens* are recognized as good producers of thermostable α-amylase, and have been widely used in commercial industries for various applications (Prakash and Jaiswal 2010). Additionally, thermostable amylases of *B. stearothermophilus* and *B. licheniformis* were used in starch processing industries (Gomes et al. 2003; Oziengbe and Onilude 2012) while *B. subtilis* was applied in food waste biodegradation (Msarah et al. 2020).

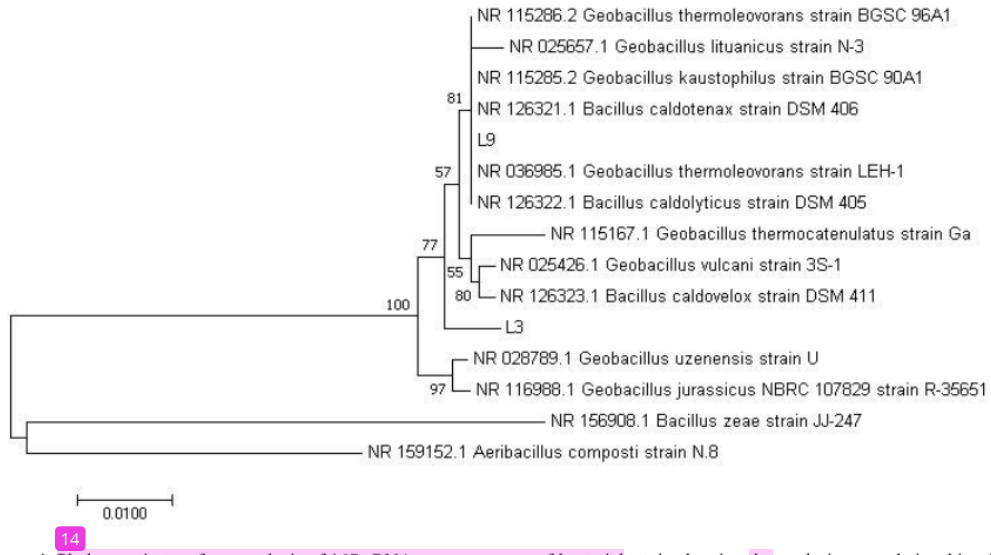

Figure 4. Phylogenetic tree from analysis of 16S rRNA gene sequence of bacterial strain showing the evolutionary relationship of the bacterial isolates within previously characterized species. *Bacillus zaea* and *Aeribacillus composti* were used as the 'outgroup'. GenBank accession numbers of 16S rRNA sequences are shown in the front. Bootstraps values expressed as a percentage of 1,000 re-samplings of the neighbor-joining dataset were set up to estimate the reliability of phylogenetic tree reconstruction

The search for novel amylase-producing thermophilic bacteria as novel sources of thermostable enzymes was interesting and important to be done. Results of this study showed that *Bacillus* sp. strain L3 and *B. caldotenax* strain L9 were thermophilic amylolytic bacteria that were successfully isolated from Likupang Marine Hydrothermal, North Sulawesi, Indonesia with the potential of thermostable amylase producers. These bacterial strains have the potential as thermostable amylase producers with the promising biotechnological application such as starch processing industries. Moreover, the information on thermostable amylase from *B. caldotenax* bacteria was not yet available.

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