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Antibacterial activity and GC-MS profile of secondary metabolites of *Bacillus subtilis* subsp. *subtilis* HSFI-9 associated with *Holothuria scabra*

MAYA DIAN RAKHMAWATIE[1,♥], NANIK MARFU'ATI[1], BESTY BARSALIPUTRI[2], AIDA ZAKIYATUL FIKRIYAH[2], STALIS NORMA ETHICA[3]

[1]Department of Biomedical Sciences, Faculty of Medicine, Universitas Muhammadiyah Semarang. Jl. Kedung Mundu Raya No. 18, Semarang 50273 Central Java, Indonesia. Tel./Fax.: +62-24-76740296, ♥email: mayadianr@unimus.ac.id
[2]Faculty of Medicine, Universitas Muhammadiyah Semarang. Jl. Kedung Mundu Raya No. 18, Semarang 50273, Central Java, Indonesia
[3]Graduate Program of Medical Laboratory Science, Universitas Muhammadiyah Semarang. Jl. Kedung Mundu Raya No.18, Semarang 50273, Central Java, Indonesia

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Abstract. *Rakhmawatie MD, Marfu'ati N, Barsaliputri B, Fikriyah AZ, Ethica SN. 2023. Antibacterial activity and GC-MS profile of secondary metabolites of* Bacillus subtilis *subsp. subtilis HSFI-9 associated with* Holothuria scabra*. Biodiversitas 24: 2843-2849.* The emergence of resistant bacteria has led to the importance of new antibacterial discovery. Before this research, the marine biota *Holothuria scabra* found in the Kodek gulf area, West Nusa Tenggara, Indonesia, was explored to isolate *Bacillus* sp. The present research was conducted to explore *Bacillus* sp. to produce secondary antibacterial metabolites, by culturing isolate using media containing starch, yeast, and peptone. Ethyl acetate was used for secondary metabolite extraction. Antibacterial activity screening was carried out by two-fold microdilution test to obtain Minimum Inhibitory Concentration (MIC) value. In this study, it was found that the ethyl acetate extract of the strain HSFI-9 can inhibit the growth of *Staphylococcus aureus*, Extended Spectrum β-Lactamase (ESβL)-*Escherichia coli*, and *Mycobacterium smegmatis* with sequential minimum inhibitory concentrations (MIC) values of 3.125; 25; and 50 µg/mL. Based on phylogenetic tree analysis, the strain HSFI-9 show 99.71 similarity with *Bacillus subtilis* subsp. *subtilis*. The ethyl acetate extract of *Bacillus subtilis* subsp. *subtilis* HSFI-9 metabolomics profile was analyzed using Gas Chromatography-Mass Spectroscopy (GC-MS). It showed five volatile secondary metabolites, with the first dominant compound being (2S, 3S)-(-)-3-propyloxiranemethanol (MW 116, $C_6H_{12}O_2$) with an area of 34.78% (database similarity index of 73.3%). This study concluded that *B. subtilis* subsp. *subtilis* HSFI-9 could be further developed as an antibacterial producer.

Keywords: Antibacterial agents, *Bacillus subtilis* subsp. *subtilis*, metabolomics, *Mycobacterium smegmatis*, volatile compounds

INTRODUCTION

Antibacterial resistance is a serious problem and has a global impact as the main cause of treatment failure in infectious disease (Varela et al. 2021). Various bacteria have developed resistance to existing antibiotics, which is associated with an increased death rate from treatment failure (Murray et al. 2022). Besides *Staphylococcus aureus* which is resistant to methicillin (Ventola 2015), other bacteria such as *Escherichia coli* are also widely reported to be resistant to β-lactam antibiotics and third-generation cephalosporin (Raphael et al. 2021). In addition, the problem of multidrug resistance of antimycobacterial continues to increase (Miggiano et al. 2020). The need for new antibacterial to replace resistant ones encourages researchers to search for drugs. A natural product is one of the potential sources of antibacterial, either from plant secondary metabolites sources (Salam and Quave 2018), or microorganisms (Schneider 2021). In particular, the search for natural products from microorganism sources can be carried out by fermentation or culturing Gram-positive or Gram-negative bacilli to produce secondary metabolites (Pham et al. 2019).

One of the Gram-positive bacilli, *Bacillus* sp., is known to produce various antibacterial secondary metabolites. An example of *Bacillus* sp. as an antibacterial producer are *Bacillus velezensis* which produce iturins and fengycins (Devi et al. 2019), *Bacillus* sp. 4040 as pumilacidins producer (de Oliveira et al. 2020), and *Bacillus subtilis* which produce bacillomycins and locillomycins (Kaspar et al. 2019). *Bacillus* sp. can be found in the soil environment, but its habitat is vast and can be found in the ocean's depths, plants or in animals (Alyousif 2022). Indonesia has a huge diversity of marine biota, and is one of the highest in the world (KKP 2017). Not to mention the unexplored marine areas for drug discovery sites, the discovery of drugs from microbial natural products still has a vast opportunity to find new drugs from unexplored area (Miethke et al. 2021).

Genomic and metabolomics approach can be used to explore *Bacillus* sp. as antibacterial producer. The approach of integrative methods between genomic and metabolomics has been proven in the discovery of new drugs (Avalon et al. 2022), for example the discovery of novel anti-quorum sensing compound (Ong et al. 2019). The genomic approach using whole genome data have the disadvantage of requiring more complex and expensive techniques (Hassler et al. 2022). Therefore, the whole genome approach can be replaced by analyzing the taxonomic similarity of *Bacillus* sp. using the 16S rRNA

gene sequencing method. The expected similarity of secondary metabolite-producing microorganisms is less than < 98% with the 16S rRNA gene database in NCBI, so it can be categorized as a new species and increases the possibility of obtaining new antibacterial agent (Stallfortha and Clardy 2014). A metabolomics approach can be carried out by profiling the secondary metabolites produced by *Bacillus* sp. using Gas Chromatography-Mass Spectrometry (GC-MS), because many volatile compounds were found due to secondary metabolite extraction from *Bacillus* sp. (Kai 2020).

Many studies have been conducted to find the antibacterial of *Bacillus* sp. isolated from Indonesian marine biota, for example endophytic *Bacillus* sp. related seagrass from Rote Ndao, East Nusa Tenggara, Indonesia, which has activity against Methicillin-Resistant *S. aureus* (MRSA) and *E. coli* (Fitri et al. 2017). In addition, *Bacillus* sp. isolates from the Savu Sea sponge symbiotic bacteria were able to produce antibacterial agents that inhibited the growth of Extended-Spectrum β-Lactamase (ESβL) *E. coli* and *Klebsiella pneumonia* (Prastiyanto et al. 2022). While in this study, *Bacillus* sp. has been isolated from the fermentation of the stomach contents and digestive system of sand sea cucumber (*Holothuria scabra*). The sea cucumber was originated from Kodek gulf area, West Nusa Tenggara, Indonesia (Hidayati et al. 2021). This *Bacillus* sp. has never been explored as antibacterial producer. Differences in the location and symbiotic organisms for the discovery of *Bacillus* sp. can lead to differences in the species of *Bacillus* sp. found, and can further affect the novelty of their secondary metabolites. Based on the initial screening test using disc-diffusion method, one of the *Bacillus subtilis* subsp. *subtilis* HSFI-9 is known to have the potential to inhibit the growth of *S. aureus* and *E. coli* (Rakhmawatie et al. 2022). Therefore, this study conduct further exploration of the isolate as new secondary metabolites producer with antibacterial activity, using 16S rRNA gene taxonomic and metabolomics approaches.

MATERIALS AND METHODS

Bacillus subtilis subsp. *subtilis* HSFI-9 extract production

The strain HSFI-9 was isolated from *H. scabra* found in the region of Marine and Land Bio Industry Research Center, National Research and Innovation Agency, Lombok, West Nusa Tenggara (Hidayati et al. 2021). Extract production was carried out by culturing *B. subtilis* subsp. *subtilis* HSFI-9 with media containing nutrients 0.4% starch (Merck 101252), 0.2% yeast (Bacto™ 212750), 0.4% peptone (Bacto™ 211677) (SYP), and 0.5% NaCl (Merck 106404) in a baffled flask (de Oliveira et al. 2020). Bacterial starter was prepared by growing 10 µL of *B. subtilis* subsp. *subtilis* HSFI-9 in 5.0 mL of Mueller Hinton Broth (MHB) (Merck 110293) medium with an incubation temperature of 37°C for 24 h. The culture process was carried out by adding 1.0 mL of starter culture *B. subtilis* subsp. *subtilis* HSFI-9 into 100.0 mL of SYP broth media (20% of the maximum volume of the baffled flask used). The culture process was carried out at 28-30°C for 3 days, using an orbital shaker with a speed of 120 rpm. After finishing the culture, the *B. subtilis* subsp. *subtilis* HSFI-9 cells was precipitated using a centrifuge at 6000 rpm for 10 min (Hettich® EBA 200). Furthermore, the supernatant separated from the bacterial cells, was extracted using ethyl acetate (1:1 v/v). After the ethyl acetate solvent had evaporated, the ethyl acetate extract of the secondary metabolite *B. subtilis* subsp. *subtilis* HSFI-9 was stored at -20°C (Rakhmawatie et al. 2021).

Antibacterial screening

Ethyl acetate extract of *B. subtilis* subsp. *subtilis* HSFI-9 was tested to assess minimum inhibitory concentration (MIC) against the test bacteria *S. aureus*, clinical isolate ESβL-*E. coli*, and *Mycobacterium smegmatis*. All of the bacteria tested were collected from the microbiology laboratory of the Faculty of Medicine, Universitas Muhammadiyah Semarang. The MIC test was conducted using a two-fold microdilution method sensitivity test in MHB media. The microplate incubation was carried out for only 16-20 h at 37°C for the sensitivity test of *S. aureus* and ESβL *E. coli* (CLSI 2020). Susceptibility test method of *M. smegmatis* to ethyl acetate extract of *B. subtilis* subsp. *subtilis* HSFI-9 was carried out using a method of Rakhmawatie et al. (2019). The maximum concentration of ethyl acetate extract of *B. subtilis* subsp. *subtilis* HSFI-9 for the antibacterial activity test is 100 µg/mL. Antibiotic control is used to see the resistance pattern of the test bacteria. Ampicillin with a maximum dose of 32 µg/mL was used as a control antibiotic in the *S. aureus* and *E. coli* sensitivity tests, while rifampicin with a maximum dose of 4 µg/mL was used as a control antibiotic in the *M. smegmatis* test.

Identification of HSFI-9

To ensure the purity of the bacteria, morphological tests were carried out on the strain HSFI-9 with Gram staining and microscopic observation at 100x objective lens magnification using a binocular microscope. DNA extraction process was performed according to the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The results of DNA extraction were measured for purity using a nanodrop spectrophotometer (NanoDrop™ One/OneC). Furthermore, the amplification of the 16S rRNA gene was carried out using universal bacterial primers 27F (5'-AGAGTTTTGATCTGGTCCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Sulistiyani et al. 2021). The PCR mix used was consisted of 12.5 µL (2x) MyTaq HS Red Mix (BIO-25048), 9.5 µL double-distilled water, 1 µL 10 µM 27F primer, 1 µL 10 µM 1492R primer, and 1 µL template DNA. Amplification was carried out under PCR conditions of 95°C for 1 min, then continued (96°C, 15 sec denaturation; 52°C, 30 sec annealing; 72°C, 45 sec elongation) 35 cycles and the final extension at 72°C in 7 min. Amplification was performed with Perkin Elmer GeneAmp PCR system 2400. PCR products were then analyzed using 0.8% agarose gel in Tris-borate-EDTA (TBE) buffer (Gomaa et al. 2007).

Sequencing of the 16S rRNA gene amplification product of HSFI-9 was carried out by PT Genetika Science

Sequence Service Company, Tangerang, Indonesia using the bi-directional sequencing method. Sequence data obtained were searched for homology or matched with online genomic databases (www.ncbi.nlm.nih.gov). Phylogenetic tree construction for taxonomy determination was carried out using MEGA 7.0 software, Neighbor Joining Method (Kumar et al. 2016).

GC-MS secondary metabolite profile of ethyl acetate extract of *Bacillus subtilis* subsp. *subtilis* HSFI-9

Ethyl acetate extract of strain HSFI-9 was prepared by dissolving it with methanol (Merck Emsure® 106009) to a concentration of 3 mg/mL, then injecting 10 µL into the column. The column used was HP-5MS Ultra Inert 30 m x 0.25 mm x 0.25 µm (Agilent 19091S), with an injector temperature of 280°C. The mobile phase uses Helium at a flow rate of 1 ml/min. The oven temperature was set at 50°C for 1 min, then increased by 10°C/min and held at 200°C for 1 min, then raised again by 5°C/min increments and held at 325°C for 1 min. The molecules were scanned in the range 50.00- 1000.00 m/z (GC-MS Agilent 7890B and Agilent MS 240 Ion Trap). Compound determination was carried out by using peak chromatogram analysis, compared with NIST 14 GC-MS database (Nas et al. 2021).

RESULTS AND DISCUSSION

Antibacterial screening activity

The process of extracting secondary metabolites from a 300 mL culture of HSFI-9 produced a total of 7.38 mg of ethyl acetate extract. After the antibacterial activity test, it was found that the ethyl acetate extract of HSFI-9 can inhibit the growth of all tested bacteria. The most potent inhibitory activity of the ethyl acetate extract of HSFI-9 was proven in *S. aureus* bacteria with a MIC value of 3.125 µg/mL (Table 1). In this study, three tested bacteria were used for the search of antibacterial compounds. *S. aureus* and *E. coli* are harmless bacteria on the mucosa, but can become dangerous when they cause sepsis or systemic infections (Sugumaran et al. 2020). *M. smegmatis* can be a substitute as the test bacteria for antimycobacterial screening. Although it is a non-pathogenic bacteria, the incidence of multidrug resistance in *M. smegmatis* has also been widely reported. However, the problem of multidrug resistance in *M. smegmatis* has the advantage of increasing the possibility of finding new antimycobacterial (Arthur et al. 2019)

Test results in determining the resistance pattern of the test bacteria to the control antibiotic concluded that only *S. aureus* ATCC 25923 met the criteria and was still sensitive to the antibiotic ampicillin. The ESβL-*E. coli* was considered to be resistant to ampicillin, a β-lactam antibiotic (CLSI 2020). Reports of resistance from *S. aureus* and *E. coli* have been reported. For example, *E. coli* isolated from minced meat were 100% resistant to ampicillin, amoxicillin-clavulanic acid, cephalotin and cefoxitin. Meanwhile, 100% of *S. aureus* was resistant to ampicillin, amoxicillin-clavulanic acid, cephalotin, and nalidixic acid (Alzaben et al. 2022). Meanwhile, rifampicin, which is not a standard drug for testing *M. smegmatis* resistance patterns, can be used to evaluate the resistance patterns of most slow growth-mycobacteria, including *Mycobacterium tuberculosis*. The clinical breakpoint value for a mycobacteria to be categorized as sensitive to rifampicin is <1 µg/mL (Huang et al. 2020). *M. smegmatis* is one of the non-tuberculous mycobacteria (NTM)) substitutes for *M. tuberculosis* for antimycobacterial screening. Naturally, *M. smegmatis* can form pores to facilitate nutrient uptake. However, *M. smegmatis* can mutate to abolish the pore formation and reduce the absorption of hydrophobic drugs such as rifampicin, erythromycin, and vancomycin to enter the target cell of the antibiotic. This caused *M. smegmatis* to develop rifampicin resistance (Saxena et al. 2021). Considering the resistance pattern of the tested bacteria, the presence of antibacterial activity from the secondary metabolites of *B. subtilis* subsp. *subtilis* HSFI-9 is expected to overcome the resistance problem.

Identification of HSFI-9

Prior to analysis of the 16S rRNA gene sequencing, the result of the Gram stain test of HSFI-9 indicated the shape of the bacilli with purple color, meaning the HSFI-9 is a Gram positive bacterium with a bacillus form (Figure 1.A) (Tripathi and Sapra 2022). Meanwhile, based on observations at 100x objective lens magnification using a binocular microscope, there is uniform growth of HSFI-9 (Figure 1.B).

Next, a molecular sequencing test of the 16S rRNA gene was carried out to confirm the species of HSFI-9. After DNA extraction process of HSFI-9, it produced DNA at a concentration of 161.1 ng/µL. The absorbance of DNA at λ 260/280 nm was 1.88, and the absorbance of λ 260/230 nm was 1.62. Nanodrop absorbance readings are used to measure the quality of extracted DNA. The absorbance ratio at λ 260/280 nm was used to establish DNA purity. In this study, the absorbance value of extracted DNA at a ratio of λ 260/280 nm of 1.88 was considered good (high DNA purity). Meanwhile, the absorbance ratio at λ 260/230 nm was done to measure the purity of nucleic acids. High purity results are stated if the absorbance value is 2.0-2.2. The absorbance value of DNA at λ 260/230 nm indicated the presence of carbohydrate and phenol contaminants that can be absorbed at λ 230 nm (Lucena-Aguilar et al. 2016). The extracted DNA of HSFI-9 was then amplified using PCR, and obtained PCR products size at about 1500 bp (Figure 2).

Sequencing results of the 16S rRNA gene from HSFI-9 was blasted using the NCBI database, and produced similarities with *Bacillus stercoris* strain D7XPN1, *B. subtilis* subsp. *subtilis* strain 168, and *B.subtilis* JCM strain 1465 (99.71%). However, based on phylogenetic analysis, *B. subtilis* subsp. *subtilis* HSFI-9 is closer especially with *Bacillus subtilis* subsp. *subtilis* strain 168 (Figure 3). The 16S rRNA sequence gene of bacterial strain HSFI-9 was submitted to NCBI GenBank and accession number assigned as OQ677993. Bacterial names consist of genus and species, and can be added with subspecies names.

Table 1. The minimum inhibition concentration (MIC) of the ethyl acetate extract of HSFI-9 and the control antibiotics

Extract/antibiotic	MIC (µg/mL)		
	S. aureus	**ESβL-*E.coli***	***M.smegmatis***
Ethyl acetate HSFI-9	3.125	25.0	50.0
Ampicillin	2.0	8.0	-
Rifampicin	-	-	2.0

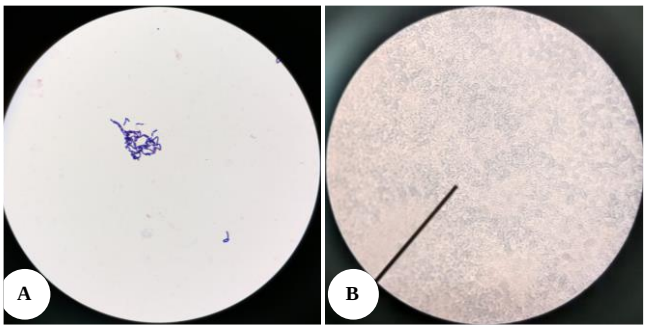

Figure 1. A. The results of Gram staining of HSFI-9 (binocular microscope at 100x objective lens magnification); B. The results of observations of HSFI-9 cells (binocular microscope at 100x objective lens magnification)

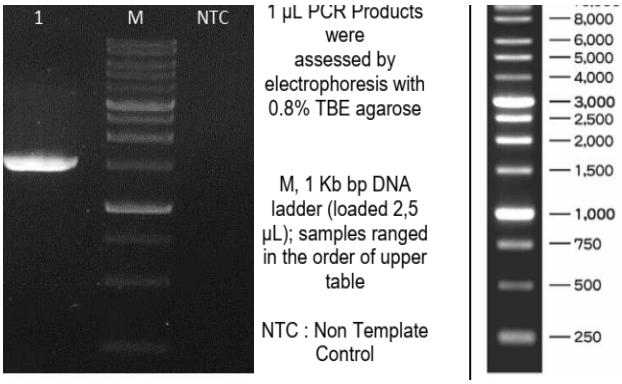

Figure 2. Electrophoresis results of the HSFI-9 gene PCR product on 0.8% TBE agarose gel

Adding subspecies names is necessary because some bacteria are considered genetically too far apart if they are called the same species, but are also considered too closely related if they are considered different species. Recent studies suggest that *B. stercoris* is also a subspecies of *B. subtilis*. Until now, apart from *Bacillus subtilis* subsp. *stercoris*, *B. subtilis* is divided into 3 other subspecies including *Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. *inaquosorum*, and *Bacillus subtilis* subsp. *spizezenii*. Genomically, *B. subtilis* subsp. *subtilis* and *Bacillus subtilis* subsp. *stercoris* is considered the closest among others. The difference between the four subspecies of *B. subtilis* has been examined in the phenotypic differences of the secondary metabolites that can be produced. According to the LC-MS chromatogram information, in *B. subtilis* subsp. *subtilis* was found peak chromatogram of secondary metabolites surfactin and subtilosin A. In *B. subtilis* subsp. *stercoris*, in addition to surfactin and subtilosin A, fengycin secondary metabolite chromatogram peaks were also found (Dunlap et al. 2020).

GC-MS secondary metabolite profile of ethyl acetate extract of *Bacillus subtilis* subsp. *subtilis* HSFI-9

The secondary metabolite profile of *B. subtilis* subsp. *subtilis* HSFI-9 ethyl acetate extract was carried out using GC-MS spectrophotometry. The results of these metabolite profiles can be used as basic information regarding the possibility of new compounds that have antibacterial activity. Based on the GC-MS results, it was possible to identify 5 compounds present in the ethyl acetate extract of *B. subtilis* subsp. *subtilis* HSFI-9. The first dominant compound was (2S,3S)-(-)-3-Propyloxirane methanol ($C_6H_{12}O_2$, BM 116) with a total area of 34.77% (Table 2).

Bacillus sp. has been known to produce secondary metabolites that have antibacterial activity. The types of compounds that can be produced by *Bacillus* sp. depend on the type of species, the nutrient media used for culture, and the method used for profiling (Khan et al. 2019). In particular, *B. subtilis* is known as a secondary metabolite producer with a broad class of compounds including peptides, polyketides, siderophores, to terpenes. Polyketide Synthase (PKS) and Non Ribosomal Polypeptide Synthetase (NRPS) secondary metabolite producing gene clusters from *B. subtilis* subsp. *subtilis* strain 168 have also been described in the antiSMASH database (https://antismash-db.secondarymetabolites.org/query.html).

Examples of lipopetide compounds produced due to stimulation of the NRPS gene cluster are surfactins, iturins, fengycins, plipastatin, bacilysin, and rhizocticin. Meanwhile, known examples of polyketide compounds from *B. subtilis* include macrolactin and kalimantacin (Harwood et al. 2018). Apart from being antibacterial, both lipopeptide and polyketide compounds from *Bacillus* sp. is known to have a variety of biological properties, including anti-biofilm, antifungal, anti-inflammatory, antiviral, antiplatelet, antitumor and anticancer properties. However, these lipopetide and polyketide compounds can usually be detected using targeted Liquid Chromatography-Mass Spectroscopy (LC-MS) because they are not volatile (Zhao et al. 2017).

Table 2. Secondary metabolite profile of ethyl acetate extract of *Bacillus subtilis* subsp. *subtilis* HSFI-9 conducted using GC-MS

Retention Time (min)	Compound name	Molecular formula	Molecular weight	% Similarity toward database	% Area
40.78; 40.98	2,3-Epoxyhexanol	$C_6H_{12}O_2$	116	769	27.70
41.45	Oxetane, 2-methyl-4-propyl-	$C_7H_{14}O$	114	696	13.07
41.55	1-Hepten-4-ol	$C_7H_{14}O$	114	703	10.43
42.10	1-Butanol, 3-methyl-, formate	$C_6H_{12}O_2$	116	695	14.02
42.44; 42.73	(2S,3S)-(-)-3-Propyloxirane methanol	$C_6H_{12}O_2$	116	733	34.78

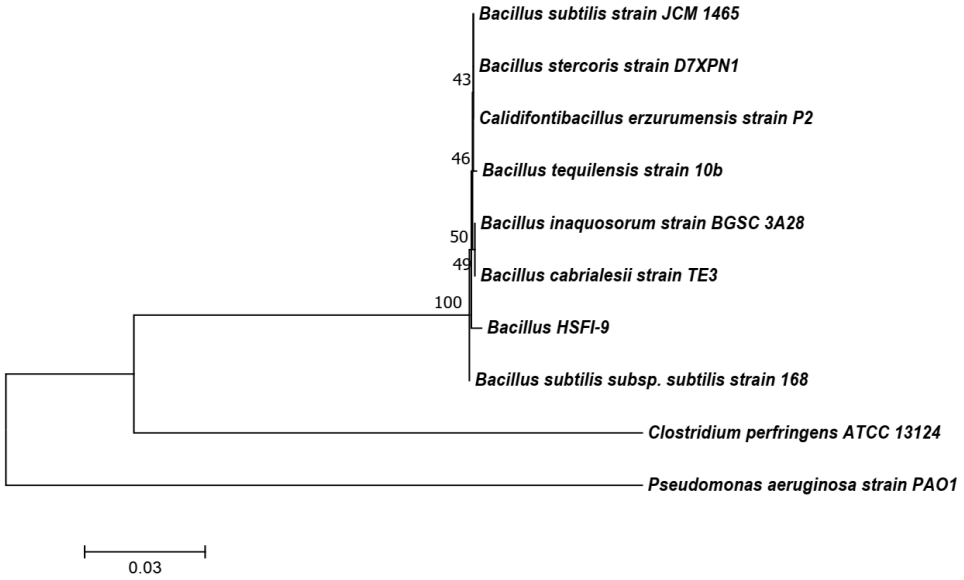

Figure 3. Phylogenetic tree of *Bacillus subtilis* subsp. *subtilis* HSFI-9 among other *Bacillus* sp.

Meanwhile, metabolite profiling can be carried out using GC-MS to detect volatile compounds. The volatile compounds found from *B. subtilis* subsp. *subtilis* HSFI-9 are different from the results of other studies (Bonifait et al. 2012; Ullah et al. 2015; Kai 2020). In this study, only 3 alcohol compounds was found (predicted as 2,3-epoxyhexanol, 1-hepten-4-ol, and 1-butanol, 3-methyl-, formate), 1 alcohol ketone compound (predicted as (2S,3S)-(-)-3-propyloxirane methanol), and 1 ketone compound (predicted as oxetane, 2-methyl-4-propyl). Five predicted volatile compounds were identified from *B. subtilis* subsp. *subtilis* HSFI-9 is not yet known for its biological activity. However, some compounds are predicted to have antioxidant or antimicrobial activity based on information from several studies. 2,3-epoxyhexanol compound can be found in green macroalga *Spongomorpha indica*, and has antioxidant activity (Swathi and Rajasekaran 2022). Meanwhile for other compound, 1-butanol, 3-methyl-, formate has antibacterial activity against *E. coli* and *S. aureus*, and also antifungal against *Aspergillus niger* (Gadhoumi et al. 2022). The dominant compound (2S,3S)-(-)-3-propyloxirane methanol) was predicted to have an antioxidant activity (Yusufzai et al. 2019).

Although in this study only five volatile compounds were identified from *B. subtilis* subsp. *subtilis* HSFI-9, until now the volatile compounds found from *B. subtilis* reached 231 compounds. The difference in the production of volatile compounds from *B. subtilis* seems to be significantly influenced by the production culture media used. Differences in the GC-MS method, including column temperature and the database used (NIST/Wiley) can also affect compound detection. The volatile compounds of *B. subtilis* known to have antibacterial activity, are benzaldehyde and acetophenone (Kai 2020). Benzaldehyde compounds can cause bacterial cell lysis (Ullah et al. 2015), whereas acetophenone inhibits bacterial cell growth by interfering with the nutrients that bacteria need to grow (Bonifait et al. 2012). However, these two groups of compounds were not found in this study. Both groups of compounds can be produced *by B. subtilis* subsp. *subtilis* 168, but using lysogeny broth media (1% tryptone, 0.5% yeast, and 0.5% NaCl) (Kai 2020). Differences in nutrient media from this study include nitrogen source (tryptone replaced by peptone) and the additional use of carbon sources (starch).

More varied volatile compounds were found from *B. subtilis* G-1, including alkanes, aldehydes, esters, sulfur containing compounds, and fatty acids with antifungal activity. The culture media comprised dextrose, glutamic acid, and various trace elements such as Mn^{2+}, Cu^{2+}, and Fe^{2+}. The GC-MS method used an initial column temperature of 230°C, which is increased by 4°C per min for 20 min to a maximum of 310°C (Shifa et al. 2015). Meanwhile, *B. subtilis* subsp. *inaquosorum* M1Sp1 which was cultured in media containing nutritional nitrogen yeast and peptone, as well as various mineral salts, different volatile compounds were also detected, such as chloromethane, ketone amide (1-(6-methyl-2-piperidyl)propan-2-o), pyrazole (3-Ethoxy-1h-pyrazole), fatty acids (n-hexadecanoic acid, cis-vaccenic acid, oleic acid), and terpene alcohol (farnesol). The extract has the activity to inhibit the growth of *S. aureus* and *E. coli* bacteria. The temperature of the GC-MS column used for analysis was 70°C which was increased by 10°C/min to 270°C (Murniasih et al. 2022). It seems that the column temperature also affects the detection of volatile compounds. In this study the maximum temperature of 330°C may be too high and affect the analyzed compounds.

In conclusion, the crude ethyl acetate extract of *B. subtilis* subsp. *subtilis* HSFI-9 has the ability to inhibit the growth of pathogenic bacteria. Therefore, the *B. subtilis* subsp. *subtilis* HSFI-9 has the potential to be developed as

an antibacterial producer. This finding can be continued with the process of fractionation and purification of the crude extract of *B. subtilis* subsp. *subtilis* HSFI-9, to obtain pure compounds and optimize their antibacterial activity. In addition to GC-MS for profiling pure volatile compounds resulting from the fractionation and purification processes of the crude extract, other spectroscopic methods such as LC-MS, High Resolution Mass Spectroscopy (HR-MS), or Nuclear Magnetic Resonance (NMR) can be used to profile pure non-volatile compounds.

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