



Isolation and Screening of Antibacterial-Producing Endophytic *Actinomycetes* from Hollyhock (*Alcea rosea*)

Somayeh Karimi¹, Fazel Pourahmad*, Mostafa Nemati¹

*Corresponding Author: Fazel Pourahmad, Faculty of Veterinary Sciences, Ilam University, P.O. Box 69315-516, Ilam, Iran

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Abstract

Actinomycete bacteria are widely recognized for their ability to produce a diverse array of bioactive compounds, particularly antibiotics, and contribute significantly to the discovery of naturally derived antimicrobial agents. This study focused on the isolation and characterization of *actinomycete* strains from hollyhock (*Alcea rosea*) plant tissues to assess their antibacterial potential against clinically relevant pathogens. The target pathogens included *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. A total of 110 bacterial isolates were obtained, with 62 isolates confirmed as *Actinomycetes* through PCR amplification of the 16S rRNA gene. Approximately 46.5% of these isolates exhibited antibacterial activity, with pronounced effects against *B. cereus* and *S. aureus*, suggesting their potential role in combating antibiotic-resistant bacteria. Genetic analysis revealed the presence of non-ribosomal peptide synthetase (NRPS) genes, indicating that the produced antimicrobial compounds may originate from NRPS pathways. However, no polyketide synthase (PKS) genes were detected, which may limit the diversity of bioactive metabolites. These findings highlight the pharmaceutical potential of hollyhock-associated *Actinomycetes* as promising sources of novel antimicrobial agents.

1. Introduction

Actinomycetes, a prominent group within the bacterial domain classified under the phylum *Actinomycetota*, are notable for their filamentous structure and significant genomic capacity to produce a wide array of secondary metabolites, including natural antibiotics. These Gram-positive bacteria are primarily found in diverse environments such as soil, marine sediments, and extreme habitats, which enhances their ability to synthesize unique bioactive compounds (Jagannathan et al., 2021; Khan et al., 2023).

Research indicates that the 10,000 identified *Actinomycetes* compounds are responsible for over 45% of all bioactive microbial metabolites, many of which serve as antibiotics in clinical settings (Izhar et al., 2023). Notably, over 70% of all naturally derived antibiotics in clinical use originate from this group (Genilloud, 2017). Their extensive genomic capacity allows for the biosynthesis of various compounds, including antibacterial, antifungal, and anticancer agents. Members of the genus *Streptomyces* are especially prolific in antibiotic production, significantly contributing to the antibiotic arsenal used to

*Corresponding Author: E-mail: f.pourahmad@ilam.ac.ir
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combat multidrug-resistant pathogens (Kim et al., 2021).

Actinomycetes thrive in diverse environments, playing essential roles across ecosystems. In soil, they contribute to nutrient cycling and the synthesis of bioactive compounds, supporting ecological balance (Jose & Jebakumar, 2014). Most *Actinomycetes* are aerobic and prefer neutral to alkaline soils, although some species can thrive in more acidic environments (Javad et al., 2021). Their adaptability to different soil conditions contributes to their ecological significance and potential for biotechnological applications, particularly in antibiotic production (Setiawati & Yusan, 2022).

Within the rhizosphere, the region surrounding plant roots, they foster plant growth and act as natural defenders against infections (Van der Meij et al., 2017). Freshwater systems benefit from their ability to enhance microbial diversity and ecological functions (Bredholt et al., 2008). In marine environments, *Actinomycetes* inhabit sediments and hypersaline habitats and produce unique secondary metabolites with distinct biochemical properties (Jensen et al., 1991; José & Jebakumar, 2014). On plants, particularly leaf surfaces known as the phyllosphere, they form symbiotic relationships that help prevent plant diseases and promote overall plant health (Wati et al., 2023).

In addition to their ecological roles, *Actinomycetes* have significant applications in medicine, agriculture, and environmental remediation, highlighting their importance in biotechnology and drug discovery (Meenakshi et al., 2024). *Alcea rosea*, commonly known as hollyhock, has been utilized for medicinal purposes since ancient times, with applications including anti-inflammatory, analgesic, and antioxidant effects (Dar et al., 2017; Ahmadi et al., 2012; Azadeh et al., 2023). The plant contains various bioactive compounds, such as flavonoids and mucilaginous polysaccharides, which contribute to its therapeutic properties (Zareii et al., 2014; Hanif et al., 2019). Many medicinal compounds found in plants are synthesized by

endophytes, microorganisms residing within plant tissues. These endophytes can produce bioactive compounds similar to those of their host plants, enhancing the plant's medicinal properties (Zhao et al., 2011; Khan et al., 2023). Endophytes are microorganisms capable of synthesizing various secondary metabolites with pharmaceutical potential, including compounds with antibacterial, antiviral, and anticancer properties (Aharwal et al., 2016; Choudhury et al., 2023; Staniek et al., 2008). This symbiotic relationship offers a rich source of novel natural products for pharmaceutical applications (Li et al., 2014). Recent advances in genome mining and coculture techniques have further enhanced the discovery of novel secondary metabolites from *Actinomycetes*, underscoring their potential as sources of new therapeutic agents (Jagannathan et al., 2021).

The present study aimed to investigate the antibacterial properties of endophytic *Actinomycetes* associated with *Alcea rosea*. By isolating and characterizing these microorganisms, we sought to assess their potential antibacterial activity against pathogenic microorganisms. This study provides a basis for further exploration of endophytic *Actinomycetes* as potential sources of novel antimicrobial agents.

2. Materials and Methods

2.1. Sample Collection and Isolation of Endophytic *Actinomycete*

Hollyhock plant samples were collected in the spring of 2020 from various regions of Ilam Province, Iran. These samples were stored in sterile plastic bags and transported to the laboratory on ice to preserve microbial integrity.

In the laboratory, the plant samples underwent a modified six-step surface sterilization process within 24 hours, as described by Quin et al. (2011). The sterilized plant parts were processed according to the method described in Hajizadeh et al. (2023).

2.2. Morphological Characterization

The preliminary identification of isolates was conducted based on morphological characteristics, including colony morphology (size, shape, color, margin, and elevation), microscopic features (Gram stain reaction, aerial and substrate mycelium formation, and spore morphology), and pigmentation (production of diffusible or non-diffusible pigments) following methods described by Cook (2003).

2.3. DNA Extraction and Molecular Characterization of *Actinomyces*

Genomic DNA was extracted from endophytic isolates using a boiling method (Tavarideh et al., 2022) and identified with *Actinomyces*-specific primers listed in Table 1 via PCR (Stach et al., 2003).

Table 1: List of Oligonucleotide Primers used in the Study

Primer name	Sequence (5'-3')	Gene	Product size (bp)	Reference
ACT235f	CGCGGCCTATCAGCTTGTTG	16S rRNA	640	Stach et al., 2003
ACT878r	CCGTACTCCCCAGGCGGGG			
A3F	GCSTACSYSATSTACACSTCSGG	NRPS	700-800	Ayuso-Sacido & Genilloud, 2005
A7R	SASGTCVCCSGTSCGGTAS			
KIF	TSAAGTCSAACATCGGBCA	PKS-I	1200-1400	Metsä-Ketelä, 1999
M6R	CGCAGGTTSCSGTACCAGTA			
PKS-II-A	TSGCSTGCTTCGAYGCSATC	PKS-II	600	Ayuso-Sacido & 89/*p]; Genilloud, 2005
PKS-II-B	TGGAANCCGCCGAABCCGCT			

2.4. Evaluation of Antibacterial Activity of *Actinomyces*

All actinomycetal isolates were fermented, and their resulting extracts were screened according to previous research without modifications (Hajizadeh et al., 2023). The antibacterial activity of the actinomycetal strains was evaluated using two Gram-negative reference strains (*Escherichia coli* PTCC 1330, *Pseudomonas aeruginosa* PTCC 1430), *Bacillus cereus* PTCC 1015, and two Gram-positive pathogens (*Bacillus cereus* PTCC 1015, *Staphylococcus aureus* ATCC 33591). The bacteria were cultured overnight at 37°C in Mueller-Hinton (MH) broth, and each culture was then adjusted to a turbidity level of 0.5 McFarland standard. A ciprofloxacin disk was used as the positive control and ethyl acetate as the negative control. Following the procedure described by Hajizadeh et al. (2023), bacterial lawns were first created on MH agar in 6 mm wells, into which 100 µL of crude extracts were added. The plates were left at room temperature for one hour before being

incubated at 37°C. After 24 hours, the inhibition zones were assessed in millimeters (mm), utilizing 100 µL of ethyl acetate as the control.

2.5. Detection of *PKS-I*, *PKS-II*, and *NRPS* genes

Genes encoding non-ribosomal peptide synthetases (*NRPS*) and polyketide synthases I and II (*PKS-I* and *PKS-II*) were detected via PCR using specific primers (Table 1). Amplifications were performed with 30 cycles of denaturation (95°C, 1 minute), annealing (58°C or 60°C, 1 minute), and extension (72°C, 1 minute). PCR products were analyzed on 1.5% agarose gels.

2.6. Statistical Analysis

All experiments were performed in triplicate. The results were presented as mean ± standard deviation, and statistical significance was determined using two-way ANOVA in GraphPad Prism version 9.0. A p-value <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Isolation and Identification of *Actinomycetes*

One hundred and ten bacterial isolates were obtained from surface-sterilized tissues of the hollyhock. The morphological characterization revealed that 70 isolates were Gram-positive, predominantly exhibiting a filamentous structure characteristic of *Actinomycetes*. This morphological trait aligns with the well-

established features of this bacterial group, known for their filamentous growth and ability to produce a variety of bioactive compounds.

To further confirm the identity of the isolates, PCR amplification of the 16S rRNA gene was performed on 62 isolates, producing a product of approximately 640 base pairs (Fig 1). The high success rate of PCR amplification suggests a high level of genetic diversity among the isolates, indicating that *Alcea rosea* harbors a rich community of *Actinomycetes*.

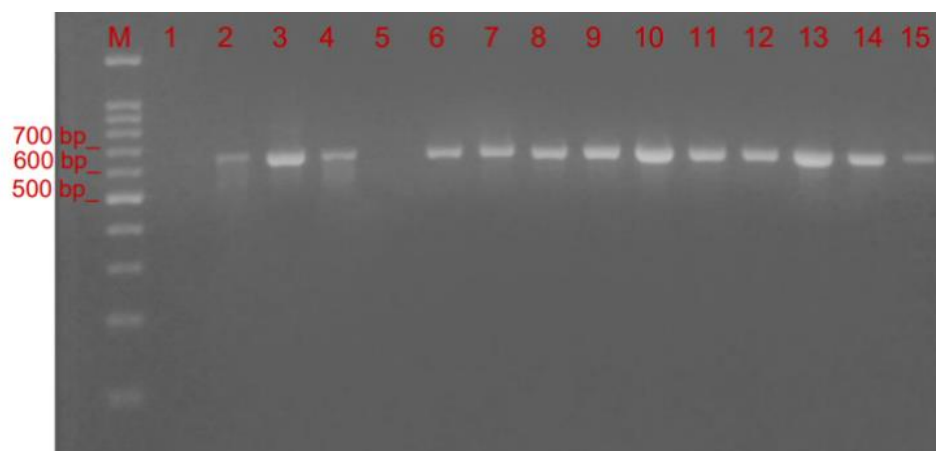


Figure 1: Agarose Gel Electrophoresis of 16S rRNA PCR Products from Bacterial Isolates

Note. Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–15: PCR products from bacterial isolates showing a 640 bp band representing the amplified 16S rRNA gene.

3.2. Antibacterial Activity

The antibacterial activity of the 62 PCR-positive actinomycete isolates was evaluated against four pathogenic bacterial strains: *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Table 2). Of these 30 isolates, 14 (46.5%) exhibited antibacterial activity against *Bacillus cereus*, 7 (23.5%) against *Staphylococcus aureus*, 6 (20%) against *Pseudomonas aeruginosa*, and 3 (10%)

against *Escherichia coli*. These findings suggest that *Actinomycetes* associated with plants like *Alcea rosea* produce bioactive compounds that are effective against Gram-positive and Gram-negative bacteria. This broad spectrum of activity aligns with previous research indicating that *Actinomycetes* are key producers of antibiotics, including streptomycin, erythromycin, and tetracycline (Roy & Banerjee, 2015).

Table 2: Antibacterial Activities of *Actinomycetes* Isolated from *Alcea rosea*

No	Strain code	<i>B. cereus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	Mean and Standard Deviation of Inhibition Zone (mm) ^a
1	Lk				> 25 mm	28.5±2.3
2	Pc		≥10<15 mm			12.0±1.5
3	Ll			≥10<15 mm		14.2±1.8
4	Lg				> 25 mm	27.8±2.1
5	Ph				> 25 mm	26.5±2.4
6	Ri			10-15 mm		18.7±1.9
7	Ln				> 25 mm	29.0±2.5
8	Po				10-15 mm	16.3±1.7
9	Pp				10-15 mm	17.0±1.6
10	Sj		≥10<15 mm			11.5±1.4
11	Ps			10-15 mm		19.2±1.8
12	Pq			10-15 mm		20.0±1.9
13	Lr	10-15 mm				22.5±2.0
14	PL ₂	10-15 mm				21.8±2.1
15	Pz ₁	10-15 mm				23.0±2.2
16	Sa ₂	10-15 mm				22.7±2.0
17	Rb ₂	> 25 mm				30.5±2.6
18	Pc ₂	10-15 mm				21.5±1.9
19	Sd ₂				10-15 mm	16.8±1.7
20	Re ₂				≥10<15 m	13.5±1.6
21	Pt			≥10<15 mm		14.0±1.7
22	Pg ₂	10-15 mm				20.5±1.8
23	Su	> 25 mm				31.0±2.7
24	Lv	> 25 mm				29.5±2.4
25	Pw	≥10<15 mm				12.8±1.5
26	Sj ₂			10-15 mm		18.5±1.8
27	Lx	≥10<15 mm				13.0±1.6
28	Py	≥10<15 mm				12.5±1.5
29	Sf ₂	10-15 mm				19.8±1.9
30	Lv ₂	10-15 mm				20.2±1.8

Note. ^a significant difference ($p < .05$) was seen between inhibition zones of *Actinomycetes* and the control.

3.3. Specific Pathogen Responses

- *Bacillus cereus*: Fourteen isolates exhibited notable antibacterial activity against *B. cereus*, with inhibition zones ranging from 12 mm to 25 mm. These findings suggest that certain *Actinomycetes* isolated from *Alcea rosea* may produce compounds effective against foodborne pathogens, which is highly relevant in the context of food safety concerns.

- *Staphylococcus aureus*: Seven isolates demonstrated activity against this clinically important pathogen, with inhibition zones between 10 mm and 20 mm. Given the increasing antibiotic resistance in *S. aureus*, these results are promising for the development of alternative treatments for infections caused by this pathogen.

- *Pseudomonas aeruginosa*: Six isolates exhibited activity against this opportunistic pathogen, with inhibition zones ranging from 8 mm to 18 mm. The ability to inhibit *P. aeruginosa*, known for its resistance to many antibiotics, further emphasizes the significance of these *Actinomycetes* in combating multi-drug-resistant bacteria.

- *Escherichia coli*: Three isolates showed moderate antibacterial activity against *E. coli*, with inhibition zones ranging from 9 mm to 15 mm. While the inhibition was less pronounced than for other pathogens, the findings suggest that these isolates could still have the potential to address *E. coli*-related infections.

Overall, the data indicate that *Actinomycetes* associated with *Alcea rosea* possess a diverse array of bioactive compounds with significant antibacterial activity. The antibacterial potential observed against foodborne and clinically relevant pathogens suggests the importance of these isolates in future antimicrobial drug discovery.

3.4. Genetic Screening for Biosynthetic Pathways

To further investigate the biosynthetic potential of the isolated *Actinomycetes*, PCR was conducted to detect genes associated with secondary metabolite production, particularly those related to non-*NRPS* and *PKS*. These pathways are crucial for the production of bioactive compounds, including antimicrobial agents.

3.5. Findings on *NRPS* and *PKS* Genes

Among the 62 PCR-positive isolates, 38 were found to possess *NRPS* genes, which are associated with the production of non-ribosomal peptides (Fig 2). This finding is significant as *NRPS* are known for their broad spectrum of biological activities, including antibacterial properties. The presence of *NRPS* genes in the isolates suggests that many *Actinomycetes* from *Alcea rosea* can synthesize bioactive compounds. This indicates their potential to produce *NRPS*, which is known for its diverse biological activities, including antibacterial, antifungal, and anticancer effects (Hur et al., 2012; Zobel et al., 2016).

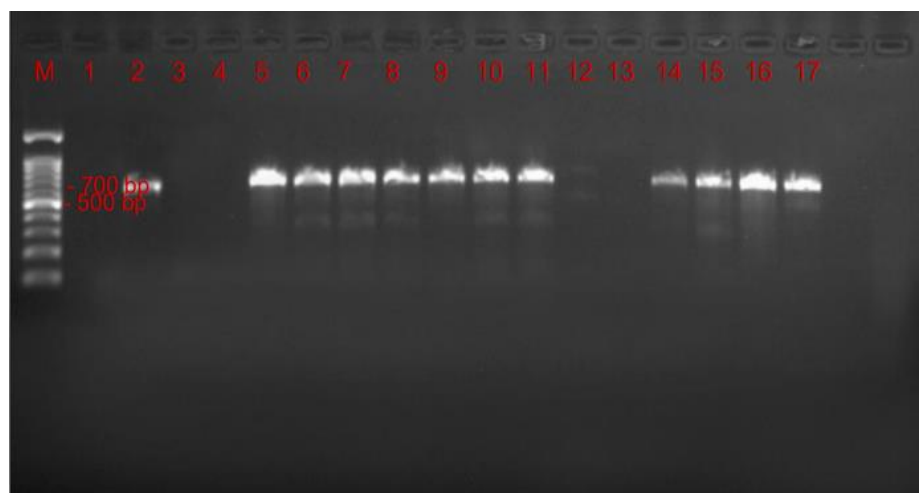


Figure 2: Agarose Gel Electrophoresis of *NRPS* gene PCR Products from *Actinomycete* Isolates

Note. Lane M: DNA size marker; Lane 1: Negative control; Lanes 2–17: PCR products from *Actinomycetes* isolates, displaying bands between 700–800 bp, indicative of the amplified *NRPS* gene.

*NRPS*s are complex multimodular enzymes that synthesize these peptides through a series of catalytic domains, each responsible for incorporating specific monomeric units into the peptide chain (Butz et al., 2008). The ability of these *Actinomycetes* to produce *NRPS*-derived compounds highlights their potential in pharmaceutical applications, particularly in developing new therapeutic agents targeting various diseases (Li et al., 2014).

The presence of *NRPS* genes in *Actinomycetes* derived from *Alcea rosea* is consistent with findings from various studies

on endophytic *Actinomycetes*, which collectively highlight their significant biosynthetic potential. A study conducted on endophytic *Actinomycetes* sourced from tropical plants revealed that 100% of the tested strains possessed *NRPS* genes. This finding underscores a robust potential for secondary metabolite production, even though some strains did not yield detectable metabolites (Janso et al., 2010). Research focusing on endophytic *Actinomycetes* associated with medicinal plants indicated that 28.9% of the isolates contained *NRPS* genes, which were correlated with broad-spectrum antimicrobial

activity (Hanh et al., 2020). Similarly, a study on endophytic *Actinomycetes* from tea plants demonstrated a high prevalence of *NRPS* genes, directly linked to their antimicrobial capabilities (Shan et al., 2018).

However, none of the isolates contained *PKS-I* or *PKS-II* genes, which are involved in the biosynthesis of polyketides (data not shown). While the absence of these genes may limit the potential for producing certain polyketide antibiotics, it does not diminish the overall relevance of these strains in the search for new antimicrobial agents. The identification of *NRPS* genes corresponds closely with the antibacterial activity observed, indicating that the bioactive compounds synthesized by these strains are likely to be *NRPS*. The lack of polyketide synthase (*PKS-I* and *PKS-II*) genes in the isolates tested implies that the bioactive compounds produced are predominantly sourced from *NRPS* pathways or other classes of metabolites that were not investigated in this study. *PKS* genes are generally linked to the biosynthesis of polyketides, which represent another important category of secondary metabolites recognized for their varied biological activities, including antibiotic effects (Ayuso-Sacido et al., 2004; Zhao et al., 2010).

The lack of *PKS* gene detection indicates that these isolates may not produce polyketide-derived compounds, which could limit the range of bioactive metabolites available from these *Actinomycetes*. Instead, the focus on *NRPS*-derived compounds may highlight a different metabolic pathway that could be explored for novel therapeutic agents (Gohain et al., 2015). This finding emphasizes the need for further investigation into other biosynthetic pathways that may contribute to the bioactivity of these isolates (Mzumdar et al., 2023).

3.6. Implications for Drug Discovery

This study highlights the significant potential of *Alcea rosea*-associated *Actinomycetes* as sources of novel antimicrobial agents. The high

incidence of antibacterial activity among the isolates indicates their potential to address the critical issue of antibiotic resistance, particularly in pathogens such as *S. aureus*, *P. aeruginosa*, and *B. cereus*. The presence of *NRPS* genes further supports the idea that these strains may produce non-ribosomal peptides, which could be developed into effective new drugs.

4. Conclusion

In conclusion, this study successfully isolated and characterized *Actinomycetes* from *Alcea rosea*, revealing their significant antibacterial properties and genetic potential for producing bioactive secondary metabolites. These findings not only contribute valuable knowledge to the field of natural product chemistry but also underscore the importance of these microorganisms in the ongoing search for novel antimicrobial agents. The promising antibacterial activity observed in this study highlights the need for continued exploration of *Alcea rosea*-associated *Actinomycetes* as potential sources for combating resistant bacterial strains and advancing drug discovery. Future studies should focus on the isolation and structural characterization of active compounds, elucidation of their mechanisms of action, and in vivo evaluation of their therapeutic efficacy in treating bacterial infections.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

S.K. and F.P. proposed and designed the research, S.K. collected samples.

S.K., F.P., and M.N. analyzed and interpreted data, S.K., F.P., and M.N. drafted the manuscript, F.P. and M.N. performed statistical analyses, F.P. and M.N. proved the final version of the manuscript.

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