



## FUNCTIONAL PROFILING OF ENZYME PRODUCING MICROORGANISMS ISOLATED FROM NEEM (*AZADIRACHTA INDICA*) LEAVES

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**ABSTRACT:** The present study aimed to isolate and comprehensively characterize enzyme producing microorganism from healthy leaves of *Azadirachta indica* collected from multiple sites in Bhopal, India. Six distinct isolates were obtained and subjected to morphological, biochemical, functional, and molecular analyses. The isolates were identified as *Cladosporium* sp., *Nigrospora oryzae*, *Streptomyces* sp., *Acremonium* sp., *Fusarium* sp., and *Curvularia* sp. Enzymatic profiling demonstrated variable production of amylase, protease, cellulase, and lipase, with *Streptomyces* sp. exhibiting the highest amylase (2.40 U/ml) and cellulase (0.47 U/ml) activities. All isolates showed positive phosphate solubilization, siderophore secretion,  $\beta$ -1,3-glucanase activity, and cellulolytic potential. Indole-3-acetic acid (IAA) synthesis ranged between 1.001 and 1.937 mg/l, with maximum production recorded in *Streptomyces* sp. Molecular identification via partial sequencing corroborated morphological classification, and sequences were deposited in the NCBI GenBank database. These findings highlight neem-associated enzyme producing microorganism as promising resources for sustainable agriculture and biotechnological enzyme production.

**Keywords:** Enzyme producing microorganism, Neem, Extracellular enzymes, Indole-3-acetic acid, Phosphate solubilization, Molecular identification.

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### INTRODUCTION

A number of microorganisms form symbiotic relationships with plants and play important roles in plant growth, development, and defense against environmental stresses and pathogens. In recent years, these microorganisms have gained considerable attention due to their ability to produce a wide range of bioactive metabolites,

extracellular enzymes, phytohormones, and antimicrobial compounds that contribute to plant fitness and offer potential applications in agriculture, medicine, and biotechnology (Strobel and Daisy, 2003; Hardoim *et al.*, 2015).

Neem (*Azadirachta indica*), a well-known medicinal tree widely distributed in tropical and subtropical regions, is valued for its diverse

pharmacological properties including antimicrobial, antifungal, anti-inflammatory, and insecticidal activities. These properties are largely attributed to its rich repertoire of bioactive compounds such as azadirachtin, nimbin, and nimbolide. The internal tissues of neem provide a unique ecological niche for diverse endophytic microorganisms, which may contribute to the biosynthesis of bioactive metabolites and enzymes of industrial significance (Jain and Lalit, 2023; Subbulakshmi *et al.*, 2012; Verma *et al.*, 2016).

Functional profiling of these microorganisms has revealed their potential in producing industrial enzymes, antimicrobial compounds, and plant growth-promoting metabolites. Several studies have reported the presence *Streptomyces*, *Fusarium*, *Cladosporium*, and *Nigrospora* in medicinal plants, demonstrating significant enzymatic activities and bioactive metabolite production (Dwivedi *et al.*, 2007; Sunitha *et al.*, 2013). These microorganism not only contribute to host plant health but also represent promising sources of novel bioactive molecules with applications in pharmaceuticals, agriculture, and environmental biotechnology.

Despite increasing global interest in these microbial diversity, systematic investigations of neem-associated enzyme producing microorganism in central India remain relatively limited, particularly regarding their enzymatic potential, functional attributes, and molecular characterization. Considering the ecological and medicinal importance of neem, exploration of its endophytic microbiota may provide new insights into microbial diversity and their potential applications.

Therefore, the present study was undertaken to isolate enzyme producing microorganism from neem (*Azadirachta indica*) leaves and to evaluate their enzymatic activities, functional characteristics, and molecular identity using molecular techniques. The findings of this research may contribute to understanding the diversity of neem-associated enzyme producing

microorganism and their potential role as sources of industrially and agriculturally important bioactive compounds.

## MATERIALS AND METHODS

### SAMPLE COLLECTION

Healthy and disease-free leaves of neem (*Azadirachta indica*) were collected from three different locations in Bhopal, Madhya Pradesh, India: Chetak Bridge, Barkheda Haat and Market Ground (BHM), and BHEL Ground. At each site, approximately 15-20 mature leaves were randomly collected from three different trees to ensure representative sampling in a sterile polyethylene bags and transported to the laboratory within 2-3 hours of collection. All samples were processed immediately upon arrival to avoid contamination and microbial degradation.

### ISOLATION OF ENDOPHYTIC ACTINOMYCETES

Isolation of endophytic microorganisms was performed following the surface sterilization protocol described by Strobel *et al.* (1996) with slight modifications. Leaves were first washed under running tap water for 5 minutes to remove dust and debris. Samples were then immersion in 70% ethanol for 1 minute and then places in 5.3% sodium hypochlorite solution for 3 minutes. Samples were then rinse with 70% ethanol for 30 seconds and finally, washing three times with sterile distilled water to remove residual sterilizing agents.

The sterilized leaves were dried on sterile filter paper under aseptic conditions. Leaves were then cut approximately 0.5–1 cm<sup>2</sup> using sterile scalpel blades. Each leaf segment was placed on Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (50 µg/mL) to suppress bacterial contamination. Plates were incubated at 28 ± 2°C for 8–10 days. Emerging colonies were subcultured on fresh media to obtain pure isolates. A control of final rinse water was plated on SDA media as a sterility control. Isolation frequency of endophytes was calculated.

## MORPHOLOGICAL CHARACTERIZATION

Preliminary identification of isolates was carried out based on colony morphology, pigmentation, texture, and growth pattern on SDA plates. Microscopic examination was performed using lactophenol cotton blue staining. Small portions of the colony were mounted on glass slides, stained, and observed under a light microscope at 40x and 100x magnification. Morphological features such as spore arrangement, mycelial structure, and hyphal branching patterns were compared with standard identification manuals of Domsch *et al.*, 1980 and Gilman, 1957.

## BIOCHEMICAL CHARACTERIZATION:

### QUALITATIVE ENZYME ASSAYS

Qualitative screening for extracellular enzyme production was carried out using substrate-specific agar media. All assays were performed in triplicate and zone diameters were measured in millimeters.

#### AMYLASE ACTIVITY

Isolates were inoculated on starch agar plates and incubated at  $28 \pm 2^\circ\text{C}$  for 48-72 hours. Plates were flooded with iodine solution, and the appearance of a clear zone around colonies indicated starch hydrolysis.

#### CELLULASE ACTIVITY

Cellulase activity was evaluated on Carboxymethyl Cellulose (CMC) agar plates. After incubation, plates were stained with 0.1% Congo red solution for 15 minutes and washed with 1 M NaCl. Clear zones around colonies indicated cellulase production.

#### PROTEASE ACTIVITY

Protease activity was assessed on skim milk agar plates. A clear halo around colonies after incubation indicated casein hydrolysis.

#### LIPASE ACTIVITY

Lipase activity was detected using tributyrin agar plates, where transparent zones surrounding colonies indicated lipid hydrolysis.

## QUANTITATIVE ENZYME ASSAYS

### AMYLASE AND CELLULASE ACTIVITY

Quantitative estimation of amylase and cellulase was carried out using the 3,5-dinitrosalicylic acid (DNS) method following Deb *et al.* (2013). 0.5 mL enzyme extract was mixed with 0.5 mL substrate solution (1% starch) and incubated at  $37^\circ\text{C}$  for 30 minutes. The reaction was stopped by adding DNS reagent, followed by boiling for 5 minutes. Absorbance was measured at 540 nm using a spectrophotometer.

### PROTEASE ACTIVITY

Protease activity was determined using the casein digestion method described by Kunitz (1965). The enzyme extract was incubated with 1% casein solution at  $37^\circ\text{C}$  for 30 minutes. The reaction was terminated with trichloroacetic acid (TCA) and absorbance of released tyrosine was measured at 280 nm.

### LIPASE ACTIVITY

Lipase activity was estimated using the titrimetric method described by Yasmin *et al.* (2006), where liberated fatty acids were titrated against 0.05 N NaOH using phenolphthalein as an indicator.

## FUNCTIONAL CHARACTERIZATION

### PHOSPHATE SOLUBILIZATION

Phosphate solubilization was evaluated using Pikovskaya's agar medium containing tricalcium phosphate. Plates were incubated at  $28^\circ\text{C}$  for 6-7 days, and the formation of clear halos around colonies indicated phosphate solubilization.

### SIDEROPHORE PRODUCTION

Siderophore production was detected using Chrome Azurol S (CAS) agar medium. Development of an orange halo zone around colonies indicated siderophore secretion.

## IAA PRODUCTION AND QUANTIFICATION

### QUALITATIVE DETECTION

Isolates were cultured in Luria–Bertani (LB) broth supplemented with 0.1% L-tryptophan and

incubated at 28°C for 72 hours with shaking at 150 rpm. Culture supernatant was mixed with Salkowski reagent, and the development of pink coloration indicated the presence of indole-3-acetic acid (IAA) by following the method of Bric *et al.*, 1991.

### QUANTITATIVE ESTIMATION

IAA concentration was measured spectrophotometrically at 535 nm. The concentration was determined using a standard calibration curve prepared with pure IAA.

### HPTLC CONFIRMATION OF IAA:

Confirmation of IAA was performed using High Performance Thin Layer Chromatography (HPTLC) following the method of Sivagnanam and Gangatharan (2017).

Stationary phase: Silica gel 60 F254 plates

Mobile phase: Hexane : Ethyl acetate (3.5 : 1.5)

Detection: UV light at 254 nm

The R<sub>f</sub> value of the sample was compared with that of the standard IAA compound to confirm its presence.

### MOLECULAR CHARACTERIZATION

#### DNA EXTRACTION AND SEQUENCING

Genomic DNA was extracted from pure isolates grown in malt extract broth for 6-7 days using a commercial bacterial DNA extraction kit following the manufacturer's protocol.

The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGAT-CMTGGCTCAG-3') and 1492R (5'-TACGGYTA-CCTTGTTACGACTT-3').

### PCR CONDITIONS INCLUDED

**INITIAL DENATURATION** 95°C for 5 min; 35 cycles of: Denaturation: 95°C for 30 sec, Annealing: 55°C for 30 sec, Extension: 72°C for 1 min, Final extension: 72°C for 7 min.

PCR products were purified and sequenced using Sanger sequencing. Obtained sequences were compared with existing sequences in the NCBI GenBank database using BLAST. The sequences were submitted to NCBI GenBank to obtain accession numbers.

### STATISTICAL ANALYSIS

All experiments were conducted in triplicate (n = 3). Results were expressed as mean.

### RESULTS

#### ENDOPHYTE ISOLATION AND COLONIZATION

A total of 63 endophytic colonies were isolated from neem leaves, representing six distinct species presented in table 1. High colonization frequency was observed across all sampling sites, with an average of 95.37%, indicating widespread endophytic association. Images of well develop plate is presented in Figure 1.

**Table 1. Colonization of endophytes in neem leaf samples**

| Species                  | Chetak Bridge | BHMG | BHEL Ground | Total |
|--------------------------|---------------|------|-------------|-------|
| <i>Cladosporium</i> sp.  | 3             | 3    | 4           | 10    |
| <i>Nigrospora oryzae</i> | 3             | 4    | 4           | 11    |
| <i>Streptomyces</i> sp.  | 2             | 4    | 4           | 10    |
| <i>Acremonium</i> sp.    | 3             | 4    | 4           | 11    |
| <i>Fusarium</i> sp.      | 3             | 4    | 3           | 10    |
| <i>Curvularia</i> sp.    | 3             | 4    | 4           | 11    |

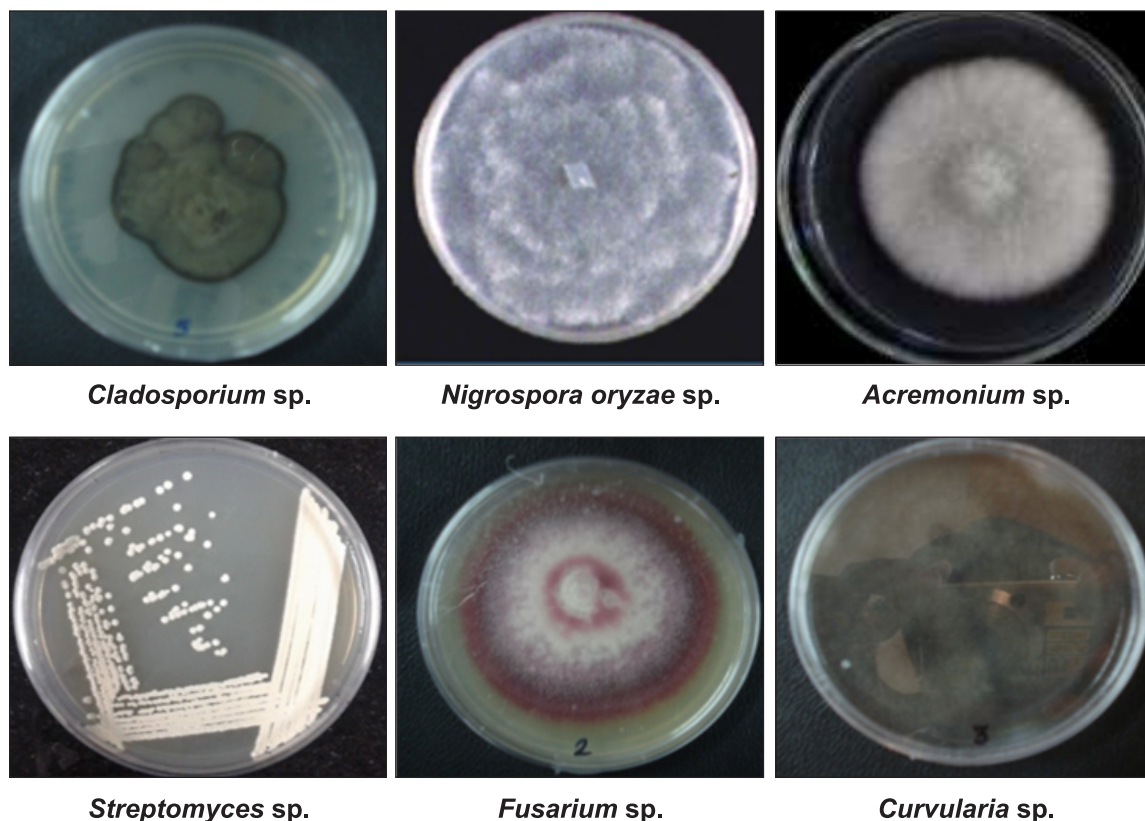


Fig. 1. Images of well develop plate

### ENZYMATIC PROFILING

Quantitative analysis revealed substantial variation in enzymatic activities among isolates. *Streptomyces* sp. demonstrated superior amylase and cellulase production,

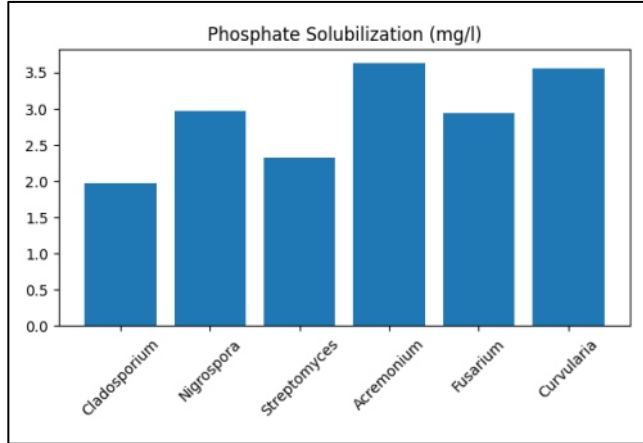
while *Cladosporium* sp. and *Curvularia* sp. exhibited notable protease activity presented in table 2. Lipase production was observed predominantly in *Cladosporium* sp., *Acremonium* sp., and *Fusarium* sp.

Table 2. Quantitative enzyme activities (U/ml)

| Isolate Organisms        | Amylase | Protease | Cellulase | Lipase |
|--------------------------|---------|----------|-----------|--------|
| <i>Cladosporium</i> sp.  | 0.00    | 1.25     | 0.26      | 0.17   |
| <i>Nigrospora oryzae</i> | 0.00    | 0.00     | 0.00      | 0.23   |
| <i>Streptomyces</i> sp.  | 2.40    | 1.71     | 0.47      | 0.00   |
| <i>Acremonium</i> sp.    | 2.10    | 0.00     | 0.43      | 0.11   |
| <i>Fusarium</i> sp.      | 2.75    | 0.00     | 0.00      | 0.06   |
| <i>Curvularia</i> sp.    | 0.00    | 1.20     | 0.00      | 0.00   |

## FUNCTIONAL ATTRIBUTES

All isolates exhibited phosphate solubilization, siderophore production (Graph 1),  $\beta$ -1,3-glucanase activity, and cellulolytic capability, confirming their plant growth-promoting potential

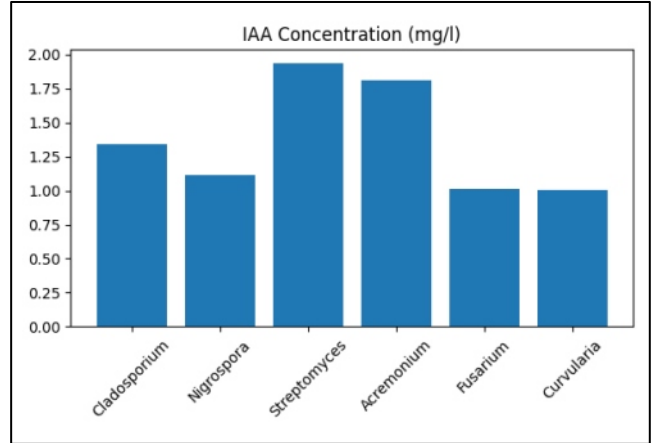


**Graph 1. Phosphate solubilization potential of isolated endophytes.**

## IAA PRODUCTION

IAA synthesis ranged from 1.001 to 1.937 mg/l, with *Streptomyces* sp. producing the highest

concentration, followed by *Acremonium* sp. is presented in Graph 2.



**Graph 2: IAA concentration produced by neem-associated endophytes**

## MOLECULAR CONFIRMATION

Partial sequencing validated the identity of isolates as *Cladosporium* sp., *Nigrospora oryzae*, *Streptomyces* sp., *Acremonium* sp., *Fusarium* sp., and *Curvularia* sp. Accession numbers of submitted sequence obtained from NCBI GenBank are presented in table 3.

**Table 3. NCBI accession numbers of such isolates**

| Isolate code | Name of Actinomycetes      | Accession No. |
|--------------|----------------------------|---------------|
| A3.i         | <i>Cladosporium</i> sp.    | OR492471      |
| A1.ii        | <i>Nigrosporaoryze</i> sp. | OR492604      |
| B3.iii       | <i>Streptomyces</i> sp.    | OR492606      |
| C3.iv        | <i>Acremonium</i> sp.      | OR492470      |
| B2.v         | <i>Fusarium</i> sp.        | OR492602      |
| B4.vi        | <i>Curvularia</i> sp.      | SUB13800117   |

## DISCUSSION

The present study demonstrated the presence of a diverse assemblage of enzyme producing microorganism of neem (*Azadirachta indica*) leaves collected from different locations in Bhopal.

A relatively high colonization frequency of these microorganism was observed, indicating that neem leaves provide a favorable ecological niche for microbial colonization. Similar findings have been reported in earlier studies of Verma *et al.*,

2016; Qin *et al.*, 2017, where medicinal plants, including neem, were found to host a rich diversity of endophytic microorganisms capable of producing biologically active metabolites and enzymes. The high colonization rate observed in the present study may be attributed to the complex chemical composition of neem tissues, which provide nutrients and protective micro-environments for isolated microorganisms.

The predominance of microbial genera such as *Streptomyces*, *Fusarium*, and *Cladosporium* observed in this investigation is consistent with previous reports describing these taxa as common endophytes associated with medicinal plants. Several studies have documented the frequent isolation of *Streptomyces* species from plant tissues, particularly from medicinal plants, where they contribute to plant growth promotion and the synthesis of bioactive compounds (Qin *et al.*, 2017). Likewise, fungal endophytes such as *Fusarium* and *Cladosporium* have been widely reported in plant tissues and are known for their ability to produce extracellular enzymes and antimicrobial metabolites (Sunitha *et al.*, 2013). The presence of these genera in neem leaves supports the hypothesis that medicinal plants harbor specialized endophytic communities with potential biotechnological significance.

Among the isolates obtained in this study, *Streptomyces* sp. exhibited the highest enzymatic activity and significant production of indole-3-acetic acid (IAA). Members of the genus *Streptomyces* are well known for their metabolic versatility and ability to produce a wide range of extracellular enzymes such as cellulase, protease, amylase, and lipase. These enzymes play important roles in nutrient cycling and have extensive industrial applications in sectors such as food processing, pharmaceuticals, and biofuel production. Previous investigations of Deb *et al.*, 2013; Qin *et al.*, 2017 have also reported high enzymatic activity in *Streptomyces* isolates

obtained from plant tissues, further supporting the findings of the present study.

The production of indole-3-acetic acid (IAA) by the isolates indicates their potential role in plant growth promotion. IAA is a major phytohormone that regulates several physiological processes including cell elongation, root initiation, and plant development. The ability of endophytic microorganisms to synthesize IAA has been widely reported and is considered an important trait contributing to plant-microbe interactions (Jain and Lalit, 2024; Bric *et al.*, 1991). The high IAA production observed in *Streptomyces* isolates in the present study suggests their potential application as biofertilizers to enhance crop productivity.

Another important finding of the study was the universal presence of phosphate solubilization and siderophore production among the isolates. Phosphate solubilization enables microorganisms to convert insoluble phosphate compounds into forms that are readily available to plants, thereby improving nutrient uptake. Similarly, siderophores are iron-chelating compounds that facilitate iron acquisition and suppress pathogenic microorganisms by limiting their access to iron. Verma *et al.*, 2016 also reported the microorganism have in plant-associated actinomycetes and contribute to their plant growth-promoting potential.

The integration of morphological, biochemical, and molecular characterization methods in the present study ensured reliable identification and functional validation of the isolates. Molecular characterization using 16S rRNA gene sequencing provided accurate taxonomic identification and confirmed the presence of actinomycetes belonging to the genus *Streptomyces*. Such polyphasic approaches combining classical microbiological techniques with molecular methods are widely recommended for the characterization of endophytic microorganisms.

Overall, the results of the present investigation highlight the ecological and biotechnological significance of neem-associated endophytic microorganisms. The enzymatic potential, plant growth-promoting traits, and metabolic capabilities exhibited by these isolates suggest that neem-derived endophytes could serve as valuable sources of industrial enzymes, biofertilizers, and novel bioactive compounds. Further studies involving genome-level analysis and large-scale metabolite screening may provide deeper insights into the functional capabilities and practical applications of these microorganisms.

## CONCLUSION

Neem leaves harbor a rich diversity of endophytic actinomycetes with remarkable enzymatic and plant growth-promoting attributes. *Streptomyces* sp. demonstrated the greatest biotechnological potential among the isolates. The study highlights neem-associated endophytes as valuable resources for sustainable agriculture and enzyme-based industries.

## FUTURE PERSPECTIVES

Further investigations should focus on large-scale enzyme production, greenhouse and field evaluations of plant growth promotion, genomic characterization of promising isolates, and formulation of microbial consortia for agricultural applications.

## REFERENCES

- Bric, J. M., Bostock, R. M., and Silverstone, S. E. (1991). Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Applied and Environmental Microbiology*, 57(2), 535–538.
- Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K., Sayem, S.M.A., 2013. Production and partial characterization of extracellular amylase enzyme from endophytic fungi. *Journal of Biotechnology* 164(1), 54–60.
- Domsch, K.H., Gams, W., Anderson, T.H., 1980. *Compendium of Soil Fungi*, Vols. 1–2. Academic Press, London, UK.
- Dwivedi, S. K., Johri, B. N., and Sharma, A. (2007). Isolation and characterization of endophytic actinomycetes from medicinal plants and their antimicrobial potential. *Indian Journal of Microbiology*, 47(3), 234–238.
- Gilman, J.C., 1957. *A Manual of Soil Fungi*, 2nd ed. Iowa State University Press, Ames, Iowa, USA.
- Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. (2015). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology*, 23(8), 463–471. <https://doi.org/10.1016/j.tim.2015.04.008>
- Jain, R. and Lalit, A., (2023) Optimization of IAA production from neem tree endophytes. *International Research Journal of Modernization in Engineering Technology and Science (IRJMETS)*, 5(10), 3096–3099.
- Jain, R. and Lalit, A., (2024), "Report of Endophytes in Neem Tree of Bhopal", *International Journal of Science and Research (IJSR)*, 13(3), 1102–1103. <https://dx.doi.org/10.21275/SR231028132823>
- Kunitz, M. (1965). Crystalline soybean trypsin inhibitor: II. General properties. *Journal of General Physiology*, 30(4), 291–310.
- Qin, S., Xing, K., Jiang, J. H., Xu, L. H., and Li, W. J. (2017). Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Applied Microbiology and Biotechnology*, 101(12), 4575–4591. <https://doi.org/10.1007/s00253-017-8276-5>

- Sivagnanam, S., and Gangatharan, M. (2017). Indole-3-acetic acid production by endophytic bacteria and its optimization using response surface methodology. *Journal of Applied Pharmaceutical Science*, 7(7), 52–59.
- Strobel, G., Daisy, B., Castillo, U., Harper, J., 1996. Natural products from endophytic microorganisms. *Microbiology and Molecular Biology Reviews* 60(2), 463–475.
- Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R. S., and Hess, W. M. (1996). Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiology*, 142(2), 435–440. <https://doi.org/10.1099/13500872-142-2-435>
- Subbulakshmi, G. K., Thalavaipandian, A., and Bagyalakshmi, R. (2012). Studies on endophytic microbial diversity of medicinal plants. *International Journal of Current Microbiology and Applied Sciences*, 1(2), 10–15.
- Sunitha, V. H., Devaki, N. S., and Srinivas, C. (2013). Extracellular enzymatic activity of endophytic fungal isolates from medicinal plants. *World Journal of Agricultural Sciences*, 9(1), 1–9.
- Verma, V. C., Gond, S. K., Kumar, A., Mishra, A., Kharwar, R. N., and Gange, A. C. (2016). Endophytic actinomycetes from *Azadirachta indica* A. Juss.: Isolation, diversity and antimicrobial activity. *Microbiological Research*, 181, 1–7. <https://doi.org/10.1016/j.micres.2015.09.006>
- Yasmin, A., Ashraf, H., and Khalid, A. (2006). Screening of lipase-producing bacterial strains and characterization of lipase from *Pseudomonas aeruginosa*. *Pakistan Journal of Botany*, 38(2), 535–542.

