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ANTIFUNGAL ACTIVITY POTENTIAL AND VOLATILE COMPOUND PROFILE OF *Bacillus* sp. HSFI-6 Extract

Potensi Aktivitas Antijamur dan Profil Senyawa Volatil dari Ekstrak *Bacillus* sp. HSFI-6

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ABSTRACT
Antifungal resistance has implications for the development of bioactive compounds. Bacillus sp. has been widely studied for the discovery of antifungal volatile compounds. This study aims to explore the antifungal activity of Bacillus sp. HSFI-6 isolated from sea cucumber (Holothuria scabra Fermented Intestine-HSFI) against Malassezia furfur, Candida albicans, and Aspergillus fumigatus. Bacillus sp. HSFI-6 was identified molecularly by 16S rRNA sequencing. Ethyl acetate extract of Bacillus sp. HSFI-6 was produced using media containing starch, yeast, peptone, and its metabolites were profiled using Gas Chromatography-Mass Spectroscopy (GC-MS). A disc diffusion antifungal assay was performed. Bacillus sp. HSFI-6 has 93.47% similarities with Bacillus albus strain MCCC 102146 and moderately inhibit C. albicans (7.00 ± 1.15 mm), A. fumigatus (9.25 ± 0.96 mm). The GC-MS metabolomics analysis showed two antifungal volatile compounds, namely cycloheptasiloxane, tetradecamethyl- and cyclononasiloxane, octadecamethyl-. These results conclude that Bacillus sp. HSFI-6 is suitable for the development of antifungal drugs.

Keywords: *Bacillus sp., Cycloheptasiloxane, Fungal infection, Metabolomics, Sea cucumber*

ABSTRAK
Resistensi antijamur secara klinis memiliki implikasi untuk pengembangan senyawa bioaktif. *Bacillus* sp. telah banyak diteliti untuk penemuan senyawa volatil dengan aktivitas antijamur. Penelitian ini bertujuan untuk mengeksplorasi aktivitas antijamur dari *Bacillus* sp. HSFI-6 yang diisolasi dari teripang laut (*Holothuria scabra Fermented Intestine*-HSFI) terhadap *Malassezia furfur, Candida albicans*, dan *Aspergillus fumigatus*. Isolat *Bacillus* sp. HSFI-6 diidentifikasi secara molekuler dengan sequencing 16S rRNA. Ekstrak etil asetat *Bacillus* sp. HSFI-6 diproduksi menggunakan kultur media berisi *starch, yeast,* dan *peptone*, kemudian metabolitnya diprofilkan menggunakan Kromatografi Gas-Spektroskopi Massa (KG-SM). Uji difusi cakram dilakukan untuk menilai aktivitas antijamur. Hasil sekuensing molekuler 16S rRNA menunjukkan bahwa *Bacillus* sp. HSFI-6 memiliki kemiripan dengan *Bacillus albus* galur MCCC 102146 (kemiripan 93,47%). *Bacillus* sp. HSFI-6 mampu menghambat pertumbuhan jamur secara sedang, baik terhadap *C. albicans* (7,00 ± 1,15 mm) maupun *A. fumigatus* (9,25 ± 0,96 mm). Hasil analisis metabolomik GC-MS menunjukkan dua senyawa volatil dari *Bacillus* sp. HSFI-6 yang berpotensi sebagai antijamur, yaitu sikloheptasiloksan, tetradekametil- dan siklononasiloksan, oktadekametil-. Hasil ini menyimpulkan bahwa *Bacillus* sp. HSFI-6 layak untuk pengembangan obat antijamur.

Kata kunci: *Bacillus sp., Infeksi jamur, Metabolomik, Sikloheptasiloksan, Teripang laut*

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INTRODUCTION

Fungal infections pose a significant challenge to vulnerable populations, such as immunocompromised patients. In these patients, *Aspergillus* sp. and *Candida* sp. can be pathogens that cause death from fungal infections. In addition, commensal fungi such as *Malassezia furfur* are usually tolerated by the immune system (Saunte et al., 2020), but in some conditions, they can have pathogenic potential and cause dermatological problems such as pityriasis or tinea versicolor (Suhara et al., 2019; Leung et al., 2022).

Fungal infections including Aspergillosis and Candidisias, can be treated with antifungals. However, increased resistance and antifungal side effects potentially pose a threat to humans (Merad et al., 2021; Putri et al., 2022). Azoles antifungal resistance in Aspergillosis therapy has the potential to cause a public health crisis (Arastehfar et al., 2021). Also, the high recurrence rate in pityriasis versicolor has the potential to lead an excessive use of antifungal drugs, which can increase the emergences of azoles-resistant *M. furfur* (Septiningrum, 2018; Park et al., 2020). Antifungal resistance has led research to shift to the discovery of antifungal bioactive compounds derived from plants, microbes, animals, and minerals (Saptarini et al., 2024).

The marine microbiome (fungi and bacteria) that live symbiotically with coral and marine animals, has been extensively studied for its important role in health. These marine microorganisms are known to produce metabolites with biological activities such as antimicrobial, anticancer, and anti-inflammatory properties (Barzkar et al., 2024). *Bacillus* sp. is a marine microorganism that is considered a source of drug discovery because it can produce compounds such as lipopeptides, enzymes, bacteriocins, polyketides, and volatile compounds that show biological activity as antimicrobials. The ability of *Bacillus* sp. to produce various classes of antibiotics has been proven from several genomic studies, and has implications for its ability to produce antifungal secondary metabolites (Abdel-Razek et al., 2020). *Bacillus* sp. is also known to produce volatile compounds such as benzaldehyde and acetophenone which have the aptitude to prevent the growth of spores and mycelia of several types of fungi (Kai, 2020).

In preliminary research, *Bacillus* sp. HSFI-6 was isolated from the intestinal fermentation of sea cucumber (*Holothuria scabra* Fermented Intestine-HSFI*)*, and was found to produce antimicrobial secondary metabolites against several pathogenic bacteria (Rakhmawatie et al., 2022). Its potential as antifungal producers has not been explored. This study aims to ascertain the antifungal activity of the secondary metabolite extract of *Bacillus* sp. HSFI-6 against the growth of *M. furfur*, *Candida albicans*, and *Aspergillus fumigatus*. An integrative approach between genomics and metabolomics can be used for new drug exploration methods. In previous research, a genomic approach was conducted using 16S rRNA gene sequences to determine the species of *Bacillus sp.* HSFI-9 as *Bacillus subtilis* subsp. *subtilis* HSFI-9. This genomic information was integrated with a metabolomics approach using Gas Chromatography-Mass Spectroscopy (GC-MS) to determine the ability of the *B. subtilis* subsp. *subtilis* HSFI-9 isolate to produce antibacterial compounds (Rakhmawatie et al., 2023b). The 16S rRNA gene sequence for studying phylogeny and taxonomy can be used as a guide in determining the potential biological activity of a new bacterial species. The metabolomics approach using GC-MS is a combination of gas chromatography analysis techniques to separate volatile compounds under high vacuum and low pressure conditions, and mass spectrometry to determine the formula and molecular weight of compounds (Hotmian et al., 2021). In this study, an integrative genomic metabolomics approach was conducted on *Bacillus* sp. HSFI-6 to explore its potential in producing antifungal compounds.

MATERIAL AND METHODS

The study was conducted from October 2024 to March 2025. The 16S rRNA gene amplification was performed at the Integrated Laboratory of Universitas Muhammadiyah Semarang, and sequencing was performed at PT. Genetika Science. Metabolite profiling with GC-MS was carried out at

the Integrated Research and Testing Laboratory of Universitas Gadjah Mada.Secondary metabolite extraction of *Bacillus* sp. HSFI-6 and antifungal activity testing were conducted at the Laboratory of Microbiology at the Faculty of Medicine, Universitas Muhammadiyah Semarang.

Materials

M. furfur (ATCC 14521), *C. albicans* (ATCC 90028), and *A. fumigatus* (ATCC 204305) were obtained from the Laboratory of Microbiology at the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Other equipments and materials are written along with a description of the research methods.

Species identification of *Bacillus* sp. HSFI-6 using 16S rRNA sequencing

Bacillus sp. HSFI-6 was cultured using Tryptic Soy Broth (TSB, Merck) media at 37°C for 24 hours, then DNA extraction is carried out using the method according to the instructions from the FavorPrepTM Tissue Genomic DNA Extraction Mini Kit (Favorgen). The DNA purity checked with NanoDrop™ (Thermo Fisher). The primers 27F and 1492R were used for amplification of the 16S rRNA gene, refer to previous research with modifications to the PCR mixture of kit PowerPol 2X Abclonal PCR Mix (Abclonal RK20718) (Algarni, 2022). Amplification starts with 45 seconds pre-denaturation at 98°C, then carried out for 30 cycles (10 seconds denaturation at 98 °C, 30 seconds annealing at 57.4 °C, 1 minutes elongation at 72 °C) and final extension at 72 °C for 5 minutes. Data assembly was performed using BLAST nucleotide, available online at https://blast.ncbi.nlm.nih.gov/Blast.cgi, and phylogenetic tree were carried out using MEGA 11.0 software (Rakhmawatie et al., 2021).

Extracts production from *Bacillus* sp. HSFI-6

The purification process of *Bacillus* sp. HSFI-6 isolated from sea cucumbers in Kodek Bay, Lombok, West Nusa Tenggara has been carried out in previous research (Hidayati et al., 2021). *Bacillus* sp. HSFI-6 was cultured using broth media contain starch, yeast, peptone, and sodium chloride in accordance with previously published working methods. The dried extract containing secondary metabolites was diluted with 5% Dimethyl Sulfoxide (DMSO, Sigma Aldrich), prepared according to the concentration of 20 mg/mL to be tested as antifungal (Akbar et al., 2016; Rakhmawatie et al., 2023a).

Metabolites profiling of *Bacillus* sp. HSFI-6 extract using GC-MS

Ethyl acetate extract of *Bacillus* sp. HSFI-6 with the concentration of 1 mg/mL was injected 1.0 µL into the column HP-5MS (Agilent 19091S). The separation and determination method for compounds was performed using the GC-MS (Agilent 7890B), according to previous research with modifications to the Helium flow rate. The initial oven temperature was 60°C (held for 2 minutes), then increased by 10°C every minutes until reached 280°C and held for 4 minutes, with a total running phase of 32 minutes. The molecules were observed in the range 40.00 - 700.00 amu and compared with NIST 17 GC-MS database. Quality control is performed by duplicating the secondary metabolite profiling process (Naveed et al., 2023).

Antifungal activity test using disc diffusion method

Antifungal test of *Bacillus* sp. HSFI-6 extract was performed using the Kirby-Bauer disc diffusion method. The test fungal suspension turbidity was adjusted to the Mc Farland standard of 0.5 (1.5 x 10^8 Colony Forming Unit (CFU)/mL). The medium used for the activity test was Mueller Hinton Agar (MHA, Merck). Specifically for *M. furfur* and *C. albicans*, MHA media was supplemented with 2% glucose (Merck) and 0.5 µg/mL methylene blue (Merck) (MHA-GMB) (Berkow et al., 2020). The test fungus was swabbed evenly with a cotton bud on the MHA. A total of 20 µL *Bacillus* sp. HSFI-6 extract (20 mg/mL) was dropped to the disc paper (Macherey-Nagel) using a micropipette according to the desired dose of 400 µg, then the disc paper was placed on the surface of the MHA. There were two control groups, ketoconazole as an antifungal control and DMSO 5% as an extract solvent

control group. *M. furfur* (ATCC 14521) incubated for 72 hours using temperature at 28-30°C, in the dark room (Rojas et al., 2017). *C. albicans* (ATCC 90028) and *A. fumigatus* (ATCC 204305) incubated for 48 hours at 37°C in incubator (Memmert) (Ballard et al., 2020). After incubation, the inhibitory zone inhibition was observed in the form of a clear zone around the disc paper (Berkow et al., 2020). The diameter of the clear zone formed is measured, and then classified as very strong (≥ 20 mm), strong (10-20 mm), moderate (5-10 mm), and weak (≤5 mm) (Rahmawati et al., 2020).

RESULTS AND DISCUSSION

Species identification of *Bacillus* sp. HSFI-6

The species indentification of *Bacillus* sp. HSFI-6 needs to be done molecularly using the 16S rRNA gene molecular sequencing. The three main processes of bacterial molecular identification are DNA extraction, amplification, and sequencing analysis. The DNA extraction stage aims to separate bacterial DNA from other cell components until pure DNA is obtained (61.93 ng/µL). The quality requirement of extracted DNA was measured by DNA purity value at an absorption ratio of 230/260 and 260/280 (Wangka et al., 2020). The results of the nanodrop readings concluded that the quality of *Bacillus* sp. HSFI-6 DNA met the requirements for DNA purity (absorbance value of 2.19 for A230/260 and 1.93 for A260/280).

The length of the 16s rRNA gene sequence of *Bacillus* sp. HSFI-6 is 1434 bp and has been stored in the NCBI database with the accession number of PZ287837. The analysis results revealed that *Bacillus* sp. HSFI-6 has similarities with *Bacillus albus* strain MCCC 102146 or *Bacillus luti* strain MCCC 1A00359 (93.47% similarity). However, based on phylogenetic analysis, *Bacillus* sp. HSFI-6 is more closely related to *Bacillus albus* strain MCCC 102146 (Figure 1). However, less than 95% value indicate that the similarity is likely only at the genus level. *Bacillus* sp. HSFI-6 can be predicted as a new species with further analysis such as Multi-Locus Sequence Analysis (MLSA) from whole genome data (Hassler et al., 2022).

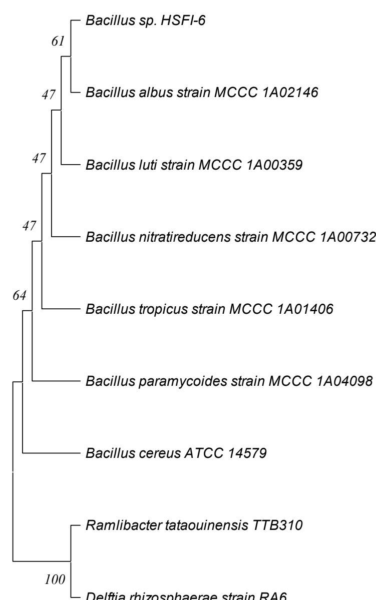

Figure 1. Phylogenetic tree analysis of *Bacilus* sp. HSFI-6 using Neighbor Joining Method

Metabolite profile of *Bacillus* sp. HSFI-6 extract

Metabolomic exploration was also conducted by profiling volatile compounds of *Bacillus* sp. HSFI-6 using GC-MS. Secondary metabolite profiles can be used to obtain predictive information for new compounds with antifungal activity. In recent years, various compounds of marine *Bacillus* sp. have a wide range of biological activities such as antimicrobial, anticancer, antiviral, antifun-

gal, antituberculosis, antimycoplasma, immunosuppressive, which show potential in the therapeutic, industrial, and agricultural fields (Xiao et al., 2022). *Bacillus* sp. isolated from marine biota are also known to yield various secondary metabolites including lipopeptides, polyketides, enzymes, bacteriocins, and volatile organic compounds (VOCs). These multiple compounds have their mechanisms for inhibiting the growth of fungi. Lipopeptides such as surfactin and iturin are known to change pressure and destroy fungi cell membranes (Wang et al., 2022). Macrolactin produce by *Bacillus amyloliquefaciens* MTCC 10456 has been proven to have valuable antifungal activity against *Malassezia* spp. (*M. furfur* ATCC 44344 and *Malassezia globose* ATCC MYA 4612). Marine *Bacillus* sp. has also been proven to produce basiliskamides A and B that very active in inhibiting *C. albicans* and *A. fumigatus* (Murniasih et al., 2022).

The GC-MS results describe the compound profile of the *Bacillus* sp. HSFI-6 extract, however several factors can influence the type of secondary metabolites produced by *Bacillus* sp. Environmental pressure associated with the aptitude to yield secondary metabolites, for example high environmental pressure will yield more secondary metabolite compounds. Other environmental factors can affect the metabolite compounds production, include pH, temperature, and the duration of culture incubation (Wendersteyt et al., 2021). In this study, the GC-MS results were able to identify 12 compounds in the ethyl acetate extract of *Bacillus* sp. HSFI-6 (Table 1). 1-Methyl propyl cyclopropane is the major constituent (55.75%) of secondary metabolites in *Bacillus* sp. HSFI-6. 1-Methyl propyl cyclopropane is one of the hydrocarbons included in terpenes, and previously identified as having toxic effects on various insects (Abdullahi et al., 2024).

Table 1. Volatile secondary metabolite profiling of ethyl acetate extract of *Bacillus* sp. HSFI-6 using GC-MS

Peak Number	RT (min)	Compound Name	Chemical Formula	MW	SI	Area * (%)	Activity
1; 2	4.23; 4.36	1-Methylpentyl cyclopropane	C_9H_{18}	126	800	55.75	Insecticidal and larvicidal activity (Abdullahi et al., 2024)
3	4.73	Cyclohexane, methyl-	C_7H_{14}	98	830	1.76	Not yet reported
10	10.39	Cyclohexasiloxane, dodecamethyl	$C_{12}H_{36}O_6Si_6$	444	784	1.16	Antibacterial (Lingfa et al., 2023)
14	12.62	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	518	727	1.61	Antibacterial, antifungal, antifouling, immunomodulatory and antitumour activities (Varshini et al., 2024)
16	14.61	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	592	783	1.31	Antibacterial (Lingfa et al., 2023)
22	16.34	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	666	760	1.29	Antifungal activity (Lutfia et al., 2021)
28; 32; 37; 41	17.88; 19.30; 20.58; 21.77	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	578	723	4.01	Antimicrobial (More et al., 2022)

Peak Number	RT (min)	Compound Name	Chemical Formula	MW	SI	Area * (%)	Activity
36; 54	20.28; 26.42	Astaxanthin	$C_{40}H_{52}O_4$	596	704	1.27	Anti-inflammatory and antioxidant activities (Bjørklund et al., 2022)
39; 43; 46; 56; 57; 59; 60	21.31; 22.69; 24.08; 26.93; 27.31; 27.61; 27.79	.psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	$C_{42}H_{64}O_2$	600	701	6.60	Antioxidant (Al Bratty et al., 2020)
44; 48; 50; 51; 52; 55; 62; 63; 64; 65	22.89; 24.47; 25.04; 25.46; 25.60; 26.76; 29.09; 30.26; 30.82; 31.26	Methyl glycocholate, 3TMS derivative	$C_{36}H_{69}NO_6Si_3$	695	694	3.33	Antioxidant, antimicrobial, insecticide agent (Arsana et al., 2022, 2024)
47; 58	24.26; 27.45	Betamethasone acetate	$C_{24}H_{31}FO_6$	434	692	1.55	Antiinflammatory (Dreyer et al., 2011)
61	28.06	Stigmastan-3,5-diene	$C_{29}H_{48}$	396	741	9.05	Antidiabetic and antiinflammatory (Abdelhamid et al., 2015; Zeb et al., 2017)

Note: RT = Retention Time; MW = Molecular Weight; SI = Similarity Index; Area = Abundance of Compounds

Antifungal activity of *Bacillus* sp. HSFI-6 extract

The disc diffusion antifungal activity test concluded that *Bacillus* sp. HSFI-6 extracts did not have antifungal activity against *M. furfur*. Nevertheless, *Bacillus* sp. HSFI-6 has moderate antifungal activity against *A. fumigatus* and *C. albicans*, with inhibitory diameter ≤ 10 mm. The positive control ketoconazole 10 µg very strongly detain the growth of *M. furfur* and *C. albicans*, also ketoconazole 30 µg has a strong aptitude to detain the growth of *A. fumigatus* (Table 2).

Table 2. Zone inhibition diameter of *Bacillus* sp. HSFI extract against tested fungi

Tested Fungi	Extracts	Dose (µg)	Mean ± SD of Zone Inhibition Diameter (mm)	Inhibition Category
Malassezia furfur	HSFI-6	400	-	-
	Ketoconazole	10	31.90 ± 2.13	Very strong
	DMSO	5%	-	-
Candida albicans	HSFI-6	400	7.00 ± 1.15*	Moderate
	Ketoconazole	10	21.50 ± 4.81	Very strong
	DMSO	5%	-	-
Aspergillus	HSFI-6	400	9.25 ± 0.96*	Moderate

Tested Fungi	Extracts	Dose (µg)	Mean ± SD of Zone Inhibition Diameter (mm)	Inhibition Category
fumigatus	Ketoconazole	30	14.83 ± 0.75	Strong
	DMSO	5%	-	-

Note: Dose of drug/extracts in microgram, except DMSO in % v/v; (-) no inhibition zone/antifungal activity;
SD (Standard Deviation); *statistical different with positive control ketoconazole (p <0.05)

In this study, *Bacillus* sp. HSFI-6 extract was not optimal to inhibit the fungal growth if compared to ketoconazole. One of the weakness of the disc diffusion method is that the disc capacity may be limited to absorb large amounts of extract solution, which may affect the results of antifungal activity tests. Variability in the rate of diffusion of active compounds into agar medium can also occur due to differences in molecular weight and solubility. When the extract penetrates the disc, the active compounds may diffuse at different rates, resulting in an uneven distribution of the antimicrobial compounds (Hossain, 2024). *Bacillus* sp. HSFI-6 extract still has ability against *A. fumigatus* and *C. albicans* that can be caused by intrinsic factors of both fungi. *Aspergillus* sp. and *Candida* sp. have cell walls with the main components of chitin and β-1,3-glucan as the targets for antifungal compounds such as lipopeptides which possibly make them more susceptible to the *Bacillus* sp. HSFI-6 extract (Garcia-Rubio et al., 2020). Meanwhile, *Bacillus* sp. HSFI-6 extract was unable to inhibit the growth of *M. furfur* (Figure 2). *M. furfur* itself has a thicker and more complex cell wall structure than other fungi, which causes suboptimal absorption of antifungal compounds. The content of Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) gives *M. furfur* the ability to differentiate self and non-self cell parts, making this fungus able to defend against antifungal compounds (Billamboz et al., 2023). The drug efflux pumps and the ability of *M. furfur* to form biofilms also contribute to its protection against antifungal compounds (Lohse et al., 2018; Sanjaya et al., 2021).

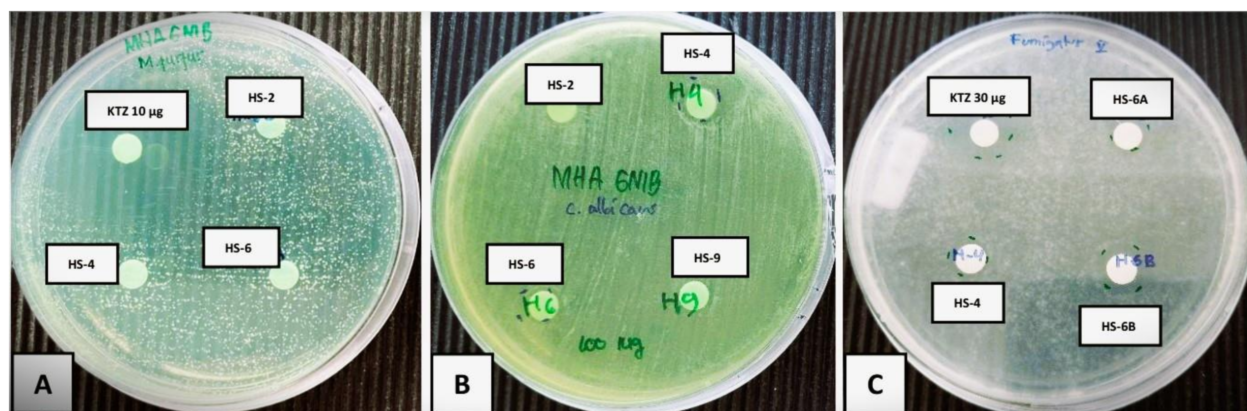

Figure 2. Disc difussion antifungal activity of *Bacillus sp.* HSFI against (A) *Malassezia furfur*, (B) *Candida albicans*, and (C) *Aspergillus fumigatus*. Note: KTZ = Ketoconazole; HS-2 = *Bacillus sp.* HSFI 2 extract; HS-4 = *Bacillus sp.* HSFI 4 extract; and HS-6 = *Bacillus* sp. HSFI 6 extract

The metabolite profile data of *Bacillus* sp. HSFI-6 extract shows several compounds that are suspected to act as antifungals, including cycloheptasiloxane, tetradecamethyl- (1.61%) and cyclononasiloxane, octadecamethyl- (1.29%) (Lutfia et al., 2021). A study proves that cyclononasiloxane, octadecamethyl in crude latex *Argemone ochroleuca* shows significant activity against several species Candida (*C. albicans, Candida glabrata, Candida krusei*, and *Candida tropicalis*) using disc diffusion method (Moustafa et al., 2013). Meanwhile, there is still no report of antifungal activity of *B. albus* or *B. luti* identity strains against *M. furfur, C. albicans,* and

A. fumigatus. However, several studies have reported that *B. albus* has antifungal activity against *Aspergillus flavus* (Rajkumar et al., 2024; Trinh et al., 2025).

Our study stated that the *Bacillus* sp. HSFI-6 extract has the potential to be developed as an antifungal agent, although it only has moderate inhibition against fungi. Moderate inhibitory activity is often sufficient for use in the development of topical anti-infective preparations (Bandyopadhyay, 2021). However, further research to maximize the effect of *Bacillus* sp. HSFI-6 extract remains to be done, for example to determine its minimum inhibitory concentration to evaluate the effective dose. This results also indicate opportunities in the pharmaceutical sector for developing antifungals with active ingredients of compound purification from *Bacillus* sp. HSFI-6 extract. Structural modification of the purified compound can also be carried out to increase its antifungal activity. Metabolomic profiling tests can be continued using the Liquid Chromatography-High Resolution Mass Spectroscopy (LC-HRMS) method for better resolution, accuracy, and identify non volatile compounds of *Bacillus* sp. HSFI-6 extract.

CONCLUSION

Bacillus sp. HSFI-6 extract has antifungal activity against *C. albicans* and *A. fumigatus* in the moderate category. Molecular sequencing analysis of the 16S rRNA gene revealed that *Bacillus* sp. HSFI-6 is similar to *Bacillus albus* strain MCCC 102146 (similarity 93.47%). The metabolomics approach GC-MS analysis shows 2 active secondary metabolite compounds from *Bacillus* sp. HSFI-6 which may act as an antifungal, including Cycloheptasiloxane, tetradecamethyl- and Cyclononasiloxane, octadecamethyl-.

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