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Antimicrobial Potential of an Actinomycete *Gordonia terrae* JSN1.9-Derived Orange Pigment Extract

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ABSTRACT

Actinomycetes are known to be a source of natural products and drugs. *Gordonia terrae*, an actinomycete pigment producer, shows potential in producing pigment with antimicrobial activity. This study aims to determine the antimicrobial activity of the active pigment fraction produced by the actinomycete *G. terrae*, assess the effects of the active pigment fraction on microbial cells, and identify the types of compounds present in the fraction. The pigment extract exhibited antimicrobial activity against Gram-positive bacteria and fungi. Specifically, it showed activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25293, and *Candida albicans* ATCC 10231. Furthermore, the study evaluated the antimicrobial activities of the active fractions, revealed the active fraction had better antimicrobial activity than the crude extract. Scanning electron microscopy (SEM) confirmed that the active fraction of the pigment causes damage to *B. subtilis* ATCC 6633 cells, inhibits the formation of filaments in *C. albicans* ATCC 10231, and alters the normal shape of the cells. LC-MS/MS results showed that the active fraction contained several compounds known for their antimicrobial activity. Among the dominant compounds identified were cholestyramine, aminopregnane, and sphinganine. Thus, this study demonstrated that the orange pigment extract derived from *G. terrae* JSN1.9 exhibits promising antimicrobial activity.

1. Introduction

Natural products became the main source for hundreds of years of treatment. Application of natural products for medicine and searching for new drugs are still active (Prateeksha *et al.* 2019). Microbes are one of the leading producers of valuable natural products. Various excellent drug was discovered from the natural products produced by microbes; microbes may produce valuable natural products like pigment (Demain 2014).

Pigments are bioactive compounds with wide applications; pigments have been discovered, showing antioxidant, anti-inflammatory, and antimicrobial properties (Ramesh *et al.* 2019). Pigment derived from microbes can be an alternative source of natural products for pharmaceutical purposes. A previous study reported a blue-green pigment from *P. aeruginosa* P1.S9 has a wide range of biological activities, including antibacterial, antioxidant, and cytotoxic (Wahyudi *et al.* 2022). Natural pigments from microbial are abundantly produced from bacteria including actinomycetes, and fungi. Among microbial pigments, actinomycete produced pigments with the potential for antimicrobial production. Actinomycetes are one of the important microbes that account for 70–80% of secondary metabolites available commercially (Parmar and Singh 2018) and can produce bioactive compounds and natural pigments (Ibrahim *et al.* 2023).

Actinomycetes produce various metabolites as pigments that are different in colors, such as blue, brown, green, orange, red, violet, yellow, black, etc. (Hemeda *et al.* 2022). Researchers have done many studies to isolate pigment-producing actinomycetes and screen for antimicrobial activity. It has been

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found that novel antimicrobial substances have been produced by actinomycetes (Baniya *et al.* 2019). Rare actinomycetes have attracted more attention with the hope of discovering new antibiotics, some antibiotics such as rifamycins, erythromycin, and teicoplanin discovered from rare actinomycetes (Ding *et al.* 2019). Orange pigment extracts derived from the rare actinomycete *Micromonospora tulbaghiae* SCA54.P2 have been reported to have antibacterial activity, especially against *Escherichia coli* ATCC 8739 (Mesrian *et al.* 2021).

Previous studies have successfully isolated a rare actinomycete named *G. terrae* JSN1.9 from rice leaves from Jasinga, Bogor, and reported antifungal activity against rice blast fungus (Harsonowati *et al.* 2017). The strain *G. terrae* JSN1.9 produce an orange intracellular pigment, and the antimicrobial potential has yet to be studied. Research on pigments from the rare actinomycetes genus in Indonesia still needs to be explored and reported, especially for its antimicrobial activity. Therefore, research on the potential of pigments as a source of compounds with antimicrobial activity must be carried out. This study aimed to determine the antimicrobial activity of the active pigment fraction derived from the actinomycete *G. terrae* JSN1.9, the effect of the active pigment fraction on microbial cells, and identify compounds that might contribute to its antimicrobial activity.

2. Materials and Methods

2.1. Materials

The materials used were pigmented actinomycetes *G. terrae* JSN1.9, this actinomycete was routinely cultured in International Streptomyces Project 2 (ISP2) medium (malt extract 10 g/L, yeast extract 4 g/L, and glucose 4 g/L). Microbes test *B. subtilis* ATCC 6633, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25293 were routinely cultured in Tryptic Soy Agar (TSA) (OXOID, United Kingdom) medium, and *C. albicans* ATCC 10231 was routinely cultured in Potato Dextrose Agar (PDA) (HIMEDIA, India) medium.

2.2. Pigment Production Media, Extraction, and UV-Vis Analysis

Pigment production was carried out on ISP2 medium. One liter of *G. terrae* JSN1.9 culture was incubated under shaking conditions (120 rpm) at 28°C for 10 days. The culture was centrifuged at 6,000 rpm for 15 minutes, and then the pellet was added with chloroform solvent and centrifuged again for 15 minutes. The supernatant was recovered, filtered, and evaporated using a vacuum evaporator at 40°C. Pigment characterization was carried out by recording the maximum adsorption. As much as 0.03 g of pigment was dissolved in 1 ml of chloroform, and its absorbance was measured at a wavelength range of 200-800 nm using UV/Visible Spectrophotometer (UV-Vis Hitachi U-2800) (Abubakar *et al.* 2022).

2.3. Antimicrobial Activity Test

The microbes test (*B. subtilis, E. coli, P. aeruginosa, S. aureus*, and *C. albicans*) were inoculated by adding 1% (v/v) inoculum into MHA/PDA medium. On the top of paper discs (6 mm) were added with 20 µL of pigment extract in various concentrations (250, 500, 750, and 1,000 µg/ml). The plates were incubated at 37°C (bacteria) and 28°C (*C. albicans*) for 18-24 hours. The inhibition zone around the paper disc indicated antimicrobial activity. The positive controls used were 100 µg/ml tetracycline (bacteria) and nystatin (*C. albicans*), while the negative control was DMSO 10% (v/v) (Mesrian *et al.* 2021).

2.4. Mobile Phase Optimization by Thin Layer Chromatography (TLC) and Bioautography Test

Pigment fractionation was carried out by the TLC method using eight single eluents (*n*-hexane, dichloromethane, chloroform, ethyl acetate, acetone, ethanol, methanol, and propanol) and silica gel as the stationary phase. A total of 10 µL (5% (w/v)) crude pigment extract was applied to silica gel G60F-254 TLC plates (2 cm × 10 cm) using CAMAG Linomat 5 (Switzerland) and eluted in a chromatographic vessel containing 10 ml eluent. The bands were observed under UV light at 254 nm and 366 nm. The best eluent was determined by observing the highest number of bands and good separation. The two best single eluents were mixed in various ratios from 1:9 (v/v) to 9:1 (v/v) as the mobile follow-up phase. The best mixed mobile phase was determined from the highest number of bands and band separation. For the bioautography test, a total of 10 µL of pigment extract (5% (w/v)) was applied to a TLC plate (2 cm × 10 cm). Next, the plate was eluted using the selected mobile phase. The plate was then placed in a Petri dish and added 15 ml of MHA/PDA medium containing 1% (v/v) of the microbe test. Plates were incubated at 37°C (bacteria) and 28°C (*C. albicans*) for 24 hours. The inhibition zone on the TLC plate indicated the active fraction of bands (Dewanjee

et al. 2015 with modifications). These bands were isolated using preparative TLC, a total (10% (w/v)) extract applied on TLC plates. The active fraction of the bioautographic results were marked, scraped, and collected from the plate.

2.5. Observation of Microbes Cell Damage

Cell damage was observed using scanning electron microscopy (SEM). Cultures of *B. subtilis* and *C. albicans* were treated with the active fraction of pigment (1,000 µg/ml) and incubated for 24 hours. The cultures were then centrifuged, and the cell pellet was washed with cacodylate buffer. The cells were treated with 2.5% glutaraldehyde for fixation. The fixation process was carried out by mixing the cells with 2% tannic acid and then washing them four times with cacodylate buffer. Subsequently, the dehydration process was carried out in five steps using alcohol. The dehydrated cells were dried using tert butanol and then attached to the SEM specimen stub. The cells were further coated with gold using an Ion Coater. The JSM-IT200 SEM was used to observe cell damage and leakage. Microbes cultures were used as negative controls, and cultures with the addition of tetracycline/nystatin were used as positive controls.

2.6. Liquid Chromatography Mass Spectrometer (LC-MS/MS) Analysis

The chemical composition of the active fractions was analyzed by Ultra Performance Liquid Chromatography (UPLC) coupled to a Xevo G2-S QTOF-MS (Waters, USA) using electrospray ionization. A mixture of distilled water + 5 mM ammonium formate (A) and acetonitrile + 0.05% formic acid (B) was used for the mobile phase. A total of 5 µL of the active fraction was injected into the High Strength Silica (HSS) LC column with the ACQUITY UPLC® HSS C18 type (1.8 µm 2.1 × 100 mm). The LC-MS/MS analysis results in chromatograms and mass spectra were then interpreted using the MassLynx V.4.1 program.

3. Results

3.1. Actinomycete Morphology

Actinomycete *G. terrae* JSN1.9 produced orange colored pigment with the best pigment color obtained during an incubation period of 10-days (Figure 1). This actinomycete has a rounded colony, convex elevation, entire margin, grows on the surface of the media, looks shiny or slick on ISP2 solid medium, bacilli cell shape, and produces an intracellular pigment.

3.2. Pigment Production Media

The actinomycete *G. terrae* JSN1.9 showed orange colonies on the ISP2 broth medium after 10 days of incubation at room temperature. After extraction using chloroform and evaporation, the crude pigment extract appeared brownish and dry, yielding 9.27±0.34% (w/v) (Figure 2).

The pigment was characterized using spectrum analysis by measuring the maximum adsorption of the pigment. The result showed a single peak, 400-500 nm, was obtained. Based on this maximum adsorption measurement, it is suggested that the pigment *G. terrae* JSN1.9 has maximum adsorption at a wavelength of 410 nm (Figure 3).

3.3. Pigment Chromatogram and Bioautography Test

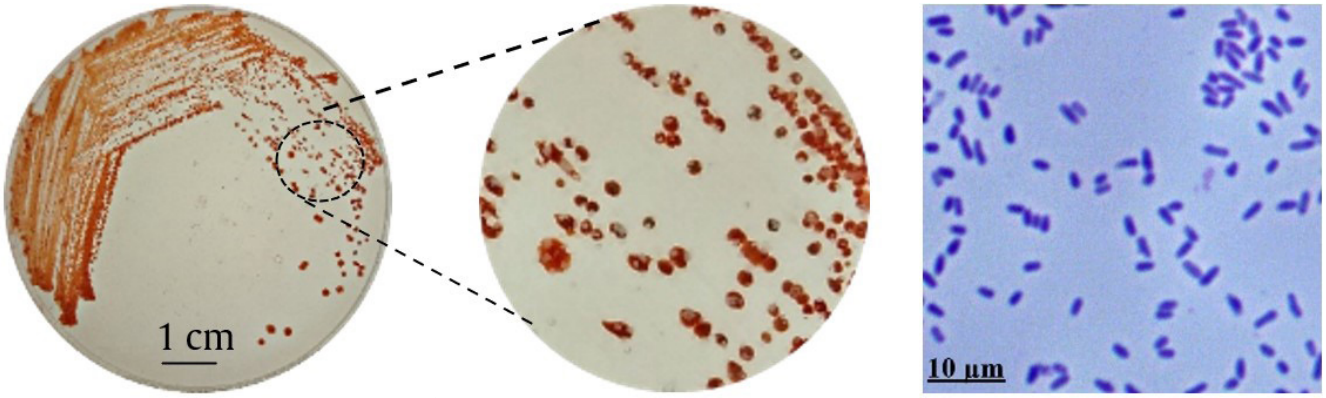
Eight single eluents were used as the mobile phase in the TLC test. Dichloromethane and chloroform were the best eluents that produced the most bands and were separated from each other. Two eluents with the most bands were mixed in various ratios of 1:9 to 9:1 (v/v); 8:2 is the best mixture ratio because it can produce as many as 13 bands. The bioautography test results showed the fraction with antimicrobial activity (Figure 4).

3.4. Antimicrobial Activity

The crude pigment extract and the active fraction showed antimicrobial activity against *B. subtilis, S. aureus*, and *C. albicans albicans* (Figure 5). The active fraction showed stronger antimicrobial activity than the crude pigment. The active fraction has the best activity against *B. subtilis* and *C. albicans* at a concentration of 1,000 µg/ml with inhibition zone diameters of 4.00±0.41 and 3.00±0.00, respectively (Table 1). Both crude pigment extract and active fraction have no activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*).

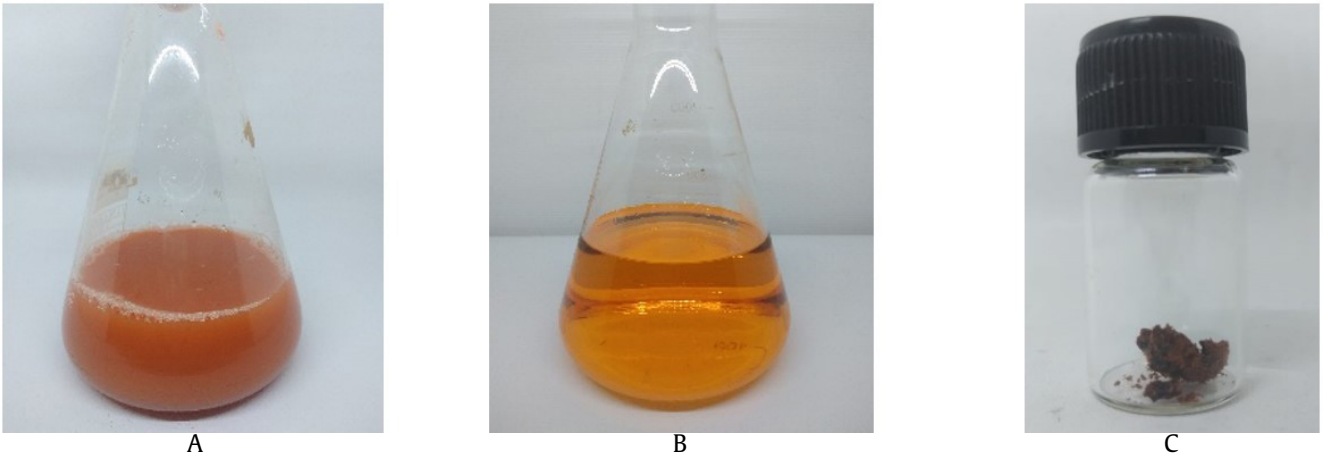
3.5. Observation the Effect of Active Fraction on Microbes Cell

SEM results demonstrated distinct differences in the morphology of both microbe cells (Figure 6). The SEM analysis showed that the untreated *B. subtilis*



A B

Figure 1. Colonies of *G. terrae* JSN1.9 on ISP2 solid medium (A) and Gram staining (B) (magnification 1.000×)



A B C

Figure 2. (A) Pigment production of *G. terrae* JSN1.9 on ISP2 medium, (B) pigment extraction result using chloroform, (C) and crude pigment extract

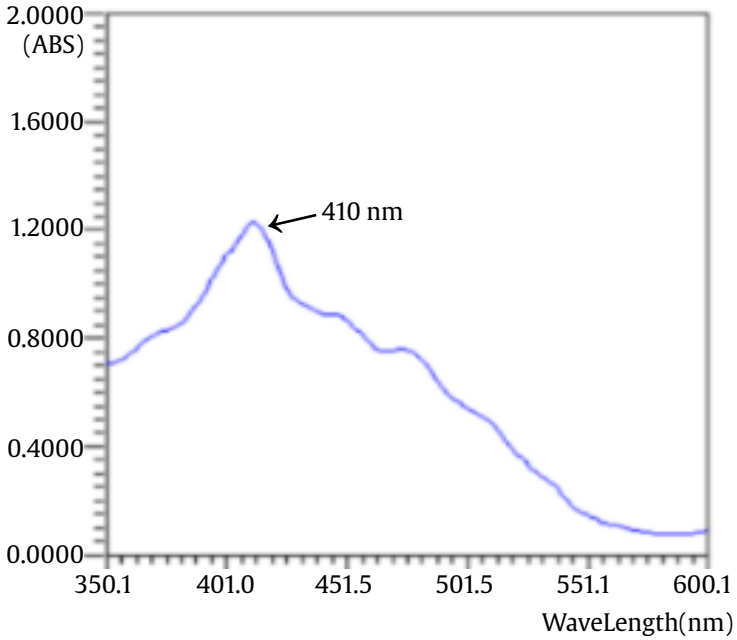


Figure 3. Maximum adsorption of *G. terrae* JSN1.9 crude pigment extract on UV-Vis spectrophotometer

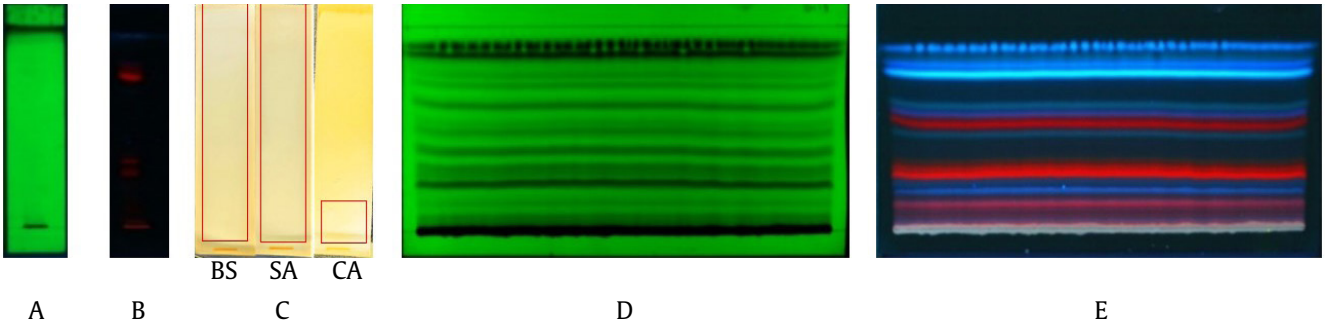


Figure 4. Chromatogram of *G. terrae* JSN1.9 pigment extract on a TLC plate. The active fraction is marked with a red box. TLC plate visualization at 254 nm (A) and 366 nm (B) at UV light, the results of the bioautographic test (C) (BS: *B. subtilis*, SA: *S. aureus*, and CA: *C. albicans*). Preparative TLC visualization at 254 nm (D) and 366 nm (E) at UV light

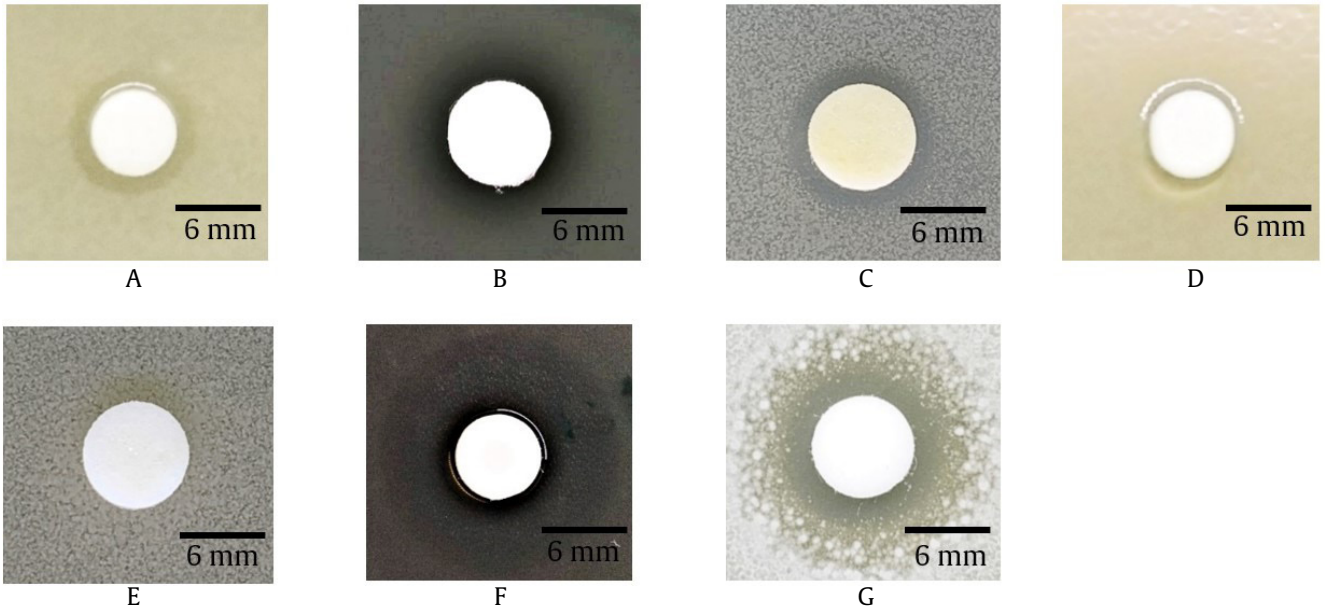


Figure 5. Antimicrobial activity test of the active fraction of *G. terrae* pigment JSN1.9 on *B. subtilis* (A), *S. aureus* (B), and *C. albicans* (C) at concentrations of 1,000 µg/ml, DMSO 10% on *B. subtilis* (D), and *C. albicans* (E), tetrasiklin 100 µg/ml on *S. aureus* (F), and nystatin 100 µg/ml on *C. albicans* (G)

Table 1. Antimicrobial activity of the active fraction of *G. terrae* pigment extract JSN1.9

Active fraction	Concentration (µg/ml)	Inhibition zone (mm)*				
		BS	EC	PA	SA	CA
G. terrae JSN1.9	250	2.00±0.00	2.00±0.00	0.00±0.00	1.50±0.58	1.00±0.00
	500	3.00±0.81	3.00±0.81	0.00±0.00	1.50±0.58	2.00±0.82
	750	3.50±0.00	3.50±0.00	0.00±0.00	2.00±0.00	2.50±0.58
	1,000	4.00±0.41	4.00±0.41	0.00±0.00	2.25±0.58	3.00±0.00
Tetracycline	100	11.00±1.41	17.00±0.82	12,75±0.29	4.00±0.81	nd
Nystatin	100	nd	nd	nd	nd	nd
DMSO 10%	100	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

nd: not determined
*BS: *B. subtilis*, EC *E. coli*, PA: *P. aeruginosa*, SA: *S. aureus*, CA: *C. albicans*
*Weak: 0.1-5.0 mm, Moderate: 6.0-10.0 mm, Strong: 11.0-15.0 mm

and *C. albicans* cells displayed smooth surfaces with a plump appearance and uniform sizes (Figure 6A1 and 6B1). However, cells treated with tetracycline and the active fraction treatment showed noticeable damage in the cells (Figure 6A2 and 6A3). The SEM results for tetracycline and active fraction treatments showed cell with wrinkled surfaces. SEM analysis for *C. albicans* cells displayed diverse cells shapes and filaments structures (Figure 6B). Untreated cells exhibited smooth surfaces and a plump appearance (Figure 6B1). On the other hand, cells treated with nystatin display cell leakage on the surfaces (Figure 6B2). When treated with the active fraction, the cells showed uneven shapes and lesser cells. Furthermore, the growth of filaments was inhibited (Figure 6B3).

3.6. Compound Identification by LC-MS/MS Analysis

The LC-MS/MS analysis of the active fraction of *G. terrae* JSN1.9 pigment showed the presence of various compounds in the active fraction of pigments (Figure 7). Dominant peaks marked various compounds. The most dominant compound based on the ten highest chromatogram peaks is cholestyramine, aminopregnane, sphinganine, dioctadecylamine, lauryldiethanolamine, and eudesmin (Table 2).

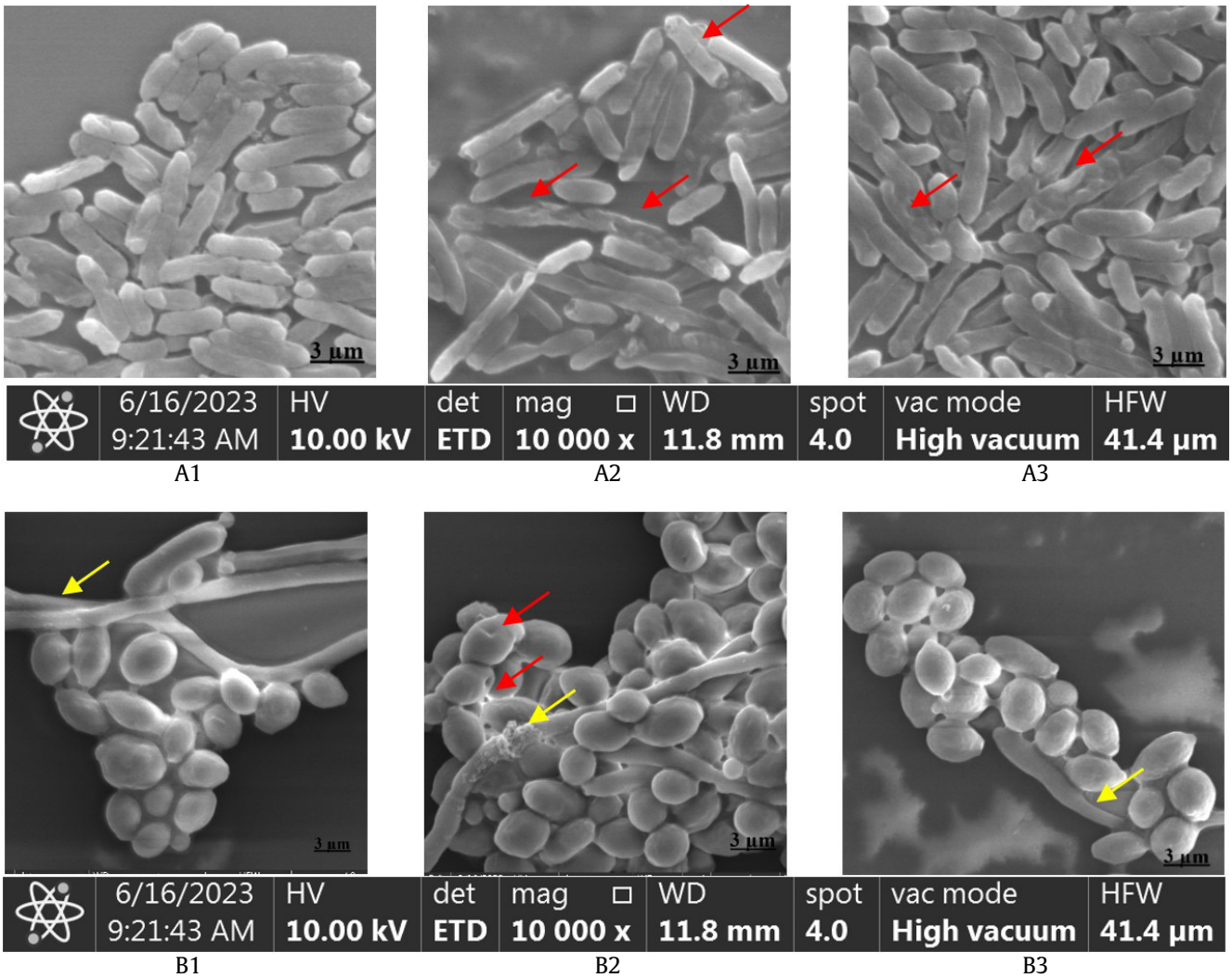


Figure 6. SEM images of *B. subtilis* (A) and *C. albicans* (B) at 10.000 magnification. (1) no treatment (negative control), (2) treatment with tetracycline/nystatin (positive control), and (3) treatment with the *G. terrae* JSN1.9 active fraction. Red arrows indicate cell damage and yellow arrows indicate filaments

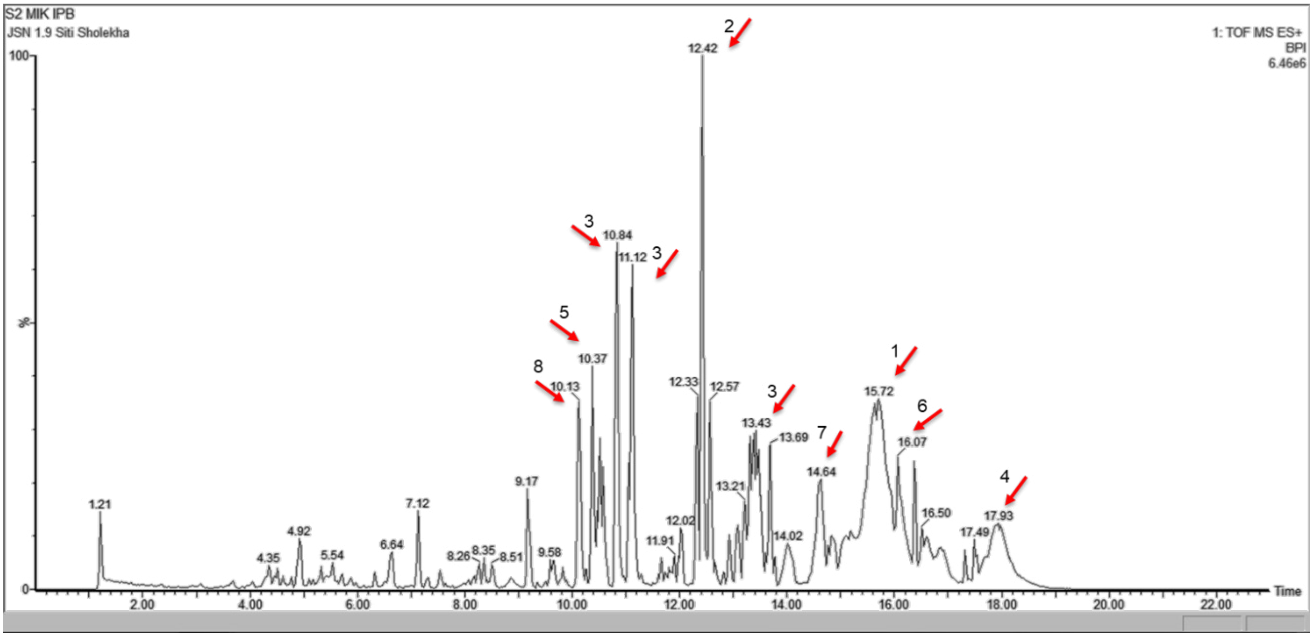


Figure 7. LC-MS/MS chromatogram of the active fraction of *G. terrae* JSN1.9. Red arrows indicate the abundance of most compounds

Table 2. Compound content in the active fraction of *G. terrae* pigment JSN1.9 as a result of LC-MS/MS analysis

Compound names	Peak area (%)	Formula	Retention time	Biological activities	References
Cholestyramine	25.64	$C_{27}H_{49}N$	15.72	Lowering high cholesterol	Morley *et al.* 2020
Aminopregnane	10.50	$C_{21}H_{37}N$	12.42	Antimicrobial	Kull *et al.* 1953
	9.85		13.39		
Sphinganine	6.17	$C_{17}H_{37}NO_2$	11.12	Antibacterial	Kunz and Kozjak-Pavlovic 2019
	5.64		10.84		
Dioctadecylamine	7.07	$C_{36}H_{75}N$	17.93	Antimicrobial	Seitz *et al.* 2021
Lauryldiethanolamine	6.02	$C_{16}H_{35}NO_2$	10.46	Antimicrobial	Chaouat et al. 2013
Unknown	5.37	$C_{39}H_{62}O_5$	16.38	-	-
Unknown	5.26	$C_{22}H_{46}O_5$	14.62	-	-
Eudesmin	3.06	$C_{22}H_{26}O_6$	10.13	Antimicrobial Anticancer	Patel and Patel 2022

4. Discussion

Microbes play a crucial role in developing natural products and medical therapies. As a rich source of natural products, microbes are considered a rich source of diverse and unique bioactive compounds (Abdel-Razek *et al.* 2020). Among these microbes, a rare genus of actinomycetes shows particular promise as a valuable source of bioactive compounds (Ma *et al.* 2021). Notably, Gordonia has been recognized for its capability of pigment biosynthesis (Loh *et al.* 2020). In this study, Actinomycete *G. terrae* JSN1.9 demonstrated the high production of an orange pigment on the ISP2 solid medium. The pigment was intracellular pigment because it did not diffuse to the ISP2 medium. The orange color was shown in the ISP2 liquid medium because the cell biomass was still mixed with the medium. Previous reports said that ISP2 is a medium that can be used for pigment production in actinomycetes (Ratte *et al.* 2022) and is the best media for higher pigment production (Ayoubi *et al.* 2018).

Pigment characterization is used to classify pigments into different groups. Based on the maximum adsorption results (Figure 3), it is assumed that the pigment produced by *G. terrae* JSN1.9 belongs to the carotenoid group, which typically exhibits colors ranging from yellow to orange. Carotenoids absorb light in the visible region of 400–500 nm (Udensi *et al.* 2022). Supporting this finding, Loh *et*

al. (2020) also reported that actinomycete *G. terrae* produced carotenoid pigment under fermentation conditions.

This study investigated the antimicrobial activity of the pigment extract from *G. terrae* JSN1.9 against various microbes, showing stronger effects on *B. subtilis, S. aureus*, and *C. albicans* (Table 1). The inhibition zone expands as the pigment concentration increases from 250 µg/ml to 1,000 µg/ml. Notably, the pigment extract exhibited selective activity, primarily inhibiting Gram-positive bacteria, which aligns with Yolmeh *et al.*'s findings (2016), where pigments from *Rhodotorula glutinis* were more effective against Gram-positive bacteria than Gram-negative bacteria. Moreover, Kazi *et al.* (2022) reported that the bioactive pigments derived from the strain BJZ10 also showed potential as antimicrobial agents against Gram-positive bacteria. Bioautography assay results demonstrated that the pigment extract of *G. terrae* JSN1.9 had a single active fraction with inhibition activity against the tested microbes, evident from the formation of an inhibition zone around the first band. The bioautography technique offers rapid screening for bioactivity, particularly for antibacterial, antifungal, antioxidant, enzyme inhibition, and other activities, facilitating targeted isolation of active compounds/fractions (Dewanjee *et al.* 2015).

The best activity of the active fraction was obtained at a concentration of 1,000 µg/ml with an inhibition zone diameter of 4.00±0.41 mm; 2.25±0.58 mm; and 3.00±0.00 mm for each test microbe *B. subtilis, S. aureus*, and *C. albicans,* respectively. The active pigment fraction of *G. terrae* JSN1.9 has higher antimicrobial activity against *B. subtilis* than previous studies by Keceli *et al.* (2013), which reported no activity from the tested orange pigment. These results proved that the pigment *G. terrae* JSN1.9 has the potential as an antimicrobial agent.

SEM results showed that administration of the active pigment fraction at a concentration of 1,000 µg/ml induced cell damage in *B. subtilis* and inhibited the formation of filaments and cell growth in *C. albicans* (Figure 6). This damage was evident through the observed shrinkage of cell-cultures, non-uniform cell shapes, and cracks in the cell walls, which can lead to cell lysis. This finding is similar to Feng *et al.* study (2019), where bacteria cells treated with an orange pigment displayed irregular and wrinkled surfaces compared to untreated cells with regular and smooth surfaces. On the other hand, the transition from yeast to filament plays a critical role in virulence and pathogenesis in *Candida* (Kadosh 2019). This transition is influenced by various inducing signals present in the environment. However, the active fraction in the tested *Candida* cells inhibited the formation of filaments, suggesting a potential reduction in pathogenicity. This result is consistent with Romo *et al.* findings (2017), where a small molecule compound named N-[3-(allyloxy)-phenyl]-4-methoxybenzamide (9029936) had potent inhibitory activity against filamentation and biofilm formation by *C. albicans* SC5314 strain.

Through LC-MS/MS analysis (Figure 7 and Table 2), several compounds were identified in the active fraction extract. Compounds detected in the LC-MS analysis were based on the literature reported to have antimicrobial activity with very diverse modes of action. Based on SEM and LC-MS analyses, the compounds belonging to the fraction actively have bactericidal activity against *B. subtilis* ATCC 6633 and fungistatic activity against *C. albicans* ATCC 10231.

The most abundant compound found in the LC-MS analysis was cholestyramine. Cholestyramine is a drug that can be used with antibiotics for therapeutic purposes (Morley *et al.* 2020). Aminopregnane was a member of the steroid class with antimicrobial activity (Kull *et al.* 1953). Sphinganine has antibacterial activity against Gram-positive and negative bacteria (Kunz and Kozjak-Pavlovic 2019), and dioctadecylamine was a compound reported antimicrobial activity (Seitz *et al.* 2021). Lauryldiethanolamine is a metabolite compound with antimicrobial activity (Chaouat *et al.* 2013). Additionally, eudesmin, present in the pigment extract, has diverse biological activities, including antibacterial, anti-inflammatory, anticancer, and anticonvulsant activities (Patel and Patel 2022). These identified compounds in the pigment extract suggest its potential as a source of various bioactive agents with promising antimicrobial properties.

In conclusion, the orange pigment produced by actinomycete *G. terrae* JSN1.9 demonstrated significant antimicrobial activity against Gram-positive bacteria (*B. subtilis* and *S. aureus*) and *C. albicans*. UV-Vis analysis confirmed that the pigment belongs to the carotenoid group. Furthermore, the active fraction exhibited stronger antimicrobial activity than the crude extract. SEM analysis clearly

revealed the noticeable differences between the treated and untreated microbe cells, displaying evident damage to the cells and inhibition of filament formation. LC-MS/MS analysis also provided insights into certain compounds that potentially contribute to the observed antimicrobial activity. Overall, this study highlights the potency of the orange pigment produced by *G. terrae* JSN1.9 as a promising antimicrobial agent. The findings underline the potential of this pigment and its active fraction for further exploration and development as a novel antimicrobial therapy.

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