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Screening of Extracellular Enzymes

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ABSTRACT

Endophytes are microorganisms that live all or part of their lives inside healthy plant tissues without obviously harming their hosts. Recent years have seen a lot of study focused on the fungus with endophytic properties. This potential for these microorganisms to produce biotechnologically useful compounds, such as antitumor agents (Taxol) and antifungal agents (quercine), in addition to factors for plant growth, toxins, and enzymes, has sparked interest in them. Some of these microorganisms may also be used as biological pest controllers for a variety of diseases and calamities. In the current study, Endophytic fungi were screened for on veld grape (Cissus quadrangularis). Using modified surface sterilization techniques, 400 segments (about 1 cm 2) of 50 segments each from the leaf and stem tissues of the different plants were screened for the enumeration of endophytic fungi. The ability of various endophytic fungus species to produce extracellular enzymes, such as Amylase, Cellulase, Laccase, Lipase, and Protease, was examined. The majority of the endophytic fungi produced positive findings in the qualitative testing.

Keywords: Endophytic fungi, Medicinal plants, Colonization frequency, Endophytic infection rates, Extra cellular enzyme.

INTRODUCTION

De Bary (1866) coined the term "**Endophyte**," which now refers to any organisms living inside plant tissues as opposed to epiphytes that are found on plant surfaces. Endophytic fungi are an intriguing class of organisms that live inside the tissues of their hosts, usually higher plants, and produce secondary metabolites that benefit plant life rather than cause any disease symptoms.Endophytic microorganisms can be categorised into two groups: those who do not produce extracellular matrix from their hosts (organisation I) and those who are able to do so in conjunction with nodules of N2-solving bacteria and mycorrhizal fungi (organization II). fungus that spend all or a portion of their life cycle colonising inter and/or intra movable gaps in stem, petiole, roots, and leaves, in the tissues of healthy vegetation, are known as endophytic fungi. These fungi typically do not show any outward signs of illness. Through the production of various secondary metabolites, nitrogen uptake, and important roles in host tolerance to heat, salinity, evolution, and plant biodiversity, those endophytes have a remarkable capacity to enhance host resistance to herbivores.

Moreover, it works to prevent infection by pathogenic organisms by rapidly invading its hosts and depleting the nutrients that pathogenic microbes need to flourish . With the formation of some poisonous chemicals that render the host inedible to insects and animals, endophytic fungus can help to protect their hosts from insects and animals . According to several studies, endophytic fungi produce the extracellular enzymes amylase, pectinase, cellulases, laccase, and protease as a defence against infections and to obtain nutrients from the host.

The enzymes' roles include hydrolyzing dietary components, obtaining nutrients from their host, and inducing pathogen defence systems [10]. Finding and using a variety of new enzymes with high stability for industrial processes is essential. Examining the variety of endophytic fungal assemblages and their capacity to synthesize extracellular enzymes in particular plants is the goal of the current study.

According to Strobel, medicinal plants possess endophytic fungi that help the host adapt to harsh climatic conditions. The healthy tissue of medicinal plants contains fungus colonies, which promotes soil nutrient uptake and alters the nutrient cycle.

In the current study, the species diversity in particular plant tissues and the distribution of endophytic fungi in *Cissus quadrangularis* (veld grape) leaves and stems were examined.

For the development of numerous secondary metabolites, bioactive substances that are beneficial to the pharmaceutical industry, endophytic fungus from the plants were a significant source. Our studies involve screening selected dominant endophytic fungi for the five different extracellular enzyme assays, including cellulase, amylase, protease, lipase, and laccase. These extracellular enzyme assays are in addition to the isolation and identification of endophytic fungal colonies from medicinal plants.

A review on Endophytic fungi and medicinal plant for screening of enzymes

Endophytes are recognised to be a rich source of certain bioactive substances that reside inside plant tissues. Ascocentrum curvifolium, Doritis pulcherrima, Dendrobiuma phyllum, Dendrobium anosmum, and Aerides falcata are five kinds of epiphytic orchids whose leaves, stems, and roots were

used in the current investigation to isolate endophytic fungus. The samples of orchids yielded 52 endophytic isolates in total, with the majority (27) coming from leaf segments, followed by roots and stems. In solid medium, the ability of every endophytic isolate to produce extracellular enzymes like amylase, protease, cellulase, pectinase, and lipase was examined.

A biomolecule produced from proteins called an enzyme speeds up chemical reactions and serves a variety of functions. With relation to its extensive use, amylase is one of the polysaccharide-degrading enzymes that has received most of the research. Maltonic amylase from Bacillus stearothermophilus has been used in the food industry for baking. Superoxide dismutase is a metalloenzyme known for its antioxidant properties against oxidative stress in the human body. Amylase is used in the feed business to increase the digestibility of fibre due to its capacity to convert starch into simple saccharides.

The protease enzyme, which is regarded as the most significant enzyme in many sectors, is responsible for carrying out proteolysis. The enzymes are used in the leather industry, the bioremediation process, detergents, and waste treatment. Nonetheless, a promising area of interest for researchers is the quest for microbial sources of new proteases in natural diversity. Aspergillus sp. 14L3S, one of these isolates, had the highest level of protease activity, as seen by the clear zone surrounding the colony, which remained at a pH of about 6.0 for 96 hours while being incubated at room temperature. The findings demonstrated that among the isolates, four fungus held high promise for application in industrial production and as a source of protease enzymes.

In the past several years, endophytic fungi have received a lot of attention as prolific producers of new bioactive natural products because of the intricate web of interactions they display with their host plants. One of them that is utilised in the food, beverage, confectionery, textile, and leather sectors to streamline the processing of raw materials is fungus. In solid media, extracellular enzymes such amylase, cellulase, laccase, lipase, pectinase, and protease were screened for in 50 fungal strains isolated from medicinal plants (Alpinia calcarata, Bixa orellana, Calophyllum inophyllum, and Catharanthus roseus). Sixty four percent of the fungus tested positive for lipase, sixty two percent for amylase and pectinase, fifty percent for lipase, thirty two percent for laccase, and only twenty eight percent for protease.

The serial dilution method was used to isolate 34 wild fungus species that were connected to waste from edible oil mills. It is stated how to quickly check fungi for the synthesis of extracellular enzymes such amylase, protease, cellulase, and lipase. Aspergillus versicolor stood out among all the species for having high amylolytic and gelatinolytic activity, while Penicillium citrinum only shown high amylolytic activity.

A family of microorganisms referred to as "endophytes" are those that develop intra- and/or intercellularly in the tissues of higher plants without causing any symptoms in the host plants. In this study, seven medicinal plants were used to source nine distinct fungal strains, including Cladosporium sp., Rhizoctonia sp., Aspergillus sp., Chaetomium sp., Biosporus sp., Fuzarium sp., Curvularia sp., and Colletotrichum sp. The synthesis of extracellular enzymes such amylase, protease, cellulose, and lipase was evaluated qualitatively and quantitatively in isolated endophytic fungi.

Epiphytic orchid Cymbidium aloifolium is utilised in the treatment of numerous human illnesses. Many bioactive substances, including extracellular enzymes, can be produced by the numerous endophytic fungi that live on orchids. The root, leaf, and flowers of C. aloifolium yielded a total of 165 endophytic fungi, which represented 22 different fungal species. Phosphatase was produced by 93% of the endophytic fungi tested for extracellular enzyme production, whereas cellulase, amylase, protease, pectinase, lipase, and laccase were also produced.

Endophytes are bacteria or fungus that inhabit the host plant and take part in a variety of biological functions without causing illness or having any other negative impacts. Endophytes are known to be a great source of secondary metabolites having pharmacological properties that may be of utility. Endophytes are bacteria or fungus that inhabit the host plant and take part in a variety of biological functions without causing illness or having any other negative impacts. Endophytes are known to be a great source of secondary metabolites having pharmacological properties that may be of utility.

This study details the identification of Penicillium-chrysogenum, a powerful extracellular-laccase-producing white-rot fungus, and the optimization of its medium utilising central-composite-rotatable-design and RSM. By statistically optimising the medium at 32 °C for 5 days, the optimal laccase activity of 6.0 U ml-1 and maximal activity of 7.9 U ml-1 were obtained. The laccase was discovered to have a molecular weight of 67 kDa. The existence of laccase is confirmed by UV-visible absorption-spectrum analysis, which exhibits peaks at 600 nm and 325 nm, respectively, corresponding to the type-I Cu(II) and type-III binuclear Cu(II) pair. DSC and TGA studies of the enzyme protein revealed a pronounced endothermic peak at 150 °C and three phases of protein denaturation.

In this investigation, extracellular lipase-producing endophytic fungi that were isolated from Handroanthus impetiginosus were to be screened (H. impetiginosus) There were 122 different endophytic fungus isolates discovered. Two isolated fungi were chosen for submerged fermentation cultivations using glucose and cottonseed oil as carbon sources after showing strong lipase activity in the plate screening. One strain produced a maximal lipase activity of 5.9 U/mL for the culture medium utilising cottonseed oil as the carbon source after 48 hours of fermentation. Preussia africana has been identified genetically as this strain. SDS-PAGE analysis was used to identify a single protein band with an apparent molecular mass of 64 kDa following lipase purification (18.5: purification factor).

Endophytic fungi, the majority of which are Ascomycota, are present in the intercellular spaces of the aerial plant parts, especially in the leaf sheaths, and occasionally even in the bark and root system without producing any visible indications of their presence. It appears that endophytic fungi have both the hydrolytic and the unique oxidative ligninolytic extracellular enzymatic systems required to break down polysaccharides and open phenyl rings in the vegetal biomass. The hydrolytic system is responsible for polysaccharide degradation and is primarily composed of xylanases and cellulases. The oxidative system breaks down lignin and opens phenyl rings. Moreover, endophytic fungi may develop into fresh sources of industrially valuable enzymes like lipases, amylases, and proteases.

MATERIALS

The laboratory equipment such as LAF and other clinical observation instruments were used and the detailed operating of such instruments are given at the report.

Scope

Industrially applicable enzymes can be identified.

Identify and Screening different types of Extracellular enzymes.

Materials Required

- Petri Plate
- Conical flask
- Inoculation loop
- Laminar air flow chamber
- Autoclave

Chemicals Required

- PDA (Potato Dextrose Agar)
- NaOCl
- Ethanol
- Distilled water
- Antibacterial Tablets
- Agar
- Glucose
- Peptone
- Sodium chloride
- $CaCl_2\,2H_2O$
- Ammonium Sulphate
- Gelatin
- Tween 20
- 1-napthol
- Iodine
- Potassium iodide
- N-Carboxymethyl Cellulose
- Congo red

Cleansing of Glassware

Corning and borosil emblem glass wares had been used. They were saved in chromic acid cleaning solution (10% Potassium dichromate in 15% Sulfuric acid) for a few hours washed thoroughly with faucet water followed by detergent solutions and rinsed with distilled water. The glassware had been dried in a warm air oven.

Sterilization

Media and glasswares were sterilized in an autoclave at 15 lbs for 15 mins at 121°C.

Media

Potato Dextrose Agar (PDA) is used for the isolation of endophytes from the chosen medicinal plants.

Media Composition

Potato	**250g**
Dextrose	15g
Agar	20 g
Distilled water	1000ml

PDA Composition

250gms of potato had been weighed, peeled and boiled in distilled water. The boiled potato extract were obtained by using filtering to that distilled water is introduced to make upto 1000ml, to this 1000ml of potato extract 20g of dextrose and 20g of agar had been introduced, pH is adjusted to 6.5 and media is then sterilized in an autoclave in 15lbs at 121°C for 15mins.

METHODS

Sample collection

The leaves and stems of Veld grape *Cissus quadrangularis* were collected. The plant parts have been thoroughly wiped clean with tap water to eliminate soil and particles. The herbs were excised and located in polyethylene zip lock covers and taken to the laboratory and processed straight away for the surface sterilization.

Isolation of endophytes

The endophytic fungi have been isolated via floor sterilization of depart, stem and root samples via the modified method of Petrini et al., (1992). all the depart, stem and root samples of plant species had been first washed very well underneath going for walks faucet water to put off dirt and debris. The surface sterilization was finished in a easy airflow bench machine. The samples have been immersed in seventy five% ethanol (v/v) for one minute accompanied by 4% sodium hypochlorite (NaOCl) (v/v) for three minutes and then immersed in 70% ethanol. The samples had been rinsed three instances in adjustments of sterile distilled water and dried on sterile tissue papers. The plant samples were reduce into 1.zero cm x 0.1cm the use of sterile scalpel. The plant segments have been located equidistantly at the potato dextrose agar(PDA) medium supplemented with the antibiotic streptomycin.The petriplates inoculated with the plant segments were incubated at 28°±2 for 4 to six weeks. Fungi growing out of the plant explants had been subcultured on separate PDA plates have been incubated at 28°C for 3 weeks. pure cultures had been then transferred to potato dextrose agar (PDA) slants and cultivated for 14 days at 28°C.

Identification Endophytic Fungi:

The endophytic fungi were discovered morphologically and microscopically. For this purpose, a small bit of fungal mycelia was remoted from pure lifestyle grown on PDA and stained with lactophenol cotton blue for visible commentary. Sporulating isolates had been recognized right down to species stage with the assist of trendy manuals Sterile isolates could not be assigned to any taxonomic group and had been looked after into morphospecies on the basis of colony surface texture, hyphal pigmentation, exudates, and increase costs, as described. Such sterile paperwork had been protected as 'species' for the analysis of the effects. Photomicrographs were inquisitive about the assist of Carl Zeiss microscope and Konica VX100 & VX200 shade films had been used.

Preparation of permanent slides

For the preparation of everlasting slides Lactophenol and Lactophenol with Cotton Blue stain were used. The slides had been sealed with DPX mountant (Nag Raj, 1993).

Stain Used Lactophenol Cotton Blue Preparation

Phenol(pure crystals)	**10g**
Lactic acid	10g
Glycerol (pure)	20g
Distilled water	10g

Lactophenol Cotton Blue Composition

Lactophenol is comfortably prepared via warming the phenol with the water until dissolved and then including the lactic acid and glycerol. The refractive index is 1.45. To the 100ml. of lactophenol, 0.05g cotton blue become added after which filtered by using using filterpaper.

Identity of the fungi and Photomicrography

With the use of selected manuals, sporulating isolates were identified down to the species stage (Guba (1961); Ellis (1971); Sutton (1980); Onions et al. (1981); and Nag Raj (1993). Sterile isolates were treated as morphospecies based on colony floor texture, hyphal coloration, exudates, and increasing charges since they could not be allocated to any taxonomic group. Such sterilised paperwork has been used as a "species" to assess the results.

Photomicrographs had been focused on the assist of Carl Zeiss microscope and Konica VX100 & VX200 coloration movies have been used.

Extracellular enzyme assay

In the current work, five distinct extracellular assays, including cellulase, amylase, protease, lipase, and laccase, were used to qualitatively analyse eight prominent endophytic fungus species from the leaves and stems of four medicinal plants. Based on the formation of a clean zone around the fungal colony after the extracellular enzyme produced by endophytic fungus digested the suitable substrate that was fed in agar medium and digested for three to five days at room temperature.

AMYLASE

GYP (Glucose Yeast Peptone) agar medium containing 2% soluble starch was used to culture the isolates.

The culture was flooded with 1% iodine in 2% potassium iodide after incubation. The colony is surrounded by the crystal transparent halos.

GYP agar media

Glucose	**1 gm**
Yeast extract	**0.1 gm**
Peptone	**0.5 gm**
Agar	**16 gm**
Distilled water	**1000ml**
pH	**6**

GYP Agar media Composition

CELLULASE

The isolates were raised in Yeast Extract Peptone Agar medium that had 0.5% N-carboxymethylcellulose added to it, and the culture was kept at room temperature.

The plates were saturated for 15 minutes with 1M NaCl and 0.1% Congo Red.

The colony's surrounding transparent halo, which denotes cellulose activity, is visible.

Yeast extract peptone agar media

Yeast extract	**0.1g**
Peptone	**0 .5 g**
Agar	**16 g**
Distilled water	**1000 ml**

Yeast extract peptone agar media composition

LACCASE

Growing the isolates on GYP agar medium supplemented with 1-naphthol (0.005%) allows for the observation of laccase activity.

The media will turn from clear to blue as a result of the isolates' laccase enzyme oxidising 1-naphthol.

GYP agar media with naphthol

Glucose	**1 gm**
Yeast extract	**0.1 gm**
Peptone	**0.5 gm**
Agar	**16 gm**
Distilled water	**1000ml**
pH	**6**
1-napthnol	**5g**

GYP agar with naphthol

PROTEASE

Isolates were sterilised and cultured on GYP agar media with 0.4% gelatin supplement.

8g of sterile gelatin dissolved in 100ml of distilled water is added to the sterilised culture media.

After incubation, saturated aqueous ammonium sulphate is poured over the culture.

The gelatin has been hydrolyzed in the media, as indicated by the clear zone surrounding the colony, and ammonium sulphate has precipitated the unhydrolyzed gelatin.

LIPASE

Growing the isolates in peptone agar media allows for the observation of the esterasic activity. Sterilized Tween 20 is added to the sterilised culture media at a final concentration of 1% (v/v).

Halos around the colony show lipase activity, which is present.

Peptone agar media

Peptone	**10 gm**
NaCl	**5 gm**
CaCl2 2H2O	**0.1 gm**
Agar	**16 gm**
Distilled water	**1000 ml**
pH	**6**

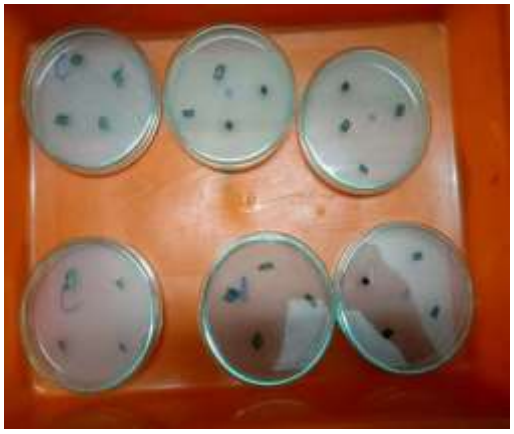
RESULTS

SCREENING OF ENDOPHYTIC FUNGI FROM SELECTED MEDICINAL PLANTS

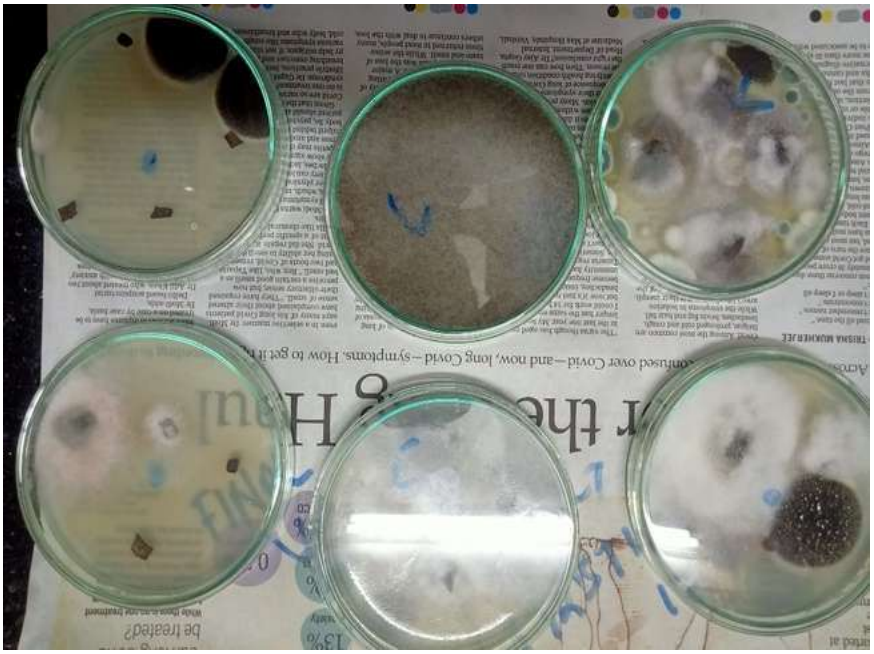
Isolation of Endophytic fungi from *Cissus quadrangularis* (Veld grape)

Altogether *Cissus quadrangularis* leaf, petiole, and stem tissues were cut into fifty segments (each measuring approximately 0.5 cm2), which were sterilised and then examined for the presence of endophytic fungi.

Veld Grape Plant (*Cissus quadrangularis)*



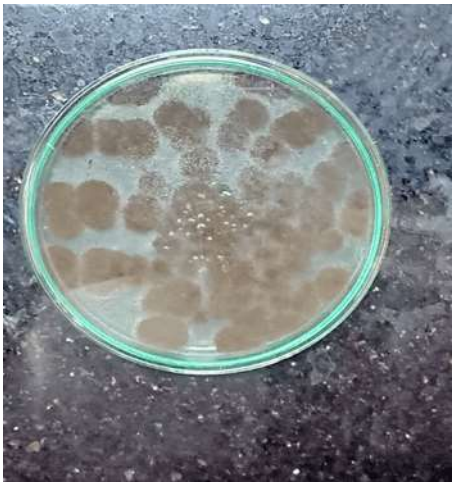
Petri plates showing that the leaves and stems of *Cissus quadrangularis* were inoculated in PDA



5.1.3 Petri plates showing the Endophytic fungal propagules emerging from surface sterilized tissues

Colletotrichum gloeosporioides

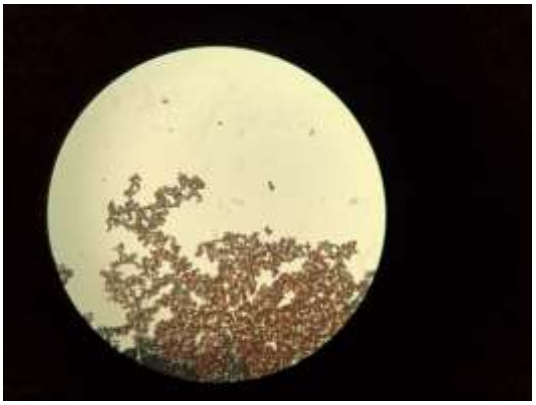
Penicillium chrysogenum



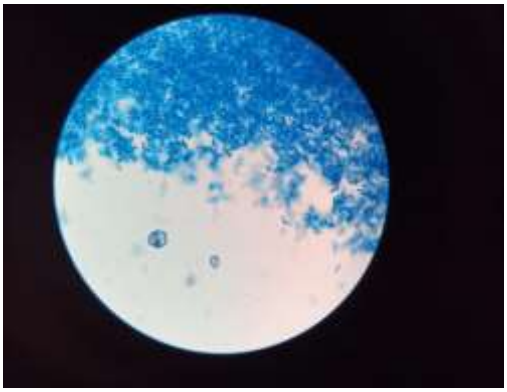
Aspergillus niger

Cladosporium Cladosporioides

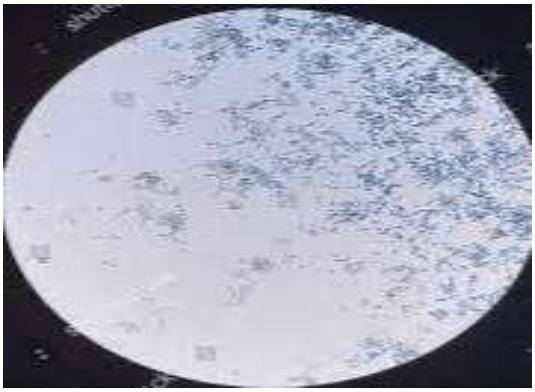
5.1.4 Petri plates showing pure cultures of Endophytic fungi



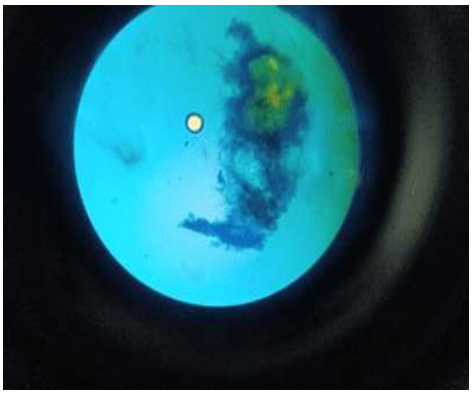
Aspergillus niger



Colletotrichum gloeosporioides



Cladosporium Cladosporioides



Penicillium chrysogenum

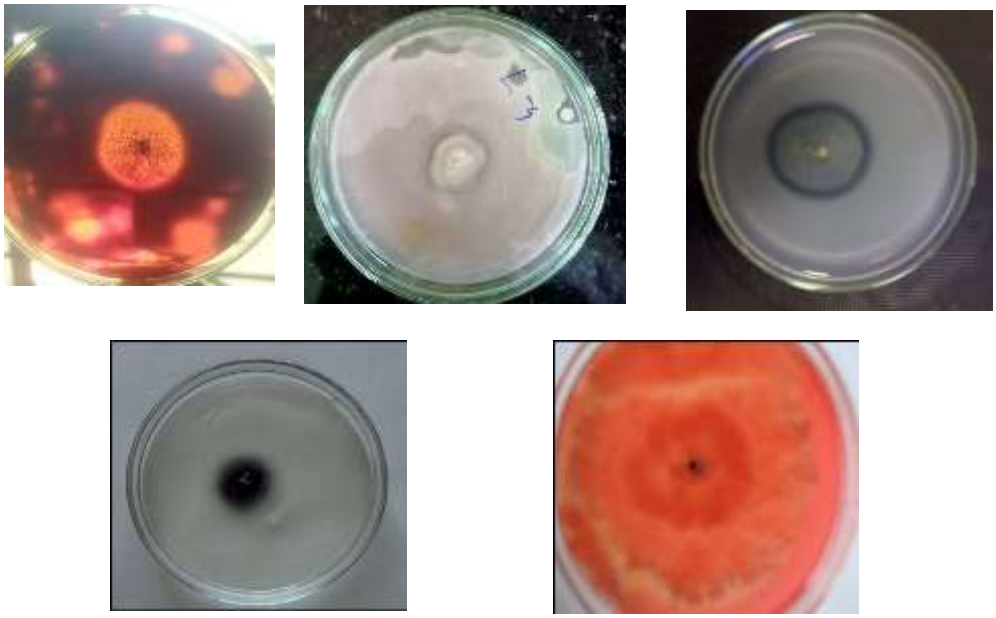
Micrographs of identified endophytic fungal species

5.2 Enzyme Assay

In the current study, endophytic fungal isolates were tested for the presence of extracellular enzymes such Amylase, Cellulase, Laccase, Lipase, and Protease that were grown on a particular medium that was previously covered in the materials and methods section. After the plant-host dies, endophytes can eat the plant's source of starch.

List of Endophytic fungi	Amylase	Cellulase	Laccase	Lipase	Protease
Cladosporium cladosporioides	+	+	-	-	-
Colletotrichum gleosporioides	+	-	-	-	+
Penicillium chrysogenum	+	-	+	+	-
Aspergillus niger	+	+	-	+	-

Table 5.2.1 List of endophytes for Enzyme assay



a) Amylase b) Lipase c) Protease d) Laccase e) Cellulase

5.2.1 Results of five different types of Endophytic fungi

Compared to bacterial amylase, the fungal amylase was more stable. A total of 4000 secondary metabolites, mostly from the Penicillium and Aspergillus species of fungi, were isolated. These chemicals were biologically active.

The increase in lipase activity suggests that cholestrol can be used as a source of energy. Our research found that endophytic fungi like *Penicillium chrysogenum* and *Aspergillus niger* exhibit lipase activity and that Tween is an appropriate substrate for the lipase enzyme assay. The most effective species for creating alkaline lipase and hydrolase for a variety of oils was *Colletotrichum gloeosporioides.*

Several terrestrial fungus produce the extracellular degradative enzyme cellulase, which is employed in the paper industry, while numerous marine fungi produce the enzyme laccase, which is involved in the degradation of lignin. Lipase activity has increased, which suggests that cholesterol can be used as a fuel source.

Our research indicates that the endophytic fungus *Penicillium chrysogenum* and *Aspergillus niger Sp* exhibit lipase activity, and that Tween is an appropriate substrate for the lipase enzyme assay.

The best plant for manufacturing alkaline lipase and oil hydrolase was shown to be *Colletotrichum gloeosporioides*.

For whatever reason, the endophytes lack a certain active enzyme that shields the host plants from harm. *Colletotrichum gloeosporioides* showed evidence of protease activity in the form of a clear zone surrounding the colony.

Enzymes derived from fungi are more stable than those from plants and animals. It is employed in the food processing, beverage, textile, and leather sectors.

Endophytic fungi are screened for a variety of metabolites, such as enzymes, antibiotics, and anti-cancer medications, which will be valuable for our future generation and lead to environmentally friendly technological advancement for a better quality of life on Earth.

CONCLUSION

The duration and level of variability of the enzyme synthesis by the endophytic fungus isolated from medicinal plants is the most important conclusion from the current investigation. This shows that the production of enzymes varies among fungus and frequently reflects the needs of its ecosystem. This might be the result of a variety of things altering in the host due to old age, environmental factors like climate and location may affect the biology of the fungi. However, choosing the organisms most appropriate for industrial purposes would benefit from understanding of the types, quantities, and properties of the enzymes produced by the endophytic fungi mentioned above. The synthesis of extracellular enzymes in liquid media by putative endophytic fungi is being quantitatively studied.

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