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Molecular Identification, GC-MS Analysis of Bioactive Compounds and Antimicrobial Activity of Thermophilic Bacteria Derived from West Sumatra Hot-Spring Indonesia

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ABSTRACT

Thermophilic bacteria are a source of bioactive compounds that have many benefits for human life. One of them is as a source of antimicrobials. This research aimed to identify and characterize the promising thermophilic bacterial isolates by analyzing bioactive compounds and their potential as antimicrobial agents. Thermophilic bacteria with the code LBKURCC were taken from the collection of the Biochemistry Laboratory of the University of Riau. Forty-four purified strains of thermophilic bacteria were tested for antimicrobial ability. These thermophilic bacteria were taken from hot springs located in the Sumatra provinces of West Sumatra and Riau. Strain LBKURCC218 isolated from Rimbo Panti hot springs in West Sumatra was chosen to further investigate antimicrobials production. Isolates of hot spring bacteria that produced the highest antimicrobial were identified by comparing the similarity of the 16S rRNA gene sequences. BLAST result and phylogenetic tree showed that the selected thermophilic bacterial strain was similar to *Bacillus paramicoides* with the similarity index of 99.93%. Analysis of bioactive compounds of the ethyl acetate extract of liquid cultures of *B. paramycoides* LBKURCC218 showed the best producer of antimicrobial compounds compared to other isolates. The most identified compounds from the ethyl acetate extract were *Dodecanoic acid*, representing 23.62% of the total compounds, followed by *11-Dodecanoic acid* at 17.84%. Ethyl acetate extract of *B. paramycoides* LBKURCC218 has a high inhibition zone against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*.

1. Introduction

Antibiotics have been used a lot to treat diseases caused by pathogenic microbial infections. Antibiotics are compounds that can inhibit the growth of pathogenic microorganisms, including bacteria and fungi (Gould 2016). The ability of microorganisms to modify themselves due to antibiotics has led to an increase in the resistance of pathogenic microorganisms to currently used antibiotics (Peterson and Kaur 2018). MRSA (methicillin-resistant *Staphylococcus aureus*) and VRSA (vancomycin-resistant *Staphylococcus aureus*) are examples of infection with antibiotic-resistant pathogenic bacteria. Cases of antibiotic resistance continue to increase every year (Scheffler *et al.* 2013). The increasing antimicrobial resistance of isolates found in dairy products due to contamination *S. aureus* and MRSA on these food products (Alghizzi and Shami 2021). According to the World Health Organization (WHO), antibiotic resistance has posed a severe threat to human health worldwide (Romandini *et al.* 2021). Based on data from WHO, at least 700,000 people worldwide die each year due to antibiotic resistance, of which around 200,000 are newborns (WHO 2021). This case is expected to increase in 2050 to 10 million (Mancuso *et al.* 2021). Data from the Indonesian Ministry of Health in 2019 shows an increase in antibiotic resistance to antibiotics Carbapenems, Fluoroquinolones, and third-generation cephalosporins for some bacteria such as *E.coli* and *K. Pneumoniae* (R.I. Ministry of Health 2020). Antibiotic resistance in Indonesia

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can also be increased by bacterial coinfection in COVID-19 patients due to the widespread use of broad-spectrum antibiotics (Prasetyoputri 2021). Search for new natural biologically active compounds and their characterization is one of the urgent tasks in modern biotechnology. One of the sources of bioactive compounds is microorganisms. Thus, they are an important source of antimicrobial compounds. Secondary metabolite compounds produced by bacteria can be antimicrobial, antitumor agents, immunosuppressant agents, herbicides, pesticides, antiparasitic agents, and enzymes. In addition, thermophilic bacteria can be used to produce bioethanol and other industrial chemicals (Zeldes *et al.* 2015; Panda *et al.* 2018; Gurumurthy *et al.* 2020). Thermophilic bacteria live optimally at 45°C between 80°C and can be isolated from various environments such as deep sea, soil, hot springs, and compost (Pandey *et al.* 2015). The provinces on the Indonesian island of Sumatra are traversed by mountainous trails and have many hot springs. These natural conditions are a source of diversity of thermophilic microorganisms. Previously, was reported the potential of thermophilic bacteria in Sumatra as a source of amylase (Ardhi *et al.* 2020), protease, and inulinase enzymes (Fachrial *et al.* 2019). Thermophilic bacteria can be used to produce bioethanol and other industrial chemicals (Zeldes *et al.* 2015; Panda *et al.* 2018; Gurumurthy *et al.* 2020). Thermophilic bacteria also have the potential to produce antibiotic compounds, such as cyclohexyl acrylate, imiloxan, tabtoxinine-lactam, and filberton (Alrumman *et al.* 2019), ktedonoketone and 2'oxosattabacin benzoidi (Igarashi *et al.* 2019), and antibiotics in the form of peptides (Esikova *et al.* 2002).

From previous studies, we have identified a thermophilic fungus, namely *Aspergillus fumigatus* LBKURCC269. Extraction of secondary metabolites of the fungus *A. fumigatus* LBKURCC269 with ethyl acetate showed antimicrobial activity against negative bacteria *Escherichia coli*, the gram-positive bacteria *Staphylococcus aureus*, and the fungal pathogen *Candida albicans* (Octarya *et al.* 2021). The present work focuses on selecting thermophilic bacterial isolates that have the most potential to produce antimicrobial secondary metabolites. The selected thermophilic bacterial ethyl acetate extract was analyzed by Gas chromatography-mass spectrometry (GC-MS) and tested for antibacterial and antifungal activity.

2. Materials and Methods

2.1. Screening for Thermophilic Bacteria that have Antimicrobial Activity from Laboratory Collections

Table 1 is the data of thermophilic bacteria from the collection of the Biochemistry Laboratory of the University of Riau. The total number of samples in this study was 50 isolates of thermophilic bacteria and coded LBKURCC (Laboratorium BioKimia Universitas Riau Culture Collection). These thermophilic bacteria were tested for antimicrobial ability against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 35218, and the fungus *Candida albicans* ATCC 10231. Pathogenic bacteria are purchased from a collection of microorganism cultures which are standard bacteria and are pathogenic for humans. *E. coli* ATCC 35218 is an Enteropathogenic *Escherichia coli* (EPEC) strain that can cause diarrhea. *S. aureus* ATCC 29213 and *C. albicans* ATCC 10231 are clinical isolates used as a standard quality control strain in laboratory testing. Bacteria were cultured in Nutrient Broth for 48 hours at 45°C. The supernatant was obtained by centrifugation for ten minutes at 10,000 rpm. The antimicrobial activity of thermophilic bacteria against microbial pathogens was tested by the disc diffusion method. Disc with a diameter of 6 mm was immersed in the bacterial culture supernatant. After 1 hour, the discs were placed on solid media containing pathogenic microbes and

Table 1. Region of origin and code of thermophilic bacteria isolate

Origin	Number of isolates	LBKURCC code
Sungai Pinang, Riau Province	17	197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213
Rimbo Panti, West Sumatera Province	10	214, 215, 216, 217, 218, 219, 220, 221, 222, 223.
Padang Gantiang, West Sumatera Province	5	224, 225, 226, 227, 228.
Pawan, Riau Province	6	229, 230, 231, 232, 233, 234.
Bukik Gadang, West Sumatera Province	6	235, 236, 237, 238, 239, 240.
Bukik Kili, West Sumatera Province	6	241, 242, 243, 244, 245, 246.

then incubated for 24 hours at 45°C. The zone of inhibition of pathogen growth in mm was used as antimicrobial activity. The following scoring system was used: Diameter of inhibition zone >15mm (++), 13-15 mm (+), <13 mm ((+/-) and absent (-) (Esikova *et al.* 2002).

2.2. Morphological Characterization of Selected Thermophilic Bacteria

The pure isolate of the most potential bacteria was incubated at 45°C for 24 hours on a Nutrient Agar medium. Single bacteria colonies were taken to observe cell shape and Gram stain test. Gram stain test was carried out referring to Al-Dhabi *et al.* 2016. Bacterial growth temperature is determined by growing bacteria in a temperature range of 35-60°C. Bacterial growth was then observed on Nutrient Agar (Merck) for 2 days.

2.3. Molecular Identification of the Promising Thermophilic Bacteria

Bacteria were grown in a liquid medium for 24 hours at 45°C. Bacterial DNA was isolated using a modified GES method (Pitcher *et al.* 1989). The mixture of PCR reaction consisted of 39.5 µL of ddH_2O, 0.5 µL of primers 27 F: (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492 R (5'--GGT TAC CTT GTT ACG ACT T-3'), 1 µL of 10 mM dNTPs, 0.5 µL of Taq-polymerase, and 5 µL of reaction buffer. A total of 47 µL of the mixture were added to 3 microliters of purified DNA. PCR was performed using either a Perkin-Elmer Cetus Thermal Cycler with a cycle profile of pre-run at 96°C for 2 minutes, denaturing at 96°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes and a total of 35 cycles was followed by a post-run at 72°C for 2 minutes (White *et al.* 1990; O'Donnell 1996). The PCR products were purified by the PEG precipitation method (Hiraishi *et al.* 1995). An automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) sequence bacterial ribosomal DNA. Sequencing data were processed using the BioEdit program. The length of the DNA fragment obtained in sequencing was 1,411 bp. The results were then analyzed for sequence homology using the mega BLAST program, accessed on the NCBI website. Sequence alignment of thermophilic bacterial strains with sequences already stored in NCBI based on the similarity of the 16S rRNA gene was carried out using Clustal W software. The resulting data were processed to obtain bacterial kinship relationships (Kumar *et al.* 2016).

2.4. Liquid Culture Fermentation and Crude Extract Extraction

Bacterial isolates that showed the highest antimicrobial activity in the previous test were selected for fermentation. Bacterial liquid culture fermentation was carried out in a Nutrient Broth (Merck) liquid medium (2 x 250 ml) at pH 7. The culture was incubated for 2 days at 45°C with a stirring speed of 150 rpm. The cultures were centrifuged at 300 rpm for 30 min to obtain cell-free extracts. Extraction with ethyl acetate was carried out by adding ethyl acetate in a ratio of 1:1 with the volume of the supernatant. Extraction was carried out by adding the volume of ethyl acetate 3 times in a separating funnel. The solvent layer was separated and evaporated at 40°C temperature by Rotavapor (Buchi R-100) under a vacuum. The extract was used as a stock for GC-MS analysis and performed antimicrobial activity tests.

2.5. Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis of the obtained ethyl acetate extract was carried out with the SHIMADZU GCMS-QP2010S. The column used was (fused silica) with 30 m x 250 µm x 0.25 µm. Three µl of the sample were injected at 300°C and the GC run times were 80 minutes. Helium gas velocity was 0.5 ml/min with a pressure of 13.7 kPa. The electron energy was 70 Ev and the measured mass was 28-600 amu. The chromatogram results were compared with the compounds in the database. Then identify the name of the components, chemical structure, weight, and molecular formula.

2.6. Antimicrobial Activity

Twenty microliters of crude extract of ethyl acetate (200 ppm) were dropped on a paper disc and then tested for antimicrobial activity. The pathogenic microbes used are bacteria and fungi. Gram-negative bacteria *E. Coli*, gram-positive *S. aureus*, and fungi *C. Albicans*. The concentration of the suspension of pathogenic microbes was 1.5 x 10^8 CFU/ml. It conforms to the 0.5 McFarland standard. The paper disc containing the extract was placed on the solid media inoculated with the test microbes. Positive control for an antibacterial was 5µL Ampicillin (100 µg/ml), antifungal was nystatin (100 µg/ml), and negative control ethyl acetate. Incubation was carried out for 24 hours at 37°C. (Alrumman *et al.* 2019). The zone of resistance formed around the disc is measured with a ruler. The experiment was done in three replications.

3. Results

3.1. Screening for Thermophilic Bacteria that have Antimicrobial Activity

The result of the primary screening showed that the isolate LBKURCC218 displayed antimicrobial activity against *E. coli, S. aureus,* and *C. albicans* (Table 2). Among a total of 50 isolates showing antimicrobial activity, isolates which were code as LBKURCC198, LBKURCC200, LBKURCC201, LBKURCC202, LBKURCC204, LBKURCC207, LBKURCC212, LBKURCC216, LBKURCC217, LBKURCC218, LBKURCC220, LBKURCC223, LBKURCC224, LBKURCC231, LBKURCC232, LBKURCC235, LBKURCC243, and LBKURCC244, displayed antimicrobial activity against *C. albicans.* In this study, three (LBKURCC206, LBKURCC218, and LBKURCC222) isolates were active against Gram-negative and Gram-positive bacteria.

One of the most potential bacterial isolates is LBKURCC218 which has the antimicrobial activity against pathogenic fungi and bacteria. The inhibition zone around the paper disc indicates the presence of secondary metabolites produced by thermophilic bacteria that function as antimicrobials. One isolate (LBKURCC218) from Rimbo Panti hot spring, West Sumatera (Figure 1) was selected for identification according to their antimicrobial activity. We further characterized the selected isolates through analyses of the 16S rRNA region, gram staining, antimicrobial activities of crude extract, and metabolic profiling by GC-MS.

3.2. Morphological Characterization of Selected Thermophilic Bacteria

LBKURCC218 thermophilic bacterial colonies were circular, convex, and yellowish-white (Figure 2A). From the gram staining test, LBKURCC218 isolate

Table 2. Preliminary screening of LBKURCC thermophilic bacteria

Isolate code	Inhibition zone category			Isolate code	Inhibition zone category		
	E. coli	*S. aureus*	*C. albicans*		*E. coli*	*S. aureus*	*C. albicans*
Sungai Pinang				Padang Gantiang			
LBKURCC197	+/-	-	-	LBKURCC224	+/-	-	+/-
LBKURCC198	+/-	-	+/-	LBKURCC225	+/-	-	-
LBKURCC199	+/-	-	-	LBKURCC226	-	-	-
LBKURCC200	-	-	+/-	LBKURCC227	-	-	-
LBKURCC201	-	-	+/-	LBKURCC228	-	+/-	-
LBKURCC202	+/-	-	+	Pawan			
LBKURCC203	+/-	-	-	LBKURCC229	-	-	-
LBKURCC204	+/-	-	+/-	LBKURCC230	-	-	-
LBKURCC205	-	-	-	LBKURCC231	+/-	-	+/-
LBKURCC206	+	+/-	-	LBKURCC232	-	+/-	+
LBKURCC207	-	-	+/-	LBKURCC233	-	+/-	-
LBKURCC208	+	-	-	LBKURCC234	+	-	-
LBKURCC209	-	+/-	-	Bukit Gadang			
LBKURCC210	-	-	-	LBKURCC235	-	-	+/-
LBKURCC211	-	+/-	-	LBKURCC236	+/-	-	-
LBKURCC212	+/-	-	+/-	LBKURCC237	-	-	-
LBKURCC213	-	-	-	LBKURCC238	-	-	-
Rimbo Panti				LBKURCC239	-	-	-
LBKURCC214	-	-	-	LBKURCC240	-	-	-
LBKURCC215	+/-	-	-	Bukit Kili			
LBKURCC216	+/-	-	+/-	LBKURCC241	-	+/-	-
LBKURCC217	+/-	-	+/-	LBKURCC242	-	-	-
LBKURCC218	+/-	+/-	+	LBKURCC243	-	+/-	+/-
LBKURCC219	-	+/-	-	LBKURCC244	+/-	-	+/-
LBKURCC220	+	-	+/-	LBKURCC245	-	+/-	-
LBKURCC221	-	-	-	LBKURCC246	-	-	-
LBKURCC222	+/-	+/-	-				
LBKURCC223	-	+/-	+/-				

Inhibition zone category: diameter of inhibition zone >15mm (++), 13-15 mm (+), <13 mm (+/-), and absent (-)

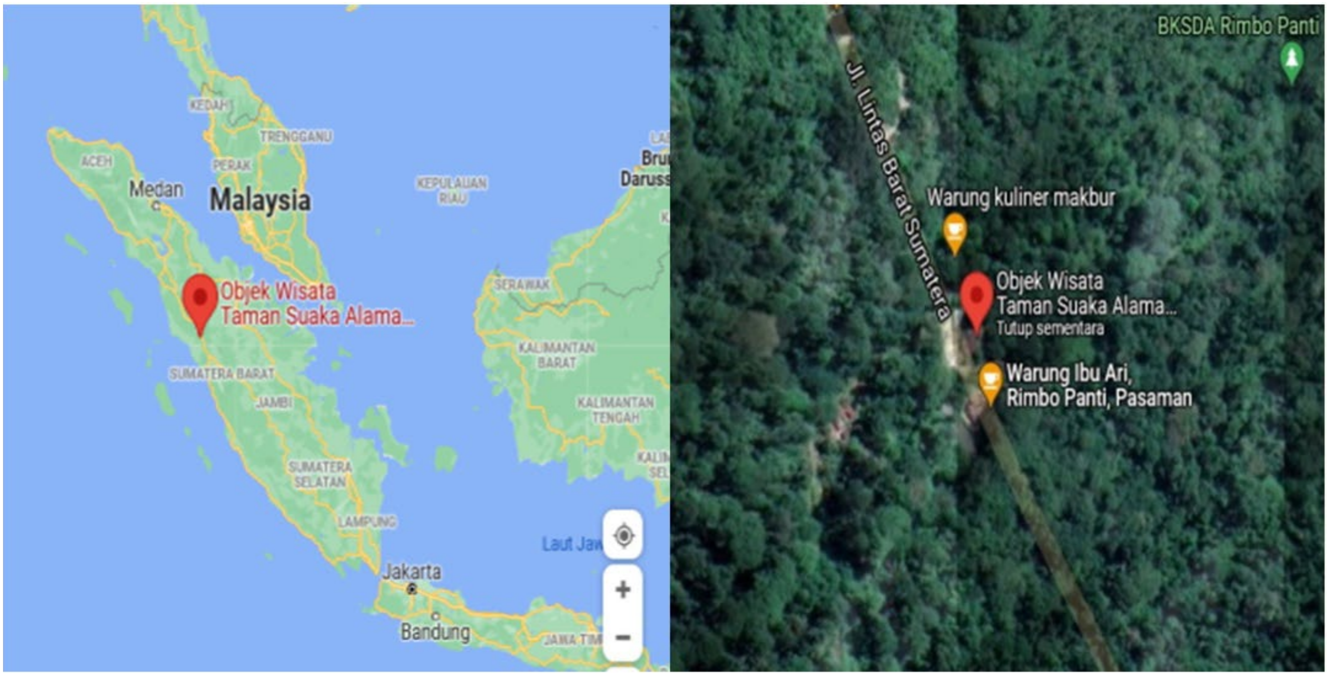


Figure 1. Location of Rimbo Panti Hot spring, West Sumatra Province, Indonesia, and natural appearance Rimbo Panti Hot spring (0°21'44.3" N 100°03'30.0" E)

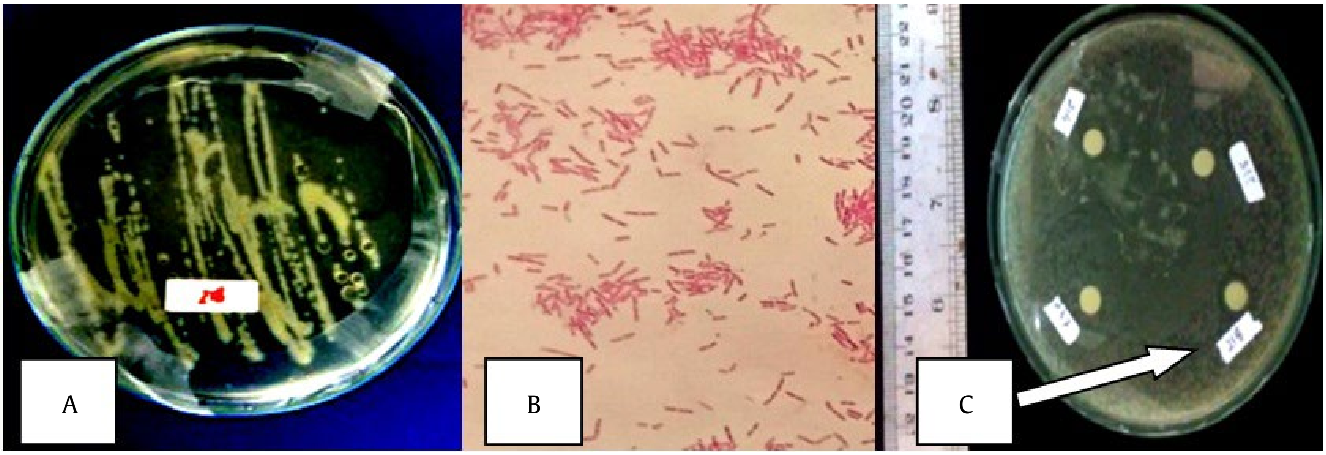


Figure 2. (A) Thermophilic bacteria LBKURCC218, (B) thermophilic bacteria LBKURCC218 gram stain, (C) zone inhibition of LBKURCC218 against *S. aureus*

was gram-positive and rod-shaped (Figure 2B). The LBKURCC218 strain can grow in a temperature range of 35 to 55°C. This strain grows optimally at 50°C (Table 3). *Bacillus* sp. LBKURCC218 was fermented at 45°C to produce secondary metabolites. Antimicrobial activity was indicated by a clear zone around the paper disc (Figure 2C).

3.3. Molecular Identification of the Selected Thermophilic Bacteria

DNA sequences of selected thermophilic bacterial with 1411 bp were used for identification based on the 16S rRNA gene. The sequence was submitted to GenBank with accession number OM802613. Analysis of thermophilic bacteria LBKURCC218 16S rRNA gene sequences with existing sequences in GenBank showed similarities to the genus *Bacillus* sp. This thermophilic bacterium showed 99.93% similarity with *Bacillus paramycoides* with accession number MW065486 (Figure 3).

Table 3. The growing temperature of bacterial isolate LBKURCC218

Temperature (°C)	Growth
35	+
40	+
45	++
50	+++
55	+
60	-

-: not grow, +: moderate growth, ++: good growth and +++: profilic growth

3.4. Analysis by Gas Chromatography-Mass Spectrometry

The GC-MS chromatogram of the ethyl acetate extract of *Bacillus paramycoides* LBKURCC218 produced 33 peaks (Supplementary Figure 1). Table 4 shows the names of the compounds extracted with ethyl acetate. Dodecanoic acid, which represented 23.62% of the total compound, was the main compound, followed by *11-Dodecanoic acid* at 17.84%. The compound detected at moderate concentration was eicosane (5.08%) followed by 4.15% of phenol 2, 6-bis(1,1 dimethyl ethyl)-4-methyl. The chemical structure of major compounds of *Bacillus paramycoides* LBKURCC218 crude extract is shown in Figure 5. Other compounds were detected in the little amount such as 1-tetradecene

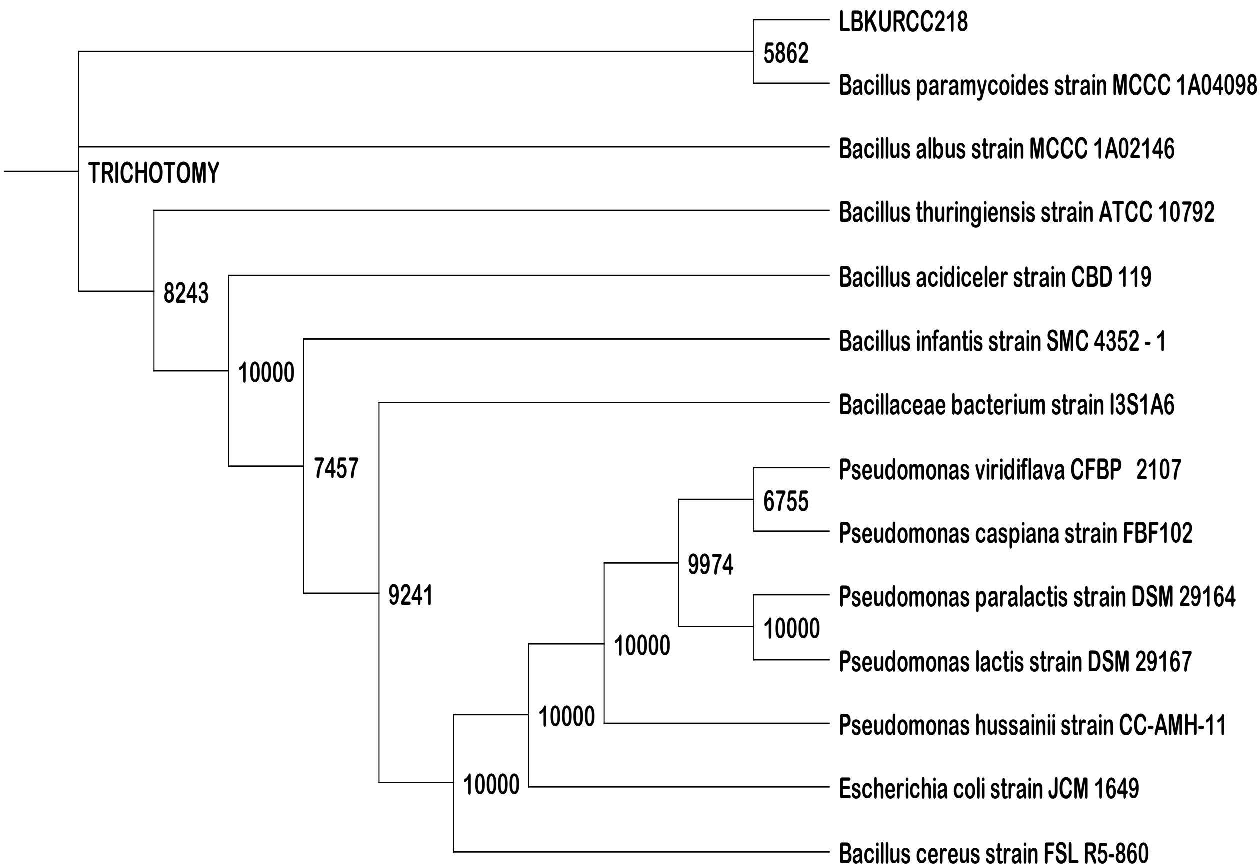


Figure 3. LBKURCC218 phylogenetic tree based on 16S rRNA gene sequences with other bacteria in GenBank

Table 4. GC-MS analysis of ethyl acetate extract *Bacillus Paramycoides* LBKURCC218

Peak	Compounds	Chemicals formula	Molecular weight	RT (min)	Area (%)	Similarity index (%)
1	Isoxazolidine	C_3H_7NO	73	8,817	1.75	70
2	Butanoic acid 2-methyl	$C_5H_{10}O_2$	102	8,896	0.89	92
3	Phenol 2, 6-bis(1,1 dimethyl ethyl)-4-methyl	$C_{15}H_{24}O$	220	27,329	4.15	75
4	Undecane, 2-methyl	$C_{12}H_{26}$	170	29,281	1.04	96
5	1-Dodecane	$C_{12}H_{24}$	168	33,820	1.72	93
6	Dodecane	$C_{12}H_{26}$	170	33,970	1.44	96
7	Dodecanoic acid	$C_{13}H_{26}O_2$	214	36,893	23.62	94
8	Decane	$C_{14}H_{30}$	198	37,045	2.40	83
9	1-Iodo-2-methylnonane	$C_{10}H_{21}I$	268	37,178	1.42	89
10	1-Tetradecene	$C_{14}H_{28}$	196	38,096	3.26	92
11	Pentadecene	$C_{15}H_{32}$	212	38,199	2.33	96
12	11-octadecanoic acid	$C_{19}H_{36}O_2$	296	40,380	17.84	92
13	Octadecanoic acid	$C_{19}H_{38}O_2$	298	40,722	1.92	94
14	Octadecane	$C_{18}H_{38}$	254	40,856	1.02	94
15	1-Hexadecene	$C_{16}H_{32}$	224	41,952	2.50	92
16	Hexadecane	$C_{16}H_{34}$	226	42,034	1.67	95
17	Naphthalene	$C_{20}H_{28}$	268	42,245	1.22	69
18	Hexadecane	$C_{16}H_{34}$	226	43,790	1.41	95
19	Methyl ester of ricnoleic acid	$C_{19}H_{36}O_3$	312	43,974	1.29	86
20	Cyclotetradecane	$C_{14}H_{28}$	196	45,483	2.17	89
21	Hexadecane	$C_{16}H_{34}$	226	45,565	2.53	96
22	Hexadecane	$C_{16}H_{34}$	226	47,194	1.88	96
23	2-Undecene	$C_{13}H_{26}$	182	47,284	0.90	75
24	Nonadecane 2-methyl	$C_{20}H_{42}$	282	47,747	1.12	89
25	1,2 Benzenedicarboxylic acid	$C_{24}H_{38}O_4$	390	48,151	2.09	83
26	Eicosane	$C_{20}H_{42}$	282	48,349	1.31	92
27	Eicosane	$C_{20}H_{42}$	282	48,802	5.08	96
28	Tetradecane	$C_{16}H_{34}$	226	49,732	0.87	87
29	Eicosane	$C_{20}H_{42}$	282	50,310	2.30	95
30	Eicosane	$C_{20}H_{42}$	282	50,785	1.47	93
31	Hexatriacontane	$C_{36}H_{74}$	507	51,371	1.14	91
32	Hexacosane	$C_{26}H_{54}$	366	51,771	2.48	95
33	Propane 2-(1,1 dimethyl ethyl) sulfonyl 12-methyl	$C_8H_{18}O_2S$	178	69,301	1.76	81

(3.26%), hexadecane (2.53%), 1-hexadecene (2.50%), hexacosane (2.48%), decane (2.40%), pentadecene (2.33%), cyclotetradecane (2.17%), Propane 2-(1,1 dimethyl ethyl) sulfonyl 12-methyl (1.76%), and isoxazolidine (1.75%).

3.5. Antimicrobial Activity

Our findings showed that ethyl acetate extract of *Bacillus paramycoides* LBKURCC218 inhibited the growth of *E. coli, S. aureus*, and *C. Albicans* (Figure 4). The inhibition diameter zone value of *Bacillus paramycoides* LBKURCC218 against *E. coli, S. aureus*, and *C. albicans* was 10.67 mm, 11.67 mm, and 23 mm (Table 5).

4. Discussion

Thermophilic bacteria from the collection of the Biochemistry Laboratory of the University of Riau have been tested for their antimicrobial activity. The thermophilic bacteria that was able to inhibit both bacterial and fungal pathogens was the LBKURCC218 isolate. The area of origin of LBKURCC218 is the hot springs of Rimbo Panti, West Sumatra Province, Indonesia. When viewed from the earth's surface, the Rimbo Panti hot spring is a dense forest. Forest areas have typical environmental conditions for hot spring bacteria to live. Existing environmental conditions are also influenced by temperature, acidity (pH), and the diversity of isolates in specific locations can affect the types of secondary metabolites produced by bacteria (Poli *et al.* 2017). The presence of microbial interactions in competition for food causes microorganisms to produce secondary metabolites that act as antibiotics. Hot springs are a habitat for thermophilic bacteria that can be used as a source of antibiotics (Panda *et al.* 2018). Thermophilic bacteria have been isolated from hot springs with antimicrobial activity in southern Saudi Arabia. There were 50 bacteria from 84 bacterial isolates with antimicrobial activity (Alrumman *et al.* 2019). Aldhabi has also isolated bacteria from sediments from hot springs at Tharban. This bacterium is a type of *Streptomyces* sp. Al-Dhabi. This strain has also been investigated, which has antimicrobial activity(Al-Dhabi *et al.* 2016).

The type of *Bacillus* isolated from hot springs was also reported by Arzu *et al.* (2012) and Oztas Gulmus and Gormez (2020). Another study reported the ability of *Bacillus* thermophilic bacteria to produce antimicrobials isolated from hot water in Jordan (Fandi *et. al* 2014) and *Bacillus subtilis* KFSB5 isolated from the Kinwat teak forest Kanse *et al.* (2014). This has increased the interest of researchers and industry to explore the potential of *Bacillus* class bacteria. Coupled with this research explores the potential of the *Bacillus* class from hot springs in Indonesia.

Table 5. Antimicrobial activity of *B. paramycoides* LBKURCC218 ethyl acetate extract using disc diffusion method

Test sample	Mean of zone inhibition against pathogenic microbes (mm)		
	E. coli	*S. aureus*	*C. albicans*
Ethyl acetate extract	23.00±0.47	11.67±0.47	10.67±0.47
Nystatin	17.33±0.47	-	-
Ampicillin	-	14.67±0.47	18.67±0.47
Negative control (ethyl acetate)	-	-	-

Microorganisms that live in hot springs have diversity and produce various types of secondary metabolites that function as antimicrobials. Bacillus-type bacteria can produce metabolites or enzymes (Kumar and Raja 2019), including antifungal and antimicrobial (Khan *et al.* 2018), (Caulier *et al.* 2019). This study also reported the ability of the thermophilic bacteria *B. paramycoides* LBKURCC218 to produce antibacterial and antifungal compounds. The inhibition zone (Figure 4) of *Bacillus paramycoides* LBKURCC218 against *Candida albicans* was 23 mm. The antifungal ability of *B. paramycoides* LBKURCC218 was higher than *Bacillus sonorensis* KKU-KS2 (15 mm) (Alrumman *et al.* 2019) and *Streptomyces* sp. Al-Dhabi (14 mm) (Al-Dhabi *et al.* 2016). While the antimicrobial activity *Bacillus paramycoides* LBKURCC218 against pathogenic bacteria *E. coli* and *S. aureus* (Table 4) had similar inhibition zones to *Bacillus sonorensis* KKU-KS2 and *Streptomyces* sp. Al-Dhabi. The antibacterial activity of *B. paramycoides* LBKURCC218 against *E. coli* was smaller than the thermophilic lactic acid bacteria *Pediococcus pentosaceus* N6 isolated from Rimbo Panti hot springs, which had an inhibition zone diameter of 20 mm (Yah *et al.* 2014).

Our study also showed that the thermophilic bacteria *Bacillus paramycoides* LBKURCC218 can produce compounds that have antimicrobial activity.

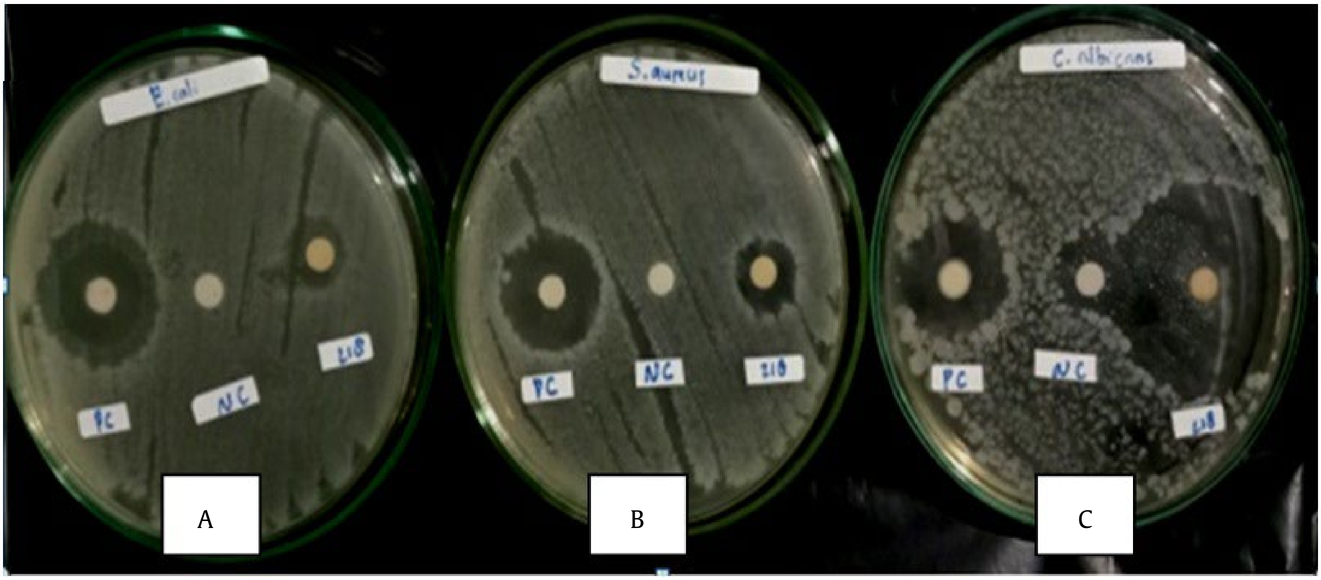
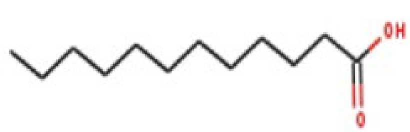


Figure 4. Inhibition zone of ethyl acetate extract strain *B. paramycoides* LBKURCC218 against microbial tested. (A) *E. coli*, (B) *S. aureus*, and (C) *C. albicans* (PC: positive control, NC: negative control, and 218: ethyl acetate extract of Bacillus paramycoides LBKURCC218)

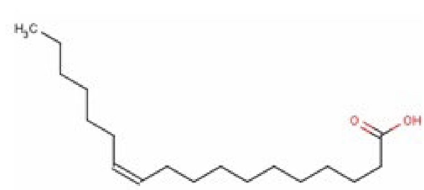
The main compound of the ethyl acetate extract analyzed by GC-MS was dodecanoic acid (Figure 5). Dodecanoic acid is a fatty acid methyl ester. Previous studies reported that this compound has antiviral, antifungal, and antibacterial properties (Özçelik *et al.* 2005; Chandrasekaran *et al.* 2008). Dodecanoic acid or lauric acid has been tested *in vitro* and *in vivo* to inhibit the growth of *Propionibacterium acnes*. This compound has a minimal inhibitory concentration (MIC) 15 times lower than benzoyl peroxide (BPO) in inhibiting *P. acnes*, *S. aureus*, and *S. epidermidis* (Nakatsuji *et al.* 2009).

Long-chain fatty acid compounds can function as antibacterial. This is because the outer membrane of bacteria is very sensitive to fatty acid compounds. The difference in the sensitivity of the outer membrane of Gram-positive and Gram-negative bacteria may be due to the impermeability of the outer membrane, which serves as an effective barrier to hydrophobic substances (Heesterbeek *et al.* 2019). This study found that the antimicrobial compound *B. paramycoides* LBKURCC218 could inhibit *S. aureus* and *E. coli* with inhibition zones of 11.67 mm and 10.67 mm, respectively. This shows that gram-negative bacteria are more resistant to fatty acids than gram-positive bacteria as reported by Klobucar *et al.* (2021). Agoramoorthy *et al.* (2007) investigated the leaves of *Excoecariaagallocha* and found the excellent antimicrobial activity of dodecanoic acid (lauric acid) against *S. aureus* and *E. coli*. This compound was also active against several pathogenic microbes such as *Micrococcus luteus, Salmonella typhimurium, Pseudomonas aeruginosa, Bacillus subtilis*, and *Klebsiella pneumonia*.

The compound 11-octadecanoic acid was the second most abundant component produced by *Bacillus* paramycoides LBKURCC218. This long-chain fatty acid is synthesized from linoleic acid by *Lactobacillus Plantarum*. Miyamoto showed that 11-octadecanoic acid has anti-inflammatory activity in the gut (Miyamoto *et al.* 2015). Extract of *Streptomyces strain KX852460* has the main components eicosane ($C_{20}H_{42}$) and dibutyl phthalate ($C_{16}H_{22}O_4$), where the extract can inhibit the fungus *R. solani AG-3*. This indicates that the two compounds above have antifungal activity (Ahsan *et al.* 2017). Aissaoui investigated the potential of thermophilic Bacillus isolated from hot springs in Algeria. These thermophilic bacteria are good producers of phenolic compounds that can be used against clinical strains (Aissaoui *et al.* 2018). The crude bioactive extracts produced in our study contain phenol 2, 6-bis(1,1 dimethyl ethyl)-4-methyl. These phenolic compounds showed antibacterial, antifungal, and antioxidant activity (Zhao *et al.* 2020).

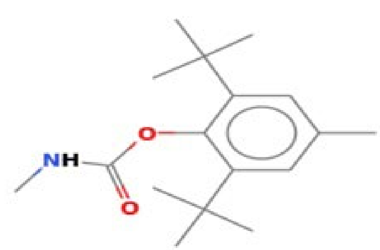


Dodecanoic acid



11-octadecanoic acid

Eicosane



4-Phenol 2,6-bis(1,1dimethylethyl)-4methyl

Figure 5. Chemical structure of major compounds of *B. paramycoides* LBKURCC218 crude extract

The first peak of GC-MS ethyl acetate extract of *Bacillus paramycoides* LBKURCC218 (Table 4) in this study was a compound similar to isoxazolidine (compound similarity index 70%). Previously, new isoxazolidine synthetic compounds have been found and have antifungal activity against plant pathogens (Ra *et al.* 2003). Isoxazolidine and γ-lactam analogs have antiviral abilities because they can damage DNA or RNA from viruses. In addition, isoxazolidine is more active as an antitumor drug than antiviral (Piotrowska *et al.* 2019).

In conclusions, this study provides new information about the thermophilic bacteria *B. paramycoides* LBKURCC218 isolated from the Rimbo hot springs in West Sumatra, Indonesia, and its potential as antifungal and antibacterial agents. The antimicrobial ability has been tested against the fungus *C. albicans* and *S. aureus* and *E. coli.* The diversity of compounds produced by this hot spring isolate was analyzed by GC-MS. The GC-MS chromatogram showed that this isolate produced general metabolites that could function as antibacterial and antifungal compounds. This study succeeded in exploring the potential of thermophilic isolates that have the potential as a source of antimicrobial compounds.

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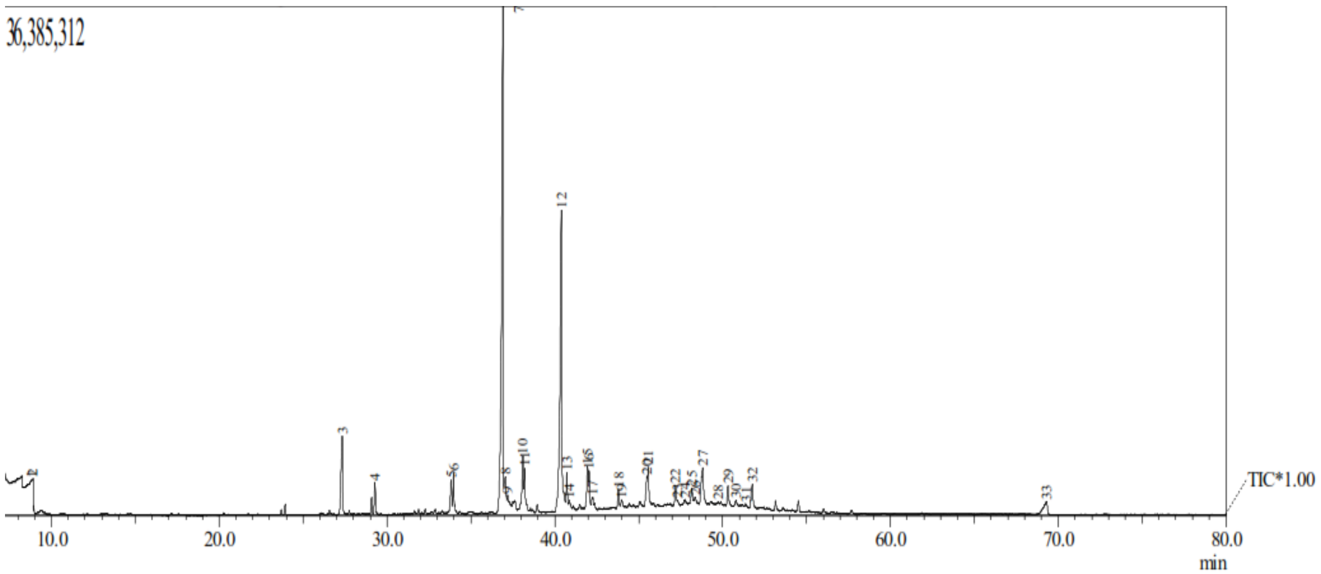

Supplementary Figure 1. Chromatogram of ethyl acetate extract of Bacillus paramycoides LBKURCC218 analyzed by GC-MS