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Enzymatic Profiling Of Endophytic Fungi: Isolation, Screening, And Optimization For Industrial Applications

Bhavika Sharma[1], Bhagyashree Deshpande[2] and Bhawana Pandey[3]

[1,2]School of Sciences, MATS University, Raipur, Chhattisgarh
[3]Department of Biotechnology and Microbiology, Bhilai Mahila Mahavidyalaya, Bhilai
Corresponding Author Email ID: bhagyashree.deshpande851@gmail.com

Endophytic fungi, microorganisms that inhabit plant tissues without causing harm, have garnered significant attention in environmental biotechnology due to their diverse biotechnological potential. These fungi play a crucial role in promoting plant health and growth, as well as contributing to the biodegradation of environmental pollutants, bio-remediation, and other industrial applications. Notably, endophytic fungi are capable of producing a wide range of enzymes, such as laccases, xylanases, proteases, cellulases, amylases, and lignin peroxidases, which are of immense value in industries such as waste management, agriculture, and pharmaceuticals. This paper presents a methodology for isolating, identifying, and screening endophytic fungi for their enzyme production potential, with a focus on optimizing growth conditions to improve production efficiency.

Keywords: Bioremediation, Endophytic fungi, Dye decolorization, Phytotoxicity control, Ligninolytic enzymes, Environmental restoration, Textile effluents, Sustainable remediation.

1.0 Introduction

Endophytic fungi, microorganisms that reside within plant tissues without causing harm, are an increasingly important focus in environmental biotechnology due to their versatile biotechnological potential. These fungi play crucial roles in promoting plant health, enhancing growth, and contributing to the degradation of various environmental pollutants (Li et al., 2020). Recent studies have highlighted the remarkable capacity of endophytic fungi to produce a variety of enzymes that are vital for industrial and environmental applications, such as the biodegradation of pollutants, bio-remediation of contaminated sites, and various other biotechnological processes (Asthana & Hawker, 2016). One of the most significant aspects of these fungi is their ability to produce enzymes such as laccases, xylanases, proteases, cellulases, amylases, and lignin peroxidases, which have broad applications in industries ranging from waste management to pharmaceuticals (Illuri et al., 2021).

The isolation and identification of these fungi are critical first steps in harnessing their potential for enzyme production. Surface sterilization techniques ensure that only the endophytic fungi

are isolated, free from external contaminants (Sahu et al., 2022). Subsequently, morphological identification of the fungal strains through techniques such as slide culture allows researchers to classify these fungi based on their cultural characteristics, including colony color, growth rate, and texture (Li et al., 2020). This identification process is integral in distinguishing species with high enzyme production potential, paving the way for further screening and optimization.

Enzyme production by isolated fungi is a key aspect of their biotechnological application. The screening process typically involves the use of specific substrates, such as gelatin for protease activity and starch for amylase activity, in media designed to encourage enzyme production (Pathak & Mehta, 2016). Once the enzymes are produced, their activity is detected through simple yet effective methods, such as gelatin hydrolysis and starch hydrolysis tests, which can be quantified by the clear zones formed around fungal colonies when the substrates are broken down (Hossain et al., 2022).

The optimization of enzyme production is another critical component of this research. Factors such as pH, temperature, and salt concentration, which influence fungal growth and enzyme production, are evaluated to maximize yield (Couto and Sanroman, 2006). Furthermore, comprehensive screening for various enzymes, including laccase, xylanase, and lignin peroxidase, is conducted to identify fungi capable of producing enzymes with industrial applications, such as waste treatment and bioremediation (Gautam et al., 2013). The study of these parameters provides a foundation for developing more efficient, sustainable methods of harnessing fungal enzymes for industrial use.

Recent studies have demonstrated the efficacy of various fungal species in dye degradation. For instance, Aspergillus flavus has been reported to effectively degrade azo dyes, suggesting its potential application in bioremediation (Ameen et al., 2023). Similarly, Trichoderma asperellum has shown significant capabilities in decolorizing triphenylmethane and sulfonated azo dyes, highlighting its role in detoxifying textile effluents (El-Rahman & Mostafa, 2022). Moreover, the white-rot fungus Bjerkanderaadusta has demonstrated a high decolorization rate of dyes such as Crystal Violet and Malachite Green, further supporting the potential of fungi in dye remediation (Shah et al., 2023).

This study explores the eco-innovative potential of endophytic fungi in bioremediation, highlighting their role in dye decolorization and their ability to reduce phytotoxicity in polluted environments. By leveraging the natural metabolic capabilities of these fungi, this approach presents a sustainable and cost-effective alternative to conventional remediation techniques. Understanding the mechanisms involved in fungal-mediated dye degradation and phytotoxicity mitigation can pave the way for the development of novel green technologies for environmental restoration.

2.0 Material and Method

2.1 Isolation of Endophytic Fungi

The fresh plant parts were collected from the Pulp and Paper Industry located in the Raipur district of Chhattisgarh (C.G.) rinsed in running water to remove dust and debris. After proper washing, leaves, stem and roots were selected for further processing via surface disinfection under aseptic conditions. The plant parts were cut into small pieces about 4 mm in size and surface disinfection was done in a series by placing them in sterilized distilled water for 1 min then into 4% sodium hypo-chlorite solution for 2 min and then in 70% ethanol for 1 min. Later, the plant samples were rinsed with sterilized distilled water and dried on a sterilized filter paper. After disinfection, all plant parts were placed in Potato dextrose agar (PDA) plates supplemented with streptomycin to inhibit the growth of bacteria. The plates were incubated at 26±1ºC for 6 to 7 days and observed daily for hyphal growth. Pure cultures were maintained on the PDA slants without antibiotics (Sahu et al., 2022).

2.2 Identification of Isolated Fungal Strain

2.2.1 Morphological Identification

Endophytic fungal strains were identified on the basis of morphological characteristics by slide culture technique. The culture which failed to sporulate after treatment was named sterile mycelia based on their cultural characteristics (i.e. colony, color, growth rate and texture) on PDA (Li et al., 2020).

2.3Screening of Enzyme-Producing Fungi

The screening of enzyme-producing fungi involves selecting strains with high enzyme production potential using specific substrates in culture media. The method follows a systematic approach, including media preparation, inoculation, incubation, and enzyme activity detection.Asthana and Hawker's medium is prepared by dissolving the required nutrients in distilled water. The medium is supplemented with either 1% gelatin (for protease activity) or 1% starch (for amylase activity) as enzyme-specific substrates. The molten sterilized medium is poured aseptically into sterile glass Petri dishes under laminar airflow to prevent contamination (Pathak & Mehta, 2016). A 2 mm fungal disc is cut from a 7-day-old culture grown on Potato Dextrose Agar (PDA) medium using a sterile cork borer. The fungal disc is carefully placed in the center of the prepared plates containing either gelatin or starch medium. The inoculated plates are incubated at 27 ± 2ºC for 5–6 days to enable sufficient enzyme production (Gupta et al., 2018).

2.4 Enzyme Activity Detection

For Protease (Gelatin Hydrolysis Test): After incubation, flood plates with mercuric chloride (HgCl) solution to detect gelatin hydrolysis. A clear zone around fungal growth indicates gelatin degradation by protease enzymes.

For Amylase (Starch Hydrolysis Test): After incubation, flood plates with iodine solution to detect starch hydrolysis. A clear zone around fungal colonies indicates amylase activity, as the starch is broken down into reducing sugars (Hossain et al., 2022).

2.5 Assay of enzyme production by isolated Fungi

The enzyme production potential of isolated fungi, a comprehensive screening will be conducted for various relevant enzymes, including laccase xylanase, protease cellulose, glucosidase amylase lignin peroxidase and manganese peroxidise. Each enzyme will be evaluated using specific assay methods, followed by optimization for enhanced production (Illuri et al., 2021).

2.6 Effect of Physicochemical Parameters on Fungal Growth and Enzyme Production

The growth and enzyme production of screened fungal isolates were optimized by evaluating their response to various physicochemical parameters, including pH, temperature and salt concentration (Sharma et al., 2014).

3.0 Result and Discussion

3.1 Endophytic Fungi Isolated from Different Sample:

This study investigated the isolation of endophytic fungi from leaves and twigs across six different sample sites (S1A, S1B, S1C, S2A, S2B, and S2C). The results showed site-dependent variations in fungal colonization, with some locations exhibiting higher fungal presence in leaves (e.g., S2B), while others had more isolates in twigs (e.g., S1B). The findings suggest that environmental conditions, plant species, and microbial interactions influence fungal distribution (Arnold &Lutzoni, 2007). Previous studies have also reported variability in endophytic fungal colonization due to factors such as plant defense mechanisms and ecological conditions (Rodriguez et al., 2009).

Table 1: Endophytic Fungal Isolates Collected from Different Sample Sites

Sample Site	No. of Isolates in Sample	
	Leaves	Twigs
S1A	4	3
S1B	3	5
S1C	4	4
S2A	4	2
S2B	5	2
S2C	3	4

On the Basis of Frequency of endophytic fungi six Endophytic Fungi were selected for further analysis and studies.

3.2 Selected Endophytic Fungi

This study identified six different endophytic fungi (EF 1 to EF 6) isolated from both leaf and twig samples across six distinct sampling sites. The results indicated that endophytic fungi display varying colonization patterns in different plant tissues, with some species preferring leaves (e.g., EF 1 and EF 3 from sites S1A and S1C) and others favoring twigs (e.g., EF 2, EF 4, EF 5, and EF 6). The presence of multiple fungal species within the same location but different plant parts suggests a complex relationship between environmental factors, plant species, and microbial interactions. These findings align with previous studies, which indicate that plant tissues can support a diverse range of fungal communities, influenced by factors such as nutrient availability, plant defense mechanisms, and habitat conditions (Rodriguez et al., 2009; Schulz & Boyle, 2005).

Table 2: List of Selected Endophytic fungi

SN	Endophytic Fungi	Sampling Site
1	EF 1	S1 A Leaves
2	EF 2	S1 B Twig
3	EF 3	S1 C Leaves
4	EF 4	S1 C Twig
5	EF 5	S2 B Twig
6	EF 6	S2 C Twig

3.3 Morphotypic identification of Endophytic fungi

Morphological identification of endophytic fungi involves examining both the colony characteristics and microscopic features to distinguish different species. Initially, fungal colonies are observed on growth media such as Potato Dextrose Agar (PDA), noting their color, shape, texture, and size. For a more detailed analysis, microscopic examination of the fungal mycelium, including hyphal structure, conidiophores, conidia (asexual spores), and any reproductive structures, is performed.

3.4 Screening of enzyme producing fungi

The preliminary screening of enzyme-producing fungi identified six endophytic fungal isolates (EF1 to EF6) with varying enzyme production profiles. All six isolates produced xylanase, indicating their potential for plant biomass degradation and industrial applications such as biorefining. Most isolates (EF1, EF2, EF4, and EF6) tested positive for protease, while cellulase activity was only present in EF2, EF4, and EF6, suggesting their potential use in bioconversion processes. Additionally, enzymes like glucosidase, amylase, laccase, lignin peroxidase, and manganese peroxidase were produced by certain isolates, pointing to their potential for bioremediation and the degradation of complex organic compounds such as lignin and phenolic compounds. These findings support the growing interest in endophytic fungi for various biotechnological applications, including bioremediation, bioconversion, and industrial enzyme production (Chadha et al., 2015; Rodriguez et al., 2009). The variability in enzyme production among isolates highlights the need for further research to identify the most promising strains for specific industrial applications.

Table 4: Screening of enzyme producing fungi

SN	Enzymes	EF1	EF2	EF3	EF4	EF5	EF6
1	Xylanase	+	+	+	+	+	+
2	Protease	+	+	-	+	-	+
3	Cellulase	-	+	-	+	-	+
4	Glucosidase	+	+	+	+	-	+
5	Amylase	-	+	+	+	-	+
6	Laccase	-	+	-	+	-	+
7	Lignin peroxidase	+	+	-	+	+	+
8	Manganese peroxidase	-	+	-	+	+	+

Dye decolorizing activity mainly depend up on Laccase, Lignin peroxidase and manganese peroxidase hence on the basis of screening of enzyme EF2, EF4 and EF6 are used for further investigation.

3.5 Assay of Enzyme production by isolated Fungi

The assay of enzyme production by endophytic fungi revealed varying levels of enzyme activity across the isolates, with EF4 exhibiting the highest activity in laccase (0.437 ± 0.02 U/mL), lignin peroxidase (0.478 ± 0.01 U/mL), and manganese peroxidase (0.501 ± 0.01 U/mL). This suggests that EF4 has a strong potential for lignin and phenolic compound degradation, making it a promising candidate for bioremediation and industrial applications. EF6 showed moderate and consistent enzyme production, with similar activity levels for laccase, lignin peroxidase, and manganese peroxidase, indicating balanced enzyme production. EF2, on the other hand, exhibited lower enzyme activity, particularly in lignin peroxidase, but still demonstrated notable activity, especially in laccase and manganese peroxidase. These results highlight the variability in enzyme production among fungal isolates, which could be influenced by genetic factors and environmental conditions. The strong activity observed in EF4 underscores its potential utility for applications requiring efficient lignin degradation, such as in bioremediation of polluted environments or the bioconversion of lignocellulosic biomass (Nigam, 2009).

Table 5: Assay of Enzyme production by isolated Fungi

SN	Endophytic Fungi	Enzyme Activity (Uml^{-1})

		Laccase	Lignin peroxidase	manganese peroxidase
1	EF2	0.354±0.01	0.311±0.001	0.316±0.03
2	EF4	0.437±0.02	0.478±0.01	0.501±0.01
3	EF6	0.401±0.0	0.398±0.04	0.404±0.001

3.6 Effect of physicochemical parameter on Endophytic fungi

3.6.1 Effect of pH on Endophytic Fungi

The effect of pH on the growth of endophytic fungi (EF2, EF4, and EF6) was assessed across a wide range of pH levels (1–14). The results revealed distinct growth patterns, highlighting EF4 as the most adaptable to varying pH conditions. EF4 exhibited the highest overall growth, with an optimal peak at neutral pH (7), and demonstrated tolerance to both acidic and slightly alkaline conditions. However, growth declined at highly alkaline pH values (11 and 14), although still better than EF2. EF6 also showed optimal growth at pH 7, with moderate tolerance to acidic and alkaline conditions. EF2, in contrast, showed the best growth at pH 7 but struggled at extremes of pH, particularly under highly acidic or highly alkaline conditions. These findings suggest that EF4 is the most resilient fungus for diverse environmental conditions, especially in neutral to slightly alkaline environments, while EF2's growth is more restricted to slightly acidic and neutral pH. These results provide valuable insights into the ecological adaptability of endophytic fungi, which can inform their use in biotechnological applications where pH may vary (Pérez et al., 2015; Patel & Jain, 2014).

Table 6: Effect of pH on Endophytic Fungi

SN	Endophytic Fungi	Growth of Endophytic fungi by Average mycelium dry weight(mg)						
		1pH	3pH	5pH	7pH	9pH	11pH	14pH
1	EF2	314.58± 0.02	454.54± 0.001	512.47± 0.014	547.21± 0.11	521.24± 0.011	465.04± 0.001	304.54± 0.021
2	EF4	587.54± 0.001	614.36± 00.03	694.25± 0.001	731.01± 0.001	651.01± 0.041	621.21± 0.01	552.21± 0.001
3	EF6	523.62± 0.11	593.58± 0.015	642.42± 0.016	687.12± 0.22	621.32± 0.034	552.12± 0.01	504.84± 0.011

3.6.2 Effect of Temperature on Endophytic Fungi

The growth of three endophytic fungi (EF2, EF4, and EF6) was assessed across temperatures ranging from 15°C to 40°C. EF4 exhibited the best overall growth, peaking at 25°C (766.01±0.02 mg), and showed resilience even at higher temperatures. EF2 and EF6 showed optimal growth at 25°C, but both declined significantly at temperatures above this point, with EF2 particularly sensitive to higher temperatures. These findings suggest that EF4 is more adaptable to a wider temperature range, while EF2 and EF6 are better suited for moderate temperatures (Kumar et al., 2016; Singh et al., 2018).

Table 7: Effect of Temperature on Endophytic Fungi

		Growth of Endophytic fungi by Average mycelium dry weight(mg)

SN	Endophytic Fungi	15ºC	20ºC	25ºC	30°C	35°C	40°C
1	EF2	424.58±0.14	454.54±0.02	578.21±0.02	554.14±0.024	501.14±0.002	466.01±0.02
2	EF4	591.54±0.22	631.36±0.02	766.01±0.02	710.12±0.002	684.01±0.10	645.21±0.03
3	EF6	555.62±0.04	578.58±0.01	649.12±0.13	617.41±0.021	581.32±0.15	512.12±0.24

3.6.3 Effect of Salt Concentration on Endophytic Fungi

The growth of three endophytic fungi (EF2, EF4, and EF6) was analyzed under varying salt concentrations (1M to 5M). EF4 showed the highest salt tolerance, maintaining strong growth up to 3M and only a moderate decline at higher concentrations (4M and 5M) (Rajwade and Deshpande, 2024). EF2 and EF6 demonstrated improved growth at lower salt concentrations, but their growth significantly declined at higher salt concentrations. EF2's growth dropped from 654.41±0.011 mg at 2M to 521.57±0.01 mg at 5M, while EF6 showed similar trends with a decrease from 713.75±0.02 mg at 2M to 501.37±0.14 mg at 5M. These results suggest that EF4 is the most resilient to salt stress, while EF2 and EF6 are more sensitive to higher salt levels(Kumar et al., 2016; Singh et al., 2018).

Table 8: Effect of Salt Concentration on Endophytic Fungi

SN	Endophytic Fungi	Growth of Endophytic fungi by Average mycelium dry weight(mg)				
		1M	2M	3M	4M	5M
1	EF2	494.25±0.004	654.41±0.011	602.34±0.05	547.54±0.01	521.57±0.01
2	EF4	678.14±0.23	776.21±0.05	724.12±0.01	601.46±0.03	524.34±0.03
3	EF6	565.69±0.00	713.75±0.02	642.78±0.04	567.24±0.02	501.37±0.14

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