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Artículos originales

Marine Actinomycete Streptomyces rubrogriseus Derived Bioactives are Effective Against Clinical Strains of MRSA

Los bioactivos derivados del actinomiceto marino Streptomyces rubrogriseus son eficaces contra las cepas clínicas de SAMR

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Resumen

Introducción: Las comunidades microbianas naturales interactúan para desarrollar relaciones mutualistas creando un entorno competitivo que estimula la producción de metabolitos secundarios que pueden desarrollarse como un fármaco potencial contra bacterias resistentes a los medicamentos. La investigación actual profundiza en la posibilidad de cocultivar actinomicetos marinos para elevar la producción de nuevos compuestos bioactivos con actividad antimicrobiana mejorada contra *Staphylococcus aureus* resistente a la meticilina (SARM).

Método: Se aisló actinomicito de sedimentos marinos, identificado como *Streptomyces rubrogresius*, se cocultivó con *S. aureus*. Se extrajeron los compuestos bioactivos y se evaluó su actividad contra variantes de SARM. Los extractos que exhibieron actividad significativa se caracterizaron adicionalmente utilizando Cromatrogafía de gases/espectrometría de masas (GC-MS).

Resultados: El estudio demostró un aumento significativo en la producción de compuestos bioactivos en cocultivo en comparación con el monocultivo. Cuando se probaron contra cepas de SARM, las zonas de inhibición obtenidas a partir de extractos de cocultivo en acetato de etilo (40 mm) revelaron diferencias sustanciales en comparación con las de los monocultivos (CIM: 10 µg/mL). El análisis GC-MS identificó composiciones químicas únicas y posibles resultados sinérgicos en el cocultivo en lugar del monocultivo.

Conclusiones: Los hallazgos de este estudio son de suma importancia ya que ayudan en el descubrimiento de nuevos antibióticos eficaces contra SARM.

Palabras clave: *Staphylococcus aureus; Streptomyces rubrogriseus,* SARM; actinomicetos marinos; Compuestos bioactivos.

Abstract

Introduction: Natural microbial communities interact to develop mutualistic relationships creating a competitive environment stimulating secondary metabolite production which may be developed as a potential drug against drug-resistant bacteria. The current research delves into the possibility of co-culturing marine actinomycetes to elevate the production of novel bioactive compounds with enhanced antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA).

Method:. Actinomyocyte was isolated from marine sediments, identified as *Streptomyces rubrogresius* was cocultured with *S. aureus.* The bioactive compounds were extracted and evaluated for activity against MRSA variants. The extracts exhibiting significant activity were further characterized using GC-MS.

Results: The study demonstrated a significant increase in the production of bioactive compounds in co-culture compared to the monoculture. When tested against MRSA strains, inhibition zones obtained from ethyl acetate extracts of co-culture (40mm) revealed substantial differences when compared with that of monocultures (MIC: 10 µg/mL). GC-MS analysis identified unique chemical compositions and potential synergistic outcomes in the coculture rather than monoculture.

Conclusions: The findings from this study are of paramount importance as they aid in the discovery of novel antibiotics effective against MRSA.

Keywords: *Staphylococcus aureus; Streptomyces rubrogriseus,* MRSA; Marine Actinomycetes; Bioactive compounds.

Highlights

It is quite hard to develop medications to compact antibacterial resistance. One possible source of a wide range of different compounds with antibiotic action is marine actinomycetes. This manuscript describes the isolation, characterization and the bioactive components from marine actinomycetes, *S.rubrogriseus* acting against MRSA. Additionally, the experiment demonstrated that co-culturing *S. rubrogriseus* and *S. aureus* resulted in a notable increase in the production of bioactive compounds.

Introduction

Marine actinomycetes, filamentous, Gram-positive bacteria found in diverse oceanic environments are known for their ability to produce a wide variety of bioactive secondary metabolites *viz.* antibiotics, antifungals, and anticancer compounds[1-4]. Recent genome sequencing initiatives have shown that actinomycetes contain a significantly higher number of secondary metabolite-biosynthetic gene clusters

(SM-BGCs) than was previously thought[5]. However, a large part of the SM-BGCs in actinomycetes were reported to remain cryptic under traditional culture conditions[6]. Thus, innovative strategies are required to activate the intrinsic biosynthetic capabilities of these actinomycetes.

To date, numerous culture-based as well as genetic engineering-based strategies, targeted genetic manipulation techniques like promoter engineering and CRISPR-mediated gene editing and non-targeted methods like ribosome engineering and activation/ inactivation of regulatory genes have made it possible to activate cryptic SM-BGCs effectively[7,8]. But compared to the aforementioned techniques increasing the production of secondary metabolites by co-culturing microorganisms has the benefit of simplicity as it does not require prior knowledge of smBGCs or tools for genetic engineering. Coculture replicates ecological pressures, such as nutrient deprivation during interspecies competition, and led to the identification of several perfect combinations of producer and inducer that effectively promote the synthesis of novel bioactive compounds.

Co-culturing actinomycetes with other bacteria is a promising approach that is projected to produce a competitive environment that may boost the production of novel compounds[9]. It also enables various microorganisms to work synergistically, utilizing their distinct metabolic pathways and abilities. The synergy between these two microbes offers a unique chance to discover bioactive compounds with potent antibacterial activity that have not yet been discovered. Therefore, our study is mainly focused on an integrative exploration of co-culturing marine actinomycetes and *S. aureus* with a primary objective of enhancing the production of novel bioactive compounds and assessing their antibacterial efficacy against MRSA strains. The background of this study is rooted in the alarmingly high prevalence of MRSA infections and the declining effectiveness of traditional antibiotics. MRSA, a beta-lactam-resistant variant of *S. aureus*, has become a powerful foe in both healthcare and community settings[10]. In addition, the discovery of key compounds with strong bioactivity against MRSA strains could pave the way for creating innovative therapeutic agents and antibiotic substitutes to address the challenges posed by MRSA infections.

Methods

Collection of sediments and pretreatment

Marine sediments were collected from 5 locations along the coastal line of Kochi, Kerala. The exploration area was located between latitudes 9° 58’ 0” N and longitudes 76° 14’ 0” E. The sediments were collected from 5 to 15 cm below the soil surface using a Van Veen grab and were transferred to the lab in sterilized polythene bags and kept refrigerated at 4 °C for further analysis. Pre-treatment of the sediments was done by adding 10 g of the wet-weight sediment to a 250 mLErlenmeyer flask containing 90 mL of 0.9 % sterile saline and kept for 30 minutes at 55 °C in an orbital shaker at 170 rpm.

Isolation of actinomycetes from sediment samples

From the treated soil samples, a volume of 1 mL was transferred aseptically and added to a test tube containing 9 mL of sterile physiological saline, mixed thoroughly, and further tenfold dilutions up to 10^{-6} were prepared. Aliquots of 1 mL from the last three dilutions were taken and spread evenly with a sterile L-rod over the three-isolation media supplemented with amphotericin (20 µg/mL) and cycloheximide (50 mg/l) as antifungal agents. The isolation media used were actinomycete isolation agar (HiMedia), Starch Casein Agar (HiMedia) and Kuster’s agar (Composition: glycerol: 10 g, casein: 0.3 g, KNO_3: 2g, K_2HPO_4: 2g, NaCl: 2g, $FeSO_4.7H_2O$: 0.01 g, $MgSO_4.7H_2O$: 0.05 g, $CaCO_3$: 0.02 g, agar: 16 g and filtered seawater: 1000 mL and pH: 7.0 ± 2.0). Three replicates were used for each dilution. The plates were incubated aerobically at 30 °C for up to 4 weeks while being periodically inspected. Colonies were identified by their physical and cultural characteristics, and the isolated actinomycetes strains were purified using the repeated streak plate method. The pure colonies were stored in the agar slants of the corresponding isolation medium.

Identification of potent Isolated Strain

The potential isolate (MS3) was examined for morphological characteristics such as aerial hyphae, the colour of aerial and substrate mycelia, colony morphology, and other microscopic characteristics.

Test microorganisms and culture conditions

Four clinical isolates of methicillin-resistant *S. aureus* (MRSA) and MRSA ATCC 33591 Strain collected from a nearby hospital were cultured on nutrient agar and incubated at 37 °C for 24 h. The nutrient broth was used to prepare the bacterial suspensions, which were then cultured for 3–5 hours at 37 °C on a rotary shaker (150 rpm). The dilution of bacterial suspensions adjusted to the turbidity of 0.5 McFarland standard (1.5×10^{8} CFU/mL) with sterile normal saline solution (NSS).

Fermentation and extraction of the bioactive compounds

Based on the primary antimicrobial activity screening by cross streak method, an isolate MS3 with promising antibacterial activity was chosen for solid-state fermentation and extraction, and the antimicrobial activity of the extracts was subsequently evaluated.

Mono and co-cultivation of samples

Solid-state fermentation of actinomycete strain MS3 was performed by inoculating 5 mL of ISP2 medium with a single pure colony and incubating for 5 days at 30 °C. Then, the ISP2 production medium (500 mL) was prepared with 50 % seawater, aseptically seeded with 1 mL of the above inoculum, and incubated on a rotary shaker at 120 rpm for 14 days at 30 °C. For the co-cultivation, the ISP2 production medium (500 mL) with actinomycetes was prepared similarly and incubated for 3 days at 30 ^{0}C. Following that the preparation of bacterial suspension was done by inoculating, the *S. aureus* ATCC 25923 strains in 5 mL seed medium (Nutrient broth) for 24 h. The cultures were incubated at 37 ^{0}C on a rotary shaker. After 24 hrs, the sample was diluted until 10^{-3} using 0.9 % NaCl, and the medium containing actinomycetes (500 mL ISP2 medium) was inoculated with 1 mL pathogenic bacteria at 10^{-1} dilution and incubated for 14 days at 30 °C on a rotary shaker at 120 rpm.

Extraction of secondary metabolites

Following incubation, each culture was aseptically filtered using the Whatman N°1 filter paper. The broth was then centrifuged at 12000 rpm for 10 min and the supernatant was extracted with an equal volume of ethyl acetate twice (1:1, v/v) using a separating funnel. The extracts of mono and coculture were then concentrated using a rotary evaporator at 40 °C. Following that, the dried extracts were weighed and dissolved in ethyl acetate and maintained as a stock solution (10 mg/mL) for further analysis.

Antimicrobial assays

The antibacterial activity of the extracts was evaluated from the 7 th to the 14 th day. The extract was added onto agar plates inoculated with MRSA, and the zones of inhibition were measured to determine the antimicrobial activity by the Kirby-Bauer disc diffusion method. Ethyl acetate was used as a negative control in all the assays.

Minimum inhibitory concentration (MIC) of the ethyl acetate extract of coculture

MIC of the ethyl acetate extract of the coculture, which showed the strongest antimicrobial activity was determined using the CLSI method with slight modifications. Briefly, 100 µl of MRSA culture adjusted to 0.5 McFarland units (1.5×10^{8} CFU/mL) was aseptically transferred into a 96-well microtiter plate, in which each well contained 100 µl of Mueller Hinton broth and equal volumes of two-fold serially diluted ethyl acetate extract of coculture with concentrations ranging from 5 to 1280 µg/mL. The inoculated plate was incubated at 37°C for 24 h. A control well was maintained which contained only sterile MHB and a positive control was prepared with MRSA culture in the same media without the extract. The assay was done twice with three replicates in each trial. The MIC was defined as the lowest concentration of the extract that produced no visible colour change after the incubation period.

Molecular Identification

The isolate with the most promising antibacterial activity was identified by the 16S rDNA sequence analysis and phylogenetic study. A single colony picked out from the plate was used to extract DNA us-

ing the conventional phenol-chloroform technique[11]. The DNA was pelleted, repeatedly washed with 70 % ethanol, and then dried for 20–30 min at room temperature. The DNA was then dissolved in TE buffer (~30 µl) and the quality of the DNA was confirmed. DNA coding for 16S rDNA regions was amplified in a 20 µl reaction volume containing 1 µl DNA (10–50 ng), 1 µl each of forward and reverse primers (10 picomoles µl-1) (27f: 5′-AGAGTTTGATC(AC)TGGCTCAG-3′; 1492r: 5′- GGTTACCTTGTTACGACTT-3′) and 10 µl Emerald Amp GT PCR master mix (Takara). The cycling conditions used for amplification were as follows: initial denaturation at 95°C for 2 min, followed by cycle denaturation at 95°C for 40 s, annealing at 55°C for 40 s, extension at 72 °C for 1.5 min for a total of 30 cycles and a final extension for 10 min at 72 °C. The Amplicons were analysed and confirmed by running 5µl PCR product on 1 % agarose gel (impregnated with ethidium bromide) at 120 V; for ~45min in 1X TAE Buffer. The PCR products were further treated with ExoSAP-IT PCR Product Clean-up Reagent and were then employed as a template for sequencing PCR performed with ABI PRISM Big Dye terminator ready reaction mix (Life Technologies, USA) using a high throughput DNA sequencer (Applied Biosystems ABI 3730 x l DNA). The sequences were quality-checked and trimmed using the software Sequencher V4.10.1 (Gene Codes Corporation, Ann Arbor, MI USA). Trimmed Sequences were searched in NCBI using the BLASTn tool and the identity of the sample was confirmed based on percentage similarity and query coverage of the nearest neighbors.

FTIR analysis

Following the secondary metabolite extraction, the ethyl acetate extracts of mono and coculture were subjected to Fourier transform and infrared (FT-IR) spectrum analysis using an FT-IR instrument (PerkinElmer, USA). The FT-IR spectrum of the active compound between the range 600–4000 cm^{-1} was scanned and then the spectrum was plotted as intensity versus wave number.

GC–MS analysis of the extract

Gas chromatography and mass spectrometry (GC-MS) were performed (Thermo Fischer Scientific, Austria) to analyze the composition of ethyl acetate extracts of monoculture and co-culture. The dimensions of the HP-5MS column were 30.0 m × 0.25 m × 0.10 µm, and high-purity helium was used as the carrier gas with a flow rate of 1 mL per minute. The MS was run in the electron ionization mode at 70eV and the source temperature was kept at 200°C. Through comparison with the NIST 11 and WILEY 9 library, the constituents in the extracts were determined.

Results

Isolation of actinomycetes from sediment samples

A total of 28 morphologically distinct actinobacterial isolates were isolated from 5 different sediment samples, and preliminary screening revealed that only 6 of the isolates possessed antimicrobial activity. Among these, isolate MS3 demonstrated broad-spectrum antibacterial as well as antifungal activity and was selected for further investigation. Among the selected isolation media, most actinobacterial isolates were grown in actinomycete isolation agar (82 %), which was followed by starch casein agar (18 %).

Morphological and biochemical characterization reveals that the genus *Streptomyces*

The macroscopic appearance of isolate MS3 showed white powdery colonies in Actinomycetes isolation agar and prominent red colour pigmentation on the bottom of the colony (Fig. 1). On Gram staining the organism appeared to be Gram-positive and cocci in nature. In the carbon source utilization test, the isolate MS3 formed abundant mycelium on the media with arabinose, cellulose, glucose, fructose, mannitol, and maltose and grew poorly in the presence of inositol and sucrose as the sole carbon source. Also, the organism tested positive for urease, melanin production, and citrate utilization and negative for starch hydrolysis. The morphology of the MS3 isolate indicated that it was closely related to the genus *Streptomyces.* The morphological (colony characters and Gram's nature) and biochemical characteristics of the isolate as observed have been summarized in Table 1.

Table 1. Morphological and Biochemical characteristics of MS3 strain

Characteristics		Carbon source Utilization test	
Gram's stain	+	Arabinose	+
Aerial mycelium	White	Cellulose	+
Substrate mycelium	Red	Glucose	+
Spore chain morphology	Spiral	Fructose	+
Multicellular complex forming ability	Yes	Inositol	-
Catalase	-	Mannitol	++
Citrate utilisation	+	Maltose	+
Indole	-	Sucrose	-
Melanin	+		
Pectinase	+		
Starch hydrolysis	-		
Urease	+		

Experiments were conducted three times

Molecular and phylogenetic analysis identified the actinobacteria as *Streptomyces rubrogresius*

Using the BLASTn tool, the 16srRNA sequence of the isolate was aligned with previously published 16S rDNA gene sequences in the NCBI GenBank database. The isolate MS3 was shown to be closely related to *Streptomyces rubrogriseus* with 99.86 % similarity, as revealed by a phylogenetic tree constructed using the neighbor-joining method in the MEGA6 software (Fig. 1C). The position of *S. rubrogriseus* among its phylogenetic neighbors is highlighted in the phylogenetic tree constructed. The 16S rRNA gene sequence data of isolate MS3 was submitted to the NCBI GenBank under the accession number OR884067 as *S. rubrogriseus.*

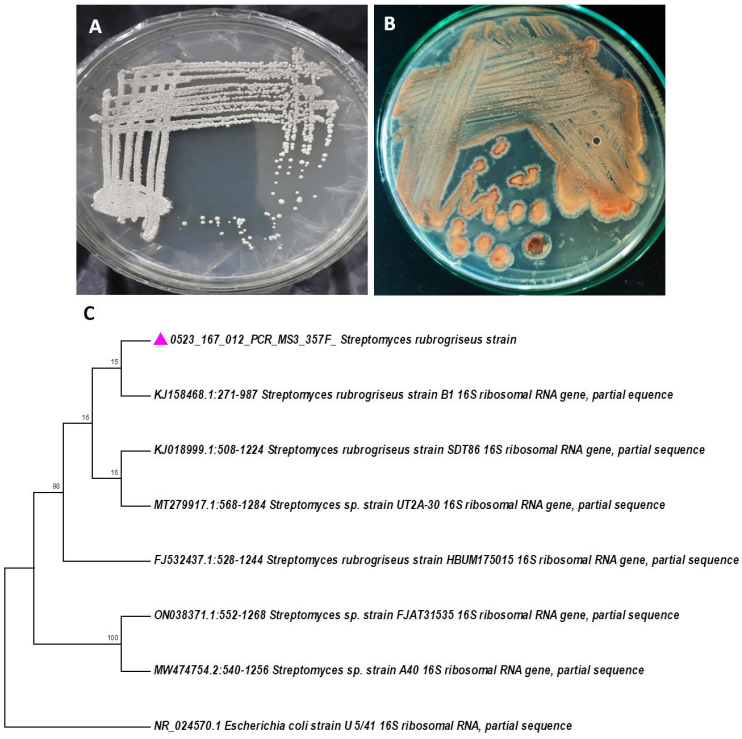

Figure 1. Morphological features and the phylogenetic tree of *S. rubrogriseus* strain using 16S rRNA analysis. A: Aerial mycelium of MS3 B: Substrate mycelium of MS3 C: Phylogenetic tree of *S. rubrogriseus*

Mono and co-cultivation of samples

The mixed fermentation was carried out for 14 days to understand the impact of the co-cultivation method on the production of secondary metabolites. The turbidity, viscosity, and colour of the medium varied over this period as a result of the interactions between the organisms. The viscosity and turbidity of the medium have increased and the colour has turned to dark red due to the production of pigments from the grown actinomycetes. The presence of actinobacteria and pathogenic bacteria was monitored and confirmed using a microscope from day 1 to day 14 and the presence of the bacteria was more difficult to detect over time in the morphological examination of the co-culture. From morphology analysis of monoculture of MS3 strain the medium remained non-turbid but the colour developed over the period as actinomycetes growth increased.

Evidence of strong antimicrobial activity

Based on the diameter of the zone of clearance when tested against 5 MRSA isolates (one ATCC reference strain and four clinical isolates), both monoculture and co-culture isolates showed inhibitory activity corresponding to a range from 31 mm to > 40mm (Fig. 2A and B) and were found to be in the susceptible range for vancomycin. The monoculture extract showed diameters of 36mm, 33mm, 33mm, 38mm, and 31mm with mean, median, and mode values of 34.2 mm, 33mm, and 33mm. Meanwhile, the coculture extract showed activity of >40mm for all the isolates. There was a substantial difference in the potential of the extracts of co-culture (5.8 %) (**Fig. 2C**). Additionally, the MIC indicated that the coculture extract possessed strong antimicrobial activity and the MIC of ethyl acetate extract of the coculture was found to be 10 µg/mL against MRSA strains. Besides, our study demonstrated that both mono and coculture showed maximum antimicrobial activity on the 14 th day.

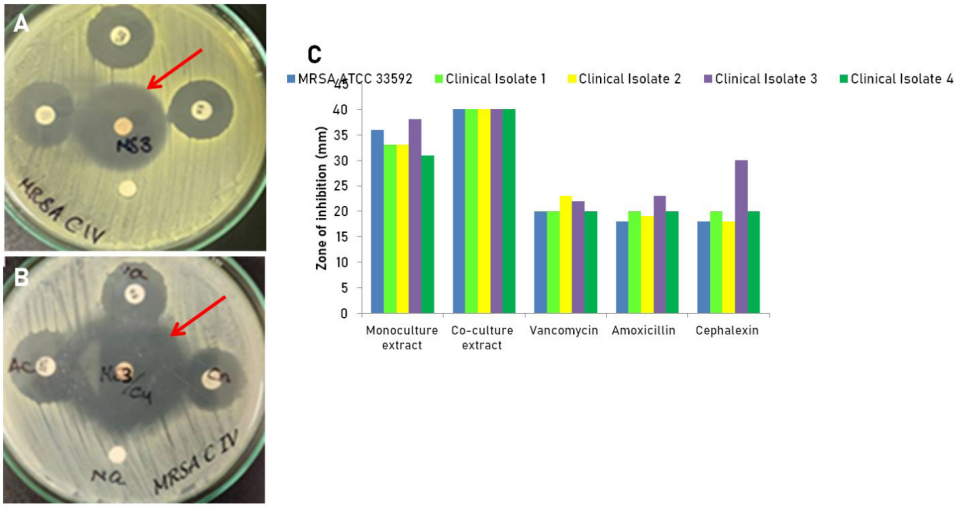

Figure 2. Antibacterial activity of the monoculture and co-culture extract using disc diffusion method against different MRSA strains. A: Effect of monoculture extract on the clinical isolate 4, B: Effect of co-culture extract on the clinical isolate 4. C: Graph showing the zone of inhibition of mono and coculture extract on MRSA ATCC 33592 strain, clinical isolate 1, clinical isolate 2, clinical isolate 3, and clinical isolate 4. The red arrow shows the extracts. Experiments were carried out three times.

FTIR analysis shows stronger absorbance for coculture extracts than from monoculture

The presence of strong absorbance peaks at 3408.92 cm^{-1} in monoculture extract and at 3374.30 cm^{-1} in coculture extracts in a Fourier-transform infrared (FTIR) spectrum is typically linked to the stretching vibration of the O-H bond. This particular peak is frequently seen in the region where the stretching

vibrations of hydrogen-bonded hydroxyl groups occur. These hydroxyl groups can be found in a variety of compounds, such as alcohols, phenols, and carboxylic acids. Alcohols often exhibit strong broad peaks in the 3200-3600 cm^{-1} range because of the O-H stretching vibration. Similarly, phenols also show strong broad peaks in this range due to the presence of hydroxyl groups. Carboxylic acids also exhibit an O-H stretching vibration peak in this region as they contain the carboxyl group (-COOH). Similarly, the absorbance peaks at 2975.80 and 2925.23 cm^{-1} in monoculture and coculture extract respectively are associated with the stretching vibration of the C-H bonds. This peak denotes the presence of alkane or alkene functional groups in the extracts. These peaks may also be produced by aromatic molecules containing C-H bonds. The distinct peaks present in the 600-1700 cm^{-1} regions are not taken into consideration as it is present in the ethyl acetate, the solvent. It is evident from the FTIR data that, despite the functional groups remaining the same, their concentration is higher in coculture than in monoculture (Fig. 3A, 3B).

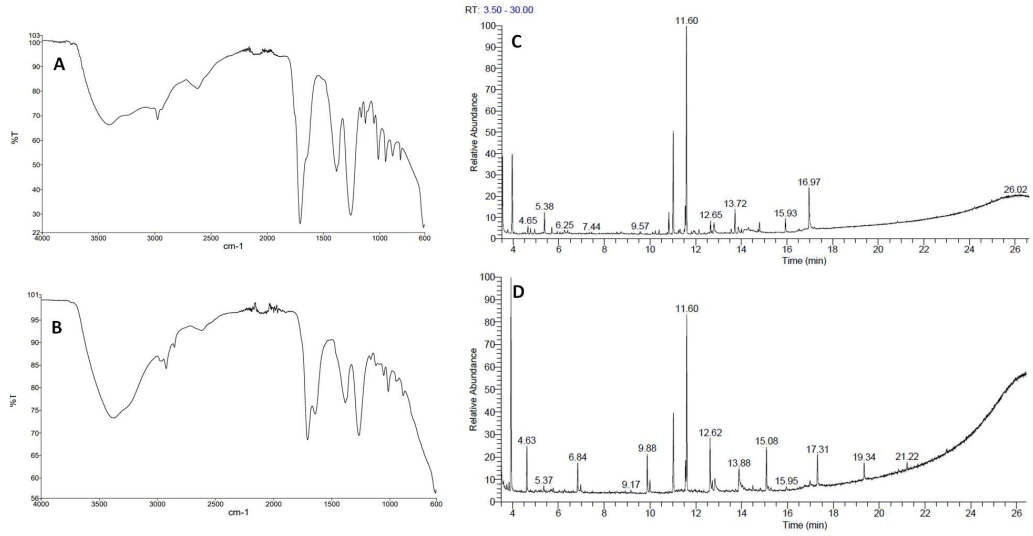

Figure 3. FTIR and GC MS analysis of mono culture and coculture extract from *S. rubrogriseus.* A: FTIR analysis of the monoculture extract. B: FTIR analysis of co-culture extract. C: GC MS TIC of of the monoculture extract. D: GC MS TIC of of the co-culture extract

GC-MS analysis shows additional compounds in coculture extract

The ethyl acetate extracts of both monoculture and co-culture were subjected to GC–MS analysis and the profiles of the fractions showed the presence of various chemical components with varying retention times and abundance (Fig. 3C, 3D). Table 2 lists the chemical components of monoculture extracts and the additional compounds present only in the coculture extracts, along with their retention time (RT), molecular weight, and molecular formula. Based on RT and peak area, the chemicals from the GC-MS study were determined. The presence of major compounds like saturated and unsaturated fatty acids, fatty acid esters, alkanes, and alkenes was confirmed in both extracts. Based on GC-MS analysis, it is evident that a higher number of compounds are present in the coculture extracts than in the monoculture of actinomycete. Ethyl acetate extracts of the co-culture of *S. rubrogriseus* and *S. aureus* showed the presence of a few additional compounds that were not present in the monoculture extracts. Also, the study confirmed the presence of a few antimicrobial compounds present only in the coculture extracts. *viz.* E-15-Heptadecenal and 10-Heneicosene (c,t) . Also, GC-MS analysis unveiled the presence of

compounds with antibacterial activity which were present in both the extracts, 2,4-Di-tert-butylphenol and α-D-Galactopyranoside, methyl.

Table 2. GC-MS analysis of the ethyl acetate extracts of monoculture of *S. rubrogriseus* and the additional compounds secreted when *S rubrogriseus* and *S. aureus* was *co*-cultured

Sl. N°	RT (min)	Compound Name	Molecular Formula	Molecular Weight (g/mol)
1.	4.65	α-D-Galactopyranoside, methyl	$C_7H_{14}O_6$	194.18
2.	5.38	2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl1,6-Dideoxy-3,4-di-c-methylhexo-2,5-diulose	$C_8H_{14}O$	174.19
3.	10.82	8,8,9-Trimethyl-deca-3,5-diene-2,7-dione	$C_{13}H_{20}O_2$	208.30
4.	11.02	2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa -2,5-dien-1-one	$C_{15}H_{24}O_2$	236.36
5.	11.54	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32
6.	11.60	Phenol, 4,6-di(1,1-dimethylethyl)-2-methyl-	$C_{15}H_{24}O$	220.35
7.	12.65	1-Cyclohexene-1-methanol, à,2,6,6-tetramethyl	$C_{11}H_{20}O$	168.28
8.	13.72	1-(2-[3-(2-Acetyloxiran-2-yl)-1,1-dimethylpropyl]cycloprop-2-enyl) ethanone	$C_{14}H_{20}O_3$	236.31
9.	13.72	4-Isopropylphenol	$C_{12}H_{20}OSi$	208.37
10.	13.72	2-Pentanone, 4-cyclohexylidene-3,3-diethyl-	$C_{15}H_{26}O$	222.37
11.	15.93	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	$C_{16}H_{22}O_4$	278.34
12.	16.97	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34
Additional compounds				
1.	9.88	1-Tetradecene	$C_{14}H_{28}$	196.37
2.	9.99	Tetradecane	$C_{14}H_{30}$	198.38
3.	15.09	E-15-Heptadecenal	$C_{17}H_{32}O$	252.43
4.	17.31	10-Heneicosene (c,t)	$C_{21}H_{42}$	294.55
5.	19.35	Nonacos-1-ene	$C_{29}H_{52}$	400.72
6.	21. 21	Tetracosan-10-yl acetate	$C_{26}H_{52}O_2$	396.70

RT-Retention time

Discussion

Natural microbial communities, where numerous species continuously interact to develop mutualistic, symbiotic, or competitive relationships so that they share nutrients or growth factors, signal-mediated interactions, or ecological pressure which creates a competitive environment stimulating secondary metabolite production.

The present study significantly contributes to the elucidation of how co-culturing microorganisms synergistically enhance the synthesis of bioactive compounds. Lately, co-culturing microbes has been the subject of numerous studies, as it is a potent technique for boosting the production of already existing ones. Co-culturing of two actinomycetes species, *Streptomyces tanashiensis* and *Streptomyces griseus* induced the accumulation of new bioactive compounds. It was found that Desferrioxamine E**,** a siderophore produced by *Streptomyces griseus*, enhanced the growth and antibiotic production of *Streptomyces tanashiensis*[12]. Similarly, when streptomyces species were co-cultured with mycolic acid-containing bacteria (MACB), several cryptic SMBGCs were activated and increased the production of compounds. Numerous antibacterial compounds like alchivemycin, prodiginine, and streptoaminals have also been found to be synthesized by co-culturing of various *Streptomyces* and MACB[13-15]. Similarly, when *Streptomyces coelicolor* was co-cultured with the bacteria *Myxococcus xanthus*, it was

reported to overproduce actinorhodin, a powerful antibiotic[16]. Similarly, the secondary metabolism of *Streptomyces lividans* was upregulated by their interaction with the fungus *Verticillium dahlia* thereby increasing the synthesis of the antibiotic compound undecylprodigiosin[17].

In another research, it was found that horizontal gene transfer between bacteria and actinomycetes has the potential to trigger the production of new secondary metabolites. Two antibiotics, designated rhodostreptomycin A (1) and B (2) were reported to be synthesized in competitive co-culture of *Streptomyces padanus* and *Rhodococcus fascians*[18]. Similarly, in co-culturing, cell-to-cell contact is not always necessary for the production of novel antibiotics as evident in the compounds produced by *Micromonospora sp*, one of the microbial strains, that were partitioned by membrane filters, which were sufficient to activate the production of the novel antibiotic keyicin[19].

Following the above studies, we observed that the co-cultivation of marine actinomycete, *S. rubrogriseus,* and *S. aureus* does promote the synthesis of potent bioactive compounds as opposed to being cultivated independently. The actinomycetes appear to mount a chemical defense in response to the interactions, which increases the synthesis of bioactive compounds. This is demonstrated by the significant difference in inhibitory zones (>40 mm) between co-culture extracts and monoculture extracts (On average 33mm). This discovery not only confirms the advantageous outcomes of co-culturing but also provides fresh insight into the potential of microbial interactions to improve biotechnological uses.

Additionally, our study effectively used GC-MS to characterize bioactive compounds, in both co-culture and mono-culture extracts offering promising prospects for further investigation into molecules with therapeutic relevance. The findings revealed unique chemical compositions and potential synergistic outcomes in the co-culture method. It is evident from our results that the presence of certain antimicrobial compounds was seen only in the co-culture extracts and could be the prime factor contributing to the enhanced antibacterial activity against MRSA strains. We have found that co-culturing has produced a few additional compounds including E-15-Heptadecenal and 10-Heneicosene (c,t). Previously E-15-Heptadecenal, isolated from the green algae *Halimeda discoidea* has proven its potency in antibacterial activity which was attributed mainly because of its potential to impede the synthesis of amino acids by bacterial cells. The compound has also demonstrated promising anti-inflammatory potential[20,21]. Besides, previous studies reported that the bioactive compound Heneicosene exhibited excellent antibacterial and antifungal activity[22]. Even though a spectrum of active compounds with diverse activities was revealed by GC-MS analysis, approximately 12 compounds were common in both co-cultivation and monoculture extracts of actinomycetes. α-D-Galactopyranoside, methyl, and Galactopyranoside-based esters have many biological activities including α-galactosidase inhibition, antidiabetic, antioxidant, antihyperlipidemic, and weak antibacterial activities[23].

In *Streptomyces* species KCA1, the active metabolite that possessed antimicrobial activity was reported as 2,4-Di-tert-butylphenol[24], which in turn was identified as a neuroprotective and an antioxidant agent[25]. Generally, natural production of Phthalic acid esters from microorganisms and plants showed broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. Similar outcomes have been seen in other studies. Roy et al. reported on the bioactive compound dibutyl phthalate, which is synthesized by *S. rubrogriseus* 321.2[26].

While the study yields encouraging results for the production of bioactive compounds by co-culturing marine actinomycetes, *S. rubrogriseus* with *S. aureus*, there are certain limitations. Even though, the study found that co-cultures produced more bioactive compounds and had higher antibacterial activity than monoculture extract, the specific mechanism underlying this synergistic effect has not been thoroughly investigated. Understanding these pathways will help to optimize co-culture protocol for optimum bioactive compound synthesis. Furthermore, the characterisation of the bioactive compounds was limited to analytical techniques like GC-MS. To fully understand the chemical structure of the mos potent compound/s and the possible therapeutic applications of these molecules, further studies involving more comprehensive analysis like NMR spectroscopy would be necessary.

Conclusions

Our study offers convincing evidence that co-culturing marine actinomycetes *S. rubrogriseus* with *S. aureus* increases the synthesis of bioactive compounds, potentially acting as a vital substitute for traditional antibiotics in the fight against MRSA strains. Future research directions have to focus on the comprehensive pharmacological investigations ought to assess the safety and effectiveness of the isolated compounds, bringing us one step closer to possible clinical trials and contributing to the resolution of the long-standing challenge of antibiotic resistance.

Data sharing statement. All data generated or analyzed during the present study are included in this published article. The 16srRNA sequence of the identified actinomycete is deposited in the GenBank with an accession number, OR884067. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

References

1. Jagannathan S V, Manemann E M, Rowe S E, Callender, M. C., Soto, W. Marine actinomycetes, new sources of biotechnological products. Marine Drugs. 2021; 19(7): 365. doi: 10.3390/md19070365.

2. Mast Y, Stegmann E. Actinomycetes: The antibiotics producers. Antibiotics. 2019; 8(3): 105. doi: 10.3390/antibiotics8030105.

3. Keikha, N, Mousavi S A, Bonjar G S, Fouladi B, Izadi A R. In vitro antifungal activities of Actinomyces species isolated from soil samples against Trichophyton mentagrophytes. Cur Med Mycol. 2015; 1(3): 33-38. doi: 10.18869/acadpub.cmm.1.3.33.

4. Ngamcharungchit C, Chaimusik N, Panbangred W, Euanorasetr J, Intra B. Bioactive metabolites from terrestrial and marine actinomycetes. Molecules, 2023; 28(15): 5915. doi: 10.3390/molecules28155915.

5. Lee N, Kim W, Hwang S, Lee Y, Cho S, Palsson B, Cho B.K. Thirty complete Streptomyces genome sequences for mining novel secondary metabolite biosynthetic gene clusters. Sci Data. 2020; 7(1): 55. doi: 10.1038/s41597-020-0395-9.

6. Kim J H, Lee N, Hwang S, Kim W, Lee Y, Cho S, Cho B K. Discovery of novel secondary metabolites encoded in actinomycete genomes through coculture. J Ind Micro Biotech. 2021; 48(3-4):kuaa001. doi: 10.1093/jimb/kuaa001.

7. Nguyen CT, Dhakal D, Pham V T T, Nguyen H T, Sohng J K. Recent advances in strategies for activation and discovery/characterization of cryptic biosynthetic gene clusters in Streptomyces. Microorganism. 2020; 8(4): 616. doi: 10.3390/microorganisms8040616.

8. Liu Z, Zhao Y, Huang C, Luo Y. Recent advances in silent gene cluster activation in Streptomyces. Front Bioeng Biotech. 2021; 9: 632230. doi: 10.3389/fbioe.2021.632230.

9. Hoshino S, Onak, H. Abe I. Activation of silent biosynthetic pathways and discovery of novel secondary metabolites in actinomycetes by co-culture with mycolic acid-containing bacteria. J Indus Microbiol Biotech. 2019; 46(3-4): 363-374. doi: 10.1007/s10295-018-2100-y.

10. Alghamdi B A, Al-Johani I, Al-Shamrani J M, Alshamrani H M, Al-Otaibi B G., Almazmomi, K, Yusof NY. Antimicrobial resistance in methicillin-resistant Staphylococcus aureus. Saudi J Biol Sci. 2023; 30(4): 103604. doi: 10.1016/j.sjbs.2023.103604.

11. Sanbrook J, Fritsch E F, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989; 11: 31.

12. Yamanaka K, Oikawa H, Ogawa H O, Hosono K, Shinmachi F, Takano H, Ueda K. Desferrioxamine E produced by Streptomyces griseus stimulates growth and development of Streptomyces tanashiensis. Microbiology, 2005; 151(9): 2899-2905. doi: 10.1099/mic.0.28139-0.

13. Onaka H, Mori Y, Igarashi Y, Furumai T. Mycolic acid-containing bacteria induce natural-product biosynthesis in Streptomyces species. Appl Environ Microbiol. 2011; 77(2): 400-406. doi: 10.1128/AEM.01337-10.

14. Traxler M F, Watrous JD, Alexandrov T, Dorrestein PC, Kolter R. Interspecies interactions stimulate diversification of the Streptomyces coelicolor secreted metabolome. MBio, 2013; 4(4): 1010-1128. doi: 10.1128/mBio.00459-13.

15. Sugiyama R, Nishimura S, Ozaki T, Asamizu S, Onaka H, Kakeya H. Discovery and total synthesis of streptoaminals: antimicrobial [5, 5]-spirohemiaminals from the combined-culture of Streptomyces nigrescens and Tsukamurella pulmonis. Angewandte Chemie. 2016; 128(35): 10434-10438.

16. Pérez J, Muñoz-Dorado J, Braña A F, Shimkets L J, Sevillano L, Santamaría R I. Myxococcus xanthus induces actinorhodin overproduction and aerial mycelium formation by Streptomyces coelicolor. Microbial Biotech. 2011; 4(2): 175-183. doi: 10.1111/j.1751-7915.2010.00208.x.

17. Meschke H, Walter S, Schrempf H. Characterization and localization of prodiginines from Streptomyces lividans suppressing Verticillium dahliae in the absence or presence of Arabidopsis thaliana. Environ Microbiol. 2012; 14(4): 940-952. doi: 10.1111/j.1462-2920.2011.02665.x.

18. Kurosawa K, Ghiviriga I, Sambandan T G, Lessard P A, Barbara J E, Rha C, Sinskey A J. Rhodostreptomycins, antibiotics biosynthesized following horizontal gene transfer from Streptomyces padanus to Rhodococcus fascians. J Am Chem Soc. 2008; 130(4): 1126-1127. doi: 10.1021/ja077821p.

19. Adnani N, Chevrette M G, Adibhatla S N, Zhang F, Yu Q, Braun D R, Bugni T S. Coculture of marine invertebrate-associated bacteria and interdisciplinary technologies enable biosynthesis and discovery of a new antibiotic, keyicin. ACS Chem Biol. 2017; 12(12): 3093-3102. doi: 10.1021/acschembio.7b00688.

20. Supardy N A, Ibrahim D, Sulaiman S F, Zakaria N A. Inhibition of Klebsiella pneumoniae ATCC 13883 cells by hexane extract of Halimeda discoidea (Decaisne) and the identification of its potential bioactive compounds. J Microbiol Biotech. 2012; 22(6): 872-881. doi: 10.4014/jmb.1111.11053.

21. Pradhan S, Dubey R C. GC–MS analysis and molecular docking of bioactive compounds of Camellia sinensis and Camellia assamica. Arch Microbiol. 2021; 203(5): 2501-2510. doi: 10.1007/s00203-021-02209-6.

22. Vanitha V, Vijayakumar S, Nilavukkarasi M, Punitha VN, Vidhya E, Praseetha PK. Heneicosane—A novel microbicidal bioactive alkane identified from Plumbago zeylanica L. Indus Crops Prod. 2020; 154: 112748.

23. Matin P, Hanee U, Alam M S, Jeong JE, Matin M M, Rahman M R, Kim B. Novel galactopyranoside esters: Synthesis, mechanism, in vitro antimicrobial evaluation and molecular docking studies. Molecules 2022; 27(13): 4125. doi: 10.3390/molecules27134125.

24. Seenivasa A, Manikkam R, Kaari M, Sahu AK, Said M, Dastager S G 2, 4-Di-tert-butylphenol (2, 4-DTBP) purified from Streptomyces sp. KCA1 from Phyllanthus niruri: Isolation, characterization, antibacterial and anticancer properties. J King Saud Uni Sci. 2022; 34(5): 102088.

25. Choi D Y, Choi H. Natural products from marine organisms with neuroprotective activity in the experimental models of Alzheimer's disease, Parkinson's disease and ischemic brain stroke: Their molecular targets and action mechanisms. Arch Pharm Res. 2015; 38: 139-170. doi: 10.1007/s12272-014-0503-5.

26. Roy R N, Laskar S, Sen S K. Dibutyl phthalate, the bioactive compound produced by Streptomyces albidoflavus 321.2. Microbiol Res. 2006; 161(2): 121-126. doi: 10.1016/j.micres.2005.06.007.