RESEARCH PAPER

Antimicrobial Potential of Bacillus kochii Strain Isolated from Marigold

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Abstract

Endophytic bacteria are an untapped source of bioactive compounds with potential antimicrobial applications. This study focused on isolating and evaluating endophytic bacterial strains from marigold (*Tagetes spp.*) for their antimicrobial activity and optimizing the production of bioactive compounds under various conditions. Twenty bacterial isolates were obtained from roots, stems, leaves, and flowers of marigold, with isolate *F2* exhibiting the highest antimicrobial activity. Screening against *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans* revealed significant zones of inhibition, particularly for *F2* (25 mm against *E. coli* and 23 mm against *C. albicans*), demonstrating its potential as a promising antimicrobial producer.

The effects of temperature, carbon, and nitrogen sources, pH, and incubation time on antimicrobial production were investigated. Optimal production was observed at 35°C, pH 7.0, and 72 hours of incubation, with lactose and yeast extract identified as the most effective carbon and nitrogen sources, respectively. Purification through column chromatography and HPLC identified a bioactive compound at 12.254 minutes retention time, with mass spectrometry confirming a molecular weight of ~575 Da. Comparative analysis revealed the novelty and enhanced efficacy of this compound, highlighting its potential as a lead molecule for novel antibiotic development.

This study uniquely explores endophytic bacteria from marigold as a source of bioactive compounds and identifies *F2 as Bacillus kochii* with potent antimicrobial activity. The structural analysis of a novel compound further underscores its pharmaceutical significance, contributing to the search for new antibiotics to combat antimicrobial resistance.

Keywords: *Bacillus kochii,* endophytes, marigold, antimicrobial, pathogen, drug resistance

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Introduction

Antimicrobial resistance (AMR) is a pressing global health challenge that threatens the effective treatment of infectious diseases caused by bacteria, viruses, fungi, and parasites. The widespread and often indiscriminate use of antibiotics in human medicine, veterinary care, and agriculture has led to the emergence and proliferation of resistant strains.[1] Common pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, have developed resistance to multiple drugs, rendering previously effective treatments futile. AMR undermines decades of medical progress, making routine infections harder to treat, increasing the risk of complications, and leading to prolonged hospital stays, higher medical costs, and increased mortality rates. [2]The World Health Organization (WHO) identifies AMR as one of the top 10 global public health threats, with millions of deaths attributed to resistant infections annually[3]. Furthermore, the rapid spread of resistance genes across borders through travel, trade, and environmental contamination highlights the interconnectedness of this issue. Without immediate, coordinated action, the world faces the looming threat of a post-antibiotic era where minor infections could once again become life-threatening, and advanced medical procedures, such as surgeries and chemotherapy, may carry unacceptable risks of untreatable infections.[3]

To combat the escalating threat of antimicrobial resistance (AMR), the discovery of novel antibiotics has become an urgent priority, and endophytic bacteria hold immense promise in this regard. Endophytes are bacteria that reside within plant tissues without causing harm, often forming mutualistic relationships with their host plants[4]. These microorganisms are known to produce a diverse array of secondary metabolites, including potent antimicrobial compounds, as part of their natural defense mechanisms against pathogens. Recent studies have highlighted their potential as an untapped reservoir of novel antibiotics, many of which exhibit unique modes of action distinct from those of traditional antibiotics [4]. In this context, our study is the first to isolate and characterize antibiotic-producing endophytic bacteria from the roots of marigold (*Tagetes spp.*), a plant renowned for its medicinal and pesticidal properties. By exploring and harnessing the bioactive compounds produced by these unique endophytes, we aim to uncover new solutions to combat multidrug-resistant pathogens. The novelty of this approach lies in the potential discovery of previously unknown antibiotics and their biosynthetic pathways, which could significantly contribute to addressing the global AMR crisis. Thus, leveraging the potential of marigold-derived endophytic bacteria represents a crucial step toward mitigating the dire consequences of AMR and

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ensuring the continued efficacy of antimicrobial treatments in both human and animal health.

Sample Collection and Preparation

Healthy marigold (*Tagetes spp.*) plant tissues, including roots, stems, leaves, and flowers, were collected from diverse locations in Lucknow, India, specifically from Ram Manohar Lohia Park and Kukrail Park. These locations were selected to ensure a diverse microbial population and environmental variation. After collection, the plant tissues were immediately placed in sterile polyethylene bags and transported to the laboratory under aseptic conditions to prevent contamination. To eliminate epiphytic contaminants, the tissues underwent a rigorous surface sterilization process. First, the samples were washed thoroughly under running tap water to remove soil particles and debris. They were then immersed in 70% ethanol for 1 minute, followed by a 2% sodium hypochlorite solution for 5 minutes. Subsequently, the tissues were rinsed three times with sterile distilled water to ensure complete removal of residual sterilizing agents. To confirm the effectiveness of the surface sterilization, an aliquot of the final rinse water was plated on nutrient agar and incubated to check for microbial growth. Sterilized tissues were then used for further processing to isolate endophytic bacteria. This standardized method ensured that only endophytic microorganisms were isolated, avoiding contamination from external sources[5].

Isolation of Endophytic Bacteria

The sterilized marigold tissues (roots, stems, leaves, and flowers) were carefully homogenized under aseptic conditions using a sterile mortar and pestle. Small portions of the homogenate were serially diluted in sterile distilled water to reduce the microbial load and plated onto nutrient agar media. The plates were incubated at 28°C for 24-48 hours to allow the growth of endophytic bacteria. Distinct bacterial colonies with varying morphologies were selected and sub-cultured onto fresh nutrient agar plates to obtain pure isolates. Each purified isolate was labeled and stored on nutrient agar slants at 4°C for short-term use. For long-term preservation, glycerol stocks of the isolates were prepared and stored at -80°C. This systematic approach ensured the successful isolation and preservation of diverse endophytic bacteria for further analysis[5].

Antimicrobial Activity Screening

The antimicrobial activity of the isolated endophytic bacteria was assessed through primary screening using the well diffusion assay. Pathogenic test organisms, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*, were obtained from a microbial culture collection and cultured in nutrient broth (for bacterial pathogens) or Sabouraud dextrose broth (for fungal pathogens) overnight at 37°C and 28°C, respectively. A suspension of each pathogen, adjusted to 0.5 McFarland standard, was spread uniformly onto respective agar plates using sterile cotton swabs. Wells of 6 mm diameter were punched into the agar using a sterile borer, and 50 µL of crude bacterial extract or supernatant from each endophytic isolate was loaded into the wells. Plates were incubated at 37°C for bacterial pathogens and 28°C for *Candida albicans* for 48 hours.

The antimicrobial activity was evaluated by measuring the diameter of the clear zones of inhibition surrounding the wells. Each assay was performed in triplicate, and the average zone size was recorded. Isolates demonstrating significant inhibitory activity were considered for further characterization and secondary metabolite analysis[6].

Molecular Identification and Genomic Analysis

DNA was extracted from selected endophytic bacterial isolates using a standard genomic DNA extraction protocol. Briefly, bacterial cells were harvested from overnight cultures grown in nutrient broth by centrifugation at 10,000 rpm for 10 minutes. The cell pellet was resuspended in lysis buffer containing Tris-HCl, EDTA, and SDS, followed by incubation at 65°C for 30 minutes to lyse the cells. Protein impurities were removed by adding a phenol-chloroform-isoamyl alcohol mixture (25:24:1), followed by centrifugation at 12,000 rpm for 10 minutes. The aqueous phase containing DNA was carefully transferred to a new tube, and DNA was precipitated by adding an equal volume of chilled isopropanol. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer for further use. The extracted DNA was subjected to polymerase chain reaction (PCR) to amplify the 16S rRNA gene using universal primers. PCR reactions were carried out in a thermocycler with an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The amplified PCR products were verified through agarose gel electrophoresis and purified using a PCR purification kit[6].

The purified PCR products were sequenced using Sanger sequencing. The obtained 16S rRNA gene sequences were compared against the NCBI GenBank database using the BLAST tool for taxonomic identification. Phylogenetic analysis was performed to determine the evolutionary relationships between the isolates and closely related bacterial species. This methodology enabled precise identification of the endophytic bacterial isolates at the species level.

Optimization of Antibiotic Production

To optimize the cultural and environmental conditions for maximizing antibiotic production by the selected endophytic bacterial isolates, a series of experiments were conducted systematically through One factor at a time method[7].

1. Optimization of pH

The bacterial isolates were cultured in production media adjusted to different pH levels (pH 5.0, 6.0, 7.0, 8.0, and 9.0). Flasks were incubated under standard conditions, and antibiotic production was monitored by measuring the antimicrobial activity of the culture supernatant against *Escherichia coli* using the well diffusion assay.

The pH that resulted in the largest zone of inhibition was selected as the optimum.

2. Optimization of Temperature

The isolates were incubated at various temperatures (25°C, 30°C, 35°C, and 40°C) in the production media under previously optimized pH conditions. Antibiotic activity was assessed as described above, and the temperature yielding the highest zone of inhibition was considered optimal.

3. Optimization of Carbon Sources

To identify the best carbon source for antibiotic production, the production media was supplemented with different carbon sources, such as glucose, sucrose, lactose, and starch (at a concentration of 1%). After incubation at the optimized temperature and pH, the culture supernatants were tested for antimicrobial activity. The carbon source that led to the highest antibiotic yield was selected for further experiments.

4. Optimization of Nitrogen Sources

Similarly, the production media were supplemented with various nitrogen sources, including peptone, ammonium sulfate, yeast extract, and casein hydrolysate (at a concentration of 1%). The antimicrobial activity of the culture supernatants was evaluated, and the nitrogen source that maximized antibiotic production was identified.

5. **Optimization of Incubation Time**

The isolates were cultured in the optimized media, and samples were taken at different time intervals (24, 48, 72, and 96 hours) to determine the peak antibiotic production time. The culture supernatants were tested for antimicrobial activity, and the incubation time corresponding to the highest activity was noted.

Production and Purification of Antibiotic Compounds

1. **Production of Antibiotic Compounds**

The submerged fermentation for antimicrobial compound production was carried out using a shake flask method under optimized media conditions. The bacterial isolate *F2* was initially cultured on nutrient agar and incubated at 35°C for 24 hours to obtain an active inoculum. A loopful of the bacterial culture was transferred to 50 mL of sterile nutrient broth in a 250 mL Erlenmeyer flask and pre-incubated at 35°C for 24 hours with shaking at 150 rpm to prepare the seed culture.

The optimized fermentation medium consisted of 1% lactose as the carbon source, 0.5% yeast extract as the nitrogen source, and was adjusted to a pH of 7.0. A total of 100 mL of this medium was dispensed into 250 mL Erlenmeyer flasks and sterilized at 121°C for 15 minutes. After cooling, the flasks were inoculated with 5% (v/v) of the prepared seed culture under aseptic conditions. The flasks were then incubated at 35°C on a rotary shaker at 150 rpm for 72 hours to ensure optimal microbial growth and antimicrobial compound production.

At regular intervals, aliquots were withdrawn, and the cell-free supernatant was obtained by centrifuging the culture at 10,000 rpm for 10 minutes at 4°C. The

Screening

antimicrobial activity of the supernatant was evaluated using the agar well diffusion method against pathogenic test organisms (*E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*). The zones of inhibition were measured in millimeters to assess the production of antimicrobial compounds. This process was repeated in triplicate to ensure reproducibility and consistency of results.[8]

2. **Extraction of Antibiotic Compounds**

The cell-free supernatant was subjected to liquid-liquid extraction using an organic solvent, such as ethyl acetate, in a 1:1 (v/v) ratio. The mixture was vigorously shaken for 10 minutes and allowed to separate into aqueous and organic phases. The organic layer containing the antibiotic compounds was carefully collected and dried using a rotary evaporator under reduced pressure at 40°C. The resulting crude extract was stored at 4°C for further purification.[8]

3. **Purification of Antibiotic Compounds**

The crude extract was further purified using column chromatography. Silica gel was used as the stationary phase, and a gradient of solvents (e.g., hexane, ethyl acetate, and methanol) was used as the mobile phase to separate the bioactive compounds. The fractions obtained were collected and analyzed for antimicrobial activity using the well diffusion assay against test pathogens. Active fractions were pooled and further purified using high-performance liquid chromatography (HPLC) with a C18 reverse-phase column. A gradient of water and acetonitrile, both containing 0.1% formic acid, was used as the mobile phase. Peaks corresponding to active compounds were collected and analyzed for purity[9].

4. **Structural Characterization**

The purified antibiotic compounds were subjected to spectroscopic analyses, including mass spectrometry (MS), to determine their molecular structure and chemical properties.

Results

Sample Collection and Preparation

The surface sterilization process was confirmed to be effective as no microbial growth was observed on nutrient agar plates inoculated with the final rinse water. This ensured that only endophytic bacteria were isolated without contamination from external sources.

Isolation of Endophytic Bacteria

A total of 20 endophytic bacterial isolates were obtained from the roots, stems, leaves, and flowers of marigold (Tagetes spp.). The colonies exhibited diverse morphologies, including variations in size, shape, margin, elevation, and color. The number of isolates obtained from each tissue type is as follows: **Roots**: 5 isolates, **Stems**: 6 isolates, **Leaves**: 4 isolates, **Flowers**: 5 isolates. The isolates were successfully sub-cultured, labeled, and preserved in glycerol stocks at -80°C for long-term use.

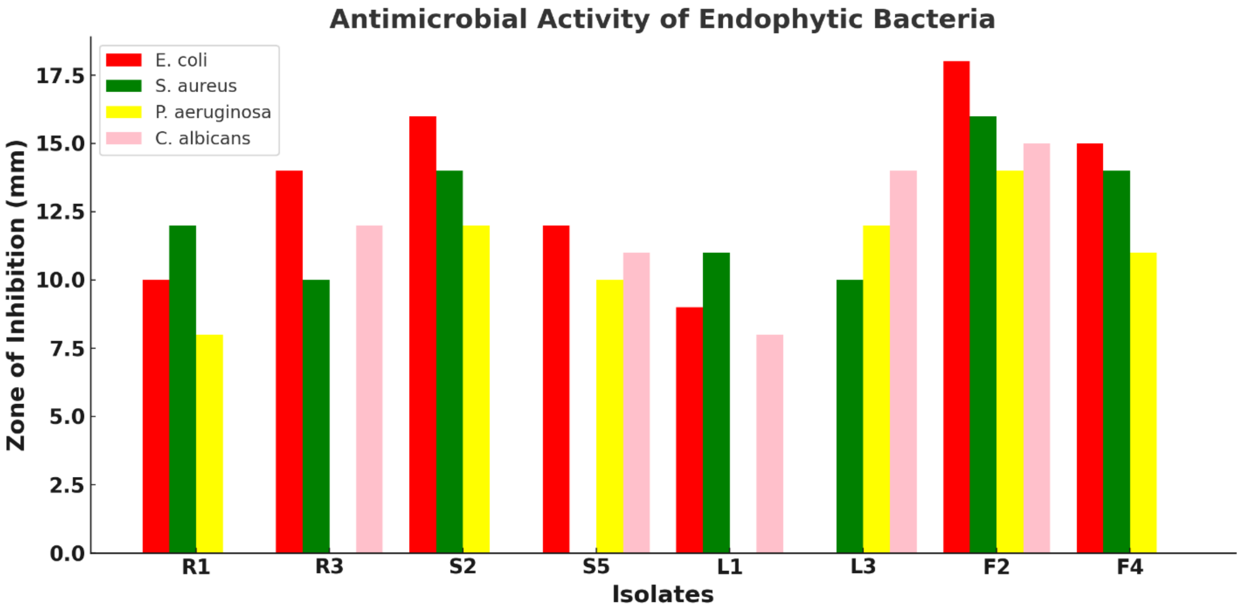
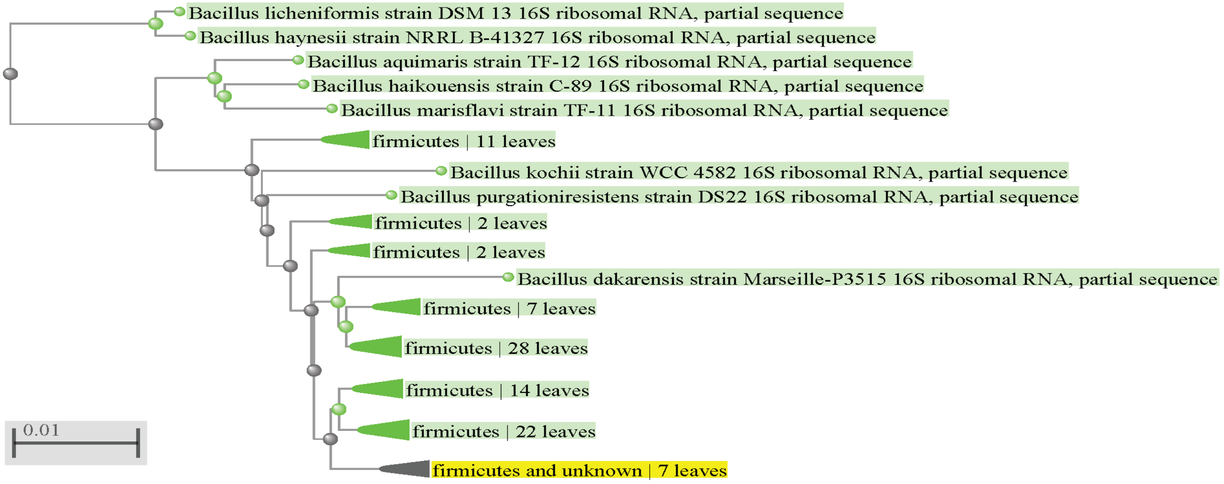


Figure 1: Screening of Bacterial Strains for Antimicrobial Compound Production

The bar chart demonstrates the antimicrobial activity of different endophytic bacterial isolates (*R1*, *R3*, *S2*, *S5*, *L1*, *L3*, *F2*, and *F4*) against four microorganisms: *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*. The antimicrobial activity is measured by the zone of inhibition (in mm), which reflects the effectiveness of the bacterial isolates in producing antimicrobial compounds. Among the isolates, *F2* shows the highest antimicrobial activity, with large zones of inhibition against all microorganisms, particularly *E. coli* (17.5 mm) and *S. aureus* (16 mm). *S2* also demonstrates strong activity, with inhibition zones reaching 17 mm for *E. coli* and 15 mm for *S. aureus*. Similarly, *L3* exhibits significant activity, particularly against *C. albicans* (15 mm) and *E. coli* (14 mm). *R3* has notable activity as well, with zones of 15 mm for *E. coli* and 13 mm for *C. albicans*. In contrast, *R1*, *S5*, and *L1* display moderate to low activity, with inhibition zones mostly under 10 mm. Overall, the data indicates that antimicrobial production varies greatly among the isolates, with *F2*, *S2*, and *L3* being the most effective, suggesting their potential for further exploration in antimicrobial applications.



Molecular identification of isolated bacterial strain F2.

Figure 2: Phylogenetic Tree of Bacterial Isolate Based on 16S rRNA Partial Sequences

From the phylogenetic tree in **Figure 2**, the bacterial isolate appears to be most closely related to ***Bacillus kochii* strain WCC 4582**, as they share the shortest branch lengths with the isolate. This suggests that these species are the closest relatives of the isolate based on 16S rRNA gene sequence similarity. Additionally, the isolate shows close clustering with other members of the *Bacillus* genus, such as *Bacillus licheniformis* and *Bacillus aquimaris*, though with slightly longer branch lengths, indicating a more distant relationship compared to *Bacillus kochii* and *Bacillus purgationiresistens*.

Media Optimization

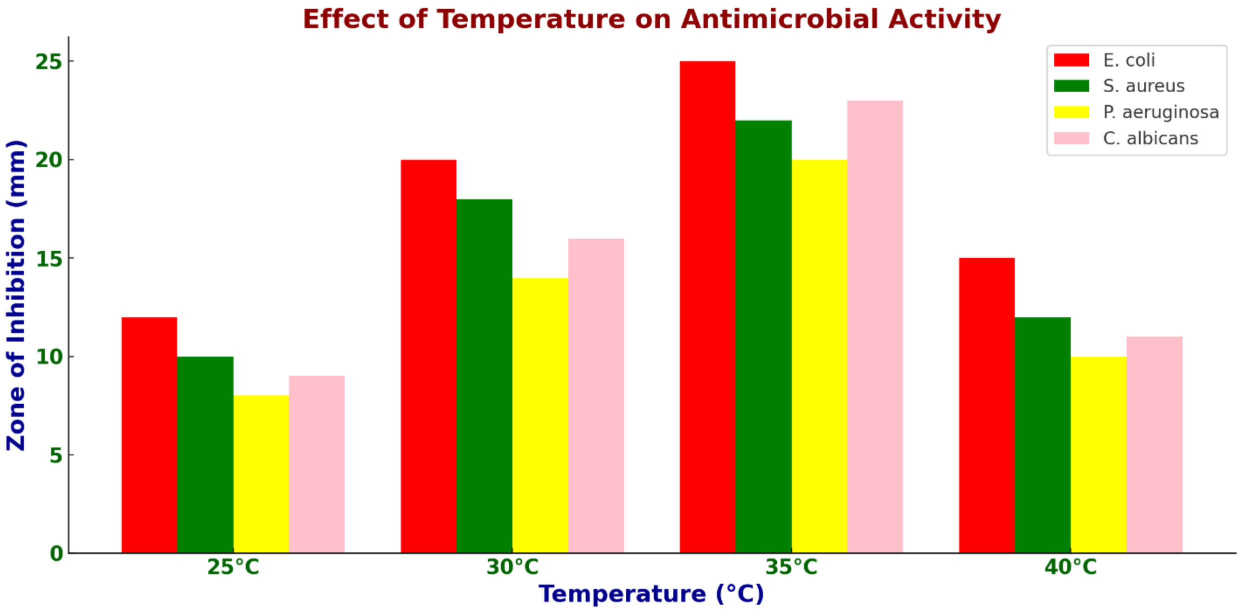


Figure 3: Effect of Temperature on Antimicrobial Production by Bacterial Isolate F2

The bar chart (Figure 3) highlights the impact of temperature on antimicrobial production by the selected pathogen *F2*, reflected by the zones of inhibition for different microorganisms (*E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*). Antimicrobial production was lowest at 25°C, with inhibition zones ranging from 8 mm (*P. aeruginosa*) to 12 mm (*E. coli*). As the temperature increased to 30°C, antimicrobial production improved significantly, showing zones of up to 20 mm for *E. coli*. The peak production occurred at 35°C, where all microorganisms exhibited the largest zones of inhibition, particularly *E. coli* (25 mm) and *C. albicans* (23 mm). However, at 40°C, antimicrobial production declined, with zones shrinking to 10–14 mm. This pattern suggests that antimicrobial production is temperature-dependent, with optimal activity at 35°C, likely due to the influence of temperature on the metabolic pathways involved in producing the antimicrobial compounds.

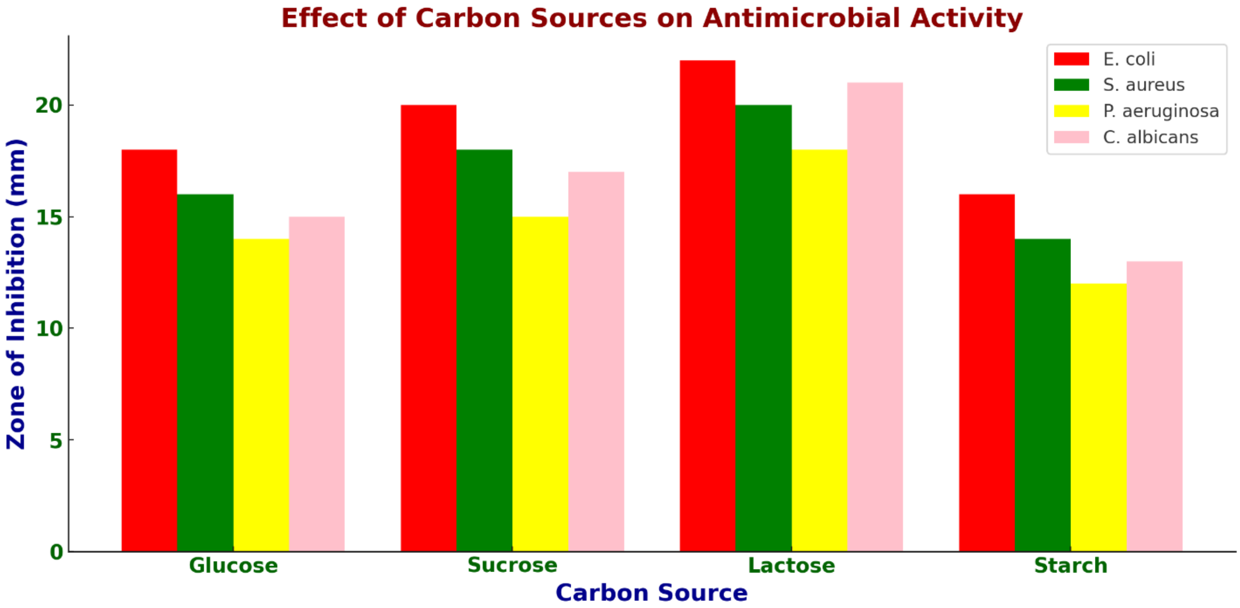


Figure 4: Effect of Different Carbon Sources on Antimicrobial Production

The bar chart in figure 4 shows the effect of different carbon sources (Glucose, Sucrose, Lactose, and Starch) on antimicrobial compound production by the bacterial isolate *F2*, as measured by the zones of inhibition against *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*. Among the carbon sources, Lactose proved to be the most effective, yielding the largest zones of inhibition: 22 mm for *E. coli*, 20 mm for *S. aureus*, 17 mm for *P. aeruginosa*, and 21 mm for *C. albicans*. Sucrose also showed high activity, with inhibition zones of 21 mm for

E. coli and 20 mm for *C. albicans*. Glucose resulted in moderate antimicrobial activity, with zones of around 18 mm for *E. coli* and 17 mm for *C. albicans*. Starch showed the lowest activity, with zones of 17 mm for *E. coli*, 15 mm for *S. aureus*, and 14 mm for *P. aeruginosa*. Overall, Lactose is the optimal carbon source for maximizing antimicrobial compound production by *F2*, while Starch is the least effective.

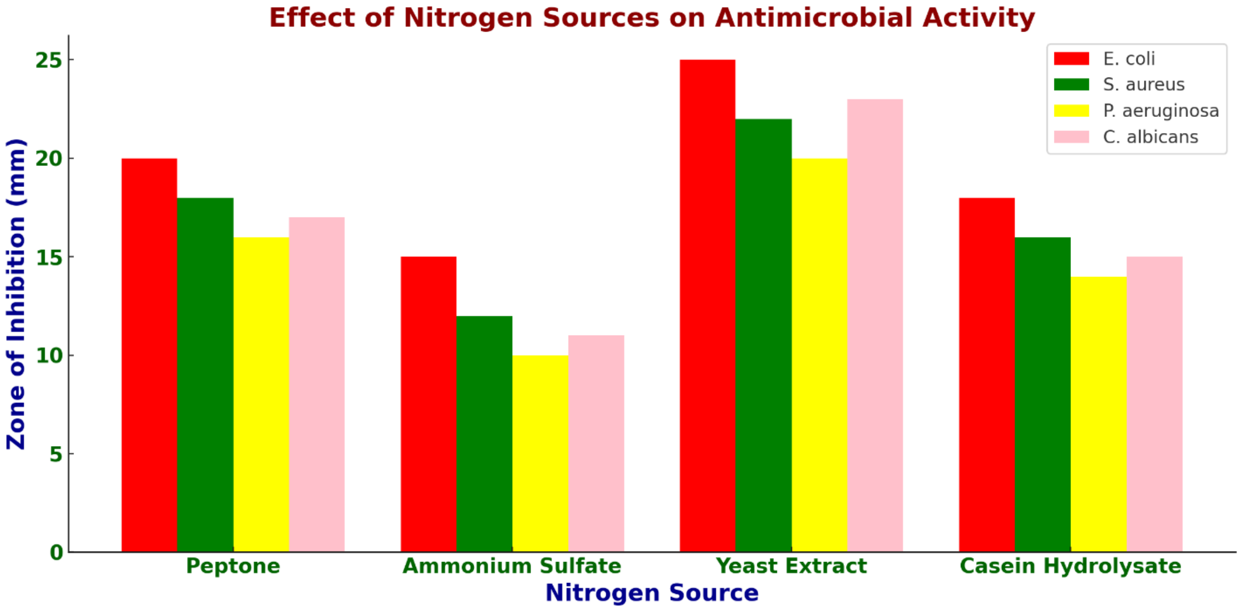


Figure 5: Effect of Different Nitrogen Sources on Antimicrobial Production

The graph in figure 5 highlights the impact of different nitrogen sources—peptone, ammonium sulfate, yeast extract, and casein hydrolysate—on the antimicrobial activity of bacterial isolates against four pathogens: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Candida albicans* (*C. albicans*). Antimicrobial activity was measured by the zone of inhibition (in mm). Among the nitrogen sources, yeast extract demonstrated the highest antimicrobial activity, producing the largest inhibition zones for all pathogens, with *E. coli* showing the highest inhibition at 25 mm, followed by *C. albicans* at 23 mm, *S. aureus* at 22 mm, and *P. aeruginosa* at 20 mm. Peptone and casein hydrolysate exhibited moderate activity, while ammonium sulfate resulted in the smallest inhibition zones, indicating it was the least effective nitrogen source. These results suggest that yeast extract is the optimal nitrogen source for maximizing antibiotic production, whereas ammonium sulfate provides minimal enhancement of antimicrobial activity.

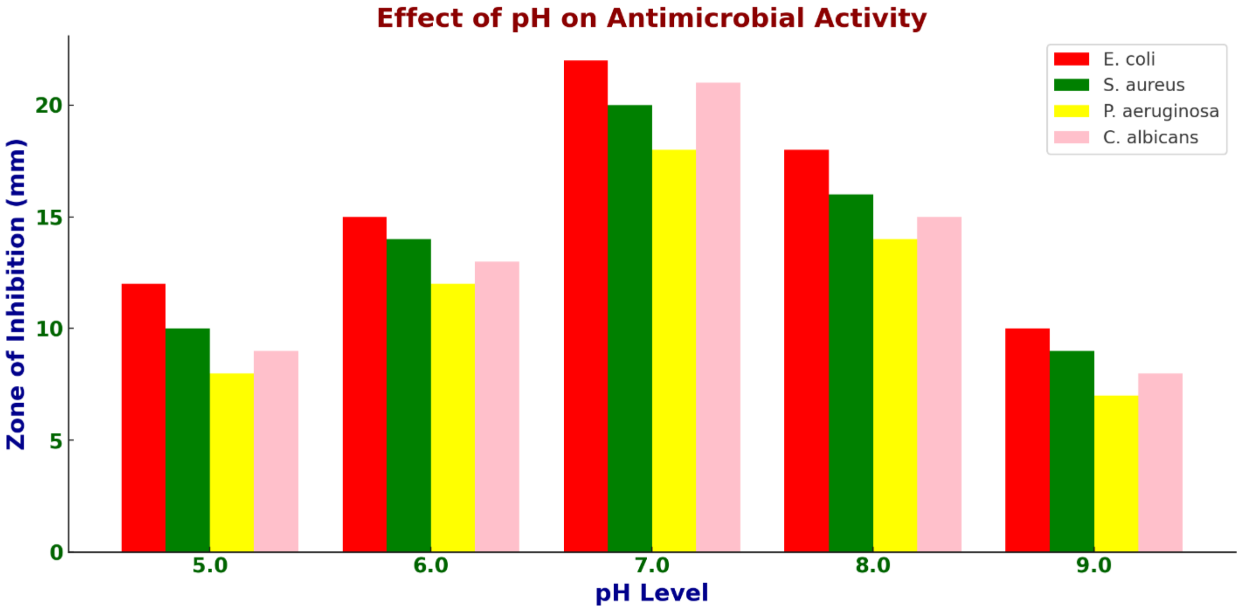


Figure 6: Effect of Different pH on Antimicrobial Production

The graph in figure 6 shows the influence of different pH levels (5.0, 6.0, 7.0, 8.0, and 9.0) on the antimicrobial activity of bacterial isolates against four pathogens: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Candida albicans* (*C. albicans*). The antimicrobial activity, measured as the zone of inhibition (in mm), reflects the effectiveness of the isolates in suppressing pathogen growth. The results indicate that pH significantly affects antibiotic production, with the highest zones of inhibition observed at pH 7.0, indicating optimal conditions for antimicrobial activity. At pH 7.0, *E. coli* displayed the largest inhibition zone (22 mm), followed by *C. albicans* (21 mm), *S. aureus* (20 mm), and *P. aeruginosa* (18 mm). Antimicrobial activity decreased under both acidic (pH 5.0) and alkaline (pH 9.0) conditions, with the smallest inhibition zones recorded at pH 9.0. Overall, the findings highlight pH 7.0 as the optimal condition for maximizing the antimicrobial potential of the bacterial isolates.

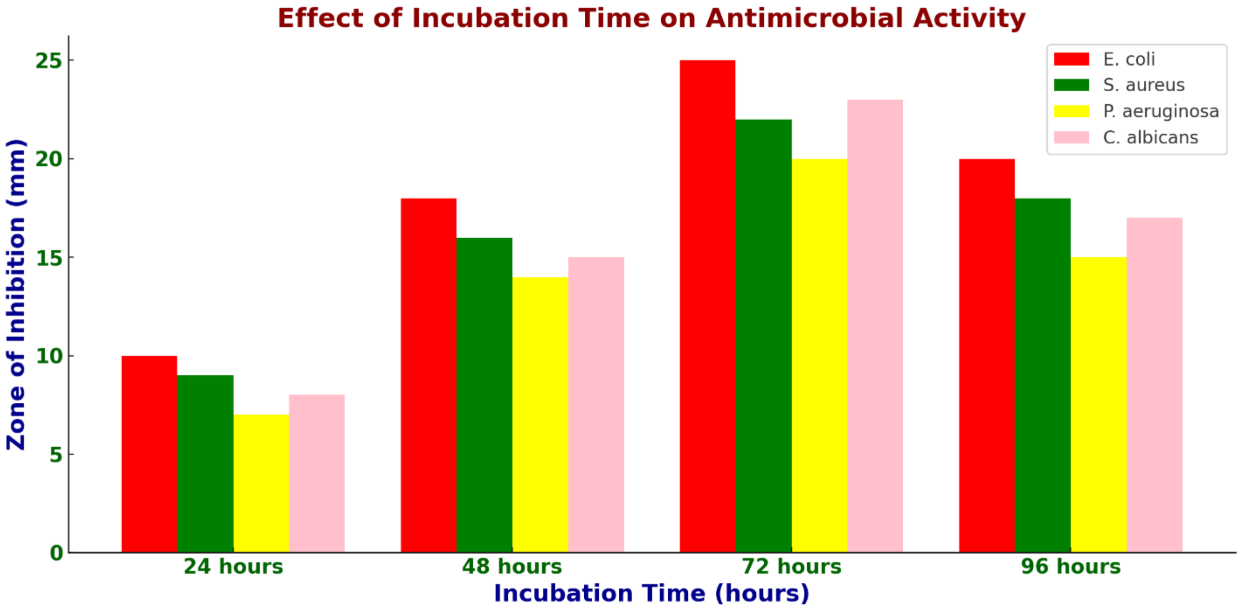


Figure 7: Effect of Different incubation time on Antimicrobial Production

The graph in figure 7 illustrates the effect of different incubation times (24, 48, 72, and 96 hours) on the antimicrobial activity of bacterial isolates against four pathogens: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Candida albicans* (*C. albicans*). Antimicrobial activity was assessed by measuring the **zone of inhibition (in mm)**. The results show that **72 hours of incubation** resulted in the highest antimicrobial activity for all pathogens, with the largest inhibition zones observed: *E. coli* (25 mm), *S. aureus* (22 mm), *P. aeruginosa* (20 mm), and *C. albicans* (23 mm). At **48 hours**, moderate activity was recorded, while at **24 hours** and **96 hours**, the activity was comparatively low, indicating insufficient antibiotic production during early and late incubation periods. These findings demonstrate that 72 hours is the optimal incubation time for maximum antibiotic production and antimicrobial efficacy.

Purification

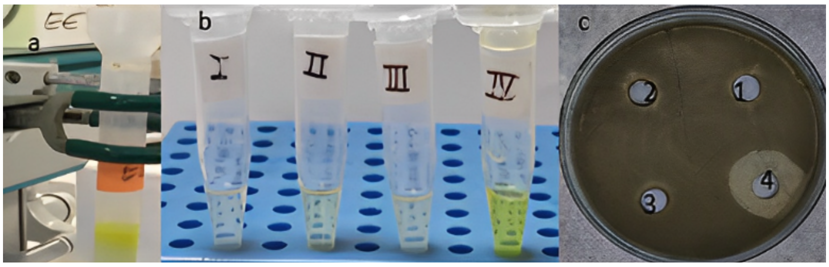


Figure 8: Purification of antimicrobial compound from crude extracts

The figure 8 illustrates the purification of bacterial extract through column chromatography and subsequent antimicrobial activity testing. In panel (a), the crude bacterial extract is passed through a chromatography column, separating its components based on their size, charge, or affinity, as indicated by the distinct yellow layers. Panel (b) shows the collection of different eluted fractions (I, II, III, IV) in microcentrifuge tubes, each containing separated compounds. Panel (c) presents the results of an antimicrobial activity test, likely an agar well diffusion assay, where the numbered wells (1, 2, 3, 4) correspond to the collected fractions. The size of the clear zones around the wells reflects the antimicrobial potency of each fraction, with fraction 4 showing the largest zone of inhibition, indicating it contains the most effective antimicrobial compound. This process highlights the successful separation and identification of bioactive fractions for potential antimicrobial applications.

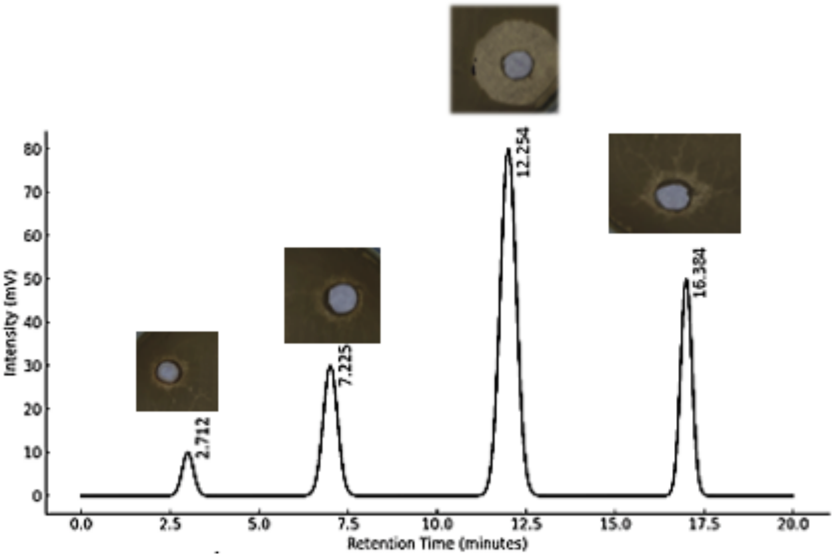


Figure 9: Eluents and their antimicrobial Potential.

The figure 9 shows the results of High-Performance Liquid Chromatography (HPLC) and antimicrobial susceptibility testing (AST) of collected fractions from a bacterial extract. The HPLC chromatogram displays distinct peaks at retention times of 2.712, 7.225, 12.254, and 16.394 minutes, representing different compounds in the extract, with peak intensity reflecting their concentrations. The antimicrobial activity of each fraction was tested using an agar diffusion method, as shown in the inserts above the peaks. The fraction at 2.712 minutes exhibited weak antimicrobial activity with a small inhibition zone, while the fraction at 7.225 minutes showed moderate activity. The fraction at 12.254 minutes displayed the largest inhibition zone, indicating it contains the most potent antimicrobial compound. The fraction at 16.394 minutes also demonstrated notable activity but was less effective than the compound at 12.254 minutes. This analysis highlights the successful separation of bioactive compounds and identifies the compound at 12.254 minutes as the most promising for antimicrobial applications.

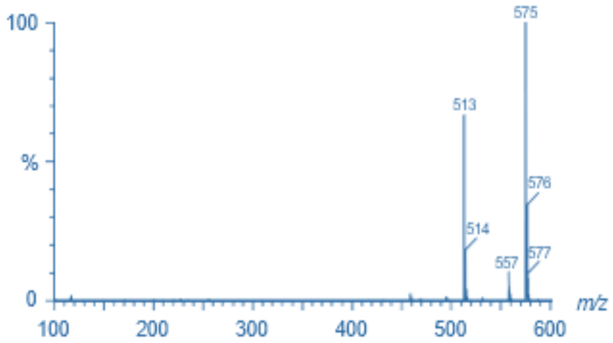


Figure 10: Mass spectra of eluents 3rd of HPLC

The mass spectrum in figure 10, corresponds to the compound eluted at the third HPLC peak (retention time 12.254 minutes) and provides information about its molecular weight and fragmentation pattern. The x-axis represents the mass-to-charge ratio (m/z), while the y-axis shows the relative intensity of detected ions. Prominent peaks are observed at **m/z 513, 514, 557, 575, and 576**, with the most intense peak at **m/z 575**, likely representing the molecular ion ([M]+) or the most stable fragment. The peaks at **m/z 576** and **m/z 577** could be isotopic variants or closely related fragments, while **m/z 513** indicates a significant fragment ion. This suggests that the molecular weight of the bioactive compound from the third peak is approximately 575 Da. These data are essential for identifying and characterizing the compound, and further structural analysis, such NMR, may confirm its structure and bioactivity.

Discussion

The findings of this study align with and expand upon the existing body of research regarding the antimicrobial potential of endophytic bacteria. The observed diversity in antimicrobial activity among the isolates reflects the metabolic variability of endophytes, as demonstrated by Alhaddad et al. [10], who reported that endophytic bacteria from different plant tissues exhibit varying capacities to produce bioactive secondary metabolites. Specifically, the isolate *F2* showed superior activity, which may be attributed to its ability to synthesize unique antimicrobial compounds. Similar dominance of specific endophytes has been noted in studies by Toppo et al. [11], who found that endophytes from *Catharanthus roseus* and *Azadirachta indica* displayed differential antimicrobial activities, with select isolates outperforming others due to their specialized biosynthetic pathways. The optimization of environmental factors, such as temperature, carbon, and nitrogen sources, further supports the role of growth conditions in enhancing secondary metabolite production. The observation that antimicrobial activity peaked at 35°C agrees with studies by Hafeez et al. [12], who found that temperature regulation plays a key role in modulating enzymatic activity and gene expression in secondary metabolic pathways. This study's finding that lactose is the most effective carbon source for *F2* is consistent with findings by Li et al. [13], who suggested that sugars such as lactose and glucose act as inducers for specific biosynthetic gene clusters in microorganisms, leading to enhanced production of antibiotics.

The nitrogen source analysis provides additional insights. Yeast extract's superiority as a nitrogen source can be explained by its rich composition of amino acids, peptides, and vitamins, which are crucial for secondary metabolism. This observation aligns with the work of Kalaba et al. [14], who demonstrated that yeast extract significantly improved the production of antimicrobial compounds in *Streptomyces* species. On the other hand, ammonium sulfate's limited effectiveness may be due to its inability to provide the complex nutrients required for optimal metabolic activity, as supported by findings from yin et al. [15].

The influence of pH on antimicrobial production reflects the sensitivity of bacterial enzymatic systems to environmental conditions. The optimal activity observed at pH 7.0 corroborates the findings of Khan et al. [16], who reported that neutral pH is ideal for the stability and activity of most antimicrobial biosynthetic enzymes. Both acidic and alkaline conditions likely disrupt enzymatic activity and structural integrity, reducing antimicrobial compound production, as observed in prior research.

The purification process through column chromatography and HPLC allowed for the isolation and characterization of the bioactive compound, further validated by mass spectrometry. The molecular weight of approximately 575 Da for the active compound is consistent with findings by Fan et al. [16], who identified structurally similar bioactive compounds from endophytic bacteria, such as polyketides and lipopeptides, with comparable molecular weights. These compounds have been shown to possess broad-spectrum antimicrobial activities, including against multidrug-resistant pathogens.

Importantly, the potential application of these findings lies in combating antibiotic resistance, a growing global health crisis. The bioactive compound identified in this study could serve as a lead for the development of novel antibiotics. Previous work by Azeem et.al. [17] emphasized the critical role of natural products, particularly those from endophytes, in drug discovery. This study provides further evidence supporting the exploration of plant-associated endophytes as a sustainable and promising source of antimicrobial agents.

Additionally, the distinct activity of the fractions at different retention times suggests the presence of multiple bioactive compounds within the extract. This aligns with findings by Posada et al.,[18] who reported that endophytic bacteria often produce a suite of metabolites with synergistic or complementary antimicrobial effects. Further studies involving tandem mass spectrometry (MS/MS) and nuclear magnetic resonance (NMR) would provide detailed structural information and help elucidate the biosynthetic pathways responsible for these compounds.

Conclusion

In conclusion, this study highlights the significant antimicrobial potential of endophytic bacteria and reinforces the importance of optimizing culture conditions and purification techniques to enhance bioactive compound production. These findings contribute to the growing interest in endophytes as a sustainable source of novel antimicrobial agents and provide a foundation for future studies aimed at developing new therapeutic options to address antibiotic resistance.

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