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Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), as the primary secondary metabolite of *Bacillus* spp., could be an effective antifungal agent against the soil-borne fungus, *Sclerotium bataticola*

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Abstract

Microbial secondary metabolites are biologically active natural compounds that exhibit a broad range of antimicrobial properties. Investigating rhizobacterial resources could lead to the development of new antimicrobial compounds to enhance biocontrol agents. In this study, six *Bacillus* isolates (KSAS15-KSAS20) were isolated from the potato rhizosphere and screened in vitro for their ability to inhibit the growth of the phytopathogenic fungus, *Sclerotium bataticola*. The antifungal efficacy of the bacterial strains was assessed in vitro using a dual culture antagonism assay. The results demonstrated that strain KSAS17 had the most significant antagonistic activity, achieving a growth inhibition rate of 31.4%. The sequencing and analysis of the 16S rRNA gene identified the bacterial isolates as *Bacillus licheniformis* (KSAS15, KSAS16, and KSAR19), *Bacillus cereus* (KSAS17 and KSAR18), and *Lysinibacillus fusiformis* (KSAR20). Gas chromatography-mass spectrometry analysis of ethyl-acetate extract of the bacterial culture filtrate revealed that the compound most likely responsible for the effects in strain KSAS17 is pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl). Furthermore, significant bioactive aliphatic and aromatic compounds were identified, including oleic acid, hexadecanoic acid, 1-tetradecanol, heptadecanoic acid, 5-fluoro-2,2-dimethylchroman-4-one, pentadecanoic acid, 3′,8,8′-trimethoxy-3-piperidyl-2,2′-binaphthalene-1,1′,4,4′-tetrone, and phthalic acid derivatives. These findings indicate that the secondary metabolites of the rhizobacteria *B. cereus* strain KSAS17 exhibit antifungal properties and may be effectively employed as a biocontrol agent for managing fungal diseases.

Keywords: *Microbial secondary metabolites; Bacillus sp.; antifungal; pyrrole derivatives compounds; GC-MS*

1. Introduction

The decreased agricultural output, increased resistance to phytopathogens, falling soil fertility, and the effects of climate change are just a few of the problems that the agricultural sector has faced in recent years [1,2]. Plant diseases cause significant losses in vegetable production around the world and pose a threat to total crop yields. Diseases damage vegetable harvests every year, resulting in losses ranging from 40% to 60% [3]. Approximately eight thousand fungal species have been identified as causative agents of plant diseases, indicating that fungi are more prevalent as soil-borne pathogens compared to bacteria. The majority of these species target roots, resulting in the complete or partial destruction of plants and crops, manifesting through symptoms such as root rot, yellowing, wilting, dieback, stunting, stem collar issues, and crown rot. *Rhizoctonia*, *Phytophthora*, *Fusarium*, *Pythium*, *Alternaria*, *Cylindrocladium*, *Sclerotinia*, and *Sclerotium* are some of the most prevalent fungi [4,5]. *Sclerotium bataticola*, which is also referred to as *Macrophomina phaseolina*, is a fungus that is transmitted through the soil and causes charcoal rot in a variety of plants, including corn, sunflowers, soybeans, potatoes, and sweet potatoes [6]. Such challenges necessitate that researchers and investigators provide effective solutions to guarantee national food security [7]. In the

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context of sustainable agriculture, disease management and the improvement of crop yield and quality are the primary challenges; therefore, it is essential to employ methods based on natural sources to prevent environmental pollution and protect the health of animals and humans [8].

Microbial natural compounds, particularly secondary metabolites from living organisms, have a wide chemical range and the potential to discover new molecules with novel activities [9]. Numerous organisms in the natural environment, including bacteria, synthesize antimicrobial compounds to compete with other organisms [10]. A multitude of these metabolites exhibit extensive antimicrobial properties against several phytopathogenic microorganisms, encompassing bacteria, fungi, viruses, and nematodes [11–13]. The *Bacillus* genus produces a wide range of antimicrobial secondary metabolites. These bacteria exhibit many metabolic pathways for natural product synthesis, which hold significant potential as biocontrol agents in agricultural applications [14]. Several *Bacillus* species, including *B. velezensis*, *B. licheniformis*, *B. mojavensis*, *B. subtilis*, and *B. amyloliquefaciens* capable of competing with various phytopathogens, enhancing plant growth, and acting as bio-fertilizers, and bio-fungicides [1,14,15]. *Bacillus* species combat fungal infections through a variety of mechanisms, including antibiotic production, lytic enzymes, siderophores, and direct competition for resources and habitats [16,17]. In addition, they produced several volatile organic compounds that could suppress fungal growth. These include alkenes, alcohols, benzenoids, terpenoids, ketones, sulfur-containing compounds, and more [18].

Volatile organic compounds (VOCs) emitted by bacteria can support plants in various ways, including enhancing growth, activating defense responses, and suppressing or eliminating harmful pathogens [19]. The VOCs produced by *B. subtilis* may help reduce post-harvest fruit diseases, as they possess the ability to hinder the mycelial growth and spore germination of specific pathogenic fungi [20]. Similarly, the VOCs of *B. subtilis* PPCB001 and *B. velezensis* PEA1 inhibited the growth of *Penicillium crustosum* and *Fusarium oxysporum*, respectively [21,22]. *Bacillus subtilis* TB09 and TB72 generated VOCs, including β-benzeneethanamine, nonan-2-one, and 2-methyl-1,4-diazine, which efficiently suppressed the anthracnose pathogen in post-harvest mangoes [23]. Furthermore, *B. amyloliquefaciens* L3 and *B. mojavensis* I4 produce VOCs with antifungal properties against post-harvest *Fusarium* wilt, *Fusarium* species, and *Rhizoctonia* species, as well as promoting growth in *Arabidopsis* plants [24,25]. Recently, *B velezensis* HNA3 exhibited antagonistic activity as a biocontrol agent against *Cladosporium cladosporioides*, *Alternaria alternata*, *Monilinia fructicola*, *P. expansum*, and *F. oxysporum* [1]. 9-Octadecenoic acid (z)-, methyl ester was the key VOC produced by HNA3 followed by heptadecanoic acid, methyl ester, and hexadecanoic acid, methyl ester. Gas chromatography-mass spectrometry (GC-MS) is an effective analytical method frequently utilized for the detection and identification of diverse compounds in biological samples [26]. These substances include microbial cells and their metabolites, such as phenolic compounds, fatty acids, and aldehydes [18,26].

In this context, the current study aims to isolate and identify certain *Bacillus* species in order to select a potential biocontrol agent against the soil-borne fungus *S. bataticola*, the causative agent of charcoal rot disease in various plants. The molecular identification of the isolated bacterial strains was conducted through 16S rRNA gene sequencing and subsequent analysis. A comparative analysis was performed utilizing GC-MS on the ethyl acetate extract derived from bacterial culture filtrate to identify bacterial secondary metabolites and volatile organic compounds linked to antifungal activity.

2. Materials and Methods

2.1. Rhizobacterial *Bacillus* spp. isolation

Samples of soil were gathered from the rhizospheres of healthy potato (*Solanum tuberosum* L.) plants in open locations throughout the Kingdom of Saudi Arabia. The intact root systems were excavated, and the loose soil adhering to the rhizosphere was carefully removed by shaking. Soil samples were collected and directly transferred to the microbiology laboratory for bacterial isolation. A soil suspension was made by combining one gram of soil sample with 10 mL of sterile distilled water and stirring it. Three plate copies were created using 200 µL of each dilution (10^{-5}, 10^{-6}, and 10^{-7}) on nutrient agar (NA) media. For 24 to 48 hours, the plates were incubated at 28 ± 2 °C. Using a single colony isolation approach, numerous colonies were separated and purified on nutrient agar plates. The samples were then stored in glycerol at -20 °C for future use [27].

2.2. Evaluating the fungicidal characteristics of *Bacillus* isolates

The *S. bataticola* fungus, responsible for charcoal

rot disease, was obtained from the Department of Plant Protection, City of Scientific Research and Technological Applications (SRTA-City), Egypt. The *S. bataticola* was cultured on potato dextrose agar (PDA) medium for 6 days at 28 °C following inoculation. The dual culture technique was employed to assess the antagonistic effects of bacterial isolates on fungal growth inhibition percentages [22,28]. In brief, fungal discs, with a diameter of 5 mm, were positioned on the left side of a petri dish that contained PDA media. Bacterial samples were introduced sequentially from the uppermost to the lowermost region of the right side of the plate. For 7 days, the plates were incubated at 28 ± 2 °C. The radius of the fungal colony that emerged from the agar disc, positioned on the left side, served as a control for measurement. The formula for the growth inhibition rate (%) = [(R-r)/R] × 100 was used to find the percentage of inhibition in mycelium growth. Here, R stands for the control plate radius measurement of the fungal colony, and r for the radius of fungal growth towards the bacterial treatment.

2.3. Molecular characterization of the selected bacterial isolates

By amplifying and sequencing the 16S ribosomal RNA (rRNA) gene, the antagonistic bacterial isolates with antifungal activity were molecularly identified. These isolates might be used as biological control agents. Using a bacterial genomic purification kit, total genetic material was extracted, quantified, and adjusted until it reached 100 ng/µL of DNA. Primers 27F and 1492R, which are universal bacterial primers, were used to amplify the 16S rRNA gene. The 20 µL PCR reaction mixture comprised 1 µL of each primer (10 pM), 1 µL of template DNA, and 10 µL of 2x PCR master mix. The PCR amplification was carried out using a thermal cycler, as previously described [29]. The amplified 16S rRNA was subjected to electrophoresis for separation on an agarose gel. After the purification of PCR results, the products were subjected to direct sequencing using the Sanger technique. The 16S rRNA sequences were analyzed using the NCBI-BLAST tool accessible at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The phylogenetic tree was constructed utilizing MEGA 11 software, applying neighbor-joining model and conducting bootstrap analysis with 2000 replications [30].

2.4. Ethyl acetate extract preparation and GC-MS analysis

To determine the secondary metabolites produced by bacterial isolates, such as VOCs, a broth culture of each strain was centrifuged at 3000 rpm for 10 minutes after growing for 48 hours in nutrient broth media. The supernatant was then collected and combined with ethyl acetate as a solvent in a 1:1 (vol/vol) ratio. The mixture was vigorously shaken using an orbital shaker at a speed of 200 rpm for 50 minutes, resulting in the separation into an ethyl acetate phase and an aqueous phase. The ethyl acetate phase was extracted from the aqueous phase utilizing a separating funnel. A rotary evaporator evaporated the ethyl acetate at 50 °C from the ethyl acetate phase, resulting in a concentrated extract. The bacteria-free nutrient broth media and an ethyl acetate extract were employed as controls. The experiment was repeated three times. The extracts containing secondary metabolites were analyzed using a GC-MS instrument (TRACE 1300 Series, Thermo, USA). Helium served as the carrier gas at a constant flow rate of 1 mL per minute, with a sample injection volume of 1 mL. The injector and hot oven were maintained at 250 °C and 110 °C, respectively, with the temperature rising by 10 °C per minute until reaching 200 °C, then by 5 °C per minute until attaining 280 °C and thereafter shutting down after 9 minutes at 280 °C. The retention times of various chemical peaks eluted from the GC column were documented. A search was conducted inside the GC-MS library for compounds exhibiting similar molecular weights and retention times after the alignment of the data with the compounds' mass spectra [18,31].

2.5. Data analysis

The data is shown as an average value (mean ± SD), with its standard deviation. The collected data underwent a one-way analysis of variance (ANOVA) utilizing CoStat software (version 6.45; https://cohortsoftware.com/costat.html; 2022) for statistical evaluation. The Tukey's honest significant differences (HSD) test was employed to assess differences and ascertain the statistical significance of the procedure at a significance level of $p \leq 0.05$. Statistical significance was indicated by letters ordered in descending order, reflecting the association (a > b > c). The analogous letters signified no substantial change.

3. Results

3.1. Isolating, screening, and selecting antagonistic *Bacillus* strains

From the rhizosphere of potato plants, seventeen bacterial isolates could be isolated and purified. In the

initial assessment for antifungal activity, only five isolates (29.4%), designated KSAS15, KSAS16, KSAS17, KSAS18, and KSAS19, showed the ability to antagonize *S. bataticola*. Of the remaining isolates (70.6%) did not influence the growth of *S. bataticola*. The KSAS20 isolate, which exhibited no antifungal activity against *S. bataticola*, was chosen for comparison of its secondary metabolites with other biocontrol isolates. Subsequently, the antifungal effectiveness of the sex bacterial isolates was evaluated. Figure 1 presents the data of growth and growth inhibition percentages exhibited by various bacterial isolates. In comparison to the control, there was no significant difference in fungal growth between isolate KSAS20 and the control, as both treatments achieved the maximum fungal growth of 8.5 cm. The greatest percentage of growth inhibition was reported with isolate KSAS17 at 31.37 ± 0.56%, followed by KSAS15 and KSAS18 with inhibition percentages of 26.67% and 26.27%, respectively. KSAS19 demonstrated a lower inhibition percentage of 16.86%, whereas KSAS20 indicated a 0% inhibition growth percentage.

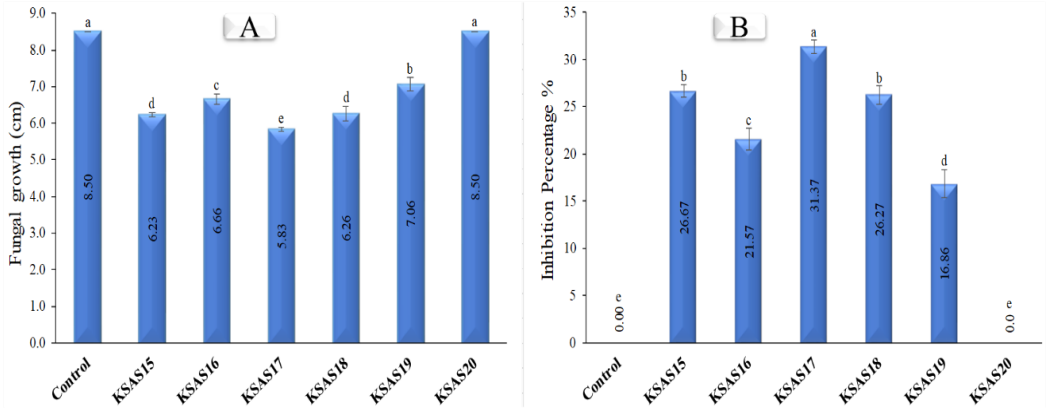

Fig. 1. Antifungal efficacy of *Bacillus* isolates KSAS15, KSAS16, KSAS17, KSAS18, KSAS19, and KSAS20 against *Sclerotium bataticola*. The isolates' impact on fungal growth is shown on the left (A), while the percentage of mycelium growth inhibition is shown on the right (B). The graph's columns represent the mean values of three replicated measurements, and the bars denote the standard deviation. Tukey's HSD test, utilizing a significance threshold of 0.05, indicates that values within each column sharing the same letter (a/b/c/d/e) do not demonstrate statistically significant differences.

3.2. Molecular characterization of bacterial isolates

In the isolation and purification of bacterial strains, only those displaying the distinct morphological characteristics of *Bacillus* spp. were chosen. The borders of these colonies displayed irregular and flattened characteristics, with appearances that were floury, waxy, dry, or creamy, and tonal qualities of white, matte, or shiny finishes. The PCR experiment produced amplicons measuring approximately 1500 base pairs for the 16S rRNA gene. The NCBI-BLAST alignment of a nucleotide sequence approximately 1450 bp in length indicated that the six isolates exhibited 98.26% to 99.79% homology with *Bacillus* spp. The three isolates (KSAS15, KSAS16, and KSAS19) demonstrated the highest similarity percentages with *B. licheniformis*, as shown in Table 1. The two isolates, KSAS17 and KSAS18, exhibited significant similarity to other *B. cereus* isolates. The isolate KSAS20 demonstrated the highest percentage identity with *Lysinibacillus fusiformis* at 99.44% (Table 1). Sequences with annotations have been added to GenBank and assigned the accession numbers PQ340921-PQ340926 for the bacterial isolates KSAS15-KSAS20, respectively (Table 1). Based on the 16S rRNA gene sequences, the constructed phylogenetic tree demonstrates that all six candidate bacterial isolates are classified within *Bacillus* spp., as they exhibit relatedness to the mentioned bacterial species in the phylogenetic analysis (Figure 2). The phylogenetic tree revealed that six isolates of *Bacillus* sp. were categorized into three primary groups. *Bacillus* strains KSAS17 and KSAS18 were in Group I and were closely related to *B. cereus* reference strains. *Bacillus* strain KSAS20 was in Group II and was linked to *Lysinibacillus fusiformis* reference strains. The *Bacillus* strains KSAS15, KSAS16, and KSAS19 were found to be identical to *B. licheniformis*, categorizing them within group III.

3.3. Identification of secondary metabolites-produced *Bacillus* strains

The GC-MS analysis showed that the culture filtrates of the six bacterial strains have 43 known compounds. These compounds included several biologically active aliphatic and aromatic compounds. Figure 3 shows the

GC-MS chromatography analysis. Table 2 lists the primary and abundant constituents, along with their respective concentrations (peak area), found in the crude extracts. The obtained data revealed that the KSAS17 that showed the highest antifungal activity produced 16 compounds.

Table 1. Molecularly identified *Bacillus* isolates: species name, sequence length, and GenBank accession number.

Strain	Species name	Sequence length	Accession number	Closest strain in GenBank	Percentage identity
KSAS15	*B. licheniformis*	1444 bp	PQ340921	*B. licheniformis*	98.89 %
KSAS16	*B. licheniformis*	1444 bp	PQ340922	*B. licheniformis*	98.26 %
KSAS17	*Bacillus cereus*	1449 bp	PQ340923	*Bacillus cereus*	99.24 %
KSAS18	*Bacillus cereus*	1453 bp	PQ340924	*Bacillus cereus*	99.79 %
KSAS19	*B. licheniformis*	1450 bp	PQ340925	*B. licheniformis*	98.55 %
KSAS20	*Lysinibacillus fusiformis*	1442 bp	PQ340926	*Lysinibacillus fusiformis*	99.44 %

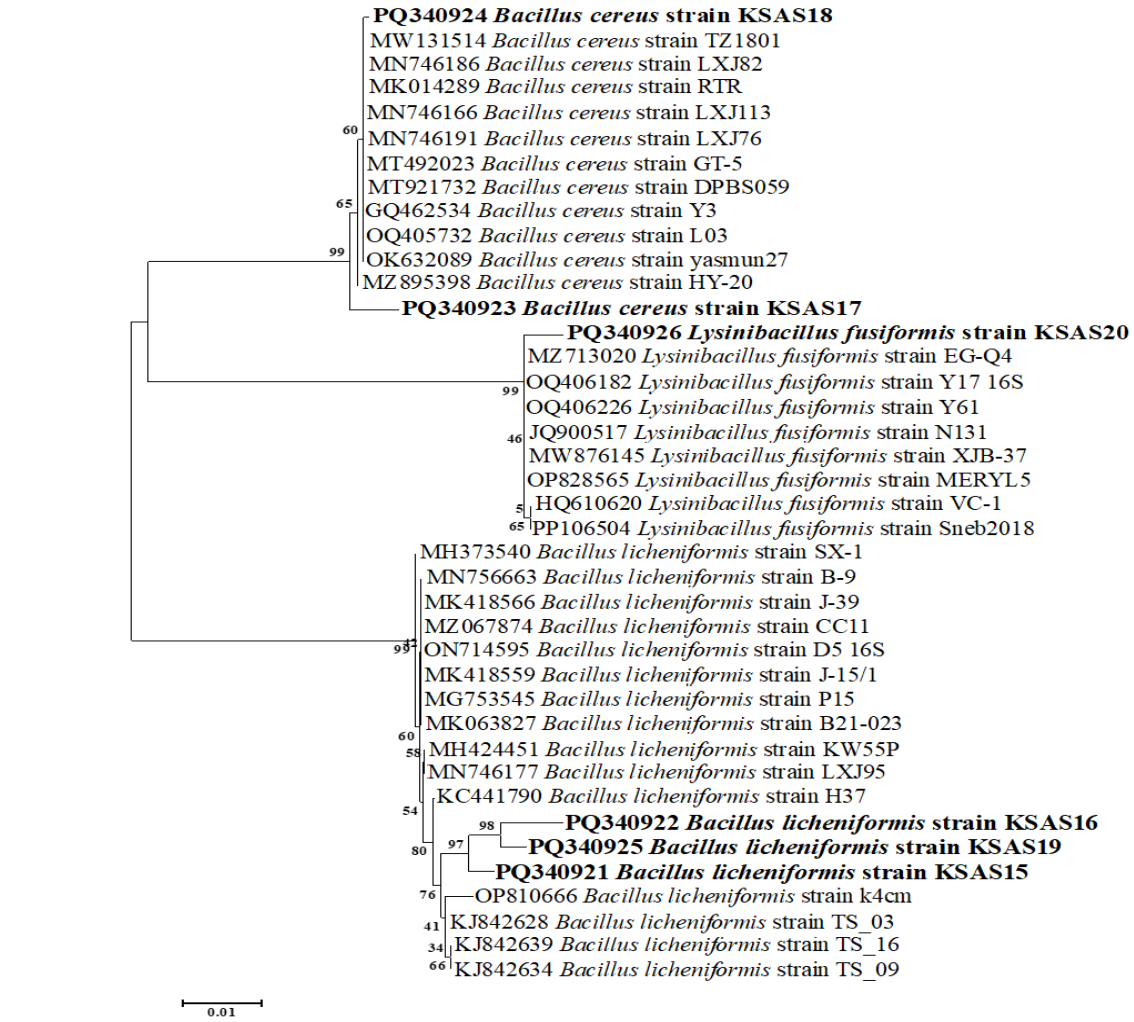

Fig. 2. A phylogenetic tree illustrates the relationships among the identified *Bacillus* strains (KSAS15, KSAS16, KSAS17, KSAS18, KSAS19, and KSAS20) and other isolates from GenBank. The phylogenetic tree is constructed from the nucleotide sequence of the 16S rRNA gene.

The loperamide; pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-; and 9,12-Octadecadienoic acid(z,z)-,2,3-bis[(trimethylsilyl)oxy]propyl ester were the highest three detected compounds with 9.05, 24.27, and 29.79%, respectively. Among the 16 compounds analyzed, four unique compounds were identified only in the secondary metabolites of this bacterial strain. These compounds include 2-cyclohexen-3-ol-1-one,2-[11-tetradecenoyl]-; 1-linolenoylglycerol, 2TMS derivative; 3',8,8'-trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone; and Methyl(1-O-retinyl-2,3,4-triacetyl-á-D-glucopyran) uronate (Table 2). Among the 16 compounds produced by KSAS15, the most abundant detected compounds were hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl

ester; hexadecanoic acid; diisooctyl phthalate; and pentadecanoic acid, with peak areas of 9.63%, 11.17%, 18.09%, and 19.89%, respectively. Similarly, the compounds 2,8,9-Trioxa-5-aza-1-silabicyclo[3.3.3]undecane, 1-ethenyl-; 10-methoxy-nb-à-methylcorynantheol; and heptadecanoic acid were exclusively identified in KSAS15, exhibiting percentages of 1.89, 5.11, and 6.09%, respectively (Table 2). Of the 19 compounds produced by KSAS18, the most significant detected compounds were hexadecanoic acid, 2,3-dihydroxypropyl ester; phthalic acid, butyl undecyl ester; pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-; 3,4-dihydro-2h-1,5-(3"-t-butyl)benzodioxepine; and diisooctyl phthalate, with peak areas of 5.85, 6.45, 6.61, 7.80, and 44.87%, respectively. Similarly, oleic acid was exclusively identified in KSAS18 at a concentration of 2.38 % (Table 2).

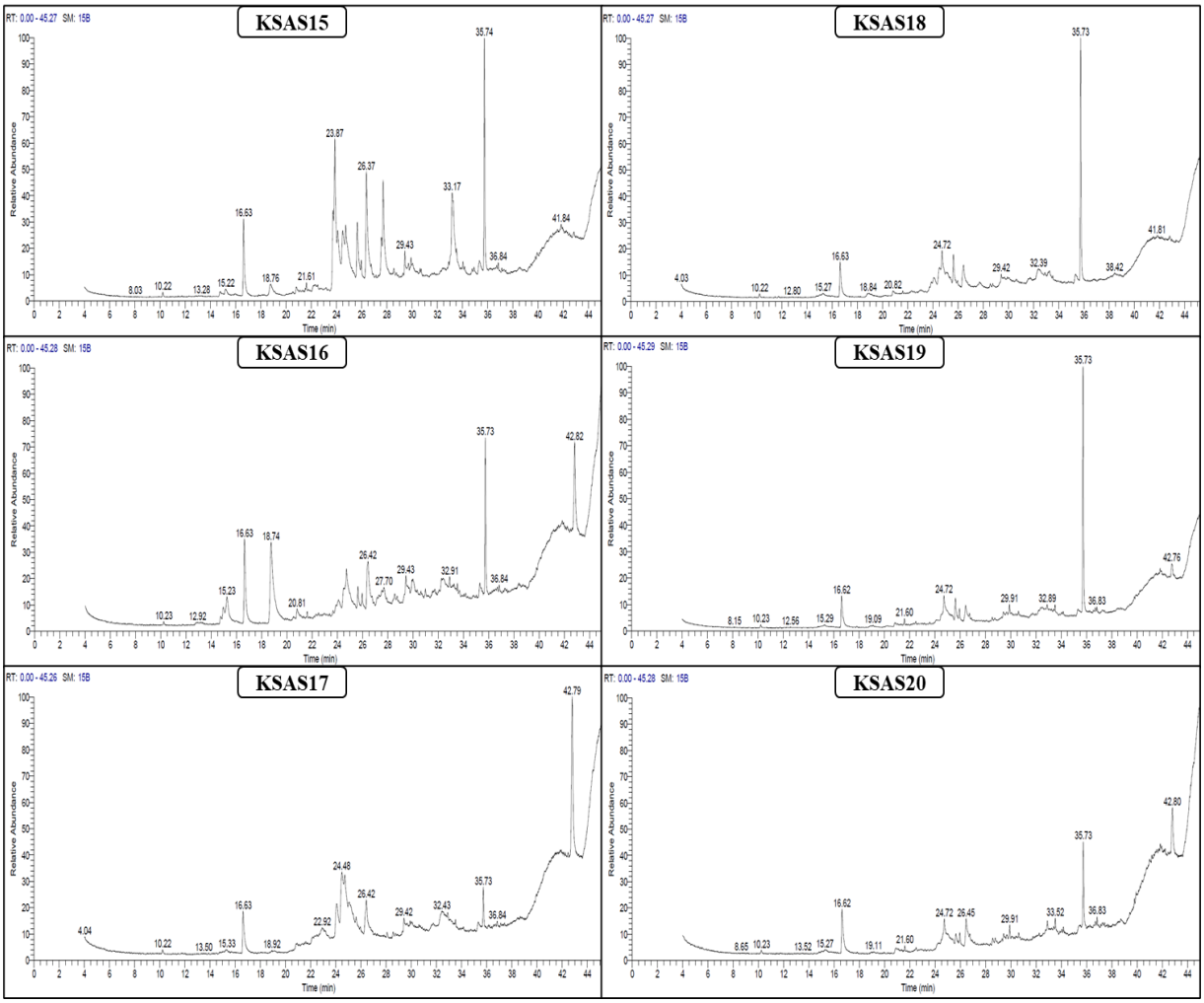

Fig. 3. GC-MS fractionation of ethyl acetate extract of the culture filtrates of *Bacillus* strains KSAS15, KSAS16, KSAS17, KSAS18, KSAS19, and KSAS20.

Among the 19 compounds synthesized by KSAS16, the most notable identified compounds are 3,4-dihydro-2h-1,5-(3"-t-butyl)benzodioxepine; diisooctyl phthalate; and 9,12-octadecadienoic acid (z,z)-,2,3-bis[(trimethylsilyl)oxy]propyl ester, exhibiting peak areas of 10.66%, 15.93%, and 23.05%, respectively. Cyclopropaneoctanoic acid, 2-octyl-, methyl ester; 3-Octadecyldihydro-2,5-furandione; and 5-fluoro-2,2-dimethylchroman-4-one were uniquely detected in KSAS16 at concentrations of 1.33%, 1.99%, and 9.46%, respectively (Table 2). Loperamide; 3,4-dihydro-2h-1,5-(3"-t-butyl)benzodioxepine; dotriacontane; and diisooctyl phthalate were the most significant compounds among the 17 compounds synthesized by KSAS19, with peak areas of 6.94, 6.96, 7.14, and 46.51%, respectively. However, only KSAS19 included the following compounds: phenyldimethylthiophene; 7,8-dihydroxydiacetoxyscirpenol; and phthalic acid, butyl 8-methylnonyl ester, at concentrations of 0.65, 0.85, and 5.10%, respectively (Table 2)

Table 2. The main constituents of secondary metabolites and their concentrations (peak area %) produced by *Bacillus* strains KSAS15, KSAS16, KSAS17, KSAS18, KSAS19, and KSAS20 that were identified through GC-MS analysis.

Detected Compound	Area %						Molecular formula	Molecular weight
	KSAS15	KSAS16	KSAS17	KSAS18	KSAS19	KSAS20		
1-Dodecanol, 3,7,11-trimethyl-	----	----	----	0.48	----	0.56	$C_{15}H_{32}O$	228
1-Hydroxycyclododecanecarbonitrile	----	4.81	----	1.00	----	----	$C_{13}H_{23}NO$	209
1-Tetradecanol	2.22	----	----	1.39	0.80	----	$C_{14}H_{30}O$	214
2-acetyl-3-(2-cinnamido)ethyl-7-methoxyindole	----	----	----	----	1.24	3.06	$C_{22}H_{22}N_2O_3$	362
3,4-dihydro-2h-1,5-(3"-t-butyl)benzodioxepine	8.69	10.66	6.92	7.80	6.96	6.40	$C_{13}H_{18}O_2$	206
3,4-dimethoxyphenethyl-n,n-bis(trimethylsilyl)amine	----	1.97	2.31	1.49	----	----	$C_{16}H_{31}NO_2Si_2$	325
3,5A,9,9-tetramethyldecahydrobenzo[2,3]cyclohepta[1,2-b]oxiren-3-ol	----	----	----	0.88	----	0.53	$C_{15}H_{26}O_2$	238
4h-1-benzopyran-4-one,2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-	----	----	----	----	1.50	6.73	$C_{18}H_{16}O_7$	344
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	2.40	----	----	3.97	3.94	3.27	$C_{17}H_{24}O_3$	276
9,12-Octadecadienoic acid(z,z)-,2,3-bis[(trimethylsilyl)oxy] propyl ester	1.51	23.05	29.79	----	----	26.73	$C_{27}H_{54}O_4Si_2$	498
Androst-4-en-3-one, 17-methoxy-,3-methoxime, (17á)-	----	----	3.69	2.33	----	----	$C_{21}H_{33}NO_2$	331
Diisooctyl phthalate	18.09	15.93	----	44.87	46.51	10.72	$C_{24}H_{38}O_4$	390
Docosane	0.64	----	----	----	4.55	2.09	$C_{22}H_{46}$	310
Dotriacontane	2.19	3.06	1.46	1.33	7.14	5.62	$C_{32}H_{66}$	450
Ergotamine	----	----	----	5.13	1.22	1.51	$C_{33}H_{35}N_5O_5$	581
Hexadecanoic acid	11.17	3.20	----	1.53	----	----	$C_{16}H_{32}O_2$	256
Hexadecanoic acid,1-(hydroxymethyl)-1,2-ethanediylester	9.63	2.29	----	3.33	----	----	$C_{35}H_{68}O_5$	568
Hexadecanoic acid,2,3-dihydroxypropyl ester	2.23	5.21	----	5.85	4.99	----	$C_{19}H_{38}O_4$	330
Isochiapin B	0.90	0.78	1.05	0.78	4.64	11.23	$C_{19}H_{22}O_6$	346
Loperamide	----	1.90	9.05	----	6.94	12.46	$C_{29}H_{33}ClN_2O_2$	476
Pentadecanoic acid	19.89	2.14	----	----	----	----	$C_{15}H_{30}O_2$	242
Phen-1,4-diol,2,3-dimethyl-5-trifluoromethyl-	----	----	1.23	----	----	1.78	$C_9H_9F_3O_2$	206
Phthalic acid, butyl undecyl ester	----	2.74	----	6.45	----	----	$C_{23}H_{36}O_4$	376
Pregn-4-ene-3,20-dione,17,21-dihydroxy-,bis(O-methyloxime)	----	5.29	1.28	3.47	----	0.42	$C_{23}H_{36}N_2O_4$	404
Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	6.31	4.29	24.27	6.61	0.97	0.79	$C_{11}H_{18}N_2O_2$	210
Rhamnetin-3-O-beta-D-glucoside	----	----	0.90	----	2.17	0.61	$C_{27}H_{30}O_{16}$	610
Tetraneurin-A-diol	----	----	1.01	----	----	0.96	$C_{15}H_{20}O_5$	280
2,8,9-Trioxa-5-aza-1-silabicyclo[3.3.3]undecane, 1-ethenyl-	1.89	----	----	----	----	----	$C_8H_{15}NO_3Si$	201
10-methoxy-nb-à-methylcorynantheol	5.11	----	----	----	----	----	$C_{21}H_{29}N_2O_2$	341
Heptadecanoic acid	6.09	----	----	----	----	----	$C_{17}H_{34}O_2$	270
Cyclopropaneoctanoic acid, 2-octyl-,methyl ester	----	1.33	----	----	----	----	$C_{20}H_{38}O_2$	310
3-Octadecyldihydro-2,5-furandione	----	1.99	----	----	----	----	$C_{22}H_{40}O_3$	352
5-fluoro-2,2-dimethylchroman-4-one	----	9.46	----	----	----	----	$C_{11}H_{11}FO_2$	194
2-Cyclohexen-3-ol-1-one,2-[11-tetradecenoyl]-	----	----	1.02	----	----	----	$C_{20}H_{32}O_3$	320
1-Linolenoylglycerol, 2TMS derivative	----	----	4.38	----	----	----	$C_{27}H_{52}O_4Si_2$	496

3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	----	----	5.90	----	----	----	$C_{28}H_{25}NO_7$	487
Methyl(1-O-retinyl-2,3,4-triacetyl-á-D-glucopyran)uronate	----	----	6.69	----	----	----	$C_{33}H_{46}O_{10}$	602
Oleic acid	----	----	----	2.38	----	----	$C_{18}H_{34}O_2$	282
Ethyldimethylthiophene	----	----	----	----	0.65	----	$C_8H_{12}S$	140
7,8-dihydroxydiacetoxyscirpenol	----	----	----	----	0.85	----	$C_{19}H_{26}O_9$	398
Phthalic acid, butyl 8-methylnonyl ester	----	----	----	----	5.10	----	$C_{22}H_{34}O_4$	362
1,3-propanediol,2-methyl-2-(1-methylpropyl)-, dicarbamate	----	----	----	----	----	0.73	$C_{10}H_{20}N_2O_4$	232
1,4-naphthalenedione,4a,5,8,8a-tetrahydro-2-methoxy-4a,8-dimethyl-	----	----	----	----	----	3.62	$C_{13}H_{16}O_3$	220

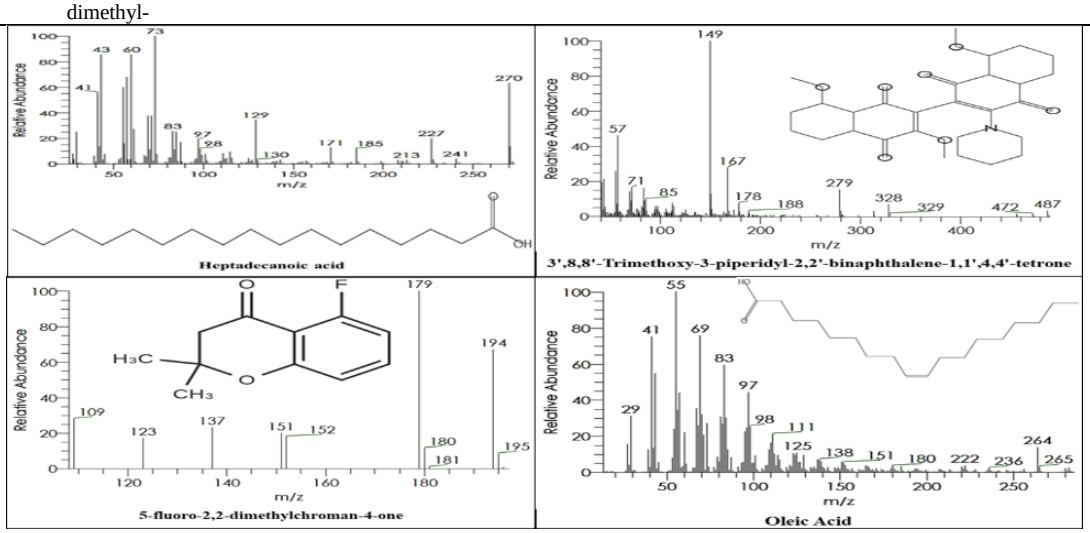

Fig. 4. Chemical structure of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-, mass spectral analysis using GC-MS and its peak area (%) in culture filtrate extract of different *Bacillus* strains.

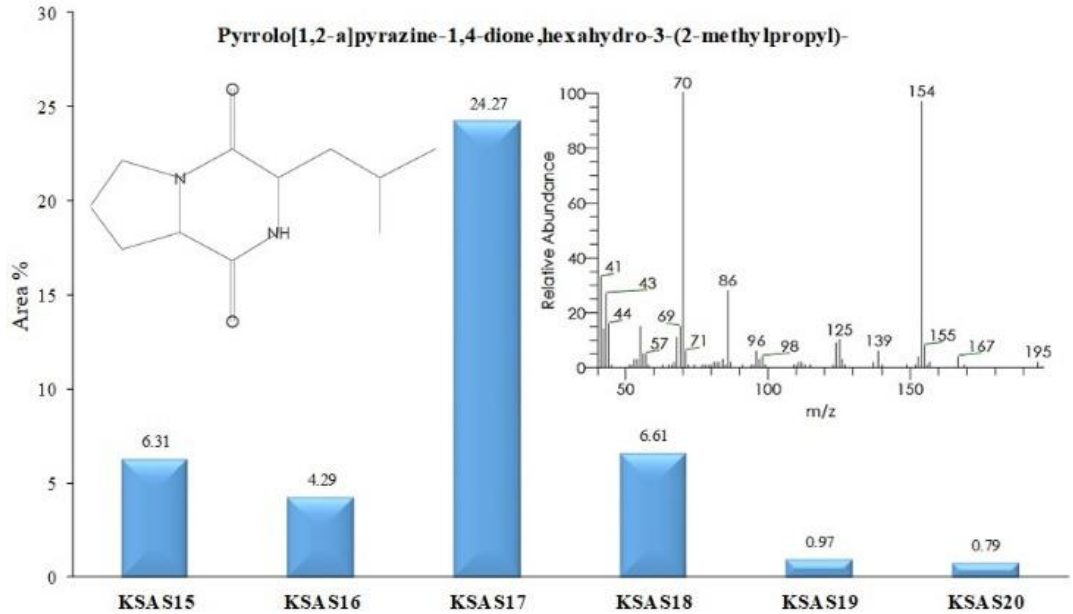

Fig. 5. Chemical structures and mass spectral analysis using GC-MS of heptadecanoic acid; 3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone; 5-fluoro-2,2-dimethylchroman-4-one; and oleic acid compound detected in the culture filtrate extract of different Bacillus strains.

The KSAS20 yielded 20 compounds, demonstrating it produced the highest number of compounds among other strains. Diisooctyl phthalate; isochiapin B; loperamide; and 9,12-Octadecadienoic acid(z,z)-,2,3-bis[(trimethylsilyl)oxy]propyl ester exhibited the highest peak areas of 10.72%, 11.23%, 12.46%, and 26.73%, respectively. Furthermore, this

strain's extract revealed the presence of unique 1,3-propanediol, 2-methyl-2-(1-methylpropyl)-, dicarbamate, and 1,4-naphthalenedione, 4a,5,8,8a-tetrahydro-2-methoxy-4a,8-dimethyl- at concentrations of 0.73% and 3.62%, respectively (Table 2).

Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl) (PPDHMP) is a pyrrolizidine chemical with a molecular formula of $C_{11}H_{18}N_2O_2$ and a molecular weight of 210. Figure 4 illustrates its chemical structure and mass spectral analysis using GC-MS. The obtained data indicated that KSAS17 yielded the highest concentration of PPDHMP at 24.27%, followed by KSAS15, KSAS18, and KSAS16 with 6.31%, 6.61%, and 4.29%, respectively (Figure 4). On the other hand, thelowest concentration of PPDHMP was reported with KSAS20 at 0.79%. Heptadecanoic acid is a saturated fatty acid with 17 carbon atoms, a molecular weight of 270 g/mol, and a chemical formula of $C_{17}H_{34}O_2$. Figure 5 presents the chemical structure alongside the mass spectral analysis. The GC-MS analysis indicated that strain KSAS15 was the sole producer, exhibiting a peak area of 6.09% among the examined bacterial strains. 5-fluoro-2,2-dimethylchroman-4-one is a cyclic-aromatic compound with the chemical formula $C_{17}H_{34}O_2$ and a molecular weight of 270. Figure 5 presents the chemical structure in conjunction with the mass spectral analysis. Strain KSAS16 was identified as the exclusive producer among the examined bacteria, exhibiting a peak area of 9.46% in the GC-MS analysis. 3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone is a phenolic molecule characterized by the chemical formula $C_{28}H_{25}NO_7$ with a molecular weight of 487. The chemical structure and mass spectrum analysis are shown in Figure 5. With a peak area of 5.90% in the GC-MS analysis, strain KSAS17 was shown to be the sole producer among the bacteria that were investigated. Oleic acid possesses a molecular weight of 282 and is classified as a monounsaturated fatty acid, represented by the formula $C_{18}H_{34}O_2$. Figure 5 presents the chemical structure and mass spectrum analysis. Among the tested strains, KSAS18 was identified as the exclusive producer, exhibiting a peak area of 2.38%.

4. Discussion

The extensive use of synthetic chemicals for control purposes causes environmental harm, adverse effects on human health, and disease resistance development [32,33]. Beneficial microbes and their secondary metabolites, which are known for their abundance, ability to produce biological control agents, and ecologically desirable features, are gaining popularity as a potential alternative to chemical pesticides [34]. The *Bacillus* genus is recognized as a biological control agent due to its several attributes, including antibiotic production, spore formation, promotion of plant growth, and soil resilience. Numerous studies have validated the efficacy of *Bacillus* strains as biological control agents for phytopathogens [14,35]. The present investigation evaluated the antifungal properties of six different *Bacillus* isolates against *S. bataticola* using the dual culture method. The results showed that the percentage growth inhibition varied from 0% to 31.37%. The KSAS17 exhibited the most significant activity, while the KSAS20 showed the least antagonistic activity. The 16S rRNA gene is widely used as a trustworthy molecular marker for phylogenetic identification because of its conserved region, which serves as a unique sequence relative to many species [31,36]. The NCBI-BLAST analysis and phylogenetic tree of the annotated sequence of the 16S rRNA gene indicated that the isolated bacterial strains are related to *Bacillus* species. Three strains (KSAS15, KSAS16, and KSAS19) were classified as *B. licheniformis*. Two strains, KSAS17 and KSAS18, were classified as *B. cereus*. The final strain, KSAS20, was classified as *Lysinibacillus fusiformis*. The findings indicate that *B. cereus* strain KSAS17 is significantly effective as a biological control agent against *S. bataticola* infestation. The production of antipathogenic secondary metabolites is the most recognized and essential mechanism via which antagonistic bacteria suppress plant diseases [37]. So, in this study, we used GC-MS to look for biologically active molecules that might have antifungal properties in the culture filtrate of the tested bacterial isolates.

Pyrrolo 1,2-a pyrazine is a category of commonly used natural antibiotics [38]. Pyrrole is an organic heterocyclic molecule characterized by a five-membered unsaturated ring structure consisting of four carbon atoms and one nitrogen atom [39]. Pyrrol derivative compounds have been documented to exhibit notable antioxidant activity, nematocidal activity, and antimicrobial activity [40–42] Pyrrolopyrazines have a broad spectrum of biologically active substances, which include antitumor, anticoagulant, antioxidant, antibacterial, and antifungal properties [43]. Such inhibition could result from interference with microbial cell membranes, reduction of enzymatic activity, inhibition of cell wall synthesis, or disruption of protein and nucleic acid synthesis [44]. The *Streptomyces* and *Bacillus* species, including *B. tequilensis*, along with the fungus *Mortierella alpina*, demonstrated effective control over multidrug-

resistant *Staphylococcus aureus*, *P. aeruginosa*, *E. coli*, and *Enterococcus faecalis* through the production of PPDHMP [45,46]. *Staphylococcus xylosus* produces PPDHMP and demonstrated antibacterial potential against *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus* [47]. PPDHMP isolated *Nocardiopsis* sp. GRG 1 demonstrates the ability to inhibit biofilm formation by *Proteus mirabilis* and *E. coli*, while also reducing the viability of established biofilms [48]. PPDHMP and Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)] can protect plants from worms and phytopathogens [49]. The GC-MS analysis of the crude extract of *B. pumilus* MMM indicates the presence of pyrrolo[1,2-a]pyrazine-1,4-dione derivatives that inhibit *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [39]. Furthermore the GC-MS analysis revealed that the antifungal efficacy of the crude extract from *Bacillus* sp. WG4 against *Pythium myriotylum* is attributed to the presence of pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro derivative compounds [50]. In addition, *B. amyloliquefaciens*-generated PPDHMP had inhibitory efficacy against *Alternaria macrospora*, a phytopathogen fungus causing leaf spot in Bt cotton [51]. As a result, the identification of PPDHMP in the KSAS17 extract at the highest concentration (24.3%) indicates that this compound may play a role in the antifungal activity observed. The results suggest that PPDHMP has the potential to inhibit fungal growth and may be developed as an antifungal biocontrol agent.

Numerous researchers have demonstrated the significant biological effects of naturally occurring aromatic compounds, including their ability to inhibit tumor growth, protect the liver from toxicity, reduce inflammation, and even kill microorganisms [52,53]. Phthalic acid, a dicarboxylic acid, is a derivative of benzoic acid and is recognized for its potential in combating fungal infections. It has been proposed that derivatives of phthalic acid may have applications in the treatment of chronic cardiovascular and cerebrovascular diseases, as well as exhibiting anti-tumor, anti-inflammatory, and antibacterial properties. The insecticidal, antibacterial, and allelopathic activities of phthalic acid esters imply that they may help plants, algae, and microorganisms compete more effectively by increasing their resistance to biotic and abiotic stress [54,55]. The butyl undecyl ester of phthalic acid, an intracellular metabolite derived from *B. subtilis* BS-01, exhibited significant antimycotic activity against *Alternaria solani* and was identified as the predominant component at 1.07% in the dichloromethane extract [55]. The present investigation revealed the presence of phthalic acid, butyl 8-methylnonyl ester, at a concentration of 5.10% in the crude extract of strain KSAS19, which has a low concentration of PPDHMP, which may confer upon KSAS19 the capacity to inhibit fungal growth at 16.9%. Plant extracts and other microorganisms, including *P. crustosum*, were found to contain the compound 3′,8,8′-Trimethoxy-3-piperidyl-2,2′-binaphthalene-1,1′,4,4′-tetrone, which has activity against certain harmful bacteria. It demonstrated a wide-ranging effect and potential action in anticancer, antimicrobial, immunomodulatory, and anti-inflammatory activities [56–60].

The GC-MS data revealed the presence of numerous compounds previously identified for their antioxidant and antibacterial characteristics, including oleic acid, hexadecanoic acid, 1-tetradecanol, and pentadecanoic acid [61]. Oleic acid was solely detected in bacterial strain KSAS18 at a quantity of 2.38%. Oleic acid, a prevalent monounsaturated fatty acid, is a natural compound that has recently gained popularity for its ability to prevent food oxidation and its significant antimicrobial potential against various fungi and bacteria [62–64]. Oleic acid, or 9-octadecenoic acid, is a beneficial omega-9 unsaturated fatty acid that significantly contributes to human health [65]. These encompass the regulation of cellular activities, the inhibition of cancer progression, inflammation reduction, oncogene expression, lowering of blood pressure, and the facilitation of wound healing [66,67]. Oleic acid derived from *Trichoderma asperellum* and *T. longibrachiatum* exhibited antifungal properties against *Fusarium xylarioides* [68]. Consequently, the production of oleic acid by KSAS18 may give this strain more antifungal activity. Similarly, hexadecanoic acid was exclusively detected in KSAS15 at a concentration of 6.09 %. Hexadecanoic acid is a carboxylic acid derivative known for its antibacterial and antifungal properties, serving as a natural preservative [69]. Hexadecanoic acid suppresses fungal growth by generating complex compounds that bind to active groups found in fungal cell walls. When hexadecanoic acid interacts with active groups in fungal cell walls, it modifies the outer ring structure (CO_2-OH) but not the core structure of chitin. This mechanism affects the integrity of the fungal cell wall, resulting in fungal cells being inhibited rather than killed [44]. Thus, the synthesis of hexadecanoic acid by KSAS15 may enhance the antifungal efficacy of this strain. 1-Tetradecanol is a straight-chain saturated fatty alcohol, often known as myristyl alcohol. It has anti-inflammatory and antibacterial properties. Because of its emollient qualities, it is a component of cosmetics like cold creams. Additionally, it serves as a chemical synthesis intermediary for other compounds, like surfactants [9,70]. Pentadecanoic acid is a specific example of a saturated long-chain fatty acid. The biofilms of various bacteria can be influenced by saturated fatty acids, which are prevalent natural molecules

consisting of carbon atoms ranging from 8 to 18. The processes by which fatty acids exert their effects vary depending on the types of cells that make up the biofilm. Some of these processes are changes in the fluidity of the cell membrane, a drop in the amount of extracellular polysaccharides, changes in the growth of fimbriae or hyphae, and the blocking of quorum sensing systems [71,72]. As a results, the synthesis of pentadecanoic acid by KSAS15 and KSAS16 may enhance the effectiveness of these strains in combating fungal infections.

5. Conclusions

The present investigation showed that the molecularly identified *B. cereus* strain KSAS17 (Accession number # PQ340923) had significant antifungal activity up to 31.4% against the causative agent of charcoal rot disease in various plants, *S. bataticola*. The GC-MS analysis of the ethyl-acetate extract from the bacterial culture filtrate identified pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) at a concentration of 24.3%, which is the molecule responsible for the antifungal action of KSAS17. Additionally, notable bioactive aliphatic and aromatic compounds were recognized, such as oleic acid, hexadecanoic acid, 1-tetradecanol, heptadecanoic acid, 5-fluoro-2,2-dimethylchroman-4-one, pentadecanoic acid, 3′,8,8′-trimethoxy-3-piperidyl-2,2′-binaphthalene-1,1′,4,4′-tetrone, and phthalic acid derivatives. The results suggest that the secondary metabolites produced by the rhizobacteria, particularly the *B. cereus* strain KSAS17, demonstrate antifungal properties and could be utilized as a biocontrol agent for the management of fungal diseases.

Conflicts of interest

There are no conflicts to declare.

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