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Antimicrobial Agents Production by Marine *Streptomyces exfoliatus* Coculture with *Staphylococcus aureus*

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ABSTRACT
In this study, marine *Streptomyces exfoliatus* was isolated from the Red Sea, Egypt, and was identified using 16s rRNA. *S. exfoliatus* was fermented on different media under different carbon source concentrations, pH, temperatures, distilled H_2O, and sea H_2O, under shaking and static conditions. The active material was isolated by chloroform and ethyl acetate extractions then separated by column chromatography and purified by preparative TLC and identified by GC-MS. *Methicillin-resistant Staphylococcus aureus* inhibited by ß-caryophyllene.

Keywords: Marine, *Staphylococcus*, Coculture, Secondary metabolites, *Streptomyces exfoliatus.*

1. Introduction
Marine oceans, seas, rivers, and lakes cover more than 70 % of the Earth's surface, comprising an enormous source of microorganisms that produce promising bioactive secondary metabolites (Subramani *et al*., 2012). The Red Sea represents a unique marine ecosystem having extremophilic living conditions, which characterizes by its high microbial diversity in comparison with the other tropical seas (Abdelfattah *et al*., 2016; Blunt *et al*., 2015). The marine environment has proven to be a rich source of diverse microbial products with relevant activities such as anticancer, anti-inflammatory, antiepileptic, immunomodulatory, antifungal, antiviral, and antiparasitic (Carroll *et al*., 2020).

Staphylococcus aureus can cause a wide range of infections in humans. The most common sites affected are skin and soft tissue; manifestations of infections in these sites include folliculitis, furuncles, and carbuncles, impetigo, mastitis, wound infections, and staphylococcal scalded skin syndrome. More serious infections include bacteremia, pneumonia, endocarditis, bone, and joint infections, and toxic shock syndrome. *S. aureus* can also be responsible for outbreaks of food poisoning (Mairi and Touati, 2020).

Actinomycetes are a rich source of bioactive natural products important for novel drug leads (Hun Kim *et al*., 2021). They are chemically rich sources of structurally diverse secondary metabolites. (Kurtböke *et al*., 2015; Hu *et al*., 2015; Isik *et al*., 2014), also economically and biotechnologically are the most valuable prokaryotes of medical or economic significance (Kin., 2006). They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics (Kurtböke *et al*., 2015; Antunes, 2014; Usha *et al*., 2010; Bredholt *et al*., 2011), antitumor compounds (Kurtböke *et al.*, 2015; Usha *et al.,* 2010), immune-suppressive agents (Kurtböke *et al*., 2015) and enzymes (Sharma, 2014). *Streptomyces* have major diversity and proven ability to produce novel bioactive compounds. The produced compounds serve as antifungal, antiviral, antitumor, and antihypertensive agents, immunosuppressants, and particularly antibiotics (Lekhak *et al*., 2018; Tian *et al*., 2017). Their genome carries numerous genes involved in the biosynthesis of secondary metabolites, including polyketides and terpenes (Virginia *et al*.,

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2014). Diverse *Streptomyces* strain that can inhibit the growth of some pathogenic bacteria. Among screened isolates, *Streptomyces exfoliatus* was the most effective against tested bacterial pathogens (Alahadeb, 2022). The co-culture strategy can not only stimulate the accumulation of diverse molecules from microorganisms but also significantly increase or decrease the yields of some original secondary metabolites compared to monoculture (Vinale *et al*., 2017). Co-culture strategy simulates the complex ecological environment of microbial life by using an ecology-driven method to activate silent gene clusters of microorganisms and tap their metabolic potential to obtain novel bioactive secondary metabolites. A series of natural products with diverse and novel structures have been discovered successfully through co-culture strategies, including fungus–fungus, fungus–bacterium, and bacterium-bacterium co-culture approaches (Xiao *et al.*, 2021). Competition between the two microbes led to genomic mutations, long-term coculture can activate silent smBGC which stimulates the production of silent and poorly expressed metabolites (Reen *et al.,* 2015), by inducing genetic mutations, which keep silent under short-term coculture (Charusanti *et al*., 2012).

2. Materials and Methods

2.1. Isolation and Identification of *Methicillin* Resistance of *Staphylococcus aureus* (MRSA)

Isolation of *methicillin-resistant Staphylococcus aureus* (MRSA) from blood, urine, sputum, wound and biological fluids supplied El Kasr El Any. Bacteria were cultivated on Mannitol agar media, M1 (Luria Bertani), and MacConkey agar media, then it was incubated at 38°C for 24 hrs.

Bacteria were identified by microscopic examination and biochemical tests. Identification of bacterial isolates was performed by VITEK-2 (Biomerieux, France) according to the published guidelines of the Clinical Laboratory Standards Institute (CLSI, 2020). The selected *MRSA2* was identified also by MALDI-TOF.

Antibiotic susceptibility testing was performed by disc diffusion method according to guidelines of the (CLSI, 2020).

2.2. Isolation and Identification of Actinomycetes

Water was collected at a depth of 5m while the soil was at a depth of 1m from three different sites at different times of the year (August 2019, 2020, and January 2020) from the marine Red Sea (Hurghada).

Isolation of actinomycetes was performed by serial dilution and plating method using starch casein agar media, starch nitrate agar, ISP 1agar, and M1 agar media. 10 g of the soil sample were suspended in 95 ml of sterilized seawater in a conical flask and incubated at 30 °C on a shaker for 24 hrs. Distilled water (9 ml) was added to each test tube from 1 to 4. The supernatant liquid from dissolved soil was added to a sterile test tube as serial dilution 10^{-1}, 10^{-2}, $10^{-3,}$ and 10^{-4} and 1 ml of spore suspension was incubated in M1 agar media and starch casein agar media at a temperature (30°C -37 °C) for 48 hrs. (Ez Eldeen *et al*., 2020). there is not a big difference in isolation from water also serial dilution and was taken 1 ml of diluted samples were on M1 agar media and starch casein agar media (prepared once by sea water and other by distilled H_2O) were incubated at a temperature of 30 °C for 48 hrs. after that subcultures isolates were numbered. Identification of selected actinomycetes was performed by 16s rRNA.

2.3. Coculture of Actinomycete -Bacteria

Screening of 40 actinomycetes isolates for antibacterial activity. Actinomycete-bacterial coculture was performed by preparing 0.5 McFarland standard (MCF) suspension from each MRSA isolate, streaking the suspension on M1 (Luria Bertani) Agar plate using a sterile cotton swab and leaving the plate to dry for 5 min. A 1cm in diameter Actinomycete disc was collected after 24 hrs. Actinomycete plate and placed in the center of the petri dish. Three Petri dishes 3X replicates were prepared for each *MRSA* to isolate, with each of 40 Actinomycete isolates, for incubation at two different temperatures at 37°C (37°C which is optimum for bacterial growth and 30°C for Actinomycetes, while ranging 28°C - 40°C optimum for Actinomycete growth). The Petri dishes were examined after 24 hrs. for both actinomycetes and bacterial growth.

2.4. Detection of Actinomycetes potency

Dual culture plate assays. A plate of M1(Luria Bertani) agar media was incubated with 1ml of 0.5 McFarland of *MRSA* bacterial suspension and was spread by a sterile cotton swab. Then was inoculated with a 1cm diameter disc of Actinomycetes in the central well of the plate and then incubated at 37°C for 24 hrs. and treated with many isolates of actinomycetes until achieved best inhibition zone diameter. The largest inhibition zone diameter was detected with *S. exfoliatus*. 5 discs of *S. exfoliatus* were inoculated together with 1ml of 0.5 McFarland standard *MRSA* bacterial suspension in 500 ml of M1 liquid media for each flask and incubated at 30°C for different incubation periods (3-6-9-12) days and other cultivation conditions (carbon source, pH, temperature, shaking and stationary) were studied for their effect on the production of the Secondary metabolite to achieve best results. The flasks were filtered and supernatants were centrifuged at 10.000 *g* for 10 min. using ethyl acetate and chloroform solvents for extraction. The extracts were dried using a rotary evaporator at temperatures of 50°C. The dried extracts were weighed and dissolved in 400 µl of its solvent and spotted on pre-coated TLC for different incubation periods.

2.5. Optimization of suitable conditions for maximum antibacterial metabolite production

I. Optimization of the incubation period.

The optimization of the incubation period was also carried out by incubating for 3-12 days at 35°C. The antibacterial activity was monitored every 3 days intervals by the disk diffusion method.

II. Optimization of temperature

The effect of culture conditions on the production of enhanced antibacterial metabolite production was studied on M1(Luria Bertani) against *MRSA* (Pathogenic bacteria which exhibited maximum activity in screening studies). The optimum temperature for the maximum antibacterial compound production was investigated on M1. Ten ml bacterial suspension was introduced in 250 ml Erlenmeyer flasks containing 100 ml of broth and incubated at different temperatures (28°C and 40°C) at pH 7. The antibacterial activity was assayed after 3 days by the disk diffusion method.

III. Optimization of pH.

The impact of pH on antibacterial metabolite production was studied at different pH, ranging from 4 to 9 at 40°C and after 3 days.

IV. Optimization of carbon source

The impact of carbon source on antibacterial metabolite production was studied at different concentrations 1X and 2X (10 g and 20 g of starch) at 40°C and after 3 days.

V. Optimization of static and shaking conditions.

The yield of secondary metabolites and growth was much better under shaking conditions.

VII. Isolation, extraction, and Estimation of the Genomic DNA of the selected Actinomycete isolate

Total genomic DNA was collected from the actinomycete by growing it in M 1 liquid media for 48 hrs. DNA isolation was performed using I-genomic BYF DNA Mini kit # 17361. The purity of isolated DNA was estimated by using gel electrophoresis. 1% of agarose in TBE buffer (10.8g Tris-base, 5.5g boric acid, 9.3g EDTA buffer, pH 8) was prepared and let to cool. 4µl of ethidium bromide was added to the gel after cooling. 2µl of loading dye (bromophenol blue) and 10µl of DNA were loaded in 1% agarose gel and then run at 80 volts for approximately 1 hour. PCR amplification was carried out using 16S rRNA primers (forward primer,16S-F 5'-AGAGTTTGATCCTGGCTCAG-3'and reverse primer, 16S-R 5'- GGTTACCTTGTTACGACTT-3') and amplification profile consists of an initial denaturation at 94˚C for 5 minutes followed by 35 amplification cycles of 94°C for 1.0 min, 52°C for 1.0 min and 72°C for1.0 min. finally, the final extension is 72°C for 10 mins (Moubasher *et al*., 2013).

VIII. Sequencing of PCR product

Purification of PCR products and sequencing of the PCR products for isolate was performed in Sigma, Biotech Research Lab, Egypt.

IX. Preparation of crude extract of *S. exfoliatus* -bacterial coculture

The broth media was filtered using filter paper Whatman No.1 to remove actinomycete mycelia and then centrifuged at 10,000 *g* for 10 min to remove the bacteria. The supernatant was transferred to a separatory funnel mixed with 2X chloroform 2 times. The funnel was strongly shaken and then left to allow partitioning. Ethyl acetate was added and the same process mentioned with chloroform was repeated. Chloroform and ethyl acetate extracts were evaporated to dryness under reduced pressure using a rotary evaporator at temperatures of 50°C, and then the two extracts were weighed separately. This extraction step was repeated with five different MRSA each giving a clear zone on solid agar media with replicates.

X. Testing the crude extract against *MRSA*

After evaporation of both extracts, they were tested against *MRSA*2 isolates using the disc diffusion method by adding 10µl of each extract on a 0.5 cm in diameter filter paper disc.

XI. Column chromatography for purification compounds

The Silica gel was suspended in chloroform for the packing of the column. The column was 50 cm long, with a diameter of 2.8 cm, with center glass at the bottom. A 376 mg ethyl acetate extract was dissolved in 3 ml of ethyl acetate and chloroform (1:1) and was passed through the column. A gradient solvent system of chloroform-ethyl acetate (2:1, 2:1.5, 1:1) was used. Each Fraction of 10 ml was collected. 30 fractions were collected and were tested using a paper disc diffusion method against *MRSA2.* Fraction No.8 was further purified using a preparative TLC, with solvent system ethyl acetate: chloroform (1:1), there were 8 bands scraped and were tested again by paper disc assay against *MRSA2*. Bands No.4 and No.6 have antibacterial effects which both showed a clear zone on *MRSA2.*

XII. Determination of MICs (minimum inhibitory concentrations)

Serial dilution for the most potent purified fraction No. 6 (7.4 mg), dissolved in 400 µl, 1:1ethyl acetate chloroform extraction 5 µl,10 µl, 15 µl, 25 µl, and 35 µl.

XIII. Gas chromatography-mass spectroscopy (GC-MS)

The identification of the chemical composition of the sample was performed by GC-MS analysis using a Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The oven temperature program used was 50- 290°C at 5°C/min, and the final temperature was held for 2 min. The injector and MS transfer line temperatures were kept at 250, and 260°C respectively. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using Autosampler AS1300 coupled with GC in the split mode. The MS data were obtained in the scan mode (40–600m/z) and EI mode operating at 70 eV. The ion source temperature was set at 200 °C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral databases.

3. Results

3.1. Effect of incubation periods on the production of the metabolites by coculture of MRSA and *S. exfoliatus.*

The results showed that coculture affected the production of metabolites. Secondary metabolites production was increased by the effect of coculture of MRSA and *S. exfoliatus.* As shown in Table 1.

Results showed that the maximum metabolites yield was achieved with the effect of coculture (MRSA 2 + *S. exfoliatus*) and (MRSA 3 + *S. exfoliatus*) during incubation periods of 3 days using ethyl acetate solvent 21 mg and 18.66 mg respectively, the least amounts of metabolites were produced under the effect of coculture (MRSA 5+*S.exfoliatus*) at incubation day 3 by using chloroform solvent MRSA 1, 2,3,4 and *S. exfoliatus* during incubation periods 3,6 and 9 days. The

data have shown that single *S. exfoliatus* produced the highest amounts of metabolites during incubation periods 3- and 6-days using ethyl acetate solvent, also single MRSA 2 and MRSA 3 during incubation periods of 6 days using ethyl acetate solvent while MRSA 2, MRSA 4 and MRSA 5 during incubation periods of 9 days using ethyl acetate solvent and MRSA 3 during incubation periods of 6 days using ethyl acetate and chloroform solvents have shown intermediate amounts of metabolites produced. In contrast, other single cultures produced the least amount of metabolites during incubation periods of 3 days MRSA1 and MRSA 5, during incubation periods of 6 days MRSA 1, MRSA2, MRSA4, and MRSA5 and during incubation periods of 9 days MRSA1 and *S. exfoliatus*.

Table 1: Effect of the incubation periods on metabolites production efficiency by coculture of MRSA and *Streptomyces exfoliatus*(mg).

		Coculture extraction weight (mg)				
Incubation period	**Solvent**	**MRSA 1 + Actinomycetes (mg)**	**MRSA 2 + Actinomycetes**	**MRSA 3 + Actinomycetes**	**MRSA 4 + Actinomycetes**	**MRSA 5 + Actinomycetes**
3 Days	**Chloroform**	1.33± 0.0015	17.3± 0.00807	1.33± 0.0015	1.67± 0.0002	0.029± 0.0004
	Ethyl acetate	1.67 ± 0.00523	21 ± 0.0002	18.66± 0.0002	3.67± 0.008	1.33± 0.0015
6 Days	**Chloroform**	1 ± 0.00	4.67 ± 0.005	1.33± 0.0015	2 ± 0.009	2.33± 0.00054
	Ethyl acetate	6.33 ± 4.445	1.33 ± 0.0015	5.33 ± 0.0005	6 ±0	4.33 ± 0.0002
9 Days	**Chloroform**	± 0	1.33 ± 0.0002	4.67 ± 0.015	1 ±0	1.33 ± 0.0015
	Ethyl acetate	3.67 ± 0.0012	6 ± 0.000	1.33± 0.00027	5.67	5.67±0

Table 1: Cont.

		Coculture extraction weight (mg)					
Incubation period	**Solvent**	**Actino control**	**MRSA1 control**	**MRSA2 control**	**MRSA3 control**	**MRSA4 control**	**MRSA5 control**
3 Days	**Chloroform**	1 ±0	1 ±0	4.8 ±0	1 ±0	2 ±0	1 ±0
	Ethyl acetate	8 ±.0002721	2 ±0	8 ±0	5.5 ±0	4 ±0	3 ±0
6 Days	**Chloroform**	2 ±0	1 ±0	1 ±0	7±.0002	1 ±0	1 ±0
	Ethyl acetate	8 ±0	3 ±0	1 ±0	7 ±0	3 ±0	3 ±0
9 Days	**Chloroform**	1 ±0	1 ±0	1 ±0	4 ±0	1 ±0	1 ±0
	Ethyl acetate	2 ±0	3 ±0	5.7 ±0	1 ±0	5.3 ±0	5 ±0

3.2. Inhibition zone diameters (mm) of coculture (MRSA 1,2,3,4,5 and *S. exfoliatus)* on MRSA 2

The optimum incubation period of coculture of MRSA2 and MRSA 3 have the highest inhibition activities during the incubation period after 3 days using ethyl acetate solvent producing an inhibition zone diameter 14 mm while coculture MRSA4 during the incubation period of 3 days using ethyl acetate solvent has an intermediate inhibition activity producing an inhibition zone of 12 mm. The coculture of MRSA 3 during an incubation period of 6 days using ethyl acetate and chloroform solvents has the least inhibition activities producing an inhibition zone of 10 mm. In the contrast, all others haven't shown any inhibition activities with no inhibition zones.

3.3. Effect of different temperatures (28°C &40°C), carbon source concentrations, and pH of coculture (*MRSA2 and S. exfoliatus*) on the production of secondary metabolites (mg).

The effect of different temperatures (28°C & 40°C), carbon source concentrations (10 g, 20 g), and pH (4, 7, 8, 9) on the production of metabolites concentrations of coculture (MRSA 2+ *S. exfoliatus).* The data have shown that the maximum metabolites yield was achieved with the effect of a temperature of 40 °C, pH 9, and 10 g of starch with ethyl acetate solvent 90 mg. In addition, other

cocultures produced an intermediate amount at a temperature of 40 °C, pH 8, and 10 g of starch with ethyl acetate solvent 32.8 mg and also the temperature of 40 °C, pH 7, and 10 g of starch with the same solvent, metabolites concentration was 24.2 mg, and all other conditions produced lesser amounts of secondary metabolites.

Table 2: Inhibition zone diameters (mm) of co-culture (MRSA 1, 2, 3, 4, 5 and *S. exfoliatus*) on MRSA2.

Time & Solvents	3 Days		6 Days		9 Days	
Organisms	Chloroform	Ethyl acetate	Chloroform	Ethyl acetate	Chloroform	Ethyl acetate
MRSA 1	- ±0	- ±0	- ±0	- ±0	- ±0	- ±0
MRSA 2	- ±0	14 ±0	- ±0	- ±0	- ± 0	- ±0
MRSA 3	10 ±0	14 ±0	_ ±0	10 ±0	- ±0	- ±0
MRSA 4	- ±0	12 ±0	- ±0	- ±0	- ±0	- ±0
MRSA 5	- ±0	- ±0	- ±0	- ±0	- ±0	- ±0

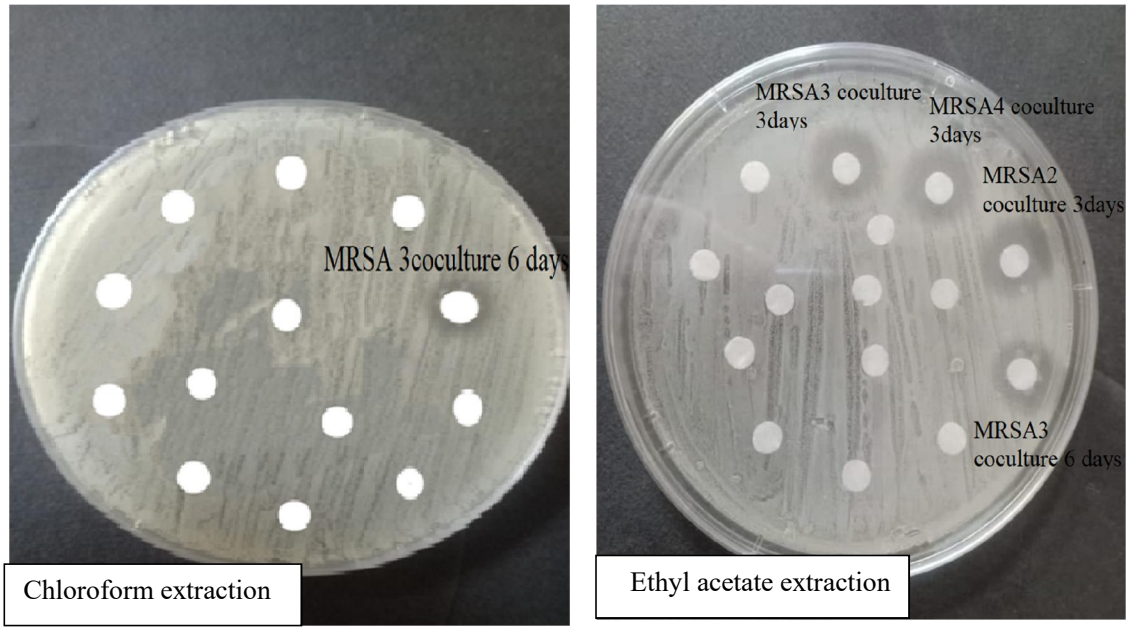


Fig. 1: Inhibition zone diameters (mm) of coculture (MRSA 1,2,3,4,5 and *S. exfoliatus)* on MRSA 2 during different incubation periods

Table 3: Effect of different temperatures (28°C &40°C), carbon source concentrations, and pH of coculture (*MRSA2 and S. exfoliatus*) on the production of secondary metabolites (mg).

Starch conc & Temperature pH	10.0 gm starch/L			
	28°C		40°C	
	$CHCl_3$	Ethyl acetate	$CHCl_3$	Ethyl acetate
4	1×10^{-1} ±0	3×10^{-1} $\pm 1 \times 10^{-4}$	2.1 ±0	$3.4 \pm 5 \times 10^{-5}$
7	1×10^{-1} ±0	1×10^{-1} ±0	$4.3 \pm 5 \times 10^{-5}$	$24.2 \pm 1 \times 10^{-5}$
8	2.2×10^{-1} $\pm 5 \times 10^{-5}$	7.4 ±0	4.5 ±0	32.8 ±0
9	2.4×10^{-1} $\pm 5 \times 10^{-5}$	6.5 $\pm 2.35 \times 10^{-3}$	5.6 $\pm 1 \times 10^{-4}$	90 ±0

Table 3: Cont.

Starch conc& Temperature	20.0 gm starch/L			
	28°C		40°C	
pH	$CHCl_3$	Ethyl acetate	$CHCl_3$	Ethyl acetate
4	1×10^{-1} ±0	1×10^{-1} ±0	1.1 ±0	2.2 ±1×10^{-4}
7	2.3 ±1×10^{-4}	2.2 ±1×10^{-4}	2.6 ±1.02×10^{-4}	8.7 ±0
8	1×10^{-1} ±0	2.6±$1.\times 10^{-4}$	2.8 ± 0	8.9 ±0
9	1.2 ±5×10^{-5}	2.9 ±1×10^{-4}	1±0	9.1 ±1×10^{-4}

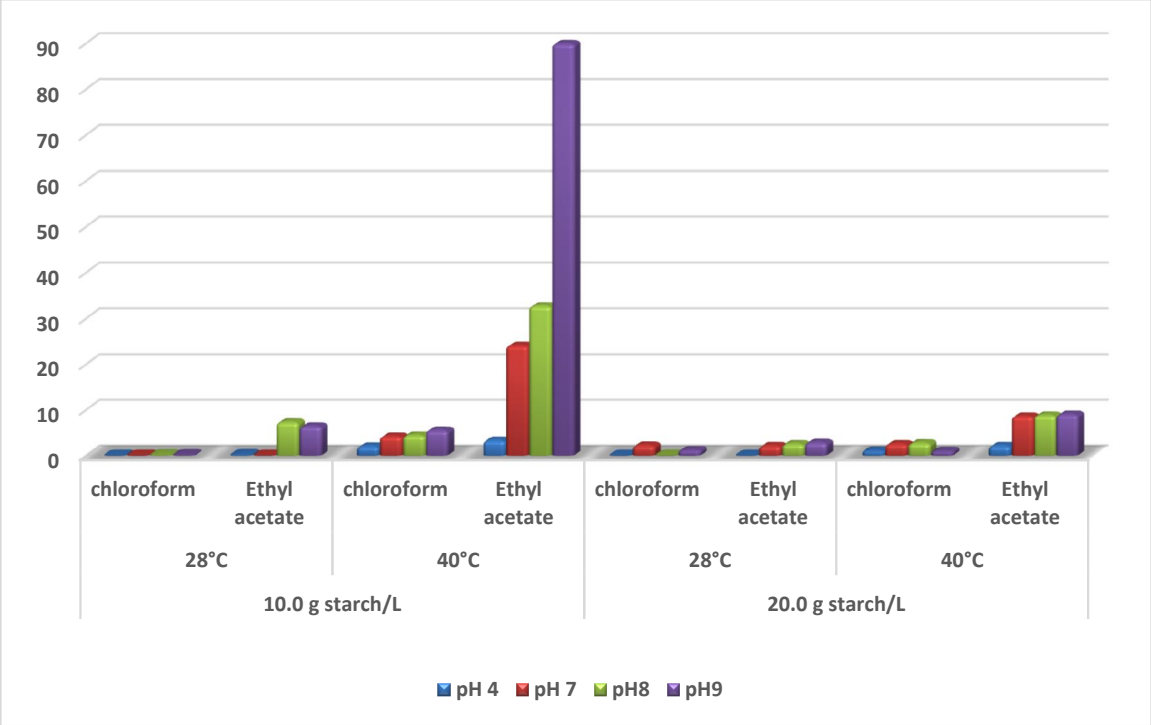


Fig. 2: Effect of different temperatures (28°C &40°C), carbon source concentrations and pH of coculture (MRSA2 and *S. exfoliatus*) on the production of secondary metabolites (mg).

3.4. Effect of different temperatures (28°C & 40°C), different pH, and carbon source concentrations of coculture (MRSA and *S. exfoliatus*) on inhibition diameters (mm) of MRSA 2.

The effect of coculture metabolites MRSA 2 at optimum conditions (carbon source, pH, temperature, shaking) on inhibition zone diameter. Secondary metabolites concentrations of coculture (MRSA 2+ *S. exfoliatus),* and yield of metabolites and their effect on inhibition activities showed that the highest inhibition zone at a temperature of 40 °C, pH 9 and 10 g of starch have shown that metabolites of coculture (MRSA 2+ *S. exfoliatus*) with inhibition zone 80 mm, while the least inhibition activity and also the least inhibition zone were at 40 °C, pH 8 and starch concentration 10 g with an inhibition zone of 60 mm and all others with no inhibition activities and also no inhibition zones using ethyl acetate solvent. In addition, other cocultures with different conditions produced an intermediate amount using the same solvent ethyl acetate, and chloroform with carbon source 10 g of starch, 28°C, and pH 8, pH 9 produced intermediate amounts of metabolites 7.4 and 6.5 by using ethyl acetate solvent and with 20 g of starch,40°C, pH 4 by using both chloroform and ethyl acetate solvent which produced 6.5 g and 9 g gradually.

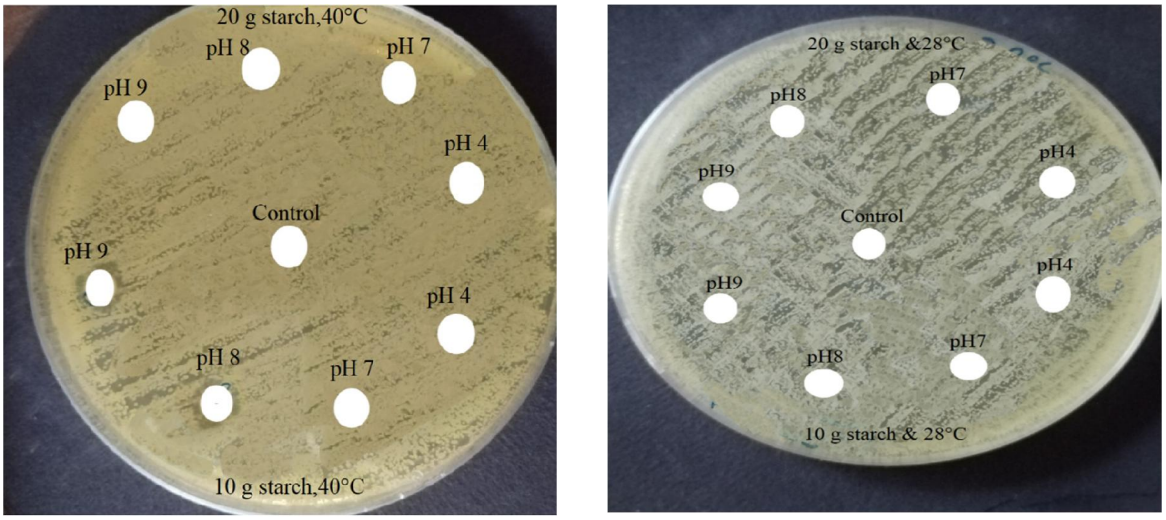


Fig. 3: Effect of different temperatures (28°C & 40°C), different pH, and carbon source concentrations of coculture (MRSA and *S. exfoliatus*) on inhibition diameters (mm) of MRSA 2.

3.5. Column chromatography for purification of compounds

After testing the antibacterial effect of each fraction, fraction No. 8 showed an inhibition zone. Fraction No.8 was purified using preparative TLC, the solvent system was ethyl acetate: chloroform (1:1). Eight bands were separated, scraped and dissolved in ethyl acetate: chloroform (1:1) and then tested using a paper disc assay against *MRSA2*. Bands No.4 and No.6 with concentrations of 0.2 and 1.85 mg/ml respectively have antibacterial effects which both showed a clear zone on *MRSA2.* Band No.6 was spotted on precoated TLC to confirm it´s a single compound.

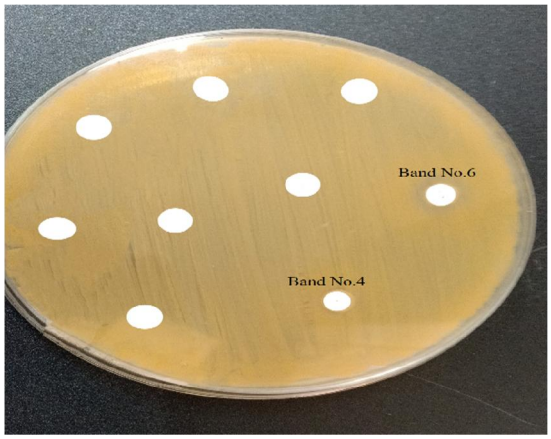


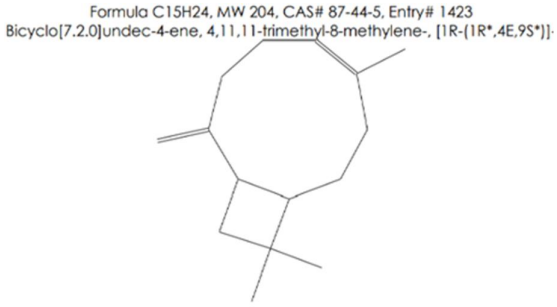
Fig. 4: Antibacterial effect of purification fraction No. 8 on MRSA 2.

3.6. Determination of MICs (minimum inhibitory concentrations)

Serial dilutions for the most potent purified fraction No. 6 (7.4 mg), were dissolved in 400 µl, 1:1ethyl acetate chloroform solvent was 0.0925, 0.185, 0.2775, 0.4625, and 0.6475 mg/ml.
MIC was 0.4625 mg/ml.

3.7. Gas chromatography-mass spectroscopy (GC-MS)

GC-MS analysis the effective compound was caryophyllene with m/z 204.



4. Discussion

Coculture is a promising approach to stimulate novel secondary metabolite production from actinomycetes by mimicking an ecological habitat where cryptic smBGCs may be activated. Coculture aims to expand the chemical diversity of actinomycetes, by categorizing the cases by the type of coculture partner. Current challenges to support the elicitation of novel bioactive compounds from actinomycetes (Hun Kim *et al.,* 2021). Cultivation conditions such as incubation period, pH, and temperature play a major role in the production of bioactive metabolites (Kiranmayi *et al*., 2011). Changes in the nature and type of nitrogen sources and carbon have been found to affect antibiotic biosynthesis in Streptomyces (Barratt and Oliver, 1994; Lounès *et al.,* 1996). Temperature, pH, incubation time, carbon, and nitrogen source directly influenced the production of bioactive metabolites (Bundale *et al*., 2015). It has been noticed that secondary metabolite production is often stimulated by slowly assimilated complex carbon sources (Bertasso *et al*., 2004). Optimal production has been accomplished by cultivating organisms in media containing slowly utilized nutrient sources (Jonsbu *et al*., 2002; Drew and Demain, 1977). Starch was found to be the best carbon source for antibiotic production by several researchers (El-Naggar *et al.,* 2003; Osman *et al*., 2011). Peptone was found to favour the production of antibiotics by other authors too (Praveen *et al.*, 2008; Chattopadhyay and Sen,1997).

Generally, in the most published literature, the optimum pH for the production of antibiotics in Streptomyces cultures has been announced to be near neutral (Oskay, 2011; Gogoi *et al.,* 2008; Singh *et al*., 2009).

From the result table (3) the best incubation period of coculture *S. exfoliatus* and *MRSA 2* was produced after 3 days in other studies antimicrobial metabolites production started after 7 days of incubation of culture broth and reached its maximum levels after 10 days and there after gradually decreased (Ripa *et al*., 2009) others after 11 days of incubations with a rare actinomycete, others maximum production of bioactive metabolite in a synthetic medium was found on the sixth day of incubation (Thakur *et al.,* 2009), other was after 5 days of incubation (Arasu *et al*., 2013), and four days of incubations (Kiranmayi *et al*., 2011), *S. exfoliatus* MT9 were optimally active at pH 8.0 and 50 °C, pH 5.0 and 60 °C, pH 9.0 and 70 °C, respectively (Choudhary *et al*., 2014). The best temperature was 40°C other studies the optimum temperature for antimicrobial metabolites production was 39°C (Ripa *et al*., 2009), The best growth and antimicrobial activity at 35°C and 32°C (Jacob *et al.,* 2017), with maximum production of bioactive metabolites were found in the culture medium at 30°C (Thakur *et al*., 2009; Arasu *et al*., 2013; Ameerah *et al*., 2015), the optimal temperature was around 25°C (Xinxuan *et al.,* 2010; Ameerah; *et al*., 2015), Some of the actinomyces having their optimum at 37°C (Haines, 1932; Ameerah *et al*., 2015). showed this phenomenon at 40° C (Haines, 1932), Waksman (1919) gives a characterization of a number of species and states that their optimum varies from 25°C to 55° C, the maximum for most species being at 40°C, and the minimum below 18-20°C. A slight tendency towards a similar branching was also observed at the lower limit of growth. the optimum pH for antimicrobial metabolites production in our study was pH 9 in other studies maximum growth, as well as increased antimicrobial metabolites, maximum production of antimicrobial metabolites was obtained when the culture medium pH was 8 (Ripa *et al.,*2009), others were obtained at pH 7 (Kiranmayi *et al.,*2011., Xinxuan *et al*., 2010).

A complex source of carbon metabolizable (starch) increased antifungal production by *Streptomyces sp. TKJ2* (Messis *et al.,* 2014). Also used starch as a carbon source in culture medium for improved antifungal production by *Streptomyces sp*. *K03-0132* and *Streptomyces nogalater NIIST A30* were able to produce an enhanced level of broad-spectrum antibacterial metabolites with starch as a carbon source (Jacob *et al.,* 2017). *S. exfoliatus* MUJA10 strain against Staphylococcus aureus ATCC29737 the highest inhibition zone was (51.33 ± 2.15 mm). The MIC value of the 'MUJA10' metabolite of *S. exfoliatus* strain against Salmonella typhimurium ATCC25566 and *E. coli 0157h7 ATCC25922* was 0.125 mg/ml. However, *Bacillus subtilis* had a MIC of 0.625 mg/ml and *Staphylococcus aureus ATCC29737* had a MIC of 2.5 mg/ml (Alahadeb, 2022). β-Caryophyllene (BCP), a natural bicyclic sesquiterpene, is a selective phytocannabinoid agonist of type 2 receptors (CB2-R). It isn't psychogenic due to the absence of an affinity to cannabinoid receptor type 1 (CB1). Receptors CB1-R and CB2-R are metabotropic receptors G protein (protein binding GTP)-coupled receptors, including in the regulation of neurotransmitters responsible for maintaining an energetic balance, in the metabolism, and in the immune response. The aforementioned receptors are bound and stimulated by endogenous cannabinoids, derivatives of arachidonic acid, including 2-arachidonoylglycerol and N-arachidonoyl ethanolamine, better known as anandamide. Both receptors are bound by proteins in various pathways, acting as mediators of cellular responses to biological molecules (Fabrizio *et al.,* 2019). β-caryophyllene isolated from *S. exfoliatus* and inhibited MRSA, from previous studies β-caryophyllene inhibited *Streptococcus mutans* biofilm which was donated by SFC bio in Korea (Hyun-Jun Yooa *et al*., 2018) and had antibacterial activity against S. aureus, in addition to potentiating the action of norfloxacin against *P. aeruginosa, and E. coli* which isolated from terminal branches of plants collected in Atlantic Forest Areas (Eduardo *et al*., 2021). β-caryophyllene isolated from the essential oil had antibacterial activity against *S. aureus* and anti-fungal activity. β-Caryophyllene also displayed strong antioxidant effects. Additionally, β-caryophyllene exhibited selective anti-proliferative effects against colorectal cancer cells (Saad *et al*., 2015).

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