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Antimicrobial peptide coding gene of thermophilic bacteria isolated from crater hot spring in mountains around West Java

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ABSTRACT

Antimicrobial peptides (AMPs) are ribosomal synthesized peptides produced by microorganisms, including thermophilic bacteria isolated from hot springs. The protein has a broad spectrum against Gram-positive and Gram-negative bacteria, making it a candidate for alternative antibiotics. This study aims to identify and characterize antimicrobial peptide coding genes of thermophilic bacteria isolated from Mount Kamojang and Tangkuban Perahu crater hot springs in West Java. Six samples of crater water were collected from different locations around Mount Kamojang and Tangkuban Perahu. All six isolates were identified using 16S rRNA gene sequencing and were identified as *Geobacillus* sp. The antagonist test of all isolates against *Streptococcus mutans* was carried out using a disk test, and it was seen that there was an inhibition zone formation in the culture of all strains. The largest inhibition zone was formed in the culture of *Geobacillus* strain TM6Sp1 which was 7.96 mm. The identification of AMP encoding genes was carried out by entering the identified genome sequence of samples into the BAGELA4 web server, and the NCBI database was used for sequence analysis and alignment. The results showed that all of the samples have the same alignment lanthipeptide Class A, which has a 100% similarity to the lantibiotic precursor. This indicates that thermophilic bacteria isolated from crater hot springs in the mountains surrounding West Java have antimicrobial activity against *S. mutans* due to their ability to produce AMPs of lanthipeptide Class A with an amplicon size of 230 base pairs.

1. INTRODUCTION

Recently, several antibiotic-resistant bacteria have been sorted out due to the misuse or overuse of the drugs [1], hence, there is an urgent need to find new sources of antibacterial agents [2,3]. Thermophilic bacteria isolated from hot springs are being used extensively in the search for alternative antibiotics. The previous studies have also focused on the production of antimicrobial peptides (AMPs) from these microbes over the past decades. Furthermore, they are regarded as valuable potential biochemical sources for the production of thermostable enzymes, antibiotics, antifungals, and anti-cancer agents [3,4]

AMPs are molecules with unique and very diverse properties, produced by all living organisms, where they function as immune factors for life continuity. These peptides also have strong antimicrobial activity against viruses, bacteria, protozoa, and fungi [4]. They react quickly and have the natural ability to combat antibiotic-resistant bacteria, such as methicillin and vancomycin. Consequently, the pharmaceutical industry has explored this potential for clinical purposes [5]. AMPs have been successfully isolated from various bacteria with different sequences, but almost all of them have the same mechanism of action. They act directly against the microbes by forming pores on the cell membrane, which helps to remove ions and important nutrients [6]. AMPs are key components of the innate immune systems, where they play an important role in the host's defense mechanism against invading pathogens. At present, they have promising clinical and industrial value [7]. Exploring new ecological opportunities to isolate bacteria that can produce strong AMPs are part of the effort to discover new and more effective agents.

Several studies have reported the production of these peptides or bacteriocins by thermophilic bacteria [8], including the *Geobacillus* species, such as *Geobacillus thermodenitrificans* and *Geobacillus stearothermophilus* [9,10]. Khalil *et al*. [9] and Sethy and Behera [10] isolated thermophilic Gram-negative rods from hot springs in the Jordan valley and tested their antibacterial activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter* sp., and *Candida albicans* with the well

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diffusion method [11,12]. Therefore, this study aims to identify and characterize the AMP coding gene of thermophilic bacteria isolated from crater hot springs in the mountains surrounding West Java with the BAGELA4 web server by screening them against Gram-positive *S. mutans* and Gram-negative *P. aeruginosa.*

2. MATERIALS AND METHODS

2.1. Collection of Water Sample and Isolation of Thermophilic Bacteria

The samples were taken from the hot spring of a crater on Mount Kamojang with an elevation of 5680 feet in Garut as well as Mount Tangkuban Perahu with an elevation of 6,837 feet in the northern part of Bandung, West Java, Indonesia. During the sampling, the temperature in Mount Kamojang crater ranged from 63°C to 95°C with a pH of 2.45–3.0, while that of Mount Tangkuban Perahu was between 45°C and 76°C with a pH of 1.0–2.0. Furthermore, thermus agar (2 g NaCl, 4 g yeast extract, 8 g peptone, 1.5% agar, and 1000 mL Aquadest) and Luria-Bertani (LB) agar (HiMedia) were used to isolate thermophilic bacteria from the samples collected [11,13]. A total of 100 mL of the water sample was filtered with a 0.45 µm membrane filter, transferred onto the agar plate surface, and then incubated at 55°C for 2–4 days. The bacterial colonies were purified on the media using the streak plate technique, and it was continuously maintained on the same medium.

2.2. Morphological Characterization

Morphological characterization of the isolate began with purification on the nutrient agar media, followed by observation of the shape of the colony and its edges, as well as its color. Microscopic observations were then carried out with the Gram staining method [14].

2.3. Molecular Characterization

The phylotype and taxonomy of the bacterial isolates were studied using the 16S rRNA gene sequencing. A single colony was inoculated on a liquid LB medium, grown at 50°C, and incubated for 16 h with rotation at 250 rpm. Subsequently, the DNA was extracted from the bacterial pellet using a genomic kit (gDNA Presto Mini). PCR amplification of the 16S rRNA gene was then carried out using the forward primers of F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primers of R1429 (5'-TAC GGY TAC CTT GTT ACG ACTT-3') [15,16]. The reaction was performed in a 20 µL volume reaction containing 10 µL of PCR Mastermix 2X (MyTaq HS Red Bioline), 1 µL of 10 µM primer, and 1 µL of the DNA template followed by the addition of nuclease-free water up to the final volume. The PCR started with 5 min denaturation at 95°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 sec annealing at 60°C, 1 min extension at 72°C, and 10 min final extension at 72°C [17]. A total of 5 µL of the product were analyzed using 1% agarose gel dissolved in 1X TAE buffer, and the gel electrophoresis was carried out for 25 min at 90 V. The presence of the DNA band was visualized and observed under UV light using a gel documentation system [18]. Each isolate's PCR product was then compared to a 1 kb DNA Ladder (Promega). A product with correct size of 700 bp was sequenced and analyzed by Genetic Science. The sequences obtained were aligned and compared with the National Center for Biotechnology Information (NCBI) databases to identify the isolate's strain [13].

2.4. Screening for Antimicrobial Activity

The disk diffusion test was used to assess the antimicrobial activity of thermophilic bacteria isolates [13]. The colonies were purified on Mueller-Hinton agar (MHA) at 55°C with the standard streaks plate technique [12]. The antibacterial activity of the purified isolate was then tested against *S. mutans* ATCC 25175 and *P. aeruginosa* ATCC 27853.

2.4.1. Preparation of supernatant and subculture of test bacteria

Bacterial inoculum was prepared by dissolving the isolate sample in MHA, which was placed in 10 mL of minimal medium (MM) [Solution 1 (g/L): K_2HPO_4: 0.23, KH_2PO_4: 0.5, NH_4NO_3: 0.3, and yeast extract: 0.5 and Solution 2 (g/L: Nitrilotriacetic acid: 200, $MgSO_4.7H_2O$: 145.44, $CaCl_2.7H_2O$: 133.78, and $FeSO_4.7H_2O$: 11.12)] until it reached 0.5 McFarland (1.5×10^8 CFU/mL) was obtained [19]. A total of 20 mL of MM were then inoculated with 2.22 mL of the inoculum in a shaking incubator for 24 h at 55°C, 150 rpm, and optimum pH 7. After 24 h of incubation, the bacterial cell and supernatant were separated through centrifugation for 15 min at 4°C and 10,000 rpm to obtain cell-free supernatant [14]. It was then evaluated for antibacterial activity against the test bacterial strains using the disk diffusion test. *S. mutans* and *P. aeruginosa* were subcultured on trypticase soya agar plates followed by incubation at 37°C for 24 h.

2.4.2. Antimicrobial activity test

The disk diffusion method was used to test the antimicrobial activity of the bacterial supernatant against Gram-positive *S. mutans* ATCC 25175 and Gram-negative *P. aeruginosa* ATCC 27853 pathogens. The supernatant was filtered with a syringe through a 0.22 µm Sartorius filter in a vial containing a sterile blank disk, which was then allowed to soak for 1 h at 4°C. The bacteria were suspended in a test tube with 9% NaCl diluent and measured to achieve 0.5 McFarland, which is equivalent to 1.5×10^8 CFU/mL. Subsequently, they were swabbed in a Petri dish containing MHA+2% glucose media for *S. mutans* and MHA for *P. aeruginosa* [12,20].The supernatant-soaked disk was placed in a Petri plate, which had been divided into several quadrants with sterile tweezers. Control experiments were carried out under similar conditions, where ampicillin disk (Oxoid) and ciprofloxacin disk served as a positive control for *S. mutans* and *P. aeruginosa*, respectively, while Aquadest was used as a negative control. The Petri plates were then incubated at 37°C for 24 h, and the antimicrobial activity was calculated in mm as the pathogen growth inhibition zone [21]. To determine the average number of inhibitions zone, the process was performed 3 times.

2.5. Identification of AMP Coding Gene

The identified thermophilic bacterial genome sequences of the samples were entered into the BAGELA4 web server (http://bagel4.molgenrug.nl) to identify and characterize the AMP coding gene for the bacteria [22], and the NCBI database was used for analysis and alignment of the coding gene sequences [23].

3. RESULTS AND DISCUSSION

3.1. Isolation and Characterization of Thermophilic Bacteria

The bacterial strains Tm6Sp1, Tm6Sp5, GE, Tm6T2(a), Lb6Sp2, and Tm6T2 (ser) were isolated from the sample. The optimum condition for the growth of bacteria was used in this study, namely, incubation temperature of 55°C with a pH of 6. The optimal temperature and pH for thermophilic bacteria range between 55°C and 65°C and 6.0 and 8.5, respectively [24].

3.1.1. Morphological-based identification

The macroscopic and microscopic examination revealed that the morphology of the bacteria varied. The macroscopic observations

revealed a variety of characteristics, namely, shape, edge, elevation, color, and the surface of the colony, as well as mucus production. Some isolates were circular with smooth colony edges (entire) and a flat surface, while others were characterized by irregular shape, spread out, creamy colonies, flat elevation, notched edges, rough surface, and mucus production. Microscopically, the isolates revealed several types of short bacilli and a few long bacilli with purple color, indicating that they are Gram-positive bacteria.

3.1.2. Molecular-based bacterial identification

Molecular genetic identification was used to identify and determine the species of the selected isolates. The 27F and 1429R primers successfully amplified almost 700 bp of the 16S rRNA target gene. Subsequently, the 16S rRNA gene sequences obtained from the six bacteria were compared to the variants in GenBank for each isolate using a BLAST search of the NCBI databases. Alignment of the 16S rRNA genome revealed 99% similarity to several species. The Tm6Sp1, Tm6Sp5, and GE isolate had more than 99% similarity to *Geobacillus subterraneus* (accession no. CP051162.1), while Tm6T2(a) and Lb6Sp2 were more than 99% similar to *Geobacillus thermoleovorans* (accession no. CP042251.1). The Tm6T2 (ser) had 99.77% similarity to *Geobacillus zalihae* (accession no. MF965181.1) (Table 1).

Based on the results of molecular-based bacterial identification, all bacteria isolated from hot springs in the craters of Mount Kamojang and Tangkuban Perahu were *Geobacillus* sp., which are often found in extremely hot environments. Several studies have also isolated thermophilic bacteria from various sources, such as Pokusaeva, which successfully identified *G. stearothermophillus* from oil wells in Lithuania, as well as Alkhalili's discovery of thermophilic bacteria *Geobacillus* sp. ZGt-1 from Zara hot springs [24,25]. More than 25 species of *Geobacillus* have been found in thermophilic environments around the world [26].

3.2. Screening for Antimicrobial Activity

The assays were performed at various time intervals because antibacterial activity can be detected between the early and late stationary phase, namely, 8 h and 24 h of incubation, respectively. This is in line with the previous report that the activity can be detected in the middle of the log phase, peaks in the initial stationary phase, and then decline afterward. Several bacterial species can still retain their antibacterial potential after death. The degradation of producer cells by specific and non-specific protease enzymes can cause a decrease in these activities [27]. The isolates' antimicrobial potential was tested against *S. mutans*, which led to the formation of an inhibition zone around the disk, but not against *P. aeruginosa*. This indicates that they all have activity against *S. mutans* (Figure 1). However, the TM6Sp1 species had the largest zone of 7.96 mm (Table 2).

Table 1: Thermophilic bacteria isolated from the hot spring in the mountain around West Java.

Isolates	Species	Accession number	Similarity (%)
Tm6Sp1	*Geobacillus subterraneus*	CP051162.1	100
Tm6Sp5	*Geobacillus subterraneus*	CP051162.1	100
Tm6T2(a)	*Geobacillus thermoleovorans*	CP042251.1	99.68
Tm6T2(ser)	*Geobacillus zalihae*	MF965181.1	99.77
Lb6Sp2	*Geobacillus thermoleovorans*	CP042551.1	99.82
GE	*Geobacillus subterraneus*	CP051162.1	99.67

The inhibition zones formed by all of the isolates indicated that *Geobacillus* sp. isolated from hot springs of Mount Kamojang and Tangkuban Perahu crater has antimicrobial activity against Gram-positive bacteria but not against Gram-negative bacteria (Figure 1). This finding is inconsistent with other studies, where *Staphylococcus haemolyticus, S. aureus, E. coli, P. aeruginosa, K. pneumoniae, Enterobacter* sp., *C. albicans,* and *P. genomospecies* had a positive result, for both Gram positive and Gram negative [10,14,23].

In recent decades, several studies have focused on the production of antimicrobial agents from thermophilic bacteria isolated from natural sources under extreme conditions to obtain new antibiotics for resistant bacteria. This study used *S. mutans* as the test bacterium because it is the main microbe that produces extracellular polysaccharide matrix in dental biofilms, which is suspected to be antibiotic resistant. This often occurs because the biofilm can form an extracellular matrix as a physical barrier, and it produces enzymes for physiological adaptation. AMP is one of the compounds used for preventing its formation and adherence to the tooth surface [7].

3.3. Identification of Antimicrobial Coding Gene

The results of the 16S rRNA gene showed that the sample is closely related to the genus *Geobacillus* sp. This indicates that the complete genome sequence of various *Geobacillus* sp. can be used for the analysis of the AMP coding gene using the BAGEL4 web-based bioinformatics tool. The analysis was carried out by downloading DNA sequences from the NCBI database and uploading them to the BAGEL4 web. The genome sequence is extremely important for the identification of a microorganism's potential to produce natural bioactive products. The majority of bacterial genomes contain a high proportion of these products. Consequently, mining them can lead to the discovery of novel compounds such as antimicrobials. Analyzing the sequences with specialized bioinformatic tools provides valuable information for identifying targets, such as antimicrobial coding genes. In this study, the isolates' sequencing data were used as the basis for identifying and characterizing the AMP coding gene [22]. The results of the BAGEL4 analysis showed that the identified genus *Geobacillus* contains sequences that are similar to geobacillin I-like, and the gene belongs to the lanthipeptide Class A. Lanthipeptides are peptides produced by polycyclic ribosomes after post-translational modification to produce the (methyl-) amino acid lanthionine. A previous study revealed that they have antimicrobial properties, and are also called lantibiotics [28]. These compounds are primarily directed against Gram-positive bacteria, and the main target is membranes and/or specific receptors. Therefore, the antimicrobial test results for isolates against Gram-negative bacteria in this study did not show any inhibition zones [29]. The lanthipeptide gene generated by bioinformatics analysis was then amplified using geobacillin F primers: 5'-CATCTTTCATCAAGTAGTTAATGATAA- '3 and geobacillin R primers: 5'-TTAGCATCGAATGCAAGAGTTACA- '3. Only four of the six thermophilic bacteria isolated were sequenced, namely, TM6SP1, TM6T2(a), TM6T2ser, and GE, while the remaining failed to show a distinct band when amplified. The size of the isolates' amplicon lanthipeptide Class A is ± 230 base pairs, as shown in Figure 2.

The amplified DNA sample was sent to genetic science for sequencing. The sequencing result for these four isolates was confirmed in the GenBank using a BLAST search of NCBI databases. The alignment of their gene sequences revealed 100% similarity to lantibiotic precursors (accession no. BAD74579.1). The sequencing result was 5'-TTG TCA AAA AAC AGG ACA ACA TTG TAC AAC CTA ATA TTA CAA GT

Table 2: Antimicrobial activity of the isolates by forming a zone of inhibition.

Indicator bacterial	Zone of inhibitions shown by the isolates (mm)					
	TM6Sp1	TM6T2 (a)	TM6T2 ser	Lb6Sp2	GE	Tm6Sp5
Streptococcus mutans	7.96	7.75	6.50	6.96	7.08	6.49
Pseudomonas aeruginosa	0	0	0	0	0	0

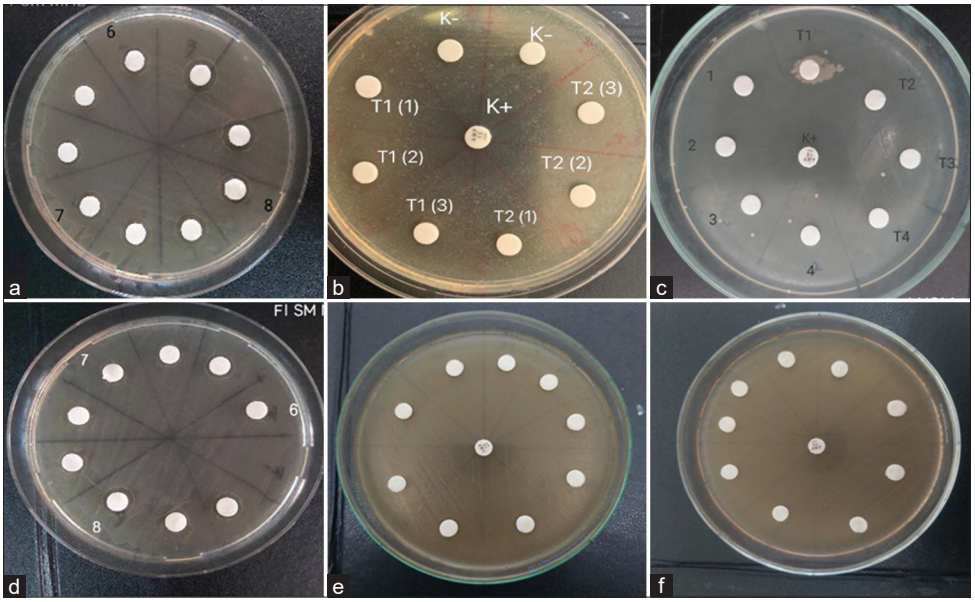

Figure 1: Antimicrobial activity of the isolates of (a): TM6SP1, (b): TM6T2 (a), (c): TM6T2 ser, (d): Lb6Sp2, (e): GE, (f): TM6SP5.

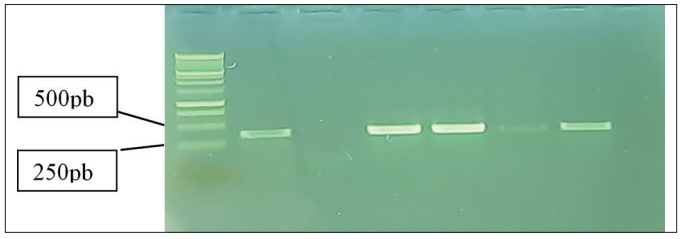

Figure2: Lanthipeptide gene amplicon size.

AAA TCT CTT TTG TAC CCC GGG TTG CAT CAC AGG CAT CTT AAT GTG CTT AAC GCA AAA TTC TTG TGT ATC TTG TAA CTC TTG CAT TCG ATG CTA A-‘3. Clustal X was then used to confirm that the four isolates have the same alignment and there are no differences between the samples. The result showed that they all have the same AMP encoding gene, namely, lanthipeptide Class A, which acts as a lantibiotic precursor. Lantibiotics are bacteriocins of Class I that contains amino acids such as lanthionine and methyllanthionine, as well as dehydrated residues formed by enzymatic reactions. During peptide maturation, they undergo post-translational modifications, which lead to the production of modified amino acids [11]. Class I bacteriocins have antibacterial activity against Gram-positive bacteria but not against Gram negative. Lanthipeptides are also produced by bacteria that are generally regarded as safe as well as pathogenic strains [29,30]. Lantibiotics have intriguing properties, which make them better alternatives to the present antibiotics [31].

Several studies have also reported their effectiveness against pathogenic bacteria, including drug-resistant strains, such as the methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) [31,32]. Furthermore, lantibiotics are not toxic to mammals, and the previous studies revealed that some of them have significant therapeutic potential [31,33]. Bacteriocins can be identified *in silico* from the genome based on homology with identified variants, motives, or genes encoding bacteriocin biosynthetic enzymes, which are typically encoded very close to the genes encoding the suspected bacteriocin precursor [34].

This study’s result is consistent with that of the previous studies, including Garg, who demonstrated that thermophilic bacteria of *G. thermodenitrificans* produced geobacillin I and II, while Pokusaeva revealed that *G. stearothermophilus* produced a bacteriocin-like substance, namely, thermosin [8,25]. Only geobacillin I and II were identified as lanthipeptides among the reported bacteriocins [8]. Egan *et al*. [35] used BAGEL 3 to conduct an *in silico* screening study, where the coding gene, as well as the gene code for lanthipeptides, was obtained. This *in silico* analysis showed that *Geobacillus* sp. has the potential to produce bacteriocins. A previous study revealed that 30–99% of *Geobacillus* produced at least one of these compounds [35]. Based on the results of this study, all samples of *Geobacillus* sp. isolated from the hot springs of the crater in mountains around West Java are putative lanthipeptide producers, which belong to the geobacillin I-like.

4. CONCLUSION

This study concludes that the crater hot springs in the mountains around West Java are considered a good source of *Geobacillus* sp., which is known that this bacterium has a good potential to produce AMPs. It could be used as a potential drug candidate in pharmaceutical industries, including to prevent the growth of *S. mutans*. All samples of this study gave antagonistic effects against *S. mutans*, but the most effective species was isolated TM6Sp1 which gave an inhibition zone

of 7.96 mm. In this study, not all samples could be sequenced because there were samples that failed to show the distinctive band at the time of amplification. Successfully sequenced isolates showed gene sequence alignment 100% similar to lantibiotic precursors when it was confirmed in GenBank using a BLAST search of the NCBI database. Thus, this study concludes that AMPs produced by *Geobacillus* sp. isolated from the study location are a lanthipeptide Class A with an amplicon size of ± 230 base pairs and are effective against *S. mutans* ATCC 25175.

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6. AUTHORS' CONTRIBUTIONS

All authors have substantially contributed to the conceptualization, design, data acquisition, analysis, and interpretation. Furthermore, all authors actively participated in compiling or revising the article, especially concerning the content of intellectual importance by agreeing to submit it to the current journal; giving final approval for the version to be published; and agreeing to be responsible for all aspects.

7. CONFLICTS OF INTEREST

The authors report no financial or other conflicts of interest in this study.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All of this research data can be accessed online.

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