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Investigation of Potent Antifungal Metabolites from Marine *Streptomyces bacillaris* STR2 (MK045300) from Western Algeria

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Abstract: Fungal infections significantly threaten public health, and many strains are resistant to antifungal drugs. Marine Actinobacteria have been identified as the generators of powerful bioactive compounds with antifungal activity and can be used to address this issue. In this context, strains of Actinomycetes were isolated from the marine area of Rachgoun Island, located in western Algeria. The isolates were phenotypically and genetically characterized. The most potent antifungal isolate was selected, and its crude extract was purified and characterized by the GC/MS method. The results revealed that the STR2 strain showed the strongest activity against at least one target fungal species tested on a panel of fungal pathogens, including *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Fusarium oxysporum*. The molecular assignment of the STR2 strain based on the 16S rRNA gene positioned this isolate as a *Streptomyces bacillaris* species. The presence of safranal (2,3-dihydro-2,2,6-trimethylbenzaldehyde) in the crude chloroform extract of *Streptomyces bacillaris* STR2 strain was discovered for the first time in bacteria using chromatographic analysis of its TLC fractions. Moreover, certain molecules of biotechnological interest, such as phenols, 1,3-dioxolane, and phthalate derivatives, were also identified. This study highlights the potential of marine actinomycetes to produce structurally unique natural compounds with antifungal activity.

Keywords: GC/MS; marine actinomycetes; polyphasic taxonomy; safranal; *Streptomyces*

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1. Introduction

Natural substances are the output of the secondary metabolism of terrestrial or marine living forms, including plants, algae, invertebrates, and, especially, microorganisms [1]. The latter occupy a fascinating place in our lives and in the pharmaceutical industry due to their ability to produce secondary metabolites with a composite structure and a wide spectrum of biological activities [2]. The first microorganism-derived antibiotic, "actinomycin", isolated from *Actinomyces antibioticus* in 1940 by Waksman and Woodruff [3], marked the beginning of an intensive quest for microorganisms with potent antimicrobial activity. This has contributed to the golden age of antibiotics. Regrettably, the magic of antibiotic therapy has dissipated, as the excessive use of antibiotics has led to the development of antibiotic resistance mechanisms in infectious bacteria. Despite the therapeutic success of synthetic antibiotics, their laborious and expensive chemical manufacture has prompted the discovery of new antibiotics from natural sources [4,5]. Actinomycetes are the most sought-after microorganisms in the pharmaceutical industry, as they produce many molecules used

in antibiotic therapy [6]. *Streptomyces* has been abundantly found in the soil, as well as in the rhizosphere. So far, the recurrent isolation of the same strains has led scientists to look for alternative ecosystems and to become more fascinated by the marine environment [7]. The oceans cover 70% of the globe and harbor diverse animal and plant life [8]. Numerous macro- and microorganisms inhabiting marine ecosystems have been studied in recent decades with the hope to expand the therapeutic arsenal and minimize the issue of the resistance of some pathogenic bacteria to the antibiotics commonly used by clinicians [9].

Scientists started to reveal the biological powers of marine microorganisms when Giuseppe Brotzu first isolated the cephalosporin-synthesizing marine fungus "*Cephalosporium acressonium* C" (currently called *Acremonium chrysogenum*) from a seawater sample in 1945 [10]. Subsequently, Burkholder et al. [11] extracted a bioactive molecule from a marine strain of *Pseudomonas* sp. Following these two findings, the bactericidal properties of marine waters were proven, and the scientists moved on to the study of other marine species.

Among marine microorganisms, cyanobacteria and actinobacteria are the most prolific producers of biologically active natural substances [12]. The first marine actinomycete strain, *Rhodococcus marinonascens*, was described in 1984 from marine bottom sediments [13]. In contrast, the first genus of obligate marine actinomycetes was described in 2005, and the name *Salinispora* was originally proposed [14]. Since then, about 7 novel genera, 48 new species of marine actinomycetes, and approximately 100 biomolecules have been discovered in aquatic systems during 2013–2017 [15]. These actinomycetes were associated with various aquatic organisms, including vertebrates such as pufferfish and invertebrates such as sponges, corals, and echinoderms [16].

Streptomyces bacillaris, of marine origin, is an actinomycete species isolated from several aquatic specimens. It is known to possess a unique structural molecule with diverse bioactivities [17]. The antibacterial and antifungal activities of biomolecules derived from *Streptomyces bacillaris* are widely recognized. The key compounds isolated from this species include lactoquinomycin A and tunicamycin. Recently, a novel antibiotic isolated from *S. bacillaris* strain MBTC38, "lactoquinomycin A", exhibited the highest activity against pathogenic bacteria, particularly against Gram-positive ones [17]. The same strain showed a moderate inhibition of the *C. albicans*-derived enzyme isocitrate lyase (ICL), which is the operative enzyme for the survival of several pathogens in the host cell [18]. Tunicamycin exhibits strong antibacterial properties by inhibiting the enzyme MurNAc-pentapeptide translocase (MraY), which is crucial for bacterial cell wall synthesis, particularly against Gram-positive bacteria such as *Staphylococcus aureus* [19].

In Algeria, most of the studies conducted on the isolation of actinomycetes have focused principally on searching for fertile soils or Saharan areas (soils and caves) [20]. Regarding the marine environment, only continental water resources, including some lakes or natural springs, have been explored [21,22].

Safranal is a natural chemical component that contributes to the distinctive aroma of saffron, a spice obtained from the flower of the *Crocus sativus* plant [23]. Chemically, it is a monoterpene aldehyde with the chemical formula 2,6,6-trimethylcyclohexa-1,3-diene-1-carboxaldehyde or other synonyms such as 2,3-dihydro-2,2,6-trimethylbenzaldehyde, 2,6,6-trimethylcyclohexa-1,3-dienyl methanal. Safranal has been demonstrated to have a wide spectrum of activities, such as an antioxidant, anti-inflammatory, antiasthmatic, anticancer, or antigenotoxic agent [24], and as an antibacterial against methicillin-resistant *Staphylococcus aureus* (MRSA) [25]. Recently, it has been reported as a secondary metabolite of several marine organisms, as well as an essential oil component of the brown alga *Dictyopteris polypodioides* [26] and the green algae *Capsosiphon tamariscifolia* and *Ulva lactuca* [27]. It was also detected in the crude extract of the marine sponge *Ircinia fusca* [6,28].

Against this background, our study aimed to isolate and identify new species of actinomycetes from Rachgoun Island, located in western Algeria, that generate distinctive biomolecules with potent antimicrobial activity.

2. Materials and Methods

2.1. Sampling and Isolation of Marine Actinomycetes Strains

Marine sediment, soil, and seawater samples were collected from Rachgoun Island in western Algeria (Beni Saf, Ain Temouchent city) (Figure 1). Marine actinomycetes were isolated in sterilized seawater using the serial dilution method. Then, 0.1 mL of each dilution was inoculated into starch–casein agar and Gauze agar medium made with 50% seawater and treated with nalidixic acid (10 μg.mL^{-1}) and cycloheximide (50 μg.mL^{-1}) to suppress Gram-negative bacterial growth and to inhibit the development of antagonist fungi and other eukaryotic microorganisms, respectively. Actinomycetes colonies were purified after 3–4 weeks of incubation in Petri plates at 30 °C by inoculating aerial mycelium over the surface of the isolation media for culture [29].

Figure 1. Localization and geographic coordinates of Rachgoun Island (Google Maps) [29].

2.2. Phenotypic Characterization of Marine Isolates

The morphological characterization of marine isolates was determined by their ability to grow in various culture media, mycelium color, and extracellular pigments as described by Shirling and Gottlieb [30].

Actinomycetes isolates were screened for the production of extracellular enzymes, including amylase, caseinase, gelatinase, mannitol, citrate assimilation, and skim milk utilization [30].

2.3. Optimal Conditions for Marine Actinomycetes Growth

The inoculation of the marine microorganisms into Bennett's medium allowed for the determination of the optimal growth conditions in terms of temperature and salinity. A range of NaCl concentrations from 0 to 10% was experimented for halo-tolerance, and 4 °C, 25 °C, 37 °C, and 44 °C were tried for temperature tolerance [29].

2.4. Phylogenetic Analysis of Isolates

2.4.1. DNA Extraction, 16S rRNA Gene Amplification, and Sequencing

The total genomic DNA of marine actinomycetes was extracted in two main steps for efficient actinomycetes genomic DNA extraction according to the previously described, albeit slightly modified, method [31].

First, the bacterial biomass was transferred in 1.5 mL Eppendorf tubes containing 250 μL of TNE extraction buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH = 8); then, the cell wall was disrupted by thermal and mechanical shocks using a stirrer (Fast Prep-24) at a speed of 4.5 m.s^{-1} for 15 sec. The second step of the extraction consisted of isolating genomic DNA with the CTAB method and standard ethanol precipitation followed by adding 3.3 μL of lysozyme (300 mg.mL^{-1}), 10 μL of proteinase K (10 mg.mL^{-1}), 65 μL CTAB/NaCl and 750 μL of a phenol–chloroform–isoamyl alcohol mixture (24:24:1 *v*/*v*). The concentration and purity of the recovered DNA were assessed spectrophotometrically using

NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and the software program ND 1000.3.8.1.

The amplification of the 16S rRNA gene was performed by PCR using universal primers 616F (5′ AGA GTT TGA TYM TGG CTC AG 3′) and 1522R (5′ AAG GAG GTG ATC CAG CCG CA 3′). Each PCR reaction was run at a final volume of 50 µL. The reaction mixture consisted of 10 µL of 5× My Taq Reaction Buffer (BIOLINE, Memphis, TN, USA) (5 mM dNTPs, 15 mM $MgCl_2$), 1 µL of each 20µM primers, 1 µL of MyTaq DNA polymerase enzyme (BIOLINE), 0.2 µL of Bovine Serum Albumin (BSA, 10%), and 1 µL of template DNA (2 ng.µL^{-1}). The reaction volume was completed with sterile ultra-pure water.

The PCR reaction was performed in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 95 °C for 1 min, 35 cycles at 95 °C for 15 sec, 55 °C for 15 sec, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplicons were examined by 1.5% agarose gel electrophoresis stained with 1 µL of SYBR Green to confirm the positive result of the PCR reaction. The DNA bands were visualized using a ChemiDoc UV transilluminator (Bio-Rad). Forward and reverse strands of the amplified DNA fragments were sequenced on an ABI 3700 capillary sequencer (Applied Biosystems, Waltham, MA, USA) using 616F, 907R (5′ CCC CGT CAA TTC ATT TGA GTT T-3), and 1522R primers.

2.4.2. Phylogenetic Analysis

Partial 16S rRNA gene sequences were edited using BioEdit version 7.2.5 for quality refinement. The taxonomic classification of the sequences was performed using the global alignment algorithm in the EzTaxon-e database [32] to ensure accurate identification. Multiple sequence alignment was conducted with CLUSTALX version 10.2.2. Phylogenetic relationships among the query sequences were reconstructed using the maximum likelihood method in MEGA version 7.0, employing the Hasegawa–Kishino–Yano model. The resulting bootstrap consensus tree, inferred from 1000 replicates, represents the evolutionary history of the taxa analyzed, with branches corresponding to partitions reproduced in less than 50% of bootstrap replicates collapsed. The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, parameter = 0.0500). This analysis involved 21 nucleotide sequences, with *Salinispora pacifica* CNT-133 (HQ642915) used as an outgroup to root the tree. There were a total of 1472 positions in the final dataset. All sequences have been deposited in the NCBI GenBank database for public access.

2.5. Solid-State Fermentation (SSF) and Extraction of Antimicrobial Products

A total of 100 µL of spore suspension (10^5 spores.mL^{-1}) from each strain was grown in AF medium [30] and M2 medium [3] for antimicrobial compound production for 7 days. After the incubation period, the agar media of each Petri dish was cut into small pieces and mixed with 50 mL of ethyl acetate or chloroform and shaken vigorously for 3 h. For the STR2 strain, 3 L of the AF agar medium was used for the extraction of antimicrobial compounds; then, the culture was filtered and evaporated. The obtained residue was solubilized in 2 mL of methanol and tested for its antimicrobial activity [29].

2.6. Antimicrobial Activity of Actinomycetes Isolates

The agar plug diffusion method first assessed the antimicrobial activity for bacteria in Muller–Hinton medium (MH), in Sabouraud medium for yeasts, and in Potato Dextrose Agar (PDA) for fungi. The agar disk diffusion method determined the antimicrobial activity of the molecules secreted by microbes in the crude extracts [33]. A panel of reference strains of different pathogenic microorganisms was assayed; it consisted of *Pseudomonas*

aeruginosa ATCC 27853, *Escherichia coli* ATCC 8739, *Klebsiella pneumonia* IBMC Strasbourg, *Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 6633 for bacteria; *Candida albicans* CIP 444, and *Candida albicans* 10231 for yeasts; and *Aspergillus fumigatus* MNHN 5666, *Aspergillus niger* ATCC 16404, and *Fusarium oxysporum* MNHN 963917 for fungi.

2.7. Purification of the Crude Extract of the S. bacillaris STR2 Strain and Antimicrobial Activity of Thin-Layer Chromatography (TLC) Fractions

A total of 100 µL of *S. bacillaris* STR2 crude extract (20 mg.mL^{-1}) was applied to a TLC plate (20 × 20, 200 µm silica gel, MERCK) and impregnated with different solvent systems with decreasing polarity, including ethyl acetate–methanol (90:10 v/v), petroleum–cyclohexane (50:50 v/v), and cyclohexane–ethyl acetate (80:20 v/v), to achieve optimal separation. After visualization with UV light under 365 nm and 254 nm, the TLC fractions were scraped, and the bioactive compounds present in the silica gel were extracted with 4-fold amounts of the best solvent system and centrifuged at 3000 g for 15 min. The supernatant was collected and tested against several microorganisms.

The antimicrobial activity of all fractions was analyzed by the radial diffusion assay (RDA) method according to Lehrer et al. [34], with minor adjustments. A double-layered gel was used in the standard Petri plate. Briefly: 15 mL of underlay melted TSB gel (42 °C) (0.03% w/v TSB, 1% w/v agar, 1 mL Phosphate-Buffered Saline 0.1 mM, pH: 7) for antibacterial activity and 15 mL of Sabouraud agar (0.5% Sabouraud broth w/v, 1% agar w/v, 1 mL Phosphate-Buffered Saline 0.1 mM, pH: 7) for antifungal activity were inoculated with 4.10^6 bacterial CFU and with 10^7 fungal spore suspension, respectively. After agar solidification, 3 mm diameter wells were punched and 10 µL of each fraction was added.

After the evaporation of the solvent in which the TLC fractions have been solubilized, the Petri dish was overlaid with 15 mL of overlay gel TSB (6% TSB w/v, 1% agar w/v, 1 mL Phosphate-Buffered Saline 0,1 mM, pH: 7) and Sabouraud agar (6% Sabouraud broth w/v, 1% agar w/v, 1 mL Phosphate-Buffered Saline 0.1 mM, pH: 7). Triton X and amphotericin B were used as positive controls, respectively, for the antibacterial and antifungal activity.

2.8. Gas Chromatography–Mass Spectrometry (GC/MS) Analysis of TLC Fractions of S. bacillaris STR2 Strain

The volatile bioactive compounds of the TLC fractions obtained from the STR2 crude extract were characterized by gas chromatography–mass spectrometry (GC/MS) analysis using an Agilent GC 7890B MS 240 (Agilent Technologies, Santa Clara, CA, USA) ion trap gas chromatography (GC) system, equipped with an MS detector and HP-5MS capillary column (30 m × 250 µm, 0.25 µm layer thickness). The injector temperature was set at 280 °C and the GC oven temperature was programmed at 40 °C for 2 min, followed by a ramp from 50 °C up to 250 °C, maintained for 20 min of analysis in full scan mode for 60 min. Helium was used as the carrier gas at a flow rate of 1 mL.min^{-1}. A total of 1 µL of sample was injected in the split mode and ionization ranged from 50 to 1000 mV. The identification of the metabolites was performed by comparing their mass spectra with those of a reference library (NIST).

3. Results

During our study, about twenty marine actinomycetes were isolated from Rachgoun Island. Among these strains, five isolates, named SM2.2, STR2, STR11, NOC1, and NOC6, showed the production of antimicrobial molecules. The cultural characteristics of the marine strains were studied according to the methods described in the International Streptomyces Project (ISP). All the isolates developed an aerial and vegetative mycelium; among them, some strains gave powdery colonies with a white or grayish color. The macroscopic characterization of the marine actinomycetes is listed in Table 1.

Table 1. Macroscopic characterization of the marine actinomycetes isolated from Rachgoun Island.

Strains	CAM					CMV					Pigmentation
	ISP1	ISP2	ISP3	ISP4	ISP5	ISP1	ISP2	ISP3	ISP4	ISP5	
SM2.2	+ White	+ IC	+ Gray-Brown	+ Gray-Brown	+ IC	+ Brown-Gray	+ IC	+ Beige-Green	++ Beige-Green	+ IC	Yellow pigment
STR2	+++ White	+++ Gray	++ White	++ White	+++ White	+++ Light Brown	+++ Gray	++ Light Yellow	++ Light Yellow	++ Light Yellow	Absent
STR11	+ White	+++ White	++ White	+++ White	++ Yellow	+ IC	+++ Orange-Brown	++ Yellow	+++ Light Ivory	++ Yellow	Absent
NOC1	+ Beige	-	++ White	++ White	+ Beige	+ Light Ivory	-	++ Orange-Brown	++ Beige	+ Light Brown	Absent
NOC6	++ White	-	+++ White	+++ White	++ Light Brown	++ Light Ivory	-	+++ Yellow	+++ Yellow	++ Brown	Absent

CAM: color of aerial mycelium; CMV: color of vegetative mycelium; IC: immature colony; ISP: International Streptomyces Project; -: no growth, +: low growth; ++: high growth; +++: very high growth.

The biochemical and physiological characteristics of the isolated marine strains are shown in Table 2. All isolates could degrade at least one useful substrate. Culture studies of the strains at various temperatures and pH concentrations allowed us to determine optimal growth conditions. The optimal temperature and NaCl concentrations for the growth of the five strains ranged from 25 °C to 37 °C. Exceptionally, the NOC6 strain could grow at 25 °C and 44 °C. Most strains could tolerate a pH degree between 0% and 9%.

Table 2. Biochemical and physiological characteristics of the isolated marine Actinobacteria.

Strains	S.C.	TSI				Mannitol	B.M	Skimmed Milk		Catalase	Starch	Gelatin	Urease	Casein
		Deep	Slant	H_2S Production	Gas Production			Coagulation	Peptonization					
SM2.2	-	+	+	-	-	-	-	+	+	+	-	+	+	+
STR2	-	+	+	-	+	-	-	+	-	+	+	+	-	+
STR11	+	-	+	-	-	-	-	+	+	+	-	+	+	+
NOC1	-	ND		-	-	-	-	+	+	-	+	+	+	+
NOC6	+	-	-	-	-	-	-	-	+	+	+	+	+	+

Strains	Growth temperature				NaCl tolerance							
	4 °C	25 °C	37 °C	44 °C	0%	3%	5%	6%	7%	8%	9%	10%
SM2.2	-	+	++	-	+++	++	++	++	+	+	-	-
STR2	-	++	+	-	+++	++	+	+	+	+	-	-
STR11	-	+++	+	-	+	+	++	++	+	+	+	-
NOC1	-	++	++	-	+++	++	++	+	-	-	+	-
NOC6	-	+	++	++	++	+++	+++	++	-	-	-	-

B.M.: bacterial motility; S.C.: starch casein; TSI: triple sugar iron; -: no growth; +: low growth; ++: high growth; +++: very high growth.Sequence analysis of the 16S rRNA gene using the EzTaxon-e database showed that three isolates belong to the *Streptomyces* genus, while two isolates are closely related to the *Nocardiopsis* genus. The origin of the isolates, their taxonomic affiliation, and the percentage of similarity (%) are mentioned in Table 3.

Table 3. Origin, isolation media used, and molecular assignment of the identified marine strains.

Source	Isolation Medium	Accession Number	Taxonomic Assignment (% Similarity)
Sediment			
STR2	Gause agar medium	MK045300	*Streptomyces bacillaris* (NBRC 13487) (100%)
STR11		MK045299	
SM2.2		MH752433	*Streptomyces chumphonensis* (K1-2) (99.50%)
Soil			
NOC1	Starch casein agar	MK045298	*Nocardiopsis prasina* (DSM43845) 100%
NOC6	Gause agar medium	MK045301	

According to the established phylogenetic analysis using the maximum likelihood algorithm, the STR2 and STR11 strains were closely related to *Streptomyces bacillaris* species, with a high degree of similarity (100%). The bootstrap value of 99% reinforces this affiliation. Regarding the isolate SM2.2, this strain is placed with the same clade as *Streptomyces chumphonensis* species, with a high value of bootstrap (81%). As for NOC1 and NOC6 isolates, their 16S rRNA sequences placed them in the same clade as *Nocardiopsis prasina* (bootstrap value >50%). The phylogenetic topology of the five marine isolates and the closely related type strains based on the phylogenetic analysis of 16S rDNA gene sequences from EzBioCloud is shown in Figure 2.

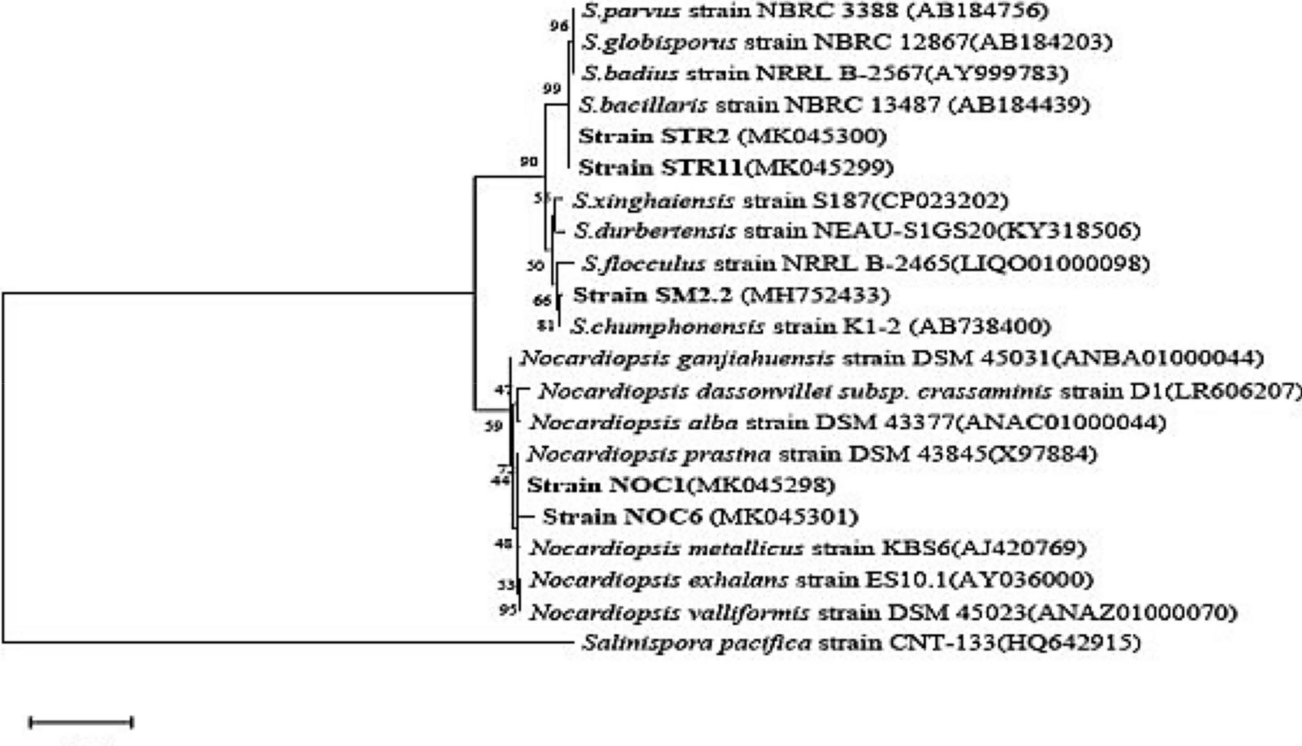

Figure 2. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains SM2.2, STR2, STR11, NOC1, and NOC6 with other genus *Streptomyces* and *Nocardiopsis species*. Bootstrap values are expressed as percentages of 1000 replicates. The 16SrRNA gene sequence of *Salinispora pacifica* CNT-133 (HQ642915) was used as the outgroup. Bar, 0.1 substitutions per nucleotide position.

Data from the in vitro antimicrobial assay of the five marine actinomycetes and the diameter of the inhibition zones (mm) are shown in Table 4. The primary screening of antimicrobial activity against bacteria, yeasts, and fungi using the agar diffusion method revealed that the AF medium seems to be favorable for the synthesis of antimicrobial substances from SM2.2, STR2, and STR11 strains, while the two isolates NOC1 and NOC6 showed the strongest antimicrobial activity in the M2 medium and seemed to be inactive in the AF medium. Solid-state fermentation and organic extraction using ethyl acetate and chloroform solvents allowed us to obtain active crude extracts from at least one marine strain. Ethyl acetate was found to be the best solvent for extracting antimicrobial substances, while chloroform seemed to be the best solvent for extracting active biomolecules from the STR2 strain.

Table 4. Diameter of the inhibition zone (mm) using plate diffusion assay.

Strains	*C. albicans* CIP 444	*C. albicans* ATCC 10231	*A. niger*	*A. fumigatus*	*F. oxysporum*	*E. coli*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*K. pneumonia*
					AF medium					
SM2.2	-	-	13	19	13	-	12	16	-	-
STR2	18	14	-	20	19	-	-	-	-	
STR11	-	-	-	18	15	-	-	-	-	-
NOC1	-	-	-	-	-	-	-	-	-	-
NOC6	-	-	-	-	-	-	-	-	-	-
					M2 medium					
SM2.2	30	-	18	17	16	-	19	25	-	-
STR2	-	-	-	16	19	-	-	-	-	-
STR11	24	20	-	14	14	-	-	-	-	-
NOC1	24	-	-	17	13.5	-	-	-	-	-
NOC6	-	-	-	-	-	-	13	15	-	-
					Ethyl acetate					
SM2.2	-	-	-	-	-	-	-	-	-	-
STR2	-	-	14	9	-	-	-	-	-	-
STR11	-	-	-	-	-	-	11	-	-	
NOC1	-	-	19	11	19	-	-	-	-	
NOC6	-	-	17	11	16	-	-	-	-	-
					Chloroform					
SM2.2	-	-	-	-	-	-	16	-	-	-
STR2	-	-	21	9	22	-	13	-	-	-
STR11	-	-	-	-	-	-	-	-	-	-
NOC1	-	-	-	-	-	-	-	-	-	-
NOC6	-	-	-	-	-	-	-	-	-	-

A: Aspergillus; B: Bacillus; C: Candida; E: Escherichia; F: Fusarium; K: Klebsiella; P: Pseudomonas; S: Staphylococcus; -: No inhibition.

Regarding antibacterial activity, no strain appeared to be active against Gram-negative pathogenic bacteria. Concerning antifungal activity, the crude extract of the STR2 strain in chloroform possesses a broad spectrum of activity against fungal pathogens, with a diameter of inhibition of 21 mm, 9 mm, and 22 mm against *Aspergillus niger*, *Aspergillus fumigatus,* and *Fusarium oxysporum,* respectively (Table 4).

Partial purification of the STR2 chloroform extract by TLC revealed the presence of seven active fractions (EB1-EB7). The antimicrobial activity of the TLC fractions against fungal pathogens (*A. niger*, *A. fumigatus*, *F. oxysporum*) and the bacterial species *B. subtilis,* assessed by the RDA method, demonstrated that all the fractions were active against at least one target strain (Table 5).

The GC/MS analyses of the TLC fractions (EB1–EB7) of the *S. bacillaris* STR2 strain revealed the presence of a mixture of biomolecules belonging to different chemical classes, including phenols, monoterpenoid derivatives, 1,3-dioxolane derivatives, and a phthalate derivative (Table 6).

Alignment of the NIST library based on retention time and molecular mass indicated a total of five chemical constituents (Table 6). Except for EB3, which contains only safranal (2,3-dihydro-2,2,6-trimethylbenzaldehyde) at 1.24%, the biomolecule 2,6-di-tert-butyl-4-chlorophenol seems to be prevalent in all TLC fractions, with a proportion ranging from 7.78 to 14.54%. The biomolecule 2,4-ditert-butylphenol was identified in each of the three TLC fractions (EB4, EB5, and EB) in modest concentrations ranging from 1.24 to 2.67%. The compounds 2(bromomethyl)-2-phenyl-1,3-dioxolane and bis (2-ethylhexyl)phthalate were identified and measured in the EB6 and EB7 fractions.

Table 5. Antimicrobial activity of TLC fractions against filamentous fungi and *Bacillus subtilis* using the RDA method.

TLC Fractions	R.F (cm)	Antimicrobial Activity of TLC Fractions			
		Bacillus subtilis	*Aspergillus fumigatus*	*Aspergillus Niger*	*Fusarium Oxysporum*
EB1	0	11	*	-	-
EB2	0.1	5	*	7	-
EB3	0.14	5	*	7	8
EB4	0.25	6	*	-	-
EB5	0.63	6	*	-	12
EB6	0.71	9	*	-	12
EB7	1	7	*	-	-
Amphotericin B		*	*	-	-
Triton X		45	21	*	*

RF: retention factor; *: untested; TLC: thin-layer chromatography.

Table 6. GC-MS profiles of TLC fractions of *S. bacillaris* STR2 strain.

TLC fractions	Major bioactive compounds	Compound Nature	RT (min)	CAS number	Percentage (%)
EB1	2,6-di-tert-butyl-4-chlorophenol	Phenol	21.38	4096-72-4	7.78
EB2	2,6-di-tert-butyl-4-chlorophenol	Phenol	21.38	4096-72-4	14.54
EB3	2,3-dihydro-2,2,6-trimethylbenzaldehyde (safranal)	Monoterpenoid	12.63	116-26-7	1.24
EB4	2,4-di-tert-butylphenol 2,6-di-tert-butyl-4-chlorophenol	Phenol	20.73 21.38	96-76-4 4096-72-4	2.67 10.41
EB5	2,4-di-tert-butylphenol 2,6-di-tert-butyl-4-chlorophenol	Phenol	20.74 21.38	96-76-4 4096-72-4	1,47 10.89
EB6	2(bromomethyl)-2-phenyl-1,3-dioxolane 2,6-di-tert-butyl-4-chlorophenol	1,3-dioxolane derivative Phenol	16.33 21.38	3418-21-1 4096-72-4	2.17 8.48
EB7	2,4-di-tert-butylphenol 2,6-di-tert-butyl-4-chlorophenol Bis(2-ethylhexyl) phthalate	Phenol Phenol Phthalate derivative	20.74 21.38 40.36	96-76-4 4096-72-4 117-81-	1.24 14.28 1.35

CAS: Chemical Abstracts Service; RT: retention time; TLC: thin-layer chromatography.

4. Discussion

Over 1 billion individuals worldwide are infected annually by opportunistic fungi belonging to the genera *Aspergillus* spp. and *Fusarium* spp. [35]. Fungal infections have been recognized as an important contributor to mortality and morbidity in immunocompromised individuals, constituting an important threat to public health [36,37].

Aspergillus spp. are widespread species of saprophytic fungi whose primary biological role is to recycle carbon and nitrogen in the environment. Aspergilli may additionally be found in indoor environments, soils, air, animal systems, and marine ecosystems [38]. Approximately twenty *Aspergillus* species that generate airborne conidia are harmful to humans [39]. *Aspergillus fumigatus* and *Aspergillus niger* are the most prevalent agents of human infections [40]. Fusariosis, an invasive fungal infection caused by *Fusarium* spp., is the second most prevalent opportunistic mold infection after aspergillosis. The most frequent *Fusarium* spp. species is the ascomycete fungus *F. oxysporum*, which is well known for its phytopathogenic abilities [41]. In human hosts, inhaled conidia produce aspergillosis infections such as craniocerebral aspergillosis of sino-nasal origin, central nervous system aspergillosis, pulmonary aspergillosis, and adrenal and hepatic aspergillosis [40]. In the case of fusariosis, a recent study suggests that *Fusarium oxyxporum* could survive in the organs of immunocompromised and immunocompetent hosts as thick-walled chlamydospores, and it is possible to develop resistance to these innate systems over time [42].

Several environmental isolates of *Aspergillus* spp. have been reported to be resistant to antifungals such asitraconazole, voriconazole, and caspofungin, which are employed for treating invasive forms of aspergillosis in human hosts [43]. The fast emergence of drug-resistant pathogens hampers clinical aspergillosis therapy. This has prompted researchers to seek out novel antifungal biomolecules to broaden the treatment arsenal [43].

A variety of potent bioactive chemicals against fungal infections have been discovered in marine species, most notably the multicellular filamentous bacterium "Actinomycetota" [44]. This bacterial phylum has been the subject of numerous studies worldwide, demonstrating that it produces a plethora of natural products useful in agriculture, biotechnology, and medicine, including two-thirds of all known antibiotics, as well as many anticancer, antifungal, and immunosuppressive agents.

The first actinomycetes isolates were discovered in soil [45], and researchers hypothesized that they are solely terrestrial. The first marine actinomycetes were discovered in 1984 [13], and since then, many marine species have been isolated from aquatic systems. In marine ecosystems, these bacteria can exist in various forms, including planktonic forms and biofilm niches. Others, on the other hand, have evolved to live in symbiosis with aquatic organisms [16]. Many scientists argue that there are substantial contrasts between aquatic and terrestrial ecosystems. By synthesizing distinct metabolites encoded by different biosynthetic gene clusters (BGCs), marine actinomycetes, as opposed to their terrestrial counterparts, generate enormous adaptation mechanisms [46].

One of the most extensively researched members of the Actinomycetota phylum and the most representative genus is *Streptomyces*. It is a surprisingly diverse genus with around 600 species, and it is responsible for the production of half of all the known antibiotics [47]. The complete genome sequence of the model strain *Streptomyces coelicolor* A3 has revealed that individual *Streptomyces* isolates can contain as many as 34 BGCs, most of which encode antibiotics [48].

There are only two kinds of natural products (NPs) with antifungal properties that have received clinical approval: echinocandins and NP polyenes. The 1,3-glucan synthase enzyme, which is essential for the production of fungal cell walls, is inhibited by fungus-derived lipopeptide NPs known as echinocandins. Amphotericin B, nystatin, natamycin, hamycin, and filipin are examples of the class of polyenes, all of which are produced by the genus *Streptomyces* and have been approved for use in treatment [49]. Fungal illnesses are becoming increasingly challenging to treat, and the majority of available antifungal medications are either synthetic or natural. Current drugs have many drawbacks, including toxicity because of their nonselective mechanisms of action, poor pharmacokinetics, and fungistatic rather than fungicidal activity, which increases the development of a resistance spectrum, in addition to the limited therapeutic options for fungal infections [49].

Overall, the isolation of novel NPs is encouraged as a means of minimizing the extent of this problem. In this context, our study was undertaken to isolate novel biomolecules from marine actinomycetes from Rachgoun Island that show activity against fungal pathogens. Antibacterial activity was also examined to verify the spectrum of activity of our isolates.

Our investigation of new NPs derived from this unexploited ecosystem allowed us to isolate five marine strains. The polyphasic taxonomy used in this study allowed for the classification of three strains into the genus *Streptomyces*, whereas two isolates were classified as *Nocardiopsis*. Analysis of 16S rRNA sequences positioned the strains STR2 and STR11 as *Streptomyces bacillaris*; the strain SM2.2 was closely related to *Streptomyces chumphonenesis*; and the strains NOC1 and NOC6 were assigned to *Nocardiopsis prasina*.

Polyphasic taxonomy requires both information from 16S rRNA sequences and phenotypic characterization for the accurate classification of the phylum Actinomycetota, in particular the genus *Streptomyces*. Several studies have demonstrated that *Streptomyces* strains are closely related by their 16S sequences but may differ in terms of biochemical profiles and physiological properties [50]. This difference can be explained by the presence of intra-species variability [51]. In our case, some variabilities in biochemical and physio-

logical characteristics were observed between strains STR2 and STR11 and between NOC1 with NOC6, which are strongly related according to their 16S sequences.

The preliminary test of antimicrobial activity allowed us to select the most promising strain to continue our study. According to our results, the marine strain STR2 showed promising antifungal activity against the filamentous fungi *Aspergillus niger*, *Aspergillus fumigatus*, and *Fusarium oxysporum* and the pathogen *C. albicans*. However, it appears to be inactive against Gram-negative bacteria. These results are in agreement with many studies showing that actinomycetes have good activity against Gram-positive bacteria but only poor activity against Gram-negative pathogens. This difference in susceptibility can be attributed to the presence of an outer membrane that can resist antibiotics through a reduced permeability mechanism [52]. In parallel, the loss of anti-Candida activity in the organic crude extracts can be explained by the inappropriate choice of organic solvents to extract anti-yeast compounds [53].

Based on the high similarity of the rRNA 16S sequence, the marine isolate STR2 was assigned to the species *Streptomyces bacillaris*. The first description of the species *S. bacillaris* was performed by Krasil'nikov in 1958, and it was assigned to *Actinomyces bacillaris*. It was later reclassified by Pridham (1970) as *Streptomyces bacillaris* [54]. It has been reported that *S. bacillaris* isolated from different terrestrial and marine environments produces structurally unique natural compounds with enormous bioactivities [54]. The *S. bacillaris* SNB-019 strain was obtained by Hu and MacMillan [55] from sand samples collected in Glaveston Bay, Texas, USA. This isolate generated a novel peptide and analogues of bafilomycin, an antibiotic known for its inhibitory action on autophagy, a process presently recognized as the first stage in carcinogenesis. Other recent investigations have demonstrated the potent antibacterial activities of *S. bacillaris* marine strains against several multiresistant pathogens belonging to the ESKAPE group (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii*) [18,56].

When the chloroform crude extract and its fractions were chemically characterized using GC/MS analysis, it was found that a variety of chemical compounds were present, of which five classes are likely to possess biological effects.

One of the most intriguing natural substances found in the purified crude extract of the STR2 strain is safranal (2,3-dihydro-2,2,6-trimethylbenzaldehyde). According to Kaiser [57], this biomolecule gives saffron its distinctive flavor and has a variety of uses in the pharmaceutical industry [24]. However, no microorganisms that produce this biomolecule have been reported in the literature; this is the first source of a safranal derivative from marine actinobacteria. It has been found in marine organisms such as green and brown algae and sponges [26–28]. According to studies, symbiotic microbes, particularly actinomycetes, may have generated many active chemicals previously discovered in marine species [58]; our data lend weight to this theory. A study by Carradori et al. [59] shows interesting antifungal activity of safranal against *Candida* spp. Another study showed good activity of safranal against *P. verrucosum* [60]. These activities of safranal can be attributed to its chemical structure, as it is an oxygenated monoterpene that can add oxygen molecules and displace or remove methyl groups. These compounds are known to have antimicrobial activity [61]. Safranal has been demonstrated to exhibit notable antifungal activity, predominantly through the disruption of fungal cell membrane integrity and the inhibition of ergosterol synthesis [62]. Recent studies have demonstrated the effectiveness of this compound against a variety of fungi, including *Candida* and *Aspergillus* [63]. Furthermore, it has been shown that its use in combination with conventional antifungal agents can enhance their efficacy [64,65]. In our study, the presence of safranal in the EB3 fraction of the purified crude extract of STR2 is probably related to its activity against *A. niger* and *F. oxysporum*.

Bis(2-ethylhexyl) phthalate is another bioactive compound that may contribute to the antimicrobial activity of STR2. This biomolecule was isolated from *Streptomyces melanosporofaciens* and *Streptomyces bangladeshensis* sp. nov. in previous studies, where it was found to have potent antibacterial and antifungal activities [66,67]. The presence of this chemical

component within the active TLC fraction (EB7) was probably responsible for its activity against *B. subtilis*.

Other chemical classes, notably the phenolic derivatives 2,4 di-tert-butyl phenol and its analogue, 2,6 di-tert-butyl-4-chlorophenol, identified in the purified fractions of the chloroform extract of the STR2 isolate, could also be contributing factors to the antibacterial activity. These substances are widely recognized to have a wide range of biological effects, such as anti-inflammatory, antitumor, antibacterial, and antifungal properties [68–70].

The bioactive compound 2-bromomethyl-2-phenyl-1,3-dioxolane, which was previously identified in the marine strain SM2.4 isolated from Rachgoun Island, was also present in the TLC fractions of the crude extract of *Streptomyces bacillaris* STR2 strain [29]. Natural dioxolide derivatives were isolated from the marine species *Penicillium* sp. and *Streptomyces tendae* [71]; these compounds had antimicrobial properties against *Candida albicans,* as well as the pathogenic microorganisms *S. aureus*, *S. epidermidis*, and *P. aeruginosa* [72].

The fatty acids (FAs) found in the chloroform crude extract of STR2, including methyl 12-methyltridecanoate, palmitic acid, methyl 15-methylhexadecanoate, methyl 14-methylhexadecanoate, and methyl 16-methylheptadecanoate, are another class of NPs that may exert promising antibacterial and antifungal properties [73]. Previous research has shown that actinomycetes can synthesize several forms of FA [29]. Moreover, Georgel, et al. [74] discovered that FA had stronger antibacterial activity in vitro than natural antimicrobial peptides.

The synergetic effect of these chemical classes is probably the result of the potent antifungal activity of the marine strain STR2 against pathogenic fungi and yeasts observed during the preliminary tests. However, the activity decreased when the compounds exerted individual effects [75].

5. Conclusions

In the present study, the marine *Streptomyces bacillaris* STR2 strain, which originates from the Rachgoun ecosystem in western Algeria, was shown to be an interesting bacterium with outstanding antimicrobial activity against several hazardous pathogenic fungi (*A. niger*, *A. fumigatus*, and *F. oxysporum*) and Gram-positive bacteria (*Bacillus subtilis*). Safranal (2,3-dihydro-2,2,6-trimethylbenzaldehyde) was discovered for the first time in a natural microbial resource. 2,6-di-tert-butyl-4-chlorophenol, 2,4-ditert-butylphenol, 2(bromomethyl)-2-phenyl-1,3-dioxolane, and bis(2-ethylhexyl) phthalate were the other active secondary metabolites with potent synergistic or distinctive effects. The present research corroborates previous findings that suggest marine *Streptomyces* are a source of new bioactive chemical discoveries.

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