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Research Article

Secondary Metabolites Production of *Bacillus* spp. Isolated from Sea Cucumbers (*Holothuria scabra*) and their Activity against *Mycobacterium smegmatis*

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ABSTRACT

New anti-tuberculosis agents are very important due to Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis problems. *Mycobacterium smegmatis* can be used to replace *Mycobacterium tuberculosis* as bacteria test to increase the velocity of anti-tuberculosis screening. To answer the need for new drugs, exploration of secondary metabolites from *Bacillus* spp. can be conducted. *Bacillus* spp. are known to produce antimicrobials, including discovery of iturins, fengycins, and pumilacidins. This study explored the *Bacillus* spp. isolated from fermented intestines of *Holothuria scabra*. The production of *Bacillus* sp. *Holothuria scabra* Fermented Intestine (HSFI) secondary metabolites was done using culture media containing starch as a carbon source, as well as yeast and peptone as a nitrogen source. Production capacity of secondary metabolites of *Bacillus* sp. HSFI was calculated, to determine its potential as an antibacterial producer. Inhibition testing of secondary metabolites of *Bacillus* sp. HSFI against *M. smegmatis* was performed using the Kirby-Bauer disk diffusion method. Based on the results of the inhibition test, it was concluded that *Bacillus* sp. HSFI-9 has the greatest potential to inhibit the growth of *M. smegmatis*, with a moderate inhibition (7.67 mm). Production of secondary metabolites from *Bacillus* sp. HSFI-9 is exceptionally good with an extract production capacity of 24.6 mg/L.

INTRODUCTION

Tuberculosis caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is still a global health concern, especially related to Multidrug-Resistant (MDR) and Extensively Drug-Resistant Tuberculosis (XDR-TB) problems. Accordingly, the search for new anti-tuberculosis drugs is very important (Miggiano et al., 2020). To increase the velocity of anti-tuberculosis screening, Rapidly Growing Mycobacteria (RGM) such as *Mycobacterium smegmatis* can be used to replace *M. tuberculosis* as a bacteria test (Lelovic et al., 2020). *Mycobacterium smegmatis* (*M. smegmatis*) and *M. tuberculosis* have similarities for mycothiol biosynthesis. Moreover, both bacteria have similar biochemical properties and genetic information. *M. smegmatis* is also a good model to study the general biological properties of mycobacteria such as physiological conditions, response to stress, and their reactivation from non-cultured conditions (Sundarsingh et al., 2020).

To answer the need for new drugs, exploration of secondary metabolites from Gram-positive bacilli such as *Streptomyces* spp. and *Bacillus* spp. can be conducted (Abdel-Razek

et al., 2020). *Bacillus* spp. are known to produce antimicrobials, including discovery of 3,5-dihydroxy-4-ethyl-trans-stilbene from *Bacillus cereus* (Kumar et al., 2014), iturins and fengycins from *Bacillus velezensis* (Devi et al., 2019), and pumilacidins from *Bacillus* sp. 4040 (de Oliveira et al., 2020).

To produce secondary metabolites, *Bacillus* sp. requires appropriate culture media. Environmental factors such as temperature and pH can also affect the production of its secondary metabolites (Al-Ansari et al., 2020). Nutrient factors in the media such as carbon and nitrogen sources greatly affect the secondary metabolites produced by *Bacillus* spp. Sources of carbon nutrients such as glucose, lactose, glycerol, and starch, are needed for the formation of biomass, growth nutrients, and increased biosynthetic energy of metabolites. Meanwhile, nitrogen sources are also needed for protein synthesis, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and adenosine triphosphate (ATP) transfer in bacterial cells. Sources of nitrogen that can be used for the production of secondary metabolites include beef extract, soybean meal, malt extract, and casein (Poernomo et al., 2020).

This study explored the gram-positive bacilli isolated from fermented intestines of sea cucumbers (*Holothuria scabra*). Based on research conducted by Hidayati et al., 2021b, there were 9 Gram positive bacilli which have been isolated, hereinafter referred to as *Bacillus* spp. *Holothuria scabra* Fermented Intestine (HSFI)-2, HSFI-4, HSFI-5, HSFI-6, HSFI-8, HSFI-9, HSFI-10, HSFI-11 and HSFI-12. One of the isolates, HSFI-5 has been identified molecularly as *Bacillus tequilensis* (*B. tequilensis*), and has been known to produce secondary metabolites that can inhibit the growth of *Staphylococcus aureus*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*) and *Saccharomyces aureus* (*S. aureus*) (Khan et al., 2019; Mardiana *et al*., 2020). However, until now, the secondary metabolite activity of *B. tequilensis* against *M. smegmatis* has not been known. Apart from *Bacillus* sp. HSFI-5, eight other species *Bacillus* sp. HSFI have not been identified so that their potential as antibacterial producers is unknown.

Production of secondary metabolites from *Bacillus* sp. *HSFI* was done using culture media containing starch as a carbon source, as well as yeast and peptone as a nitrogen source. With the aim of identifying more effective treatment against *M. tuberculosis*, it is important to know the ability of the culture media to stimulate *Bacillus* spp. HSFI to produce secondary metabolites that have antibacterial activity. Production capacity of secondary metabolites of *Bacillus* sp. HSFI were also calculated to determine the potential of *Bacillus* spp. HSFI as an antibacterial producer. The efficiency of developing new antibacterial agents is expected to increase along with the higher secondary metabolite production capacity of *Bacillus* spp. HSFI.

METHODS

Material and chemicals

Mycobacterium smegmatis mc155 (American Type Culture Collection (ATCC) 700084) was obtained from the Microbiology Laboratory of the Faculty of Medicine, Universitas Muhammadiyah Semarang. *Bacillus* spp. HSFI are held in a collection of the Microbiology Laboratory, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang. *Bacillus* spp. HSFI bacteria were isolated from the fermented intestine of sea cucumbers (*Holothuria scabra*) taken from the territorial waters belonging to Balai Bio Industri Laut, National Research and Innovation Agency, Mataram, Indonesia. The media used in this research included Mueller Hinton Agar (MHA) (Oxoid), Starch (Merck), Yeast (BD Difco), Peptone (BD Difco), and Sodium Chloride (NaCl) (Merck). Other materials used included dimethyl sulfoxide (DMSO) (Sigma), ethyl acetate (Merck), Rifampicin (PT. Phapros Tbk), H_2SO_4 (Merck), $BaCl_2$ (Merck), and blank disk paper (Oxoid).

Production and extraction of secondary metabolites from *Bacillus spp*. HSFI

The secondary metabolite production process was initiated by preparing 10 µL (using inoculation loop) of 24–48-hour old *Bacillus* spp. HSFI growing in MHA media. The culture process used 150 mL of Starch Yeast Peptone (SYP) broth media containing 1% starch, 0.4% yeast, 0.2% peptone, and 0.5% NaCl, and the resultant was placed in a 500 mL baffled flask (Duran®). An orbital shaker (Ohaus) was used to increase oxygen aeration during culture, at a speed of 120 rpm. Culture incubation was done at room temperature 28-30 °C for 3 days (Rakhmawatie et al., 2021).

After the culture process was completed, the supernatant was separated from the bacterial cells using a centrifuge (Hettich) at 6,000 rpm for 10 minutes. Furthermore, secondary metabolites from the supernatant were extracted using ethyl acetate (1:1 v/v). The ethyl acetate phase obtained was allowed to dry for approximately ± 24 hours in a fume hood (ChemFast). The end result of this process is a

thick ethyl acetate extract of *Bacillus* spp. HSFI, which is ready to be diluted to a certain concentration using 5% DMSO solvent. The total extract production was calculated by weighing of the ethyl acetate extract produced by each species of *Bacillus* spp. HSFI. Extract production capacity was calculated by dividing the weight of the ethyl acetate extract with total culture volume (mg/L).

Antibacterial activity test of ethyl acetate extract of *Bacillus spp.* HSFI against *M. smegmatis*

Inhibition strength of ethyl acetate extract of *Bacillus* spp. HSFI against *M. smegmatis* was measured using the Kirby-Bauer disc diffusion method. *Mycobacterium smegmatis* (*M. smegmatis*) suspension with a cell density of 1.5 x 10^8 colony forming unit (CFU)/mL was swabbed onto MHA using a cotton bud (CLSI, 2020; Hudzicki, 2009). After that step, paper discs containing 20 µg ethyl acetate extract of *Bacillus* spp. HSFI were placed on the surface of the MHA that had been smeared with *M. smegmatis*. Paper discs containing 5 µg rifampicin were used as control antibiotics. Petri dishes were incubated at 37°C for 24 hours. After incubation, the activity of the ethyl acetate extract of *Bacillus* spp. HSFI was seen from the formation of an antibacterial inhibition zone around the disc (National Centre for Disease Control, 2019). The inhibition strength was calculated and interpreted into four categories, namely > 20 mm, very strong; 10-20 mm, strong, 5-10mm, moderate; and < 5 mm, and no response (Ouchari et al., 2019).

Data analysis

The difference in the diameter of the inhibition zone between ethyl acetate extract of *Bacillus* sp. HSFI, rifampicin and 5% DMSO control was analyzed for significance. The normality of the data was first assessed with the Shapiro–Wilk test at a significance level of 0.05. A value of $p < 0.05$ was interpreted as indicating significant correlation at a confidence interval (CI) of 95%. Nonparametric Kruskal-Wallis followed by Mann-Whitney tests were used for data not normally distributed.

RESULTS AND DISCUSSION

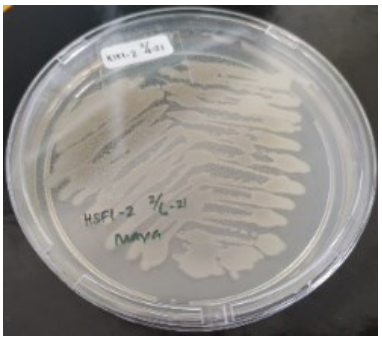
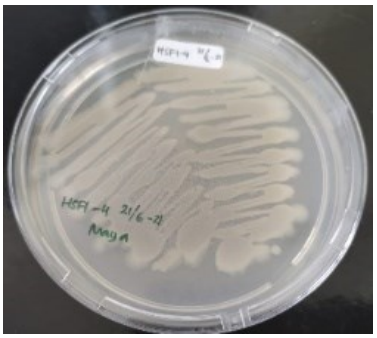
Bacillus spp. is known to produce secondary metabolites but in low fermentation yields, therefore its production capacity is very important to evaluate. Optimization of the culture for secondary metabolite production, either on a small or large scale, is essential to achieve efficiency in drug development (Kaspar et al., 2019). In this study, the use of SYP broth culture media with the addition of NaCl caused each species of *Bacillus* spp. HSFI to produce extracts in different quantities. The largest extract production capacity was achieved by *Bacillus* sp. HSFI-9 with a total production of 24.6 mg/L, and the smallest production capacity by *Bacillus* sp. HSFI-12 with a production amount of 2 mg/L.

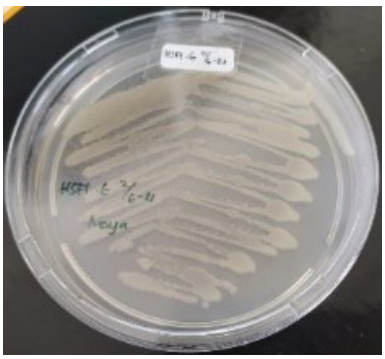
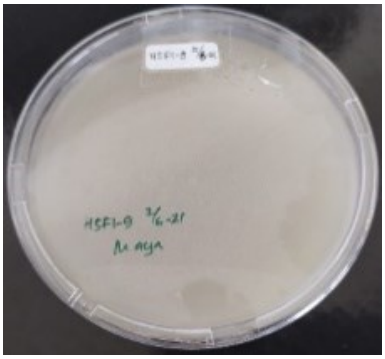
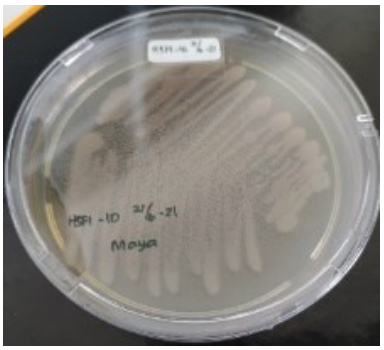
The production capacity of *Bacillus* sp. HSFI-9 is exceptionally good, especially when compared to other species of *Bacillus* spp. HSFI. However, when compared with *Bacillus velezensis* (*B. velezensis*) strain RP137, the production capacity of *Bacillus sp.* HSFI-9 is very small. The *B. velezensis* strain RP137 can produce antibacterial secondary metabolites with a production capacity of 131 mg/L. The production of secondary metabolites by *B. velezensis* strain RP137 was done by culture using rice starch as a carbon source and potassium nitrate as a nitrogen source (1.5%), as well as additional sea salt (1%) (Pournejati and Karbalaei-Heidari, 2020). In addition to the differences in the type of nitrogen source used for the culture, the use of sea salt when cultivating *B. velezensis* strain RP137 was replaced with a lower concentration of NaCl (0.5%) in the culture of *Bacillus* spp. HSFI.

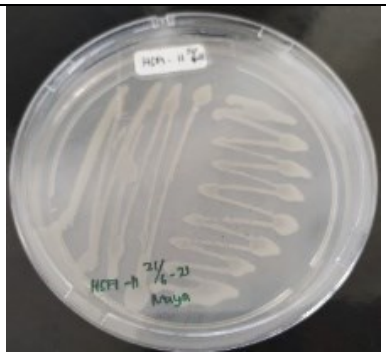
Ethyl acetate was used as a solvent for extracting the secondary metabolites of *Bacillus* spp. HSFI because it is volatile, has low toxicity, and is not hygroscopic. In addition, ethyl acetate is a semi-polar solvent that is able to attract compounds with a wide polarity range from polar to semi-polar (Warditiani, 2022). The antimicrobial compounds that have been discovered vary among the compounds in terms of polarity. Increased polarity of a compound can reduce its activity as an antibacterial agent. Compounds that are polar are stated to be more active as antifungals (Nazemi et al., 2017). Meanwhile, compounds that are known to be active as antimycobacterial vary from polar to semi-polar. The more non-polar a compound is, then the antimycobacterial activity of a compound will decrease (Obakiro et al., 2022). Mycobacteria have a cell wall structure containing peptidoglycan which is hydrophilic (polar) (Alderwick et al., 2015). However, mycobacteria also have a typical structure of mycolic acid in their cell walls which are hydrophobic (non-polar) because they consist of long chains of fatty acids (Nataraj et al., 2015). As a result, compounds that are too hydrophilic (polar) cannot penetrate mycolic acid, and those

that are too hydrophobic (non-polar) cannot penetrate the peptidoglycan. The search for antimycobacterial compounds can be devoted to semi-polar compounds. Apart from ethyl acetate, other solvents that can be used for the extraction of semi-polar compounds from bacterial secondary metabolites are ethanol, methanol, or acetone. The ethyl acetate itself has been successfully used for the extraction of antimycobacterial compounds from the secondary metabolites of *Streptomyces* spp. (Rakhmawatie et al., 2021). In this research, based on the extraction results, there are differences in the color of the ethyl acetate extract produced by each species of *Bacillus* spp. HSFI. The growth characteristics of each *Bacillus* sp. HSFI, along with the production capacity of the extract, as well as the color of the extract produced by *Bacillus sp.* HSFI are summarized in Table 1.

Table 1. Bacterial growth characteristics, extract color, and production capacity of *Bacillus* sp. HSFI

Bacillus sp.	Growth of *Bacillus* sp. in Mueller Hinton Agar	Growth Characteristics of *Bacillus* sp. (Hidayati et al., 2021a)	Color Extract of *Bacillus* sp.	Extract Production Capacity of *Bacillus* sp. (mg/L)
HSFI-2		Circular shape, white color, and umbonate elevation	Light brown	4.0
HSFI-4		Irregular shape, cream color, convex elevation	Light brown	7.1
HSFI-5		Filamentous shape, white cream, crater-form elevation	Light brown	7.0

HSFI-6		Irregular shape, cream color, convex elevation	Brown	6.7
HSFI-8		Circular shape, cream color, convex elevation	Brown	9.3
HSFI-9		Circular shape, cream color, umbonate elevation	Light Brown	24.6
HSFI-10		Circular shape, cream color, convex elevation	Dark brown	7.0
HSFI-11		Circular shape, cream color, convex elevation	Brown	2.6

			
HSFI-12		Circular shape, cream color, convex elevation	Brown	2.0

Testing of antibacterial activity of secondary metabolites of *Bacillus* spp. HSFI was performed using the Kirby-Bauer disk diffusion method. This method is considered reliable at describing the ability of a drug to inhibit bacterial growth (Djaenuddin and Muis, 2015). However, the diameter of the zone of inhibition of bacterial growth resulting from this method can be influenced by several aspects, including the growth rate of the test bacteria, the rate of drug diffusion, and the degree of sensitivity of the test bacteria (Soleha, 2015). The sensitivity of *M. smegmatis* to the control antibiotic rifampicin is still good. The inhibition zone diameter of the *M. smegmatis* formed after administration of 5 µg rifampicin discs was 11.3 mm. Rifampicin was concluded to have strong activity in inhibiting the growth of *M. smegmatis.* Meanwhile, from the screening results, it was found that *Bacillus sp.* HSFI-9 has the potential to produce secondary metabolites that inhibit the growth of *M. smegmatis* (Figure 1), with a mean of inhibition zone diameter 7.67 mm. Ethyl acetate extract of *Bacillus* sp. HSFI-9 had moderate inhibition against the test bacteria *M. smegmatis*.

In addition to the use of the antibiotic control rifampicin, 5% DMSO was used to ensure that the solvent did not affect the activity of the ethyl acetate extract of *Bacillus* spp. HSFI in inhibiting the growth of *M. smegmatis.* Dimethyl sulfoxide is used because of its ability to dissolve polar or non-polar substances (de Brito et al., 2017). Secondary metabolites of *Bacillus* sp. HSFI-9 were found as the strongest in inhibiting the growth of *M. smegmatis*. However, when compared with the diameter of the inhibition zone produced by the control 5% DMSO solvent, the secondary metabolites of other *Bacillus* spp. HSFI can be considered for optimization The results of the Kruskal-Wallis test, followed by the post hoc Mann-Whitney test, concluded that there was a significant difference in the mean diameter of the *M. smegmatis* inhibition zone (p<0.05) between the 5% DMSO and the extract of *Bacillus* sp. HSFI-2, HSFI- 4, and HSFI-6 (Table 2).

Table 2. The mean ± standard deviation of the inhibition zone diameter of the ethyl acetate extract of *Bacillus* sp. HSFI against *M. smegmatis*

Group	N	Mean ± SD Inhibition Zone Diameter	Description of the inhibition category (Ouchari et al., 2019)
HSFI-2	18	1.33 ± 1.41[b]	No response
HSFI-4	18	1.19 ± 1.09[b]	No response
HSFI-5	18	0.56 ± 1.10	No response
HSFI-6	18	1.44 ± 1.20[b]	No response
HSFI-8	18	0.61 ± 0.90	No response
HSFI-9	18	7.67 ± 2.03[a, b]	Moderate
HSFI-10	18	0.78 ± 1.20	No response
HSFI-11	18	0.78 ± 1.17	No response
HSFI-12	18	0.44 ± 1.10	No response
Rifampicin 5 µg	9	11.33 ± 0.50	Strong
5% DMSO		0.00	No response

Note: DMSO, dimethyl sulfoxide; SD, standard deviation; [a]Mann-Whitney analysis showed significant difference with rifampicin ($p < 0.001$) and [b]Mann-Whitney analysis showed significant difference with 5% DMSO ($p < 0.05$)

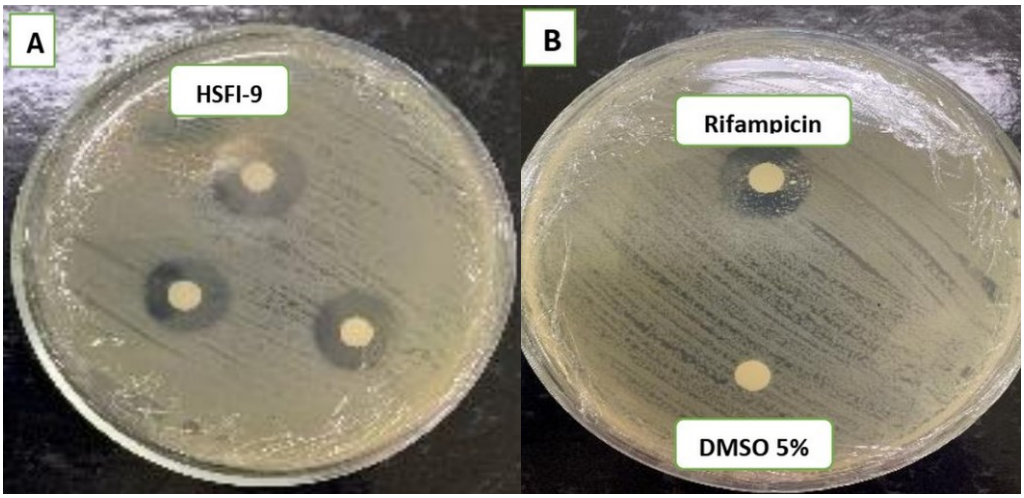


Figure 1. The results of the Kirby-Bauer disk diffusion test of (A) ethyl acetate extract of *Bacillus* sp. HSFI 9 and (B) rifampicin and 5% DMSO on the growth of *M. smegmatis.*

The *Bacillus* sp. HSFI-5 used in this study has been identified as *B. tequilensis* (Hidayati et al., 2021b). Unfortunately, *B. tequilensis* HSFI-5 was unable to produce secondary metabolites capable of inhibiting the growth of *M. smegmatis*. Whereas based on another research, the *B. tequilensis* strain ZMS-2 (Khan et al., 2019) and *B. tequilensis* strain BSM-F (Poernomo et al., 2020) are able to produce secondary metabolites that can inhibit the growth of *S. aureus*, *E. coli*, *K. pneumonia*, and *Bacillus licheniformis*. This could be due to *M. smegmatis* having different characteristics compared to these bacteria. *Mycobacterium smegmatis* has acid-resistant characteristics and has a cell wall with mycolic acid and high lipid content, so it requires relatively non-polar compounds to penetrate it (Sánchez and Kouznetsov, 2010). In addition to differences in the susceptibility of the tested bacteria, there were also differences in the culture process of *B. tequilensis* to produce secondary metabolites. The *B. tequilensis* BSM-F uses glycerol as a carbon source and casein as a nitrogen source. Meanwhile, *B. tequilensis* ZMS-2

was cultured with media containing 0.5% glucose as a carbon source, 1% skim milk and 0.1% peptone as a nitrogen source, and minerals were added to produce alkaline proteases that have antibacterial activity.

From this research, it can be concluded that differences in carbon and nitrogen nutrients can affect the production of secondary metabolites of *Bacillus* spp. However, differences in the antibacterial activity of secondary metabolites produced by *Bacillus* spp. can also be influenced by different species or even strains. Each species of *Bacillus* spp. has differences in its secondary metabolite-producing gene clusters. The Non Ribosomal Peptide Synthetase (*NRPS*) and Polyketide Synthase (*PKS*) are two gene clusters that have been extensively studied from *Bacillus* spp. Activation of the *NRPS* and *PKS* genes can be influenced by environmental factors of culture such as temperature, pH, and nutrient media (Al-Ansari et al., 2020). For example, *Bacillus subtilis* can synthesize various peptide compounds due to the role of various *NRPS* genes. In the case of surfactin and plipastatin production, both compounds are produced from *Bacillus subtilis* due to the activation of the NRPS gene, but their production is due to the activation of different operons, which are operon *srfA* in surfactin production and operon *ppsA* in plipastatin production. Likewise with the *PKS* gene, the different compounds produced by *B. subtilis* can depend on the type of multimodular enzyme that is activated (Harwood et al., 2018; Kai, 2020).

In general, based on the results of this study, the ethyl acetate extract of *Bacillus* sp. HSFI-9 can be continued for the drug development process. The metabolite profiling process is needed to determine the compounds of the ethyl acetate extract of *Bacillus* sp. HSFI-9. For the efficiency of drug development, optimization of culture can be done to increase the production capacity of secondary metabolites of *Bacillus* sp. HSFI.

CONCLUSIONS

The results of the Kirby-Bauer disk diffusion test of ethyl acetate extract from nine *Bacillus* spp. HSFI concluded that *Bacillus* sp. HSFI-9 has the potential to inhibit the growth of *M. smegmatis*. *Bacillus* sp. HSFI-9 cultured using starch, yeast, peptone, and NaCl nutrients was able to inhibit *M. smegmatis* with a zone inhibition diameter of 7.67 mm (moderate inhibition). Production of ethyl acetate extract from *Bacillus* sp. HSFI-9 is exceptionally good with an extract production capacity of 24.6 mg/L.

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CONFLICT OF INTEREST

None

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