ORIGINAL ARTICLE

Enhanced Antimicrobial Activity and Cytotoxicity of Egyptian *Streptomyces rochei* RS2 through Optimized Fermentation Conditions: Insights from MTT Assay

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ABSTRACT

Background: *The escalating prevalence of drug resistance and cancer necessitates the development of novel antimicrobial and anticancer agents.* ***Objective:*** *This study focuses on the isolation, characterization, and bioactivity assessment of actinomycetes-derived compounds with antimicrobial and cytotoxic potential.* ***Methodology:*** *Fifty-five soil samples from diverse regions in Egypt were initially identified based on morphological characteristics and the VITEK® 2 system. Three isolates were further confirmed using PCR and sequencing* ***Results:*** *Among these, three isolates (RS1, RS2, RS3) exhibited significant antimicrobial activity. Molecular characterization via PCR amplification and phylogenetic analysis confirmed RS2 as closely related to Streptomyces rochei. Optimization of fermentation conditions (pH 7, 28–31°C, 5–7 days incubation) significantly enhanced bioactive compound production. LC-MS analysis of RS2 crude extract identified antimicrobial compounds (Candicidin, Novobiocin, Erythromycin, Chloramphenicol) and anticancer agents (Mithramycin, anthracycline). The extract demonstrated robust antibacterial activity, with inhibition zones of 19 mm (E. coli), 21 mm (B. subtilis), 14 mm (S. aureus), and 12 mm (Pseudomonas sp.). Cytotoxicity assays revealed dose-dependent inhibition of HepG2 liver carcinoma cells, with an IC50 of 79.91 ± 0.24 µg/mL and 97.46% inhibition at 1000 µg/mL. Morphological changes in HepG2 cells indicated apoptosis induction at higherconcentrations.* ***Conclusion:*** *These findings underscore Streptomyces rochei RS2 as a promising source of novel antimicrobial and anticancer compounds, highlighting its potential for addressing drug-resistant pathogens and cancer therapy.*

INTRODUCTION

Actinomycetes are free-living, unicellular, gram-positive bacteria predominantly found in soil and aquatic environments [1]. These microorganisms are prolific producers of bioactive compounds, contributing approximately 45% of the 22,500 known bioactive metabolites, with bacteria and fungi accounting for 17% and 38%, respectively [2]. Actinomycetes are characterized by their ability to form distinct pigmentation on culture media, a feature often utilized for identification purposes [2]. Among actinomycetes, the genus *Streptomyces* is particularly notable, recognized by the World Health Organization as a major source of broad-spectrum antibiotics. However, the overuse and misuse of antibiotics have led to the emergence of widespread antibiotic resistance, posing a significant global health challenge in the 21st century [3].

Streptomyces species are of immense pharmaceutical interest due to their production of secondary metabolites with antibacterial, antifungal, and anticancer properties[4]. This makes them a valuable resource in the search for novel therapeutic agents to combat antibiotic resistance and cancer. Egyptian soil, with its diverse environmental conditions, harbors a rich diversity of actinomycetes, including *Streptomyces rochei*, a promising candidate for antimicrobial and anticancer applications. Molecular identification of *S. rochei* was confirmed through PCR and phylogenetic analysis, while LC-MS analysis revealed the presence of bioactive compounds such as anthracyclines, Candicidin, Novobiocin, Erythromycin, and Mithramycin, known for their antimicrobial and cytotoxic activities [5].

Further optimization of large-scale fermentation could facilitate the development of these bioactive molecules for clinical trials and future drug development [6]. This research positions *S. rochei* as a valuable candidate for biotechnological applications, supporting its role in the discovery of new antibiotics and anticancer therapies.

METHODOLOGY

Isolation of Actinomycetes from Soil Samples

Fifty-five Actinobacteria isolates were collected from diverse ecological environments in Cairo, Giza, and El-Gharbia, Egypt. Soil samples were obtained from a depth of 10–20 cm, targeting the rhizosphere zone due to its high microbial diversity [7]. The samples were air-dried, sieved, and stored at 4°C for microbial analysis. Serial dilutions were prepared, and Actinobacteria were isolated using starch nitrate agar (SNA) medium, followed by incubation at 29°C for 7–10 days[7].

Morphological and Biochemical Characterization of Isolates

Colony morphology, including size, shape, color, and texture, was recorded for each isolate. Gram staining was performed to confirm the Gram-positive, filamentous structure typical of Actinobacteria. Biochemical tests, including oxidase, iodine, glucose, and catalase assays, were conducted using the VITEK® 2 system [8].

Genomic DNA Isolation for Molecular Identification of Streptomyces

Genomic DNA was extracted from three Streptomyces strains (RS1, RS2, and RS3) using the QIAamp DNA Mini Kit (Qiagen, USA). The protocol involved cell lysis, treatment with RNase A and proteinase K, bead beating, heat incubation, and DNA purification. The purified DNA was eluted for downstream applications [9].

PCR Amplification of 16S rRNA Gene

The 16S rRNA gene was amplified from genomic DNA using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR was performed using EmeraldAmp GT PCR Master Mix (Takara, Japan) in a thermal cycler (Eppendorf, USA) under the following conditions: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min. PCR products were confirmed by 1% agarose gel electrophoresis[10].

DNA Sequence Determination

DNA sequencing was performed using the dideoxy chain termination method on an automated Biosystems sequencer at Synergy Scientific Services. Sequencing was conducted at the Animal Research Centre [11].

Phylogenetic Analysis and Species Identification

The 16S rRNA secondary structure was predicted using RNAstructure version 5.7 software (Animal Health Institute, Egypt), and restriction sites were identified using the NEB Cutter online tool version 2.0[12].

Crude Extract Preparation

The culture filtrate of the selected strain (RS2) was extracted with ethyl acetate in a 1:1 (v/v) ratio. The mixture was shaken vigorously, and the organic layer was collected using a separating funnel. The solvent was evaporated using a rotary evaporator[13].

Antimicrobial Activity of Crude Extracts

The agar well diffusion method was used to assess antimicrobial activity. Nutrient agar plates were inoculated with microbial suspensions, and wells (6–8 mm diameter) were punched aseptically. A volume of 100 µL crude extract was added to each well, and plates were incubated overnight at 37°C. Inhibition zones were measured and recorded [14].

Preliminary and Secondary Screening for Antimicrobial Activity

Preliminary screening was conducted using the cross-streak method, where actinomycetes isolates were inoculated against pathogenic microorganisms, including *Staphylococcus aureus* (ATCC-6538), *Pseudomonas aeruginosa* (ATCC-9027), *Candida albicans* (ATCC-10231), *Aspergillus brasiliensis*(ATCC-16404), and *Bacillus subtilis* (ATCC-6633), prepared to 0.5 McFarland standard [15]. For secondary screening, the culture filtrate was centrifuged, and the supernatant was tested using the agar well diffusion method [16].

Medium Optimization for Antimicrobial Activity

The RS2 isolate was grown in SNA medium under varying conditions of pH (5–8), temperature (28–31°C), and incubation time (3–11 days) using response surface methodology (RSM). Optimization was performed in an orbital shaker (150 rpm) at 30°C to maximize the zone of inhibition against pathogens [17].

Cytotoxicity Assay (MTT Protocol)

Cytotoxicity was assessed using the MTT assay. Cells were seeded in a 96-well plate, incubated at 37°C for 24 hours, and treated with serial dilutions of the crude extract. MTT reagent was added, and formazan crystals formed by viable cells were quantified by measuring optical density [18].

LC-MS Analysis of Bioactive Compounds

LC-MS analysis was performed using the Thermo/Finnigan Surveyor system in positive ion mode to determine the molecular weight of bioactive compounds. Analysis was conducted at the Central Research Center, Giza, Egypt [19].

Statistical Analysis

Data were analyzed using SPSS version 25. Results are expressed as means ± standard deviations (SDs). Multiple comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

RESULTS

Morphological and Biochemical Characterization of Isolates

Fifty-five isolates were subjected to morphological and biochemical characterization using the Vitec®2 system. Among these, seven were identified as actinomycetes, with three exhibiting antimicrobial activity. The three Streptomyces isolates, designated as RS1, RS2, and RS3, displayed filamentous morphology with branched hyphae and formed powdery colonies of varying sizes. These colonies exhibited distinct pigmentation: yellow for RS1, gray for RS2, and green for RS3. All three isolates were oxidase-positive, catalase-positive, and capable of hydrolyzing starch, as confirmed by a positive iodine test. Glucose utilization varied among the species: *Streptomyces mutabilis* RS1 and *Streptomyces rochei* RS2 demonstrated vigorous metabolic activity with effective glucose utilization, whereas *Streptomyces microflavus* RS3 showed weak or no glucose utilization, highlighting species-specific metabolic differences.

Molecular Identification of Selected Isolates

Molecular identification of the three isolates was performed, and the strains were deposited into GenBank under the following accession numbers:

Streptomyces mutabilis RS1: PP112248 (https://www.ncbi.nlm.nih.gov/nuccore/PP112248.1/)

Streptomyces rochei RS2: PP112262 (https://www.ncbi.nlm.nih.gov/nuccore/PP112262)

Streptomyces microflavus RS3: PP112264 (https://www.ncbi.nlm.nih.gov/nuccore/PP112264)

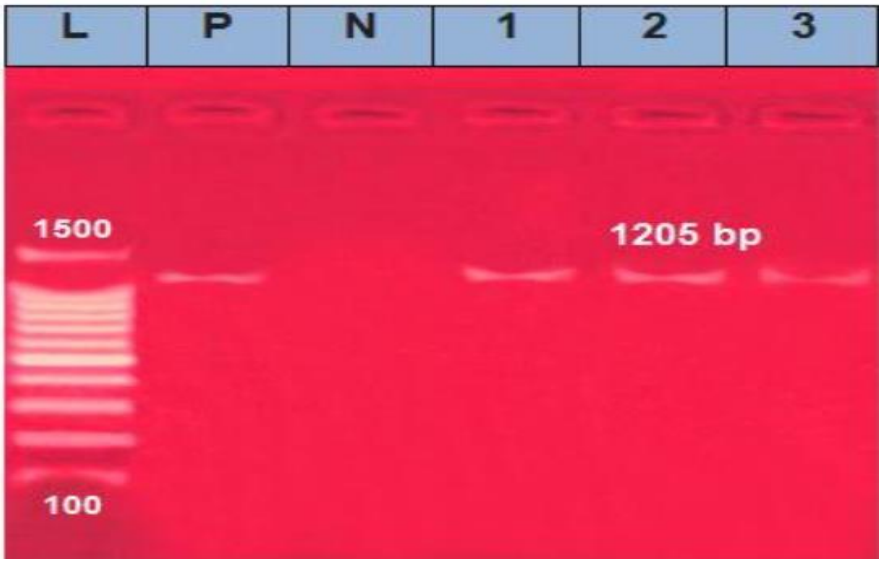


Fig. 1. PCR Product for Strains Exhibiting Antimicrobial Activities. Gel electrophoresis confirmed successful PCR amplification of antimicrobial-associated genes. The marker lane (M) displayed DNA size variation, while the positive control (P) showed a strong band, and the negative control (N) exhibited no bands, confirming PCR specificity. Lanes 1, 2, and 3, corresponding to RS1, RS2, and RS3, respectively, displayed sharp bands, indicating successful amplification of the target gene with high specificity and efficiency.

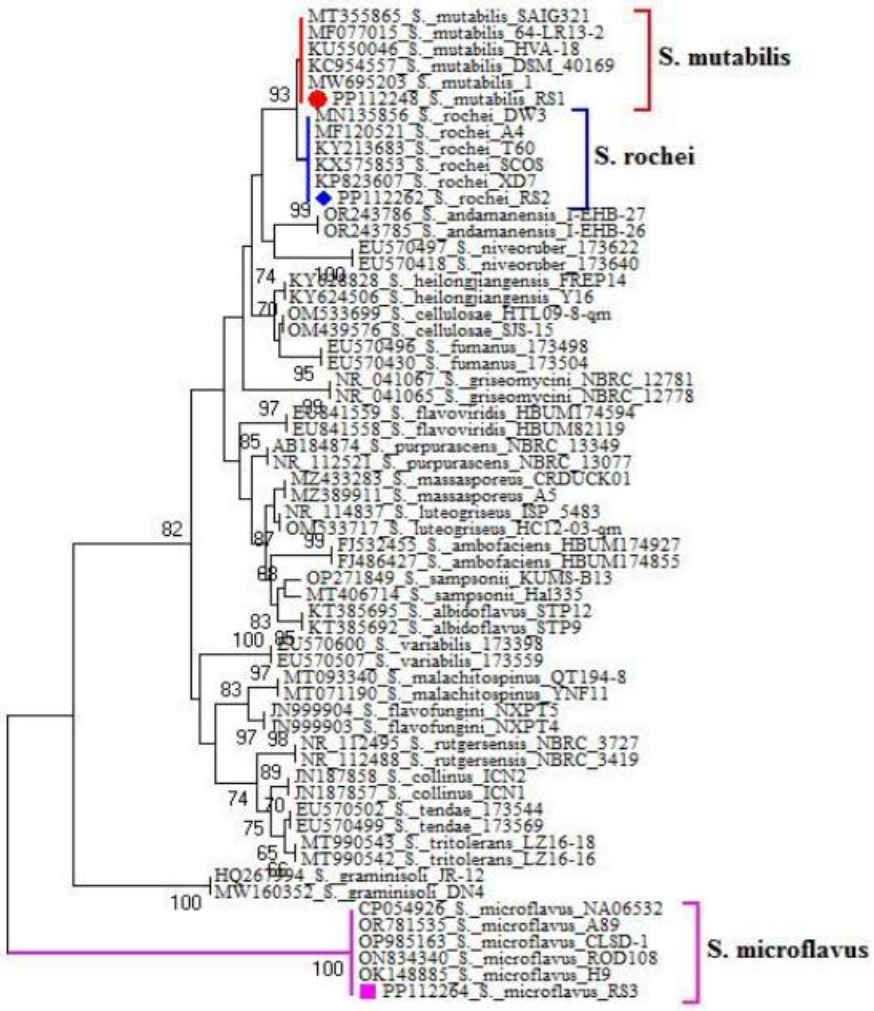


Fig. 2: Phylogenetic Tree of the selected Isolate of Streptomyces. A neighbor-joining tree constructed from 1000 bootstrap replicates revealed evolutionary relationships among the isolates. Streptomyces microflavus RS3 showed 100% similarity, Streptomyces rochei RS2 showed 99% similarity, and Streptomyces mutabilis RS1 showed 93% similarity to their respective reference strains. The scale bar indicates 0.005 substitutions per nucleotide position.

Preliminary and Secondary Screening

All isolates were screened for antimicrobial activity against pathogenic microorganisms. Only three isolates (RS1, RS2, and RS3) exhibited activity, with Streptomyces rochei RS2 demonstrating the highest inhibitory effects (Table 1).

Table 1: Preliminary Screening of Active Actinomycetes Using the Cross-Streak Method against Microbial Pathogens

Pathogen	RS1	RS2	RS3
Staphylococcus aureus (ATCC6538)	++	++++	++
Pseudomonas aeroginosa (ATCC9027)	-	+++	-
Candida albicans (ATCC10231)	-	-	-
Aspergillus brasiliensis (ATCC16404)	-	-	-
Bacillus subtilis (ATC6633).	++	++++	+

Key: ++++ = strong activity, +++ = moderate activity, ++ = weak activity, - = no activity.

Streptomyces rochei RS2 exhibited strong antibacterial activity against Staphylococcus aureus and Bacillus subtilis , moderate activity against Pseudomonas aeruginosa , and no activity against Candida albicans or Aspergillus brasiliensis . RS1 and RS3 showed weaker activity, with no effect on Pseudomonas aeruginosa , Candida albicans , or Aspergillus brasiliensis . These results highlight RS2 as the most promising strain for further investigation.

Table 2: Antimicrobial Activities of Crude Extract of Streptomyces rochei RS2 Strain

Pathogens	Zone of inhibition by mm
S. aureus (ATCC-6538)	14
PS. aeroginosa (ATCC-9027)	12
C. albicans (ATCC-10231)	-
B. subtilis (ATCC-6633)	21
E-coli (ATTC-8739)	19
Aspergillus niger (ATCC16404)	-

The crude extract of Streptomyces rochei RS2 demonstrated significant antimicrobial activity, particularly against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* , as well as Gram-negative bacteria including *Pseudomonas aeruginosa* and *Escherichia col*i . No activity was observed against *Candida albicans* or *Aspergillus niger*.

Medium Optimization of Streptomyces rochei RS2 Strain

Among the three active strains, Streptomyces rochei RS2, cultivated in SNA medium, exhibited the highest inhibitory activity against pathogens. Optimization studies using Response Surface Methodology (RSM) revealed that RS2 achieved the largest inhibition zones under optimized conditions.

Media Optimization against *E. coli*

The influence of pH, temperature, and incubation time on the inhibition zone against E. coli was evaluated. The maximum inhibition zone of 30.035 mm was observed at pH 7 and 31°C on day 6.

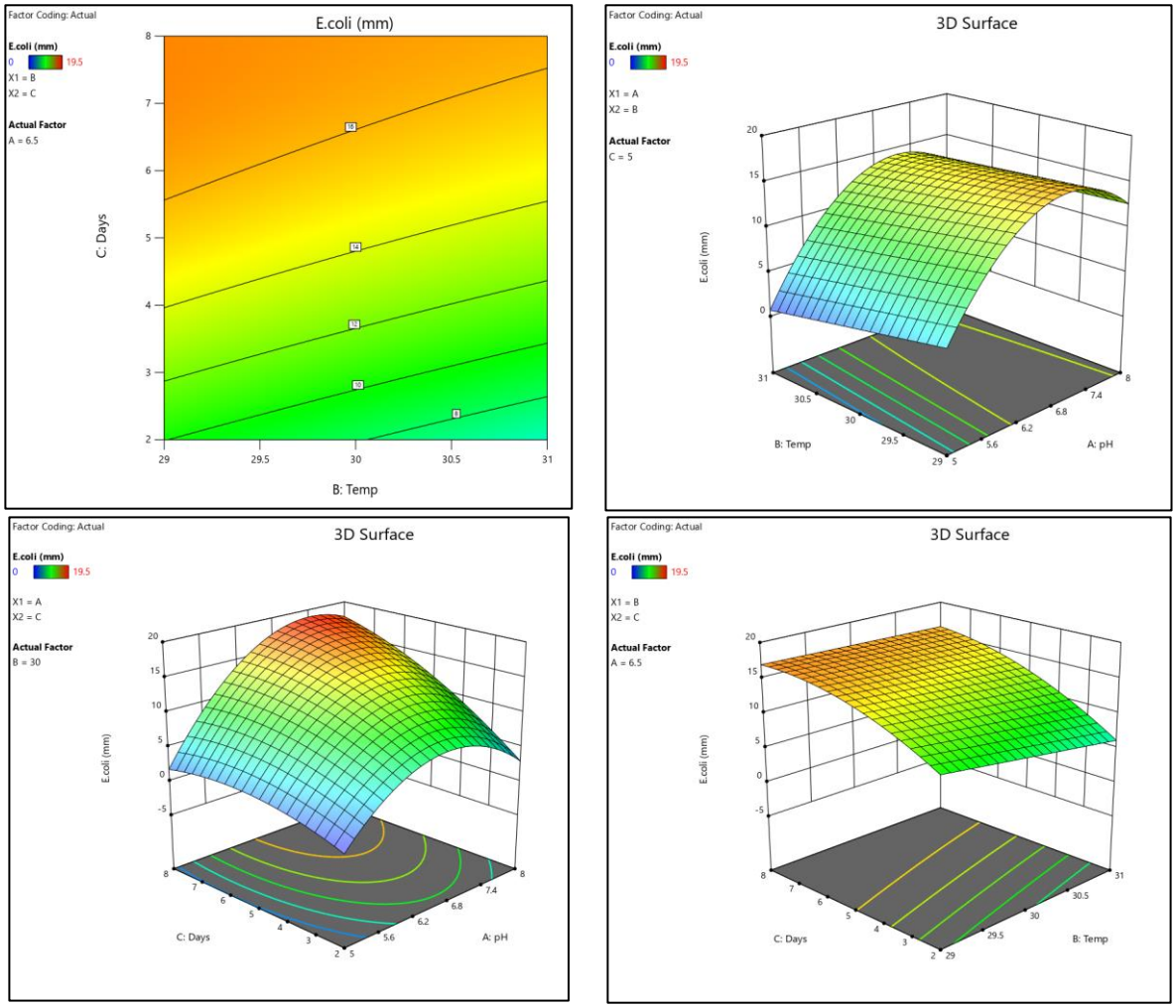


Fig. 3: Media Optimization Streptomyces *rochei RS2 strain against E.coli*

Media Optimization Against B. subtilis

Similarly, the inhibition zone against B. subtilis reached 21 mm at pH 7 and 31°C on day 6.

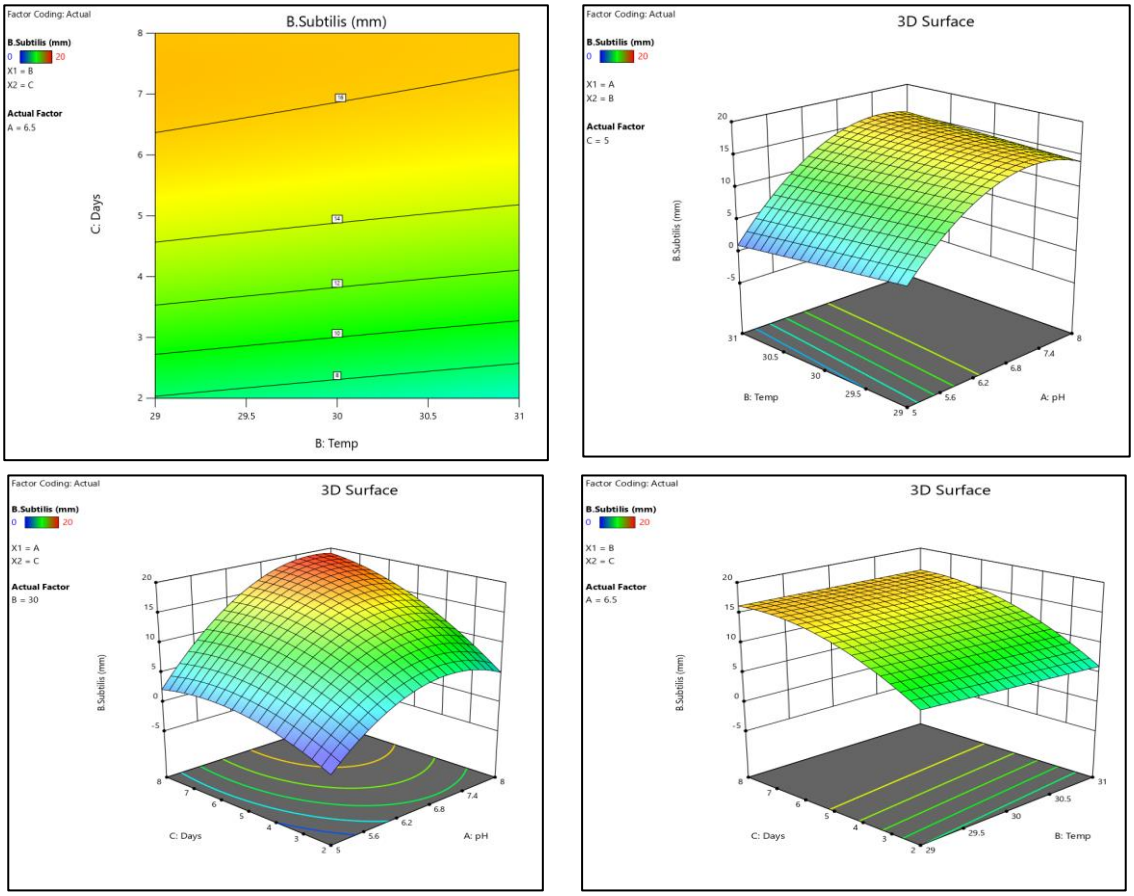


Fig. 4: Media Optimization Streptomyces *rochei RS2 strain against B. Subtilis*

Cytotoxicity of Crude Extract on Hepatocellular Cells

The crude extract exhibited dose-dependent cytotoxicity against HepG2 cells, with an IC50 value of 79.91 µg/mL (Table 3).

Table 3: Functional Assay (MTT) for Viability and Cytotoxicity Against HepG2 Cells

ID	ug/ml	Mean±SE O.D	Viability %	Toxicity %	IC50 ± SD
HepG2	--------	0.018 ± 0.001	100	0	ug
1	31.25	0.018 ± 0.001	99.307	0.693	79.91 ± 0.24
	62.5	0.717 ± 0.002	51.939	48.061	
	125	0.375 ± 0.005	17.544	82.456	
	250	0.126 ± 0.006	14.081	85.919	
	500	0.101 ± 0.001	3.139	96.861	
	1000	0.022 ± 0.001	2.539	97.461	

The crude extract exhibited dose-dependent cytotoxicity against HepG2 cells, with an IC50 value of 79.91 µg/mL (Table 3). The data indicate a dose-dependent reduction in cell viability, with the highest toxicity observed at 1000 µg/mL and the lowe.

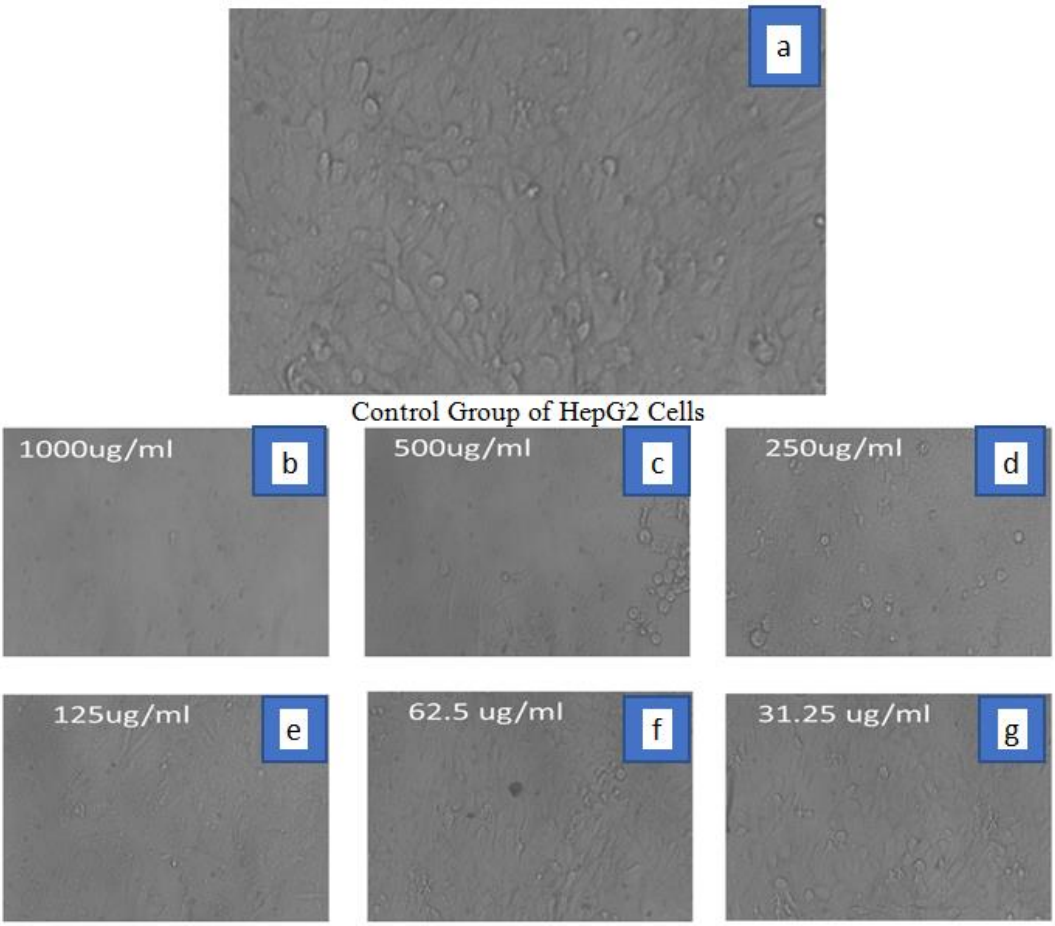
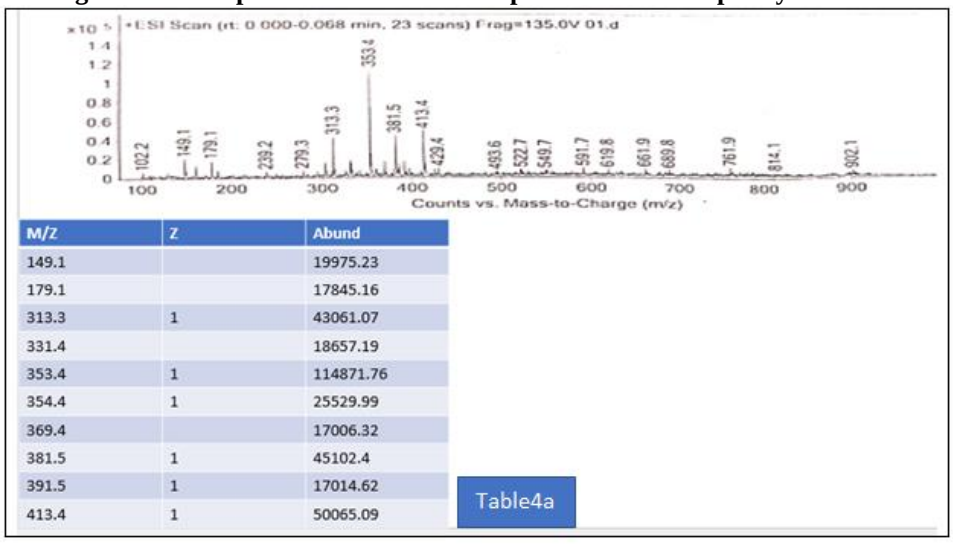


Fig. 5. Cytotoxicity and Viability of HepG2 Cells Treated with Crude Extract. a. control cells. b. tretement with crude extract at conc. 1000µg/ml, c. at conc. 500µg/ml, d. at conc. 250µg/ml, e. at conc. 125µg/ml, f. at conc. 62.5µg/ml and g at conc.31.25µg/ml.

LC-MS Analysis of Bioactive Compounds from *Streptomyces rochei*

LC-MS analysis identified several bioactive metabolites in the crude extract of Streptomyces rochei RS2, including candicidin, Actinomycin D, Streptomycin , Rifamycin ,novobiocin, erythromycin, and mithramycin, which are known for their antimicrobial and anticancer properties.

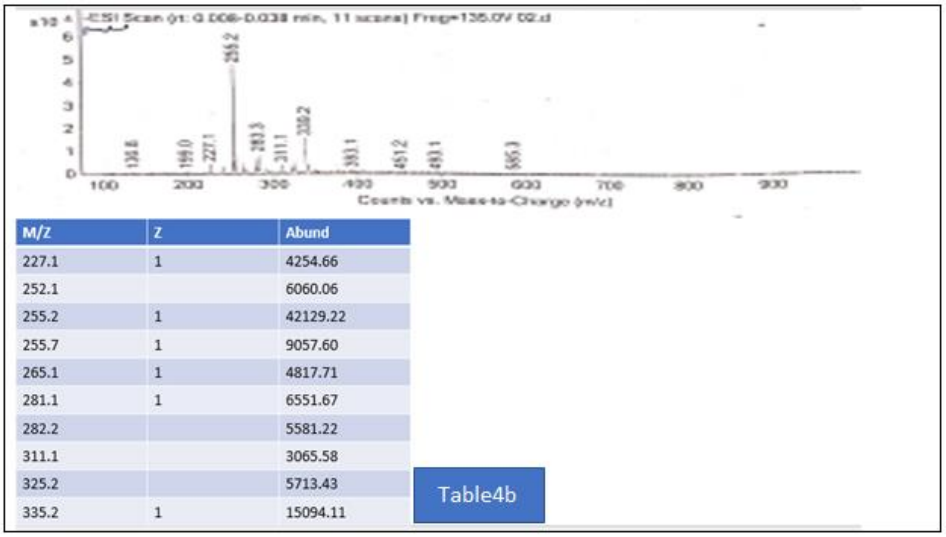
Fig. 6. LC-MS Spectra of Bioactive Compounds from Streptomyces rochei



M/Z	Z	Abund
149.1		19975.23
179.1		17845.16
313.3	1	43061.07
331.4		18657.19
353.4	1	114871.76
354.4	1	25529.99
369.4		17006.32
381.5	1	45102.4
391.5	1	17014.62
413.4	1	50065.09

Table4a

Fig. 6a: (Table.4a) Peaks at m/z 313.3 (Actinomycin D), 353.4 (Streptomycin), 381.5 (Doxorubicin-like compound), and 413.4 (Rifamycin) were identified.



M/Z	Z	Abund
227.1	1	4254.66
252.1		6060.06
255.2	1	42129.22
255.7	1	9057.60
265.1	1	4817.71
281.1	1	6551.67
282.2		5581.22
311.1		3065.58
325.2		5713.43
335.2	1	15094.11

Table4b

Fig. 6b: (Table.4b) Peaks at m/z 313.1 (Candicidin), 353.2 (Novobiocin), and 282.2 (Mithramycin) were detected.

These findings underscore the potential of *Streptomyces rochei* RS2 as a source of novel therapeutic agents with both antimicrobial and anticancer activities.

DISCUSSION

The crude extract of *Streptomyces rochei* demonstrated potent inhibitory activity against the proliferation of HepG2 hepatocellular carcinoma cell lines, highlighting its potential as an anticancer agent. Studies have indicated that maintaining a pH of 7.0±2 optimizes antimicrobial production in *Streptomyces rochei*, as this pH range enhances the synthesis of secondary metabolites by influencing enzymatic activities critical to antibiotic production [19]. Consistent with these findings, the present study revealed that optimal conditions for antimicrobial compound production by *Streptomyces* species occur at 28–31°C and pH 7.0, with maximum activity observed on day 6 of incubation. Higher concentrations of the extract disrupted the HepG2 cell layer, leading to cell death and detachment, indicative of significant cytotoxicity. The dose-dependent cytotoxic effects of

actinomycete-derived compounds on HepG2 cells underscore their potential for anticancer applications, warranting further investigation into their mechanisms of action and therapeutic index[20].

The reliability of molecular identification methods, including PCR amplification and phylogenetic analysis, was confirmed in this study. The phylogenetic tree constructed from 16S rRNA sequences identified three distinct clusters corresponding to *Streptomyces mutabilis*, *Streptomyces rochei*, and *Streptomyces microflavus*. Bootstrap analysis supported the robustness of the tree, aligning with previous molecular identification studies of *Streptomyces* strains [21].

Three-dimensional surface and contour plots illustrated the influence of pH, temperature, and incubation period on the antimicrobial activity of *Streptomyces rochei* RS2 against *Escherichia coli*. The first plot revealed that a pH range of 6.5–7.0 and an incubation period of 5–7 days maximized antimicrobial activity, with the highest inhibition observed at pH 6–7. The second plot highlighted temperature as a critical factor, with the 28–31°C range being optimal for metabolite production [22]. These findings align with previous studies on *Streptomyces exfoliatus* strain 'MUJA10', which exhibited high inhibitory potential against human pathogens. 16S rRNA gene analysis confirmed its similarity to *Streptomyces exfoliatus* A156.7 [23]. In the present study, *Streptomyces rochei* RS2 demonstrated enhanced growth and antimicrobial production at higher pH values, suggesting its suitability for large-scale antibiotic production, particularly against Gram-positive bacteria such as *Bacillus subtilis*. Media optimization studies revealed that the highest inhibition zones against *B. subtilis* were achieved at pH 6.5–7.0, an incubation period of 5–7 days, and a temperature range of 29–31°C, emphasizing the importance of optimized conditions for maximizing antibiotic yield [23].

The observed differences in inhibition zones between *B. subtilis* and *E. coli* can be attributed to the structural differences in their cell walls. The absence of an outer membrane in Gram-positive bacteria like *B. subtilis* renders them more susceptible to antibiotics targeting cell wall biosynthesis, whereas Gram-negative bacteria like *E. coli* exhibit greater resistance due to their outer membrane barrier. This underscores the potential of *Streptomyces rochei* as a broad-spectrum antibiotic producer effective against both Gram-positive and Gram-negative bacteria. Additionally, variations in membrane protein profiles between the bacillary and L-forms of bacteria, influenced by medium salt concentrations, further highlight the complexity of bacterial resistance mechanisms [24].

The results of the functional assay (MTT) demonstrated the dose-dependent cytotoxic effects of the crude extract on HepG2 cells, with an IC50 value of 79.91µg/mL. While lower concentrations exhibited minimal cytotoxicity, higher concentrations significantly reduced cell viability, suggesting a potential therapeutic window for anticancer applications. These findings align with previous studies on *Streptomyces rochei* M32, isolated from the Western Ghats ecosystem, which exhibited broad-spectrum antimicrobial activity against drug-resistant and enteric pathogens [25]. Further research is necessary to fully elucidate the therapeutic potential and safety profile of these compounds.

CONCLUSION

The current study establishes *Streptomyces rochei* as a prolific producer of antimicrobial and anticancer compounds. LC-MS analysis identified several bioactive metabolites, including candicidin, novobiocin, erythromycin, chloramphenicol, and mithramycin, which exhibited broad-spectrum antibacterial and cytotoxic activities. The crude extract demonstrated significant inhibition against both Gram-positive and Gram-negative bacteria and induced apoptosis in HepG2 carcinoma cells, with an IC50 of 79.91 µg/mL. Optimal conditions for antimicrobial production were achieved at pH 7.0, 28–31°C, and an incubation period of 5–7 days. These findings highlight the potential of *Streptomyces rochei* as a source of novel antibiotics and anticancer agents.

Recommendations

Future research should focus on optimizing fermentation conditions, validating the therapeutic potential of identified compounds, and exploring the genetic and biochemical pathways involved in their biosynthesis. Such efforts will facilitate the development of these compounds for clinical and industrial applications.

Conflict of Interest

The authors declare no conflicts of interest. All experiments and analyses were conducted independently and without external influence.

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