BIODIVERSITAS
Volume 21, Number 10, October 2020
Pages: 4859-4865

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d211052

Characterization of BSL6 isolates isolated from honeybee hive and to determine its antibacterial activity

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Manuscript received: 11 September 2020. Revision accepted: 26 September 2020.

Abstract. *Fitri L, Yasmin Y, Fauziah, Septiani DA, Suhartono. 2020. Characterization of BSL6 isolates isolated from honeybee hive and to determine its antibacterial activity. Biodiversitas 21: 4859-4865.* This study was aimed to characterize BSL6 isolate of bacteria isolated from honeybee hive, and to determine its antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. In addition, the Minimum Inhibitory Concentration (MIC) value of BSL6 isolate was determined against *S. aureus*, and content of secondary metabolic compounds was also determined. Characterization was conducted in this study on the basis of morphological observations, physiological tests, and 16S rDNA. The method used in determining MIC value was completely randomized design (CRD) then analyzed by ANOVA test using SPSS application and followed by Tukey test. The analysis conducted to determine the content of secondary metabolites was descriptive type. The results showed that BSL6 isolate morphologically and physiologically belonged to the genus *Bacillus*, and 16S rDNA test results showed that the isolate had the highest similarity with *Bacillus siamensis* strain 64X-5. BSL6 isolate was able to inhibit the growth of *S. aureus* with inhibition zone diameter of 17.87 mm. MIC value of BSL6 against *S. aureus* was at a concentration of 12.5%. The content of secondary metabolite compounds from BSL6 extract was saponins.

Keywords: BSL6, characterization, honeybee hive, Minimum Inhibitory Concentration (MIC), secondary metabolites

INTRODUCTION

Honeybee hive is composed of propolis, a natural product derived from plant resins collected by honeybee. Propolis has been used as medicine by the public for centuries. Propolis has attracted attention of researchers in recent years because of its potential as a medicinal and cosmetic product (Lofty 2006). Research by Dantas Silva et al. (2017) showed that the properties of various Brazilian propolis extracts have antimicrobial, antioxidant, and cytotoxic activity.

The hive of honeybee (*Apis cerana*) has been investigated and proven to have antimicrobial activity. Part of the hive has different chemicals compounds which can act as an antimicrobial agent. This shows that honeybee hives has the potential to be used as an antibiotic to inhibit growth and to kill various pathogenic bacteria so that the quality of honey still maintained. Honeybee hive compounds with antimicrobial activity can be used as a source of natural antimicrobials (Reybroeck et al. 2012). A study conducted by Yuliana et al. (2015) showed that honeybee hive had antimicrobial activity against pathogenic microbes with minimal inhibitory concentration off 1% (v/v).

Microbial resistance is the inability of antimicrobials to kill microbes. Until now, microbial resistance towards antibiotics is increasing continuously. Antibiotic resistance occurs when bacteria change in certain ways that reduce or eliminate the effectiveness of antibiotics used as drugs (Bisht et al. 2009). This lead to the emergence of new pathogenic microbes that are resistant to antibiotics. All of these become the primary reason for the search of new, inexpensive and sustainably available antibiotics in large quantities.

Previous research results succeeded in obtaining BSL6 isolate from honeybee hive, however, the characterization of the isolate and its antibacterial ability had not been carried out yet. Based on this, this study was carried out to characterize BSL6 isolate based on its morphologically, biochemical test and at molecular using16S rDNA amplification and to determine the ability of this isolate to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. This study also aimed to determine minimum inhibitory concentration (MIC) value and secondary metabolite content of BSL6 which might have antibacterial properties.

MATERIALS AND METHODS

Bacterial isolation

A total of 1 gram piece of hive was soaked with sterile distilled water for one minute, then the surface was soaked with 70% alcohol for three minutes and soaked again with sterile distilled water for one minute. Beehive was cut using a sterile knife and mashed using mortar and pestle, then added with 5 mL of sterile distilled water are and homogenized using vortex for three minutes. A total of 100 µL of the solution from the suspension was taken using micropipette and spread into NA medium. The sample was

incubated at 37ºC for 48 hours (Promnuan et al. 2009). The isolate obtained then purified and identified.

Bacteria identification

Identification was conducted by referring to Bergey's Manual of Determinative Bacteria (Holt et al. 1994) and the Manual for the Identification of Medical Bacteria (Cowan and Steels 1993). Characterization of bacteria isolates obtained was carried out by macroscopic, microscopic, biochemical test, and 16S rDNA test. Macroscopic characterization was carried out by observing morphology of bacterial colony including shape, margin, color, and elevation of the colony. Biochemical test of BSL6 isolate was conducted for further identification. Biochemical tests conducted include catalase test, motility test, indole test, Methyl Red (MR) test, Simmons Citrate test, and TSIA test.

Identification of BSL6 by analysis of 16S rRNA gene

DNA Genome isolation of BSL6 was carried out using Wizard Genome Purification Kit (Promega, USA). Amplification was performed using PCR Thermal Cycler T100. The primers used for PCR process were universal primers pair Bact 27F (forward) (5 'AGA GTT TGA TCM TGG CTC AG 3') and UniB 1492R (reverse) (5 'GGT TAC CTT GTT ACG ACT T 3').

Sequencing process was carried out after amplification, by sending PCR DNA product to 1st Base via PT. Genetics Science. The sequenced DNA chromatogram was edited using DNA Baser Assembler v5.15.0 software. The results of DNA sequencing were then analyzed using the Basic Alignment Search Tools Nucleotide (BLAST-N) program through NCBI online media to find the similarity of the 16S rRNA gene nucleotide sequence with other microorganisms found in GenBank. Then processed using Molecular Evolutionary Genetics Analysis Version X (MEGA X) application (Rau et al. 2018).

Phylogenetic tree analysis

Phylogenetic tree analysis was carried out in two stages, namely primary sequences of Bact 27F and UniB 1492R on BSL6 isolates from the sequencing using DNA Baser Assembler application. Then the sequences that had similarities to BSL6 sequences were aligned using MEGA X. The results of the alignment then constructed with phylogenetic tree using MEGA X with the Neighbor-Joining (NJ) approaching method with bootstrap value of 1000 replicates.

Antimicrobial activity test

Test bacteria (*S. aureus* and *E. coli*) were incubated for 24 hours at 37ºC in slanted Nutrient Agar (NA) medium. The tested bacteria were then taken using inoculation loop and added into 0.9% NaCl solution. Furthermore, as much as 1 mL of bacteria suspension was taken and spreaded on the surface of Mueller Hinton Agar (MHA) medium and let it settle for 5 minutes so that bacteria would stick to the medium.

Honeybee hive bacteria isolates were taken using cork borer and transferred into MHA medium upside down so that test bacteria facing towards the medium and then incubated at room temperature for 24 hours. Observations were carried out by measuring the diameter of clear zone or inhibitory zone formed using a caliper.

Bacteria extraction

Total 5 discs (5 mm diameter) of NA medium having inoculated with BSL6 were taken with the help of a cork borer, put into Erlenmeyer flask containing 150 mL of Nutrient Broth (NB) medium, and then incubated in orbital shaker for 48 hours at a speed of 100 rpm at room temperature. The cell biomass was separated using filter paper then centrifuged at 3000 rpm for 60 minutes to obtain supernatant. The supernatant obtained was filtered again using filter paper to obtain crude extract of the bacteria which would be used for further tests (Duniaji et al. 2016).

Preparation of bacterial extract concentrations

A total of 25 mL of BSL6 extract was taken in a sterile Erlenmeyer flask then added with 25 mL of sterile NB medium so that the extract with a concentration of 50% in 50 mL was obtained. Furthermore, the concentration was continued to obtain concentration of 25%, 125% and 6.25%.

Minimum Inhibitory Concentration (MIC) test

Antibacterial activity of beehive bacteria extract was carried out by turbidity measurement method using spectrophotometer analysis. Minimum inhibitory concentration value was determined using pour plate method. The isolate was tested against *Escherichia coli*. The density of *E. coli* suspension was 10^6 CFU/mL. Preparation of the suspension at this density was carried out using McFarland 0.5 method consisting of 9.95 mL of 1% H_2SO_4 solution and 0.05 mL of 1.175% BaCl solution, which was equivalent to bacteria density of 10^8 CFU/mL (Sutton 2011).

The suspension of tested bacteria was made by taking *E. coli* from NA medium which had been incubated for 24 hours in a tube containing 10 mL of 0.9% NaCl and then homogenized using vortex. The homogenized suspension then equalized its turbidity by measuring it using spectrophotometer with a wavelength of 630 nm. Absorbance value of 0.08-0.1 was equivalent to McFarland solution of 0.5 or 10^8 CFU/mL. The bacteria suspension that has been made then diluted by taking 0.1 mL of the bacterial suspension and put in a tube containing 9.9 mL of 0.9% NaCl solution and then vortexed, so that the bacterial density is 10^6 CFU/mL (Oonmetta-aree et al. 2005).

A total of 14 test tubes containing 8.8 mL sterile NB medium were prepared. The first tube was added with 1 mL of sterile NB media and 200 µL of *E. coli* suspension. The second tube was added with 1 mL of Amoxicillin antibiotic and 200 µL of *E. coli* suspension. The 3rd - 7th tube was added with 1 mL of BSL6 extract with a concentration of 100%, 50%, 25%, 12.5%, and 6.25% respectively, then each tube was added with 200 µL of *E. coli* suspension. This treatment was repeated 3 times.

All tubes were vortexed and 2 mL of each treatment was taken in a cuvette to measure Optical Density (OD)

value using spectrophotometer with a wavelength of 630 nm. All tubes were incubated at 37°C for 18 hours. The OD value was measured again after incubation by taking 2 mL of each treatment in a cuvette and measuring the OD value using spectrophotometer with a wavelength of 630 nm. The difference between OD values of the suspension before and after incubation were compared (Munfaati et al. 2014).

Furthermore, pour plate method was used to calculate the number of colonies that have grown from the incubated suspension. All bacterial suspensions that had been incubated were taken as much as 10 µL each for dilution, then put into a 1.5 mL Eppendorf tube that already contained 990 µL of 0.9% NaCl. The dilution was done up to 10^{-6}. A total of 100 µL from the 10^{-6} dilution from each treatment were taken and put into a sterile petri dish, and then Plate Count Agar (PCA) medium was poured into the petri dish and waited until it solidified. Furthermore, it was incubated at 37°C for 24 hours. The number of colony growths in PCA medium was calculated using Colony Counter. The data obtained then analyzed using one way ANOVA test through SPSS application to see significant differences and to determine the value of MIC (Munfaati et al. 2014).

RESULTS AND DISCUSSION

Characterization of Isolate

Based on the observations bacteria isolates isolated from bee hive had circular colony shape, smooth margin, cream color, and flat elevation. Morphological characterization of bacteria was characterized based on Prescott's Microbiology book (Figure 1).

Microscopic characterization was carried by using Gram staining, which aimed to observe the shape of bacterial cells and divide the bacteria into Gram positive and Gram negative bacteria. The results showed that BSL6 was belong to Gram positive bacteria and had Coccobacilli cell shape. The results of biochemical test of BSL6 could be seen in Table 1

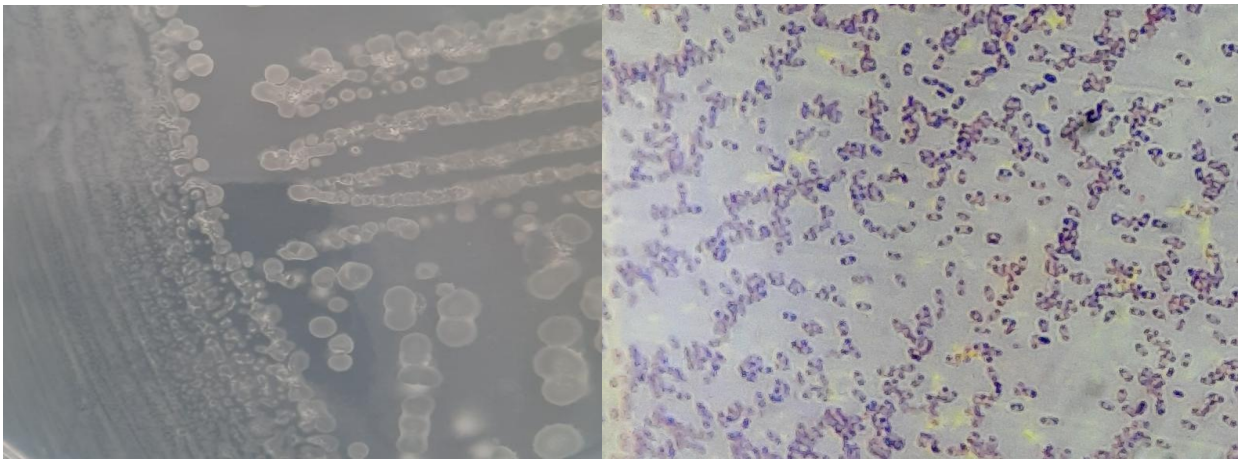
Based on the characteristics observed, BSL6 isolate belonged to the genus *Bacillus*. Cowan and Steels (1993) stated that bacterium from genus *Bacillus* has rod-shaped and straight cells, some in the form of coccobacilli on non-selective agar media, with size of 0.5-2.5 x 1.2-10 µm, and often arranged in pairs or chains, with round or square ends. The physiological abilities were diverse, usually non-motile, if motile by peritrichous flagella, catalase and oxidase-positive, negative methyl red, negative indole, negative nitrate and optimum growth at 30-37°C. *Bacillus* sp. are aerobic or facultative anaerobes, very sensitive to heat, pH, and salinity. *Bacillus* sp. was widespread in various habitats, several species were pathogens to vertebrates or invertebrates.

The bacteria obtained were in accordance with several previous studies. Piccini et al. (2004) also found various types of bacteria belong to the genus *Bacillus* from the beehive of *Apis* sp. Perez et al. (2013) also added that the beehive was dominated by bacteria from *Bacillus* sp.

Molecular identification based on partial sequences of 16S rRNA showed that BSL6 had the highest similarity with *Bacillus siamensis* strain 64X-5, *Bacillus subtilis* strain MDA1, *Bacillus subtilis* strain MDA2, *Bacillus amyloliquefaciens* strain 15535, and *Bacillus amyloliquefaciens* strain ORE3 with a query score of 2412, e-value of 0.0 and identity percentage value of 99.77%.

Table 1. Biochemical test of BSL6

Test	BSL6
Catalase	-
Motility	-
Indole	-
MR	-
Simmons Citrate	+
TSIA:	
Glucose	+
Glucose dan Sucrose	-
H_2S	-
Gas	-



A **B**

Figure 1. Macroscopic and microscopic morphology of BSL6 isolates in NA medium. A.Macroscopic; B.Microscopic

Table 2. Alignment data of several organisms from BLAST with BSL6

Description	Max score	Total score	Query cover	E value	Ident (%)	Accession
Bacillus siamensis strain 64X-5	2412	2412	99%	0.0	99.77	MN462850.1
Bacillus subtilis strain MDA1	2412	2412	99%	0.0	99.77	MN493719.1
Bacillus subtilis strain MDA2	2412	2412	99%	0.0	99.77	MN493754.1
Bacillus amyloliquefaciens strain 15535	2412	2412	99%	0.0	99.77	MN630201.1
Bacillus amyloliquefaciens strain ORE3	2412	2412	99%	0.0	99.77	MN685260.1

According to NCBI (2019), *B. siamensis* strain 64X-5 is a soil-borne bacteria. According to Sumpavapol et al. (2010), *B. siamensis* is a bacterium from the genus *Bacillus* which belong to Gram-positive, facultatively anaerobic, rod-shaped bacteria, with a size of 0.3-0.661 µm, 5-3 µm, and 5 µm. *B. siamensis* cells can be single, paired, and sometimes in the form of short chains. The ellipsoidal endospores of this bacteria are produced in the middle or subterminal bulging sporangia. Colonies of *B. siamensis* are creamy white, slimy, clear, raised, have entire margins, with colony diameter of 3-4 mm after 2 days incubation at 37ºC in TSA media.

B. siamensis in liquid media was in the form of a thin film on medium surface and the rest was shaped like clouds in the media. This bacteria produced catalase but did not produce oxidase. Voges-Proskauer test results showed positive results in reducing nitrates, positive results in hydrolysis of casein, aesculin, DNA gelatin, and starch. *B. siamensis* could grow at a temperature ranged from 4°C to 55°C and the optimal temperature was 37°C. *B. siamensis* could grow with 0-14% (w/v) NaCl and could grow optimally without NaCl. *B. siamensis* could grow in environments with pH 4.5 and 9.0 and could grow optimally at pH 6-7 (Sumpavapol et al. 2010).

Claverie and Notredame (2007) stated that the expectation value (E-value) was the value used to determine the similarity of sequencing results with the data contained in GenBank. Identical sequences have zero e-value. If the e-value is closer to one, the lower the level of similarity between the sequencing results and the data contained in GenBank. According to Bosshard et al. (2003), in identifying species of bacteria, there were three criteria from the query cover value, namely the first isolates could be classified as the same species if the query cover value was ≥99%, the second isolate could be in the same genus if the cover query value ranges from 95% to 99% and the third isolate could be classified in the same family if the query cover value was ≤95%. Meanwhile, according to Drancourt et al. (2000); Kadaikunnan et al. (2015), identification could be seen from the value of identity percentage. Identity percentage value ≥97% indicated that identification up to genus level, identity percentage value> 99% indicated that identification up to species level, and identity percentage value <97% indicated a low homology level from sequencing results with sequences contained in the database.

Based on phylogenetic tree analysis using Neighbor-Joining (NJ) approach with a bootstrap value of 1000 replicates, BSL6 isolate formed a monophyletic group with one strain from the genus *Bacillus*, namely *Bacillus amyloliquefaciens* strain ORE3. BSL6 isolate had the closest relationship with the *B. amyloliquefaciens* strain ORE3 with a bootstrap percentage value of 100% (Figure 2.).

Antibacterial activity test

Antibacterial activity test was conducted to observe and to measure inhibition zone of bacterial isolates isolated from honeybee. The results showed that BSL6 isolate was not able to inhibit the growth of *E. coli*, but was able to inhibit the growth of *S. aureus* with inhibition zone of 17.87 mm. (Figure 3). According to Lay (1994), the ability of antimicrobial agents to inhibit microbial growth could be determined by measuring inhibition zone formed around the disc.

BSL6 isolate had strong antibacterial activity against tested bacteria. Based on the study by Davis and Stout (1971), the result showed that the measurement of antibacterial inhibition zone could be divided into several categories, namely very strong if the inhibition zone diameter was >20 mm, strong (sensitive) if the inhibition zone diameter was 10-20 mm, intermediate if the inhibition zone diameter was 5-10 mm, and weak (resistant) if the inhibition zone was <5 mm. Bhorgin and Uma (2014) stated that the size of inhibition area was influenced by the growth rate of microorganisms, the ability and diffusion rate of active ingredients in the medium, the sensitivity of microorganisms towards active substances, and the thickness and viscosity of the medium.

Table 3. The average results of BSL6 treatment against *Staphylococcus aureus*

Treatment	*Staphylococcus aureus*	
	Colony	OD
K+	$0.0^{a} \pm 0.0$	$0.0710^{a} \pm 0.0030$
K-	$321.0^{a} \pm 41.0$	$0.1295^{a} \pm 0.0025$
6.25%	$354,.0^{a} \pm 229.0$	$0.1340^{a} \pm 0.0620$
12.5%	$80.0^{a} \pm 20.0$	$0.1910^{a} \pm 0.0060$
25%	$54.0^{a} \pm 17,.0$	$0.1890^{a} \pm 0.0110$
50%	$0.0^{a} \pm 0.0$	$0.1860^{a} \pm 0.0100$
100%	$0.0^{a} \pm 0.0$	$0.1705^{a} \pm 0.0055$

Note: different superscripts (a and b) indicated significant differences ($p<0.05$)

Table 4. The content of secondary metabolite compounds in BSL6 isolate

Metabolite Content	Reagent	BSL6	Observation
Alkaloids	Mayer	-	There is no cream-colored precipitate
	Wagner	-	There is no brown precipitate
	Dragendorff	-	There is no red precipitate
Steroids	Liebermann-Burchard Test	-	There is no green color
Terpenoids	Liebermann-Burchard Test	-	There is no red color
Saponins	Aquadest	+	Foamy
Flavonoids	HCl dan Logam Mg	-	There is no reddish color
Phenolics	$FeCl_3$	-	There is no green color
Tannins	$FeCl_3$	-	There is no green color

Note: (+) showed positive result and (-) showed negative result

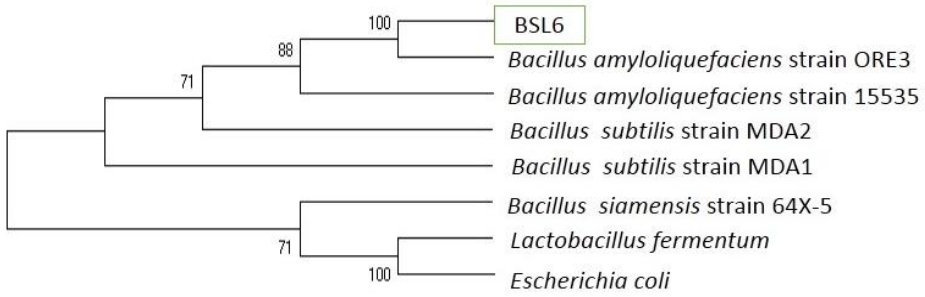


Figure 2. The results of phylogenetic tree reconstruction of BSL6 with several organisms from BLAST

BSL6 isolate was only able to inhibit the growth of *S. aureus*. This isolate produced secondary metabolites which were more suitable for Gram-positive bacteria than Gram-negative bacteria Dharmawan et al. (2009) stated that inhibition zone was formed because this isolate produced secondary metabolites. Inhibition zone was influenced by the difference in polarity of the compounds contained in bacteria.

Minimum Inhibitory Concentration (MIC)

Table 3 showed that BSL6 extract treatment affected total colony and OD value of *S. aureus*. However, if based on statistical analysis, BSL6 was not significantly different from total colony and OD values of *S. aureus*. The difference in superscripts values indicated that there were significant differences between each treatment.

Based on Table 3 all concentrations of BSL6 extract showed an effect towards total colonies of *S. aureus*, but concentration of 6.25% (354.0a ± 229.0) had a higher mean value than negative control (321.0a ± 41.0) which indicated that concentration of 6.25% had no effect on the total colonies of *S. aureus*. Meanwhile, there was no significant difference in OD value because all concentrations showed the same superscript as positive control. MIC value of BSL6 against *S. aureus* was analyzed at a concentration of 12.5%. The concentration at 12.5% had mean value of 80.0a ± 20.0. The results of a study carried out by Soelama et al. (2015) showed that MIC value obtained with a concentration ratio of 1:2 (v/v) was at a concentration of 6.25%, the higher the concentration of the extract used, the greater the ability to inhibit the tested bacteria.

BSL6 extract with concentration of 6.25% could not inhibit the growth of *S. aureus*. This was presumably because at this concentration there were not too many secondary metabolites and there were still BSL6 bacterial cells, so that when the pouring plate was done BSL6 bacterial cells could still multiply which caused the total colony to be larger than the negative control. Bacterial cells that were still present in BSL6 extract were suspected because the extraction was not carried out with ethanol solvents, so that the BSL6 bacterial cells did not die. According to Nofiani et al. (2009), extraction using ethanol as the solvent could cause cell lysis. Lysed cells may cause secondary metabolite compounds to diffuse into the ethanol solvent.

This is in line with the study of Wachidah et al. (2016), where the effectiveness of an antibacterial was influenced by the concentration of substance given, the higher the concentration of the honey beehive bacterial extract solution given, the higher the active antibacterial substances in the solution, so that the ability to inhibit bacteria would be even greater.

Figure 3. Antibacterial activity test results of BSL6 isolate against *S. aureus*. Note: Honeybee hive bacteria isolate indicate by red arrow, Inhibition zone formed indicate by yellow arrow

Secondary metabolite test

The results of secondary metabolite testing of BSL6 extract were shown in Table 4. This qualitative data showing the content of BSL6 secondary metabolites. The testing of secondary metabolite content in BSL6 was carried out in order to determine secondary metabolite compounds that act as antibacterials against *S. aureus*. Based on the results, only saponins contained in BSL6.

The results of the secondary metabolite test showed that BSL6 isolate contained saponins because bacterial extract formed foam when adding with distilled water and be shaken. The mechanism of saponins as antibacterials was that they could cause protein and enzyme leakage within cells. According to Madduluri et al. (2013), saponins could be antibacterial compounds because their surface-active agents were similar to detergents, consequently, saponins could reduce surface tension of bacterial cell walls and damage membrane permeability. According to Ningsih et al. (2016), saponins could diffuse through outer membrane and susceptible cell walls, then bound to cytoplasmic membrane so that they could disrupt and reduce the stability of cell membrane. This led to cytoplasmic leakage and resulted in the death of bacteria. Antimicrobial agents that interfere with cytoplasmic membranes are bactericidal.

Other secondary metabolite compounds such as alkaloids, steroids, terpenoids, flavonoids, phenolics, and tannins did not form. This was presumably because the bacterial extract used in secondary metabolite test was a liquid extract, which is an extract with low yield due to a large amount of water solvent contained and not evaporated, so that it was possible that the secondary metabolite compounds contained in bacteria extract were not expressed.

The conclusion that could be taken based on this study was that BSL6 isolate morphologically and physiologically belonged to the genus *Bacillus*, and 16S rDNA test results showed that the isolate had the highest similarity with *B. siamensis* strain 64X-5. BSL6 isolate was able to inhibit the growth of *S. aureus* with inhibition zone diameter of 17.87 mm. MIC value of BSL6 against *Staphylococcus aureus* was at a concentration of 12.5%. The content of secondary metabolite compounds from BSL6 extract was saponins.

ACKNOWLEDGEMENTS

The author would like to thank the Ministry of research and Technology/National Research and Innovation Agency (Indonesia) for the financial support through Basic Research Funding fiscal year 2020 (83/UN11.2.1/PT.01.03/ DRPM/2020).

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