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Enhancement of Anti-MRSA Activity in Endophytic *Streptomyces* drozdowiczii Ca2- 5 Through OSMAC and Light-Regime Optimization

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Abstract

Background: Multidrug-resistant bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), pose a major global health threat and cause hundreds of thousands of deaths annually. Endophytic *Streptomyces* strains are promising sources of bioactive metabolites; however, many of their biosynthetic gene clusters remain silent under standard laboratory conditions.

Objectives: This study aimed to activate silent biosynthetic pathways in an endophytic *Streptomyces* isolate using the one strain–many compounds (OSMAC) strategy to enhance the production of metabolites with anti-MRSA activity.

Methods: An endophytic *Streptomyces* strain was isolated from medicinal plants in Iran. The OSMAC approach was applied by systematically varying key culture parameters, including nutrient composition, pH, aeration, incubation time, and the physicochemical conditions of the medium. Light regimes were also optimized to better simulate the bacterium's natural habitat. The antibacterial activity of the extracts was evaluated against MRSA ATCC 43300 using the standard agar well diffusion method.

Results: Optimization of culture conditions significantly increased the production of secondary metabolites with potent anti-MRSA activity. The largest inhibition zones were achieved under specific combinations of nutrient limitation, a slightly acidic pH, and defined light exposure, compared with standard laboratory conditions.

Conclusions: This study highlights the OSMAC strategy as an effective and cost-efficient method for activating silent biosynthetic pathways in endophytic *Streptomyces* by manipulating culture conditions. The optimized approach provides a valuable framework for discovering novel anti-MRSA compounds from actinobacteria.

Keywords: Streptomyces, MRSA, OSMAC Strategy, Phenotypic Screening, Bioactivity Enhancement

1. Background

Multidrug-resistant bacteria cause infections that are increasingly difficult to treat with existing antibiotics (1). Among these bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is of particular concern and poses a serious risk to vulnerable populations, including older adults, children, and immunocompromised individuals (1-4). According to the Centers for Disease Control and Prevention (CDC), antimicrobial resistance was responsible for more than 1.27 million deaths globally in 2019, with MRSA contributing to a substantial proportion of these cases each year (5, 6). These alarming statistics underscore the

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urgent need to discover and develop new antimicrobial agents (7, 8).

Endophytic actinomycetes, particularly members of the genus *Streptomyces*, are prolific producers of bioactive compounds, including antibiotics, antifungals, anticancer agents, and immunosuppressants (9-11). Despite their remarkable biosynthetic potential, many of their secondary metabolite gene clusters remain silent under standard laboratory conditions (12, 13). Various strategies have been used to activate these cryptic biosynthetic gene clusters (BGCs), including genetic engineering (14-16), co-cultivation with other microbes (17, 18), and the OSMAC approach. The OSMAC strategy relies on altering culture conditions, such as nutrient sources and physical parameters, thereby enabling a single strain to produce diverse secondary metabolites without genetic manipulation.

2. Objectives

This study aimed to isolate endophytic *Streptomyces* from Iranian medicinal plants and optimize key physicochemical parameters, including nutrient sources, aeration, incubation time, and light regime, to maximize anti-MRSA activity.

3. Methods

3.1. Sample Collection

In May 2024, medicinal plants, including *Zataria multiflora* [IPNI: 461954 - 1] and *Allium sativum* [IPNI: 528796 - 1], which are well known for their traditional use in the treatment of MRSA-associated infections (19, 20), were collected from different altitudes across Mount Damavand (coordinates: 35°57′04″N, 52°06′32″E), Iran. Plant samples were immediately placed in sterile plastic bags, kept under cold conditions, and transported to the Microbiology Laboratory of the Pasteur Institute of Iran for the isolation of endophytic *Streptomyces* and further processing (21).

3.2. Isolation of Streptomyces From Plant Samples

Plant samples were surface-sterilized using a previously described multistep protocol (22). Sterilized root and stem tissues were aseptically cut into approximately 4 × 4 mm segments and plated onto selective media, including R3A (22), ISP4, and GMA. Plates were incubated at 28 °C for 4 days, and microbial growth was monitored daily. Colonies exhibiting typical *Streptomyces*-like morphology were purified by repeated subculturing, grouped according to similar morphological characteristics, and screened for antimicrobial activity.

3.3. Initial Antimicrobial Activity Assay

A single colony of each isolate was inoculated into 100 mL of Mannitol Soya Flour (MSF) broth (23), containing 2 g mannitol, 2 g soya flour, and 10 mM CaC_2. Cultures were incubated at 28 °C for 7 days on a rotary shaker at 200 rpm. After incubation, cultures were centrifuged at 4000 rpm for 20 minutes using a Gyrozen 1524 centrifuge to separate the biomass from the supernatant. Both fractions were tested for antimicrobial activity using the agar well diffusion method against a lawn of *Staphylococcus aureus* (ATCC 43300). The inoculum was standardized to a 0.5 McFarland turbidity standard. Briefly, 25 µL of each sample was pipetted into 6-mm wells in agar plates, followed by overnight incubation at 37 °C. Several isolates showed notable antimicrobial activity, with one isolate (Ca2 - 5) exhibiting particularly strong activity against MRSA. Notably, the cell-free supernatant displayed stronger inhibitory activity than the biomass, indicating that it was a promising candidate for further study.

3.4. OSMAC-Based Optimization of Antimicrobial Activity

To explore the metabolic potential of the selected *Streptomyces* strain, the OSMAC approach was applied using 96 different culture media formulations (Table 1 in Supplementary File) to evaluate antimicrobial activity. Each 100 mL culture contained 2 g of nitrogen sources (soybean meal, peptone, or yeast extract) and 2 g of carbon sources (mannitol, glucose, starch, or chitin), supplemented with trace elements, including 10 mM $CaCl_2$. Cultivation conditions were diversified by varying shaking speed (0 - 200 rpm), incubation temperature, and light exposure, with dark conditions achieved by wrapping flasks in aluminum foil. After incubation, cultures were centrifuged at 4000 rpm for 20 minutes, and the antimicrobial activity of the supernatants was assessed against MRSA (ATCC 43300) using the agar well diffusion method with a 0.5 McFarland inoculum standard. Inhibition zones (mm) were measured after overnight incubation at 37 °C using a Scan 4000 scanner. All 96 conditions were initially screened once, and active conditions were retested in two independent experiments to confirm the reproducibility and consistency of antimicrobial activity.

3.5. Molecular Characterization

3.5.1. Genomic DNA Extraction

Genomic DNA was extracted using the boiling method (24). Briefly, bacterial cultures grown in Tryptic Soy Broth (TSB) were centrifuged at 15,000 × g for 10 minutes. The pellet was washed once with sterile water, resuspended in sterile water, and boiled at 100 °C for 10 minutes. After centrifugation, the supernatant containing DNA was collected and stored at -20 °C until use.

3.5.2. PCR Amplification of the 16S rRNA Gene

The 16S rRNA gene was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCCGCA-3′), purchased from Bioneer (USA). PCR was performed in an Eppendorf 96 AG thermal cycler (model 22331, Hamburg, Germany) under the following conditions: initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 68 °C for 1 minute, with a final extension at 68 °C for 5 minutes. PCR products were visualized using SYBR Safe DNA Gel Stain after electrophoresis on a 1% agarose gel at 90 V for 1 hour and purified using the GeneJET PCR Purification Kit.

3.5.3. Sequencing of the 16S rRNA Gene

Sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) on an ABI 310 DNA sequencer, according to the manufacturer's instructions. Both forward (27F) and reverse (1525R) primers were used. The obtained sequences were analyzed and edited using CLC Main Workbench software (version 7.0.3). Consensus sequences were exported in FASTA format and compared with sequences in the NCBI GenBank database using BLASTn to determine sequence similarity.

3.5.4. Phylogenetic Tree Analysis

Sequences showing high similarity were retrieved and aligned using MEGA X software. A maximum-likelihood phylogenetic tree was constructed to illustrate evolutionary relationships, with bootstrap analysis based on 200 replicates.

3.6. Statistical Analysis

All assays were performed in seven technical replicates, and results were expressed as mean ± SD. A value of 6 mm was considered the baseline (no inhibition). Data normality was assessed using the Shapiro-Wilk test. Differences among groups were analyzed using one-way or two-way analysis of variance, whereas nonparametric data were evaluated using the Kruskal-Wallis test. Associations between cultivation conditions and antimicrobial activity (active vs inactive) were analyzed using the chi-square test. Statistical significance was set at $P < 0.05$.

4. Results

4.1. Antimicrobial Activity of Endophytic Streptomyces Isolates

A total of 20 endophytic *Streptomyces* strains were successfully isolated from surface-sterilized root and stem tissues of medicinal plants collected from Mount Damavand, Iran. The isolates were initially characterized based on morphological features, including colony color, size, and texture, and were subsequently screened for antimicrobial activity.

The 20 isolates were cultured in ISP4 medium and then inoculated into 100 mL of MSF broth in Erlenmeyer flasks. Cultures were incubated at 30 °C with shaking at 200 rpm for 7 days. Three isolates (Ca2 - 1, Ca2 - 4, and Ca2 - 5) exhibited significant antibacterial activity against MRSA. Isolates with no activity against the test organisms were excluded from further experiments. Isolate Ca2 - 5 was selected for OSMAC optimization to enhance its anti-MRSA activity.

4.2. Effects of OSMAC Conditions on Anti-MRSA Activity

Antimicrobial activity was evaluated using the agar well diffusion method. Inhibition zone diameters were classified as follows: <6 mm, no activity; 6 - 10 mm, weak activity; 11 - 15 mm, moderate activity; 16 - 20 mm, strong activity; and > 20 mm, very strong activity. Isolate Ca2 - 5 was cultured under 96 different OSMAC conditions by systematically varying carbon and nitrogen sources, trace elements, and physical parameters, including shaking speed, incubation time, and light conditions, as shown in Table 1 in Supplementary File. These modifications resulted in significant differences in anti-MRSA activity, with the statistical analysis presented separately. The highest anti-MRSA activity was observed with soybean-mannitol, peptone-mannitol, and yeast extract-mannitol combinations. Mannitol was the most effective carbon source, whereas yeast extract was the most potent nitrogen source, particularly when combined with mannitol, starch, or glucose (Figures 1 and 2). Optimization of shaking speed indicated that 200 rpm produced the highest antimicrobial activity (Figure 3). Peak activity was observed between days 3

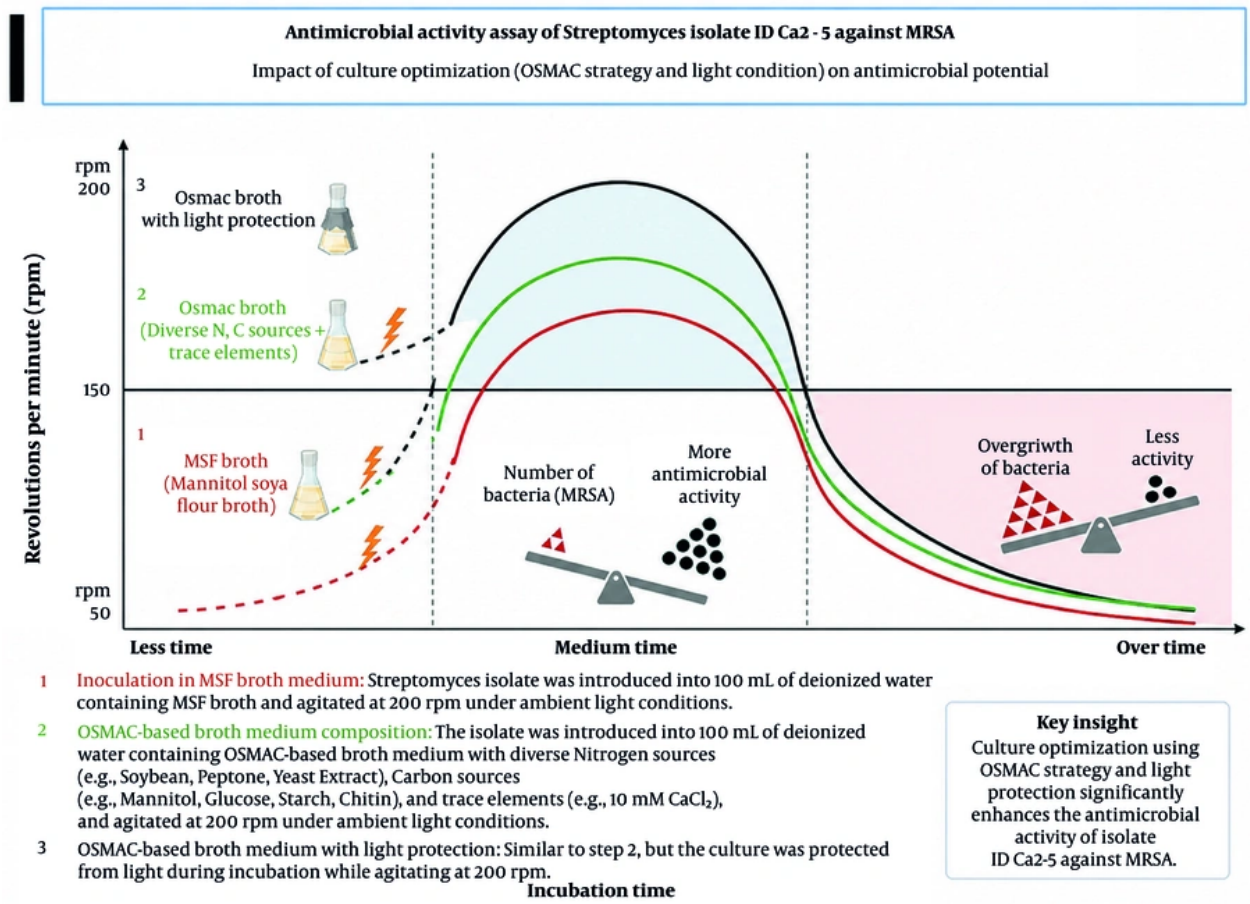

Figure 1. Distribution of nitrogen sources (soybean, peptone, and yeast extract) and carbon sources (mannitol, starch, glucose, and chitin) across different incubation conditions for isolate Ca2 - 5. The pie chart shows the breakdown of sources into three primary groups, each divided into seven subgroups corresponding to incubation periods from day 1 to day 7. These subgroups are categorized under light and dark conditions at four shaking speeds (50, 100, 150, and 200 rpm).

and 5 of incubation (Figure 1). In addition, dark incubation, achieved by wrapping flasks with aluminum foil, significantly enhanced anti-MRSA activity compared with that under light conditions (Figures 1 and 2), likely through regulation of secondary metabolite biosynthesis. The overall OSMAC optimization workflow is summarized in Figure 4.

4.3. Molecular Identification of Streptomyces Strains

Molecular identification was performed based on 16S rRNA gene sequencing (Figure 5). The sequence of isolate Ca2 - 5 was most closely related to *Streptomyces drozdowiczii* NBRC 101007^T (GenBank accession AB249957), showing 100% identity with 98% query coverage over an approximate sequence length of 601 bp. Isolate Ca2 - 1 was closely related to *Streptomyces poonensis* JCM 481, showing 98% identity with 95% query coverage over an approximate sequence length of 695 bp. Isolate Ca2 - 4 was most closely related to *Streptomyces flavovirens* NBRC 3716^T (GenBank accession AB184834), showing 99.44% identity with 94% query coverage over an approximate sequence length of 669 bp. The 16S rRNA gene sequences were deposited in GenBank under accession numbers PV998000.1 (Ca2 - 1), PQ773515 (Ca2 - 4), and PQ773516 (Ca2 - 5).

4.4. Statistical Analysis

Multifactorial statistical analysis revealed that shaking speed and carbon source were the most significant determinants of antimicrobial activity ($P < 0.001$), whereas nitrogen source had a moderate effect ($P < 0.05$). Light conditions had a minor or context-

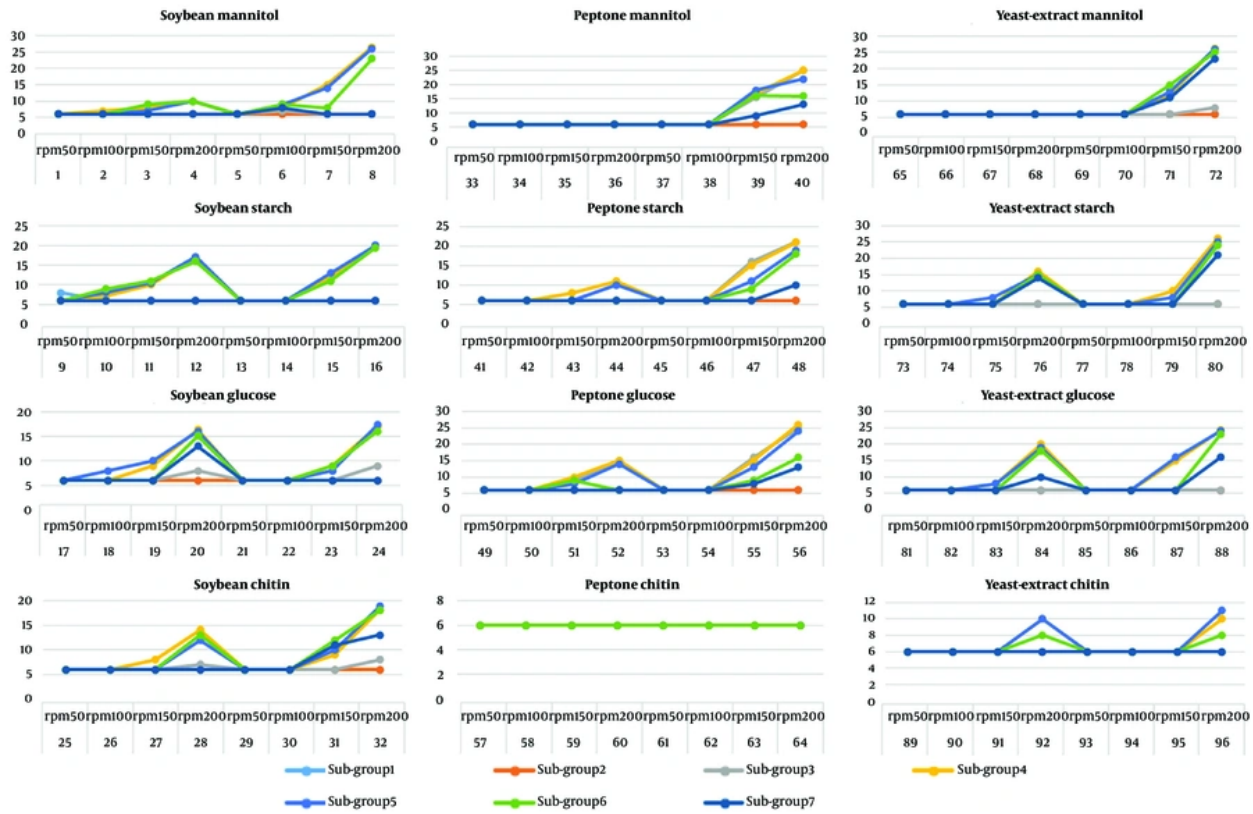

Figure 2. Antimicrobial activity of isolate Ca2 - 5 against MRSA on day 4 at 200 rpm under light and dark conditions. Panel A shows results under light incubation, and panel B shows results under dark incubation. Numbers 1 - 12 represent different nitrogen and carbon source combinations: 1) soybean-starch, 2) soybean-glucose, 3) soybean-mannitol, 4) soybean-chitin, 5) peptone-starch, 6) peptone-mannitol, 7) peptone-glucose, 8) peptone-chitin, 9) yeast extract-mannitol, 10) yeast extract-chitin, 11) yeast extract-glucose, and 12) yeast extract-starch.

dependent influence. A strong interaction between rpm and carbon source was observed, indicating that metabolite production in *Streptomyces* Ca2 - 5 is highly condition dependent. Overall, 43.7% of OSMAC conditions exhibited detectable antimicrobial activity against MRSA ATCC 43300, confirming the successful activation of cryptic biosynthetic pathways.

5. Discussion

Alarming projections suggest that, without rapid intervention, approximately 10 million lives could be lost due to antimicrobial resistance (AMR) by 2050 (25). Despite the encouraging discovery of approximately 1600 new natural products (NPs) annually over the past two decades (26), the repeated identification of known compounds remains a substantial impediment in the field. Advances in genomic analysis have revealed a disparity between the number of gene clusters identified through bioinformatics analyses as being involved in the production of secondary metabolites and the number of chemically characterized secondary metabolites (27, 28). BGCs typically comprise co-localized genes encoding enzymes responsible for secondary metabolite production (29, 30). Genome sequencing has revealed cryptic BGCs in microbes that are not associated with chemically characterized molecules (31). These BGCs are believed to be dormant or inactive under laboratory conditions (30).

The production of secondary metabolites (SMs) requires cellular energy and resources, making it a regulated process activated in response to specific environmental conditions (3, 13). Various approaches, such as modifying growth conditions without genetic manipulation, can trigger global physiological changes and enhance SM production (32). These approaches, derived from fermentation optimization practices, have

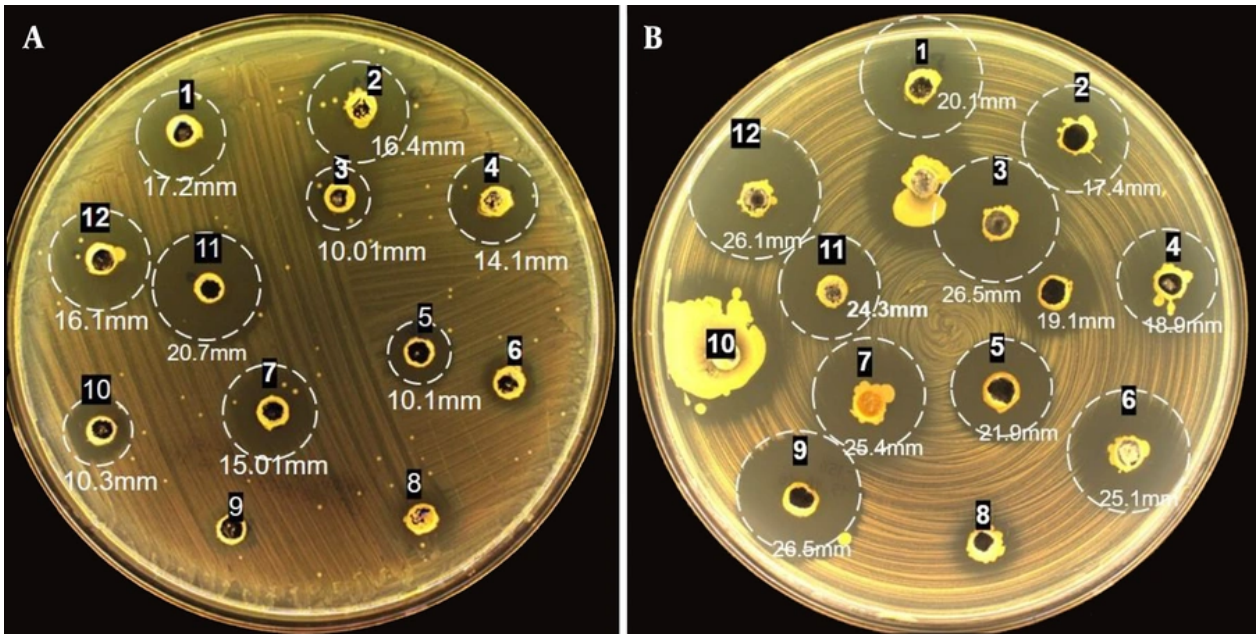

Figure 3. Comparative analysis of the antibacterial activity of isolate ID Ca2 - 5 against MRSA at varying agitation speeds: A) 50 rpm, B) 100 rpm, and C) 150 rpm. Within each panel, specific configurations are outlined, representing various combinations of nitrogen and carbon sources: 1) soybean-starch, 2) soybean-glucose, 3) soybean-mannitol, 4) soybean-chitin, 5) peptone-starch, 6) peptone-mannitol, 7) peptone-glucose, 8) peptone-chitin, 9) yeast extract-mannitol, 10) yeast extract-chitin, 11) yeast extract-glucose, and 12) yeast extract-starch.

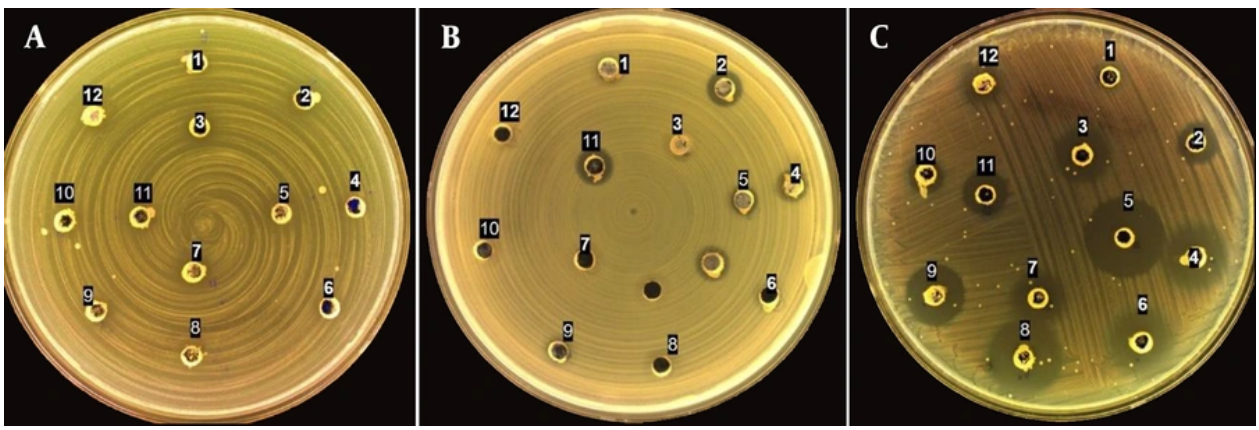

Figure 4. Graphical abstract illustrating the effect of culture optimization on 3 the antimicrobial activity of *Streptomyces* isolate ID Ca2 - 5 against MRSA.

shown that altering cultivation parameters, such as nutrients, trace elements, physical factors (pH and temperature), and chemical elicitors (sublethal antibiotic concentrations and communication molecules), can affect SM production in microorganisms. The influence of nutrient composition on secondary metabolite production varies substantially among microbial species.

Enhanced production of antimicrobial metabolites was achieved using the OSMAC approach, with culture

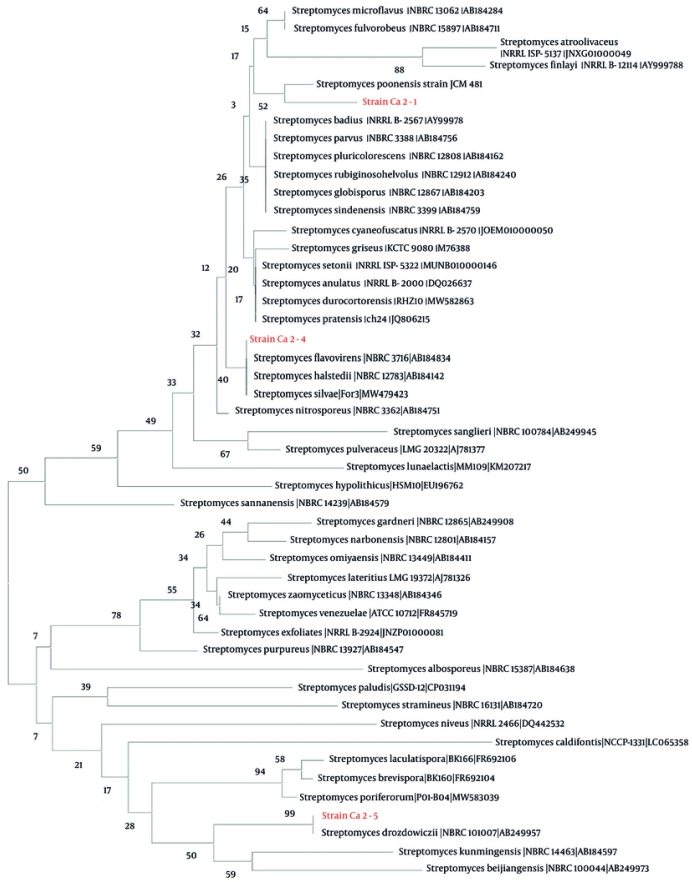

Figure 5. Phylogenetic analysis of *Streptomyces* isolates based on DNA sequencing. The genetic sequences of the isolates are detailed below. Isolate Ca2 - 5 showed 100% sequence identity with *Streptomyces drozdowiczii* NBRC 101007(T) (GenBank: AB249957). Isolate Ca2 - 1 showed the highest sequence similarity to *Streptomyces poonensis* JCM 481, while Ca2 - 4 showed 99.44% similarity to *Streptomyces flavovirens* NBRC 3716 (GenBank: AB184834).

conditions designed to mimic the natural plant-associated habitat. The results demonstrated that shaking speed, incubation time, carbon and nitrogen sources, and particularly light conditions, had a significant effect on anti-MRSA activity. Increased agitation speeds (150 and 200 rpm) markedly enhanced antimicrobial activity by improving oxygen transfer, nutrient distribution, and the expression of secondary metabolite biosynthetic genes, whereas low agitation (50 rpm) resulted in oxygen limitation and reduced metabolite production. The optimal incubation period was 4 - 6 days, corresponding to the stationary growth phase; shorter periods limited biomass formation, whereas longer periods led to nutrient depletion and the accumulation of toxic by-products.

Among the tested substrates, soybean meal showed the strongest effect, especially when combined with mannitol or glucose at higher shaking speeds. Yeast extract also performed well, whereas chitin exhibited weak activity due to its poor degradability. These findings highlight the critical role of the carbon-to-nitrogen (C/N) ratio and strain-specific substrate utilization in the OSMAC strategy. Moreover, visible light exerted an inhibitory effect on antibiotic production, with dark incubation significantly enhancing anti-MRSA activity compared with light conditions. Statistical analysis indicated that shaking speed was the most significant factor influencing antimicrobial production ($P < 0.001$), whereas light conditions had a weaker, context-dependent effect. These results demonstrate the strong dependence of metabolite production on culture

conditions in *Streptomyces* Ca2 - 5 and are consistent with the natural ecology of *Streptomyces*, which inhabits dark environments such as soil and plant tissues (33).

5.1. Study Limitations

Despite promising anti-MRSA activity, this study has limitations. The identification of isolate Ca2 - 5 was based solely on 16S rRNA analysis, and genome-based methods are required for definitive species-level classification. In addition, although OSMAC conditions enhanced bioactivity, the active metabolites were not isolated or characterized, indicating that enhanced bioactivity was observed rather than the discovery of novel antimicrobial compounds.

5.2. Conclusions

This study demonstrates the potential of endophytic *Streptomyces* as a promising source of antimicrobial metabolites. By optimizing environmental and cultivation parameters, including nutrient sources, rotation speed, and light exposure, we enhanced antimicrobial metabolite production. Key findings demonstrated that soybean and mannitol significantly boosted antimicrobial activity, whereas dark incubation may better mimic the low-light conditions encountered in plant-associated microenvironments. These optimizations provide a framework for advancing bioactivity-enhancement efforts and addressing the growing global challenge posed by multidrug-resistant pathogens.

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Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

AI Use Disclosure: The authors declare that no generative AI tools were used in the creation of this article.

Authors' Contribution: M. B. contributed to methodology, investigation, data curation, formal analysis, and original draft preparation. A. H. S. contributed to methodology, formal analysis, software, validation, and manuscript review and editing. S. A. contributed to methodology, formal analysis, validation, and manuscript review and editing. H. S. R. contributed to conceptualization, project administration, validation, supervision, and manuscript review and editing. S. Y. contributed to conceptualization, validation, supervision, and manuscript review and editing.

Conflict of Interests Statement: The authors declare that they have no known financial or personal conflicts of interest that could have influenced the work reported in this paper.

Data Availability: The datasets supporting the findings of this study are available from the corresponding author upon reasonable request. Furthermore, the sequence data generated during this research have been deposited in the National Center for Biotechnology Information (NCBI) repository and can be accessed at [https://www.ncbi.nlm.nih.gov/] (https://www.ncbi.nlm.nih.gov/) under accession numbers PQ773515, PQ773516 and PV998000.1.

Ethical Approval: This study involved Streptomyces samples collected from natural aquatic environments under academic approval from Hamadan University of Medical Sciences (IR.UMSHA.REC.1404.472: https://ethics.research.ac.ir/IR.UMSHA.REC.1404.472).

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