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Molecular identification of thermophilic bacteria with antimicrobial activity isolated from hot springs in North Sumatra, Indonesia

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Abstract. *Ginting CN, Piska F, Harmileni, Fachrial E. 2023. Molecular identification of thermophilic bacteria with antimicrobial activity isolated from hot springs in North Sumatra, Indonesia. Biodiversitas 24: 752-758.* Microorganisms such as thermophilic bacteria are promising sources of antimicrobial compounds due to their abundance, which has yet to be widely investigated. Therefore, this study aims to identify thermophilic bacteria isolated from natural hot springs, Sidebuk Debuk, in North Sumatra, Indonesia. The bacteria were isolated from sediments and hot springs water. Isolated 10 bacteria were characterized and identified based on their morphological and biochemical properties, which included gelatinase activity, motility, citrate utilization, triple sugar iron agar test, catalase test, cell shape, color, and Gram staining. Antimicrobial activity was tested using the disc diffusion method against *Escherichia coli* and *Staphylococcus aureus*. The most potential isolates were identified molecularly by amplification of 16S rRNA genes using universal primers. The sequences result were trimmed and assembled using BioEdit and submitted to BLAST to determine the homology of the bacteria. The isolate ITU9 was identified as *Bacillus megaterium* and exhibited the best antimicrobial activity with an inhibition zone of 9.5 mm against *S. aureus* during 72 h of incubation. This research showed that the thermophilic *B. megaterium* strain ITU9 was successfully isolated from Sidebuk Debuk hot springs and had stable antimicrobial activity for 72 h against *E. coli* and *S. aureus*.

Keywords: 16S rRNA, antimicrobial, *Bacillus megaterium* ITU9, Sidebuk Debuk

INTRODUCTION

The treatment of infectious diseases caused by pathogenic bacteria and fungi is the most common problem encountered in the health sector and can only be treated with antibiotics (Moshafi et al. 2011). The secondary metabolites produced by microorganisms are compounds with low molecular weight and can kill or inhibit the growth of other microorganisms (Singh and Nair 2021). Its consumption causes antimicrobial resistance in pathogenic bacteria. This problem has become a concern of WHO, not only at the level of public health services but also in agriculture and livestock. In 2014, WHO reported that antibiotic resistance is a severe challenge threatening the world. It was also stated that the globe had entered the post-antibiotic era, marked by the fear of the inability of existing antibiotics to treat infectious diseases (Dahesihdewi et al. 2019). A 2017 report by the World Health Organization (WHO, Geneva, Switzerland) confirmed that the entire world would run out of this product as the existing drugs in clinical use were developed through modifications to classes and were identified to have short impact cycles (Aljeldah 2022).

The leading causes of this resistance are mutations in bacteria and irrational or inappropriate consumption (Odonkor and Kennedy 2012). Antibiotic resistance is a complex problem that requires a global solution to replace ineffective medication. The cost of developing a new drug is approximately 2.7 million dollars, which takes many years. Hence, various options are currently being explored. Natural sources such as archaea, bacteria, fungi, and plants can be used to isolate new compounds. Antibiotics are one of the most significant commercially exploited secondary metabolites produced by bacteria and employed in a wide range (Patel and Fadaei 2016).

The genus *Bacillus* is one of the most abundant bacteria in the soil and is capable of producing various antibiotic compounds with several chemical properties (Moshafi et al. 2011). It produces antimicrobial metabolites as polypeptides with low molecular weight through ribosomal and non-ribosomal mechanisms. Of the antimicrobial compounds produced were almost 167, where 66 and 23 were derived from *Bacillus subtilis* and *B. brevis*, while the remaining were from other species (Stoica et al. 2019). *Bacillus paramycoides* isolated from Rimbo Panti hot springs, West Sumatra, Indonesia, was reported to show antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (Octarya et al. 2022).

Thermophilic bacteria are organisms that have not been widely studied, specifically related to their ability to produce antimicrobial metabolites. They have optimum growth above 45°C and are commonly discovered in hot springs adjacent to volcanic environments, topsoil, or even in artificial thermal environments such as composting facilities (Ovando-Chacon et al. 2020). Numerous

researches on the molecular identification of antimicrobial-producing bacteria have been reported worldwide. However, rarely have antibacterial properties of the thermophilic bacterium *Bacillus megaterium* been identified in the research.

In previous research, it was reported that six unidentified bacterial isolates from Mount Merapi showed antimicrobial activity against *E. coli*, *S. aureus*, and *Candida albicans* (Yulianti et al. 2014). In another research, it was stated that thermophilic bacteria from the soil in the Cisolok geyser were discovered as *Streptomyces cellulosae* and have antimicrobial activity against *Bacillus subtilis*, *S. aureus*, and *Kocuria rhizophila* (Sari et al. 2021). This study aimed to isolate thermophilic bacteria from Sidebuk Debuk hot springs in North Sumatra, Indonesia, determine antibacterial activity and conduct molecular identification of isolates with the most potent antibacterial activity.

MATERIALS AND METHODS

Research area

Sampling was performed at natural hot springs Sidebuk Debuk in Semangat Gunung Village, Merdeka Subdistrict, Karo District, North Sumatra, Indonesia. The samples were hot water and sediment was obtained using Ekman Grab. A total of 10 g of sediment and 10 mL of hot water were put into each sterile plastic tube, after which the pH and temperature were measured. Subsequently, the sample was stored in a cool box and taken to the laboratory for bacterial isolation (Konieczna et al. 2011). The sampling coordinates are at 3°13'24.97728 N" and 98°31'1.38792 E".

Bacterial isolation and characterization

Approximately 1 g of sediment and 1 mL of hot water samples were inoculated into 9 mL of each nutrient broth separately in a test tube and incubated at 55°C for 24 h. This was continued with serial dilution, spread on nutrient agar plates, and incubated at 60°C for 48 h. Bacterial colonies that grow on the agar surface were purified by stretching them to a new medium in a Petri plate and incubated for 48 h at 55°C (Ambeng et al. 2019). The isolates were characterized morphologically and biochemically, including elevation, margin, color, Gram staining, catalase test, motility, triple sugar iron agar test, simmon citrate, and gelatinase. Observation of elevation, edge, and color was conducted visually, while the isolate was characterized through Gram staining by taking cultured bacterial colonies using a loop, placed on a glass object, and fixed. The bacteria were stained by dripping about 2-3 drops of gentian violet, allowed to stand for 1 min, and rinsed under running water. The samples were dripped with 2-3 drops of iodine, waited for about 1 min, and washed with running water. Subsequently, it was followed by drops of 96% alcohol, which were allowed to stand for 30 sec, and washed with running water. A total of 2-3 drops of safranin was added, allowed to stand for 10 sec, rinsed with running water, and dried. The stained bacteria were observed under a microscope with 100x magnification with the addition of immersion oil (Li et al. 2020).

The catalase test was performed by dripping H_2O_2 onto the bacterial isolate on a slide. The result is positive when bubbles appear after the process. Furthermore, the motility test was conducted by inserting a straight loop containing bacteria into the indole motility sulfite media, which was incubated at 55°C for 24-48 h. The results showed motility in bacteria as indicated by the traces of their movement. The carbohydrate fermentation test and the triple sugar iron agar analysis were performed by scratching the media with bacterial isolates on the surface of the triple sugar iron agar media and stabbing it in the middle. Additionally, the plates were incubated at 60°C for 24-48 h, and changes that occurred were observed. The result was positive when there was a change in the media color to orange or yellow, and also H_2S could be formed, which was indicated by a black color. Simmon citrate test was conducted by taking the bacterial suspension using scratching a loop on the media in a zig-zag way, after which it was incubated at 60ºC for 18-24 h. A positive result was indicated by a color change from green to blue (Saimin et al. 2020). Gelatinase test was also performed by inoculation of bacterial isolates by piercing the middle of the gelatin media vertically using a needle, incubating for five days at 50ºC. Furthermore, the culture media was stored in the refrigerator for 15 min. A positive gelatinase test was indicated by the form of the medium, which remains liquid even when stored in the refrigerator (Prihanto et al. 2020).

Production of antimicrobial compounds

Selected isolate ITU 9 was tested for their antibiotic activity, and the inoculum was prepared in a nutrient broth medium. Subsequently, the composition of the synthetic media, including 5.0g of L-glutamic acid, 0.5g of KH_2PO_4, 0.2g of $MgSO_4.7H_2O$, 0.01g of $MnSO_4.H_2O$, 0.01g of NaCl, 0.1g of $FeSO_4.7H_2O$, 0.01g of $CuSO_4.7H_2O$, and 0.05g of $CaCl_2.2H_2O$ in 1 L of distilled water was used as a production medium. A 1% glucose solution was sterilized using a 0.2 mm syringe filter and added to the Erlenmeyer flask. The inoculum was incubated in a shaker incubator for 72 h at 50°C and 150 rpm. Finally, samples were collected every 24 h, centrifuged at 10,000 rpm for 10 min, and the supernatant was used for antimicrobial testing (Muhammad et al. 2009).

Antimicrobial activity

The disc diffusion method was applied to test the antimicrobial activity, with *E. coli* ATCC25922 and *S. aureus* ATCC25923 being used as indicator bacteria. Their colonies were dissolved into 9 mL of physiological NaCl and homogenized using a vortex. A 0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175% $BaCl_2.2H_2O$ with 9.95 mL of 1% H_2SO_4. Furthermore, the suspension turbidity was adjusted at 1.5×10^8 CFU/mL using a McFarland 0.5. These pathogenic bacteria are swabbed evenly on the surface of the nutrient agar media using a sterile cotton swab. The sterile discs were dipped in the supernatant (crude antibiotic) with tweezers, attached to the surface of the nutrient agar, and incubated at 50°C in an inverted petri dish for 24 to 72 h. The inhibition zone was

measured using a digital caliper (Hudzicki 2012).

Molecular identification of thermophilic bacteria

Genomic DNA isolation was performed using the Quick-DNA Fungal/Bacterial miniprep kit (Zymo Research, D6005) and quantified by nanodrop. The procedure is briefly presented as follows one loop of the bacterial isolate was cultured in nutrient broth for 18 h. The resulting pellet was then resuspended in the ZRBashingbead™ lysis tube using phosphate buffer saline of pH 7 and 750 µL of BashingBead™ buffer into the tube, after which it was centrifuged at 10,000 x g for 1 min. Subsequently, 400 µL of the supernatant was transferred to a filter in a collection tube and centrifuged again at 8000 g for 1 min. Following the addition of 1200 µL lysis buffer to the collection tube, 800 µL of the solution was transferred to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 min. The collected solution was discarded, and the previous process was performed. Two hundred microlitters of DNA pre-wash buffer were added to the Zymo-Spin™ IICR column in a new collection tube and centrifuged at 10,000 x g for 1 min. Furthermore, 500 µL gDNA wash buffer was added and centrifuged at 10,000 x g for 1 min. The Zymo-Spin™ IICR column was transferred to a clean 1.5 mL microcentrifuge tube, and 100 µL DNA elution buffer was added directly to the column matrix, after which it was centrifuged at 10,000 x g for 30 sec to elute the DNA. Subsequently, 16S rRNA gene amplification was performed using 27F/1492R primer. Pre-denaturation at 95°C for 90 sec was followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 90 sec, with a final extension at 72°C for 3 min. A 1 µL of PCR products was assessed by electrophoresis with 0.8% TBE agarose and 1 Kb bp DNA being used as a marker. These products were sequenced using the Bi-directional sequencing method. The result was trimmed and assembled using the BioEdit program; then, it was submitted to BLAST to determine the homology of the microorganism (Fachrial et al. 2019). Phylogenetic analysis was conducted by first aligning sequences from the BLAST search with the 16S rRNA gene of ITU 9 using clustalW. This was followed by phylogenetic analysis and tree generation using the program MEGAX (Kumar et al. 2018).

RESULTS AND DISCUSSION

Research area

The sampling location is about 50 km from Medan city with the coordinates of 3°13'24.97728 N" and 98°31'1.38792 E". The pH and temperature were measured at 3 points (Table 1). It was found that there were no significant differences in temperature and pH at each point around the sampling location (Table 1).

Bacterial isolation and characterization

In the present investigation, four bacteria were isolated from sediment and six bacteria were isolated from water. These isolates were characterized morphologically and biochemically (Table 2).

Antimicrobial activity

Antimicrobial activity was performed using *E. coli* ATCC25922 and *S. aureus* ATCC25923 as indicator bacteria. It was determined by measuring the clear zone around the discs. Antimicrobial activity of the isolates is shown in the following figure (Figure 1).

ITU9 has exhibited higher activity compared to other isolates. Its average antimicrobial activity against *E. coli* and *S. aureus* was 7.9 mm and 9 mm, respectively, which was the strongest (Figure 2).

Molecular identification of ITU9

The BLAST results of ITU 9 against the NCBI Database showed that the isolate belongs to *B. megaterium* with accession number OP942339, query cover reaching 100%, and percentage identity of 100% confirmed by phylogenetic analysis, which indicates that the isolate was a bacterium with the genus *Bacillus* (Figure 3).

Table 1. pH and temperature measurement results

	Site 1	Site 2	Site 3
Temperature (°C)	59	60	60
pH	6.6	6.4	6.6

Table 2. Biochemical and morphological characteristics of isolates

Isolate number	Biochemical characteristics					Morphological characteristics			Source
	Gelatin test	Motility test	Citrate test	TSIA test	Catalase test	Cell shape	Color	Gram staining	
ITU 1	-	+	-	+	+	Bacillus	Opaque	Negative	Water
ITU 2	-	+	-	+	+	Bacillus	Opaque	Negative	Water
ITU 3	+	-	-	+	+	Coccus	Opaque	Negative	Water
ITU 4	-	+	-	+	+	Coccus	Opaque	positive	Water
ITU 5	-	+	-	+	+	Bacillus	Opaque	Positive	Water
ITU 6	-	+	-	+	+	Coccus	Opaque	Positive	Water
ITU 7	-	+	-	+	+	Bacillus	Opaque	Positive	Sediment
ITU 8	-	+	-	+	+	Coccus	Opaque	negative	Sediment
ITU 9	-	+	-	+	+	Bacillus	Opaque	Positive	Sediment
ITU 10	-	+	-	+	+	Coccus	Opaque	negative	Sediment

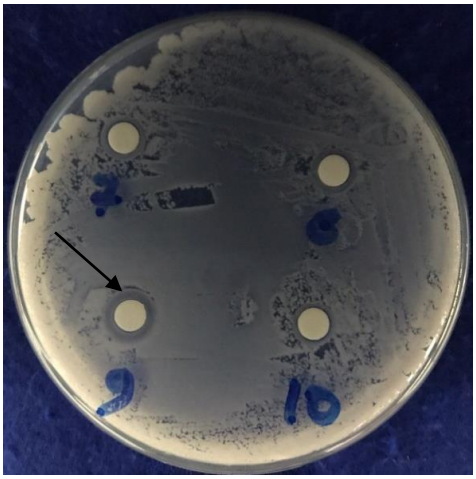

Figure 2. Antimicrobial activity of ITU 9 against *Staphylococcus aureus* for 72 h, with a disc diameter of 5 mm indicated by an arrow

Discussion

Natural geothermal hot springs can be found in various parts of the earth. On the other hand, natural aquifers are filled with boiling water from the outside world when a volcano erupts and leaves a hole. The physical and chemical conditions, and specific pH of natural hot springs, reflect the biodiversity of microorganisms (Mane and Nadaf 2022). In this research, pH can be categorized as neutral, and the isolated microorganisms are thermophiles with a temperature tolerance ranging from 46°C to 75°C (Pandey et al. 2015). Bacillus species are often found in natural hot springs with a pH range from acidic to alkaline and temperatures from 40°C to 85°C and an acidic to alkaline pH. Previous research showed that *Bacillus licheniformis*, *Bacillus* sp, *Bacillus subtilis*, *Geobacillus kaustophilus*, and *Weizmania coagulans* were isolated from various hot springs in Eastern and Southern Anatolia regions of Turkey, with pH of 5 to 8.5 (Ulucay et al. 2022).

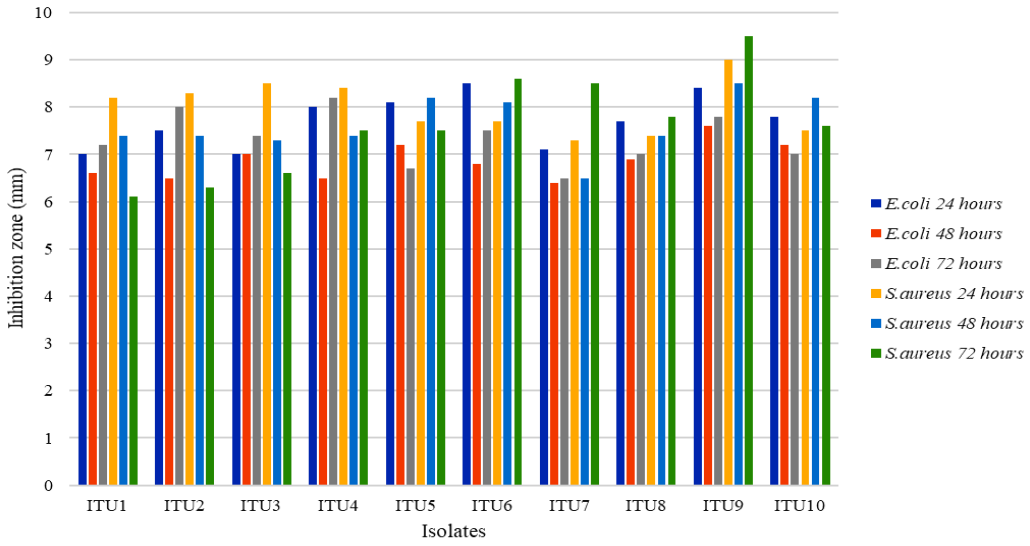

Figure 1. Antimicrobial activity of 10 thermophilic bacteria isolates against *Escherichia coli* and *Staphylococcus aureus* (including disc diameter of 5 mm)

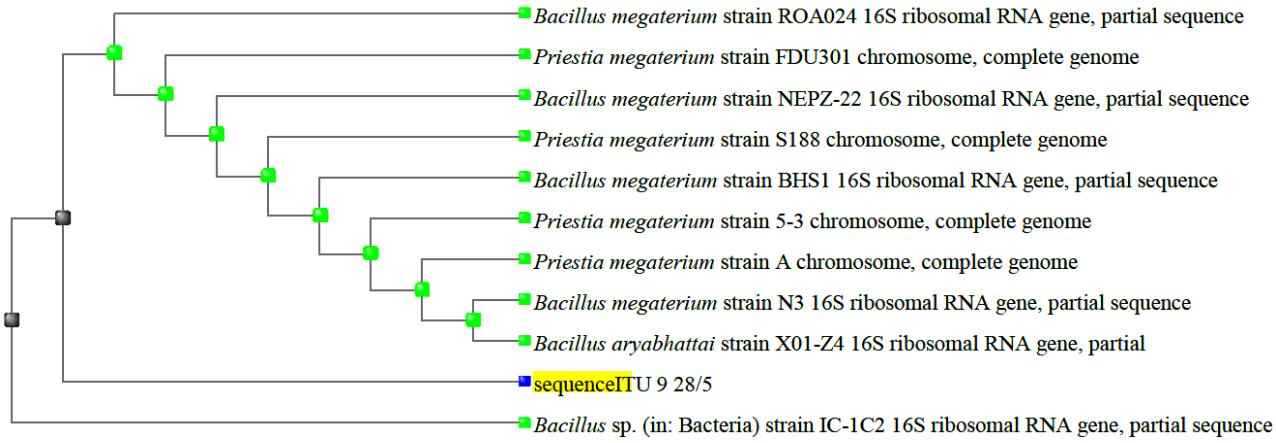

Figure 3. Phylogenetic Tree of ITU9 using Neighbor-Joining method using MEGA X

Secondary metabolites with antimicrobial or antifungal activity are generally classified as peptides. Various species of actinomycetes, fungi, and bacteria produce these antibiotic peptides. Microorganism produces secondary metabolites at the end of the stationary phase based on their growth stage. In this research, isolates were incubated for 24 to 72 h. Furthermore, bacteria enter the stationary phase after 1 to 3 days of incubation under ideal conditions in the laboratory (Llorens et al. 2010). Therefore, it is suggested that *B. megaterium* ITU 9 produces a secondary metabolite in the form of a peptide with antimicrobial activity, such as bacteriocins which Gram-positive and Gram-negative bacteria could secrete. Bacteriocins are ribosomally synthesized and have a relatively narrow spectrum of antimicrobial activity. They are a group of proteins that can be classified into several classes based on microbial target, mode of action, release, and mechanism of immunity. Furthermore, bacteriocins have several different modes of action, including inhibition of cell wall synthesis, inhibition through DNAse and RNAse activity, and, most commonly, the formation of pores on the target cell membrane (Darbandi et al. 2022).

Bacillus megaterium is a probiotic with rich enzyme production and strong resistance to pathogenic bacteria (Yao et al. 2020). FAO/WHO defines probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host." Furthermore, there are several requirements for the strains to be included in the composition of probiotic preparations. One is to exhibit antimicrobial activity against pathogenic, toxigenic, and saprophytic microorganisms (Denkova et al. 2017). Probiotics, which do not contribute to antibiotic resistance but reduce the risk, are commonly used in animal feed production. *Bacillus* probiotics produce spores that can withstand extreme conditions and tolerate industrial aerobic conditions, while *Lactobacillus* and *Bifidobacterium* are highly susceptible to extreme environmental conditions (Tran et al. 2022). *Bacillus* is the largest producer of antibiotic compounds, including bacteriocin (Esikova et al. 2002). *B. megaterium* strain 22 isolated from soil has been reported to produce bacteriocins that have antimicrobial activity against food spoilage microorganisms, including *Salmonella typhi* and *S. aureus*. Finally, they were also produced in the stationary phase and had stability against heating for 15 min (Khalil et al. 2009).

The previous investigation also stated that three isolates of *B. megaterium*, strains INA 01083, VKPM B-603, and VKPM B-605, have antimicrobial activity against Gram-negative and Gram-positive bacteria, including *S. aureus*, *Bacillus subtilis*, *B. mycoides*, *B. pumilis*, *E. coli*, and *Pseudomonas aeruginosa*. The chromatographic analysis, UV-Vis, and IR spectra showed five different antibiotics, with 3 being polypeptides (Malanicheva et al. 2012). *B. megaterium* strain T04 isolated from the Rach Lang stream in Vietnam has antimicrobial activity against several microbes, including *Candida albicans*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus sciuri*, and *Micrococcus luteus* with an incubation period of 24 to 72 h, as well as an inhibition zone from 3.3 mm to 18.7 mm (Nguyen and Thu 2015). It was also discovered that *B. megaterium* produced bacteriocins, which displayed a broad spectrum of antimicrobial activity against food-spoilage microorganisms. According to Khalil (2009), strain 19 produced bacteriocins that effectively inhibited the growth of *Salmonella typhimurium* and *S. aureus*. This antimicrobial activity has a stability of about 100°C for 15 min at low or neutral pH. Finally, the molecular weight of bacteriocins has been estimated at 6,512 kDa (Khalil et al. 2009).

Bacillus megaterium can inhibit *S. aureus* and *E. coli*, but the activity against *S. aureus* was stronger than against *E. coli*. This statement is supported by the results of the research reported by Musikasang et al. (2012). In their study, the bacteriocins produced from selected lactic acid bacteria showed solid inhibitory activity toward many Gram-positive bacteria. This may be due to differences in cell wall structure between *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). This also follows the results from Alkhalili et al. (2016) reported. It was stated that the thermophilic bacteria isolate ZGt-1 had more potent antimicrobial activity against *Bacillus subtilis* (Gram-positive) than Gram-negative. This is because the peptidoglycan layer in the cell wall of Gram-negative bacteria is protected by an outer membrane consisting mainly of lipopolysaccharides. In addition, some Gram-negative bacteria can develop specific mechanisms to eliminate the effects of antimicrobial peptides under certain conditions. The outer membrane is a distinguishing feature of Gram-negative bacteria, while Gram-positive bacteria lack this organelle. The only known function of the outer membrane is to serve as a protective barrier, and it is generally true that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria. Furthermore, *B. megaterium* strain ITU9 is a Gram-positive bacterium. In one report, it was mentioned that as bacteriocins produced by Gram-positive bacteria usually possess little or no activity against Gram-negative pathogens, Gram-negative bacteriocin producers seem to have more significant potential concerning controlling such pathogens (Dobson et al. 2012).

There are several mechanisms of antimicrobial metabolites produced by *Bacillus* strains, namely metabolites that target the cell wall, intracellular processes, and the plasma membrane. Out of the 47 produced by *Bacillus*, 23 are known to target cell plasma membranes (Tran et al. 2022). Another research reported that *B. megaterium* isolated from the oral microflora has a structure similar to bacitracin. This cyclic polypeptide compound effectively inhibited Gram-negative and positive bacteria (Al-Thubiani et al. 2018). Bacitracin is a mixture of related cyclopeptides. Bacitracin A is the most active antimicrobial form with a molecular weight of 1422.7 Da, consisting of 12 amino acid residues, 4 of which are D-derivatives. Bacitracin acts by inhibiting the synthesis of cell walls (Aslanli et al. 2022). Bacitracin interferes with the dephosphorylation of C55-isoprenyl pyrophosphate and bactoprenol pyrophosphate. Both of these lipids function as membrane carrier molecules that transport the building blocks of the peptidoglycan bacterial cell wall outside of the inner membrane; thus, their dephosphorylation leads to

membrane damage (Khattak et al. 2022).

Research has reported that *B. megaterium* produces class III bacteriocins (>30kDa) with phospholipase activity, such as megacin. The A-group megacin include the inducible A-216 and A-19213 produced by *B. megaterium* 216 and ATCC 19213. They showed phospholipase A2 activity in converting phospholipids to the corresponding lysophospholipids (Abriouel et al. 2011). Megacins are classified into three categories based on inducibility, mode of action, and spectrum of activity. The megacin A can be induced through treatment in the logarithmic phase with low levels of mitomicyn C or UV irradiation. Meanwhile, megacin C is not inducible, has a limited spectrum of activity compared to A, and causes activation of DNAse activity in sensitive bacteria. Finally, megacin B shows an intermediate spectrum of activity (Von Tersch and Carlton 1983).

In addition to peptides, antimicrobial compounds produced by *B. megaterium* can be organic. *B. megaterium* belongs to Gram-positive bacteria and naturally occurs in the soil. These microorganisms are not pathogenic strains; hence, they do not threaten human and animal health. This Gram-positive bacteria can synthesize organic acids (Ciopińska et al. 2019). *B. megaterium* L2 produced five organic compounds: erucamide, behenic acid, palmitic acid, phenylacetic acid, and β sitosterol. It was also reported that β sitosterol had the most potent antimicrobial activity against the bacterium *Ralstonia solanacearum* with a MIC value of 15.6 mg/mL (Xie et al. 2021). The mode of action is due to the ability of these acids to penetrate bacterial cell membranes and to acidify cell cytoplasm, thereby inhibiting bacterial growth. Other mechanisms also proposed that organic acid could reduce ATP production by uncoupling electron transport or interrupt nutrient uptake by disturbing bacterial cell membranes (Kovanda et al. 2019).

In conclusion, thermophilic bacteria are microorganisms that contain abundant bioactive compounds, including antimicrobial substances. A total of 10 isolates of the bacteria were successfully isolated from SidebukDebuk hot springs in North Sumatera. Furthermore, they exhibited activity against *E. coli* and *S. aureus*, with ITU 9 showing the best antimicrobial activity among the other 9. This research suggested that the antimicrobial compound produced is expected to be a peptide. The isolate was molecularly identified as *B. megaterium* strain ITU 9 with accession number OP942339. Therefore, further research is needed to purify and characterize the antimicrobial compounds produced by *B. megaterium* ITU9.

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