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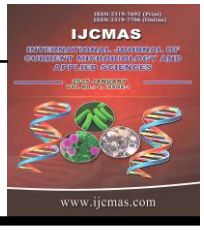

Original Research Article

Extracellular Enzymatic Activities of Endophytic Fungi Isolated from Various Medicinal Plants

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A B S T R A C T

The term "endophytes" includes a family of microorganisms that grow intra-and/or intercelullarly in the tissues of higher plants without any symptoms on the plants in which they reside. Endophytic fungus are now extensively studied as sources of new bioactive products. In this work nine different fungal strains such as *Cladosporium sp.*, *Rhizoctonia sp.*, *Aspergillus sp.*, *Chaetomium sp.*, *Biosporus sp.*, *Fuzarium sp.*, *Curvularia sp.*, *Cladosporium sp.*, *Colletotrichum sp.* were isolated from seven medicinal plants. Isolated endophytic fungi were screened qualitatively and quantitatively for production of extracellular enzymes such as amylase, protease, cellulose and lipase. Out of the nine fungus screened *Rhizoctonia sp*.showed highest production of amylase enzyme i.e. 0.26U/ml , productivity of protease was highest in *Biosporus sp*. (11U/ml), *Colletotrichum sp*. Showed better yield of cellulase i.e.0.013U/ml and *Cladosporium sp.* (0.72 U/ml) showed greater production of lipase**.** The strong enzymatic activities of the endophytic extracts shows a high potential for clinical microbiology and therapeutic applications.

Keywords

Endophytic fungi, extracellular enzymes, medicinal plants

Introduction

The term endophyte (Greek: endo = within + phyte=plant) include all organism that during a variable period of their life colonies the living internal tissues of their hosts. Endophytic fungi are one among these endophytes living inside the plants without causing any apparent symptoms [1]. Nearly one million endophytic species present ubiquitously in all plants [2]. The Bary mentioned term endophytes for first time in 19th century [3]. Endophytic fungus was first identified by *Freeman* in 1904, and was isolated from *Lolium persicum* (Persian darnel) [4]. Endophytic fungi are relatively unexplored producers of metabolites useful to pharmaceutical and agricultural industries. A single endophyte produce several bioactive metabolites. As a result, the role of endophytes in production of various natural products with greater bioactivity have received increased attention [5]. Endophytes can influence soil stability directly by their mycelia networks in the soil as well as indirectly altering roots and physical conditions of the host plants[6] and also endophytes are the chemical

synthesizers inside plants [7]. The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis[8]. Like other organisms invading plant tissues, endophytic fungi produce extracellular hydrolases as a resistance mechanism against pathogenic invasion and to obtain nutrition from host. Such enzymes include pectinases, cellulases , lipases, laccase from the endophytic fungus *Monotospora* sp., xylanase, -1, 4-glucanlyase , phosphotases and proteinase [9]. Hydrolytic enzymes (amylase, cellulase and laccase) with various industrial applications are also of major interest. Although hydrolyases are typically isolated from soil-borne fungi such as *Aspergillus sp., Penicillium sp.* and *Rhizopus sp*.,endophytes producing these enzymes present an interesting alternative[10]. Endophyte fungi have been most extensively studied for their ability to produce antibacterial, anticancer, antioxidants, anti-diabetic and immunosuppressive compounds [11]. Extracellular enzyme synthesis in endophytic fungi for penetrating the host plant cell wall, as well as biocontrol has been demonstrated previously[5]. In our present investigation, we isolated endophytic fungi from seven different medicinal plants viz *Cladosporium sp., Rhizoctonia sp., Aspergillus sp., Chaetomium sp., Biosporus sp., Fuzarium sp., Curvularia sp., Cladosporium sp., Colletotrichum sp.* i.e. *Azadirachta indica* , *Citrus limon* , *Gossypium hirsutum* , Son-chafa, *Datura stramonium* , *Gossypium hirsutum , Datura stramonium , Phyllanthus emblica* , and *Piper betle* respectively were evaluated for their enzymatic activities such as amylase, protease, cellulose, and lipase. Enzymatic activity is assessed quantitatively and qualitatively for these listed endophytic fungi.

Materials and Methods

Sources of Endophytes:

The fungi used in this study are the Endophytic fungi isolated from different medicinal plants, *Azadirachta indica*, *Citrus limon*, *Gossypium hirsutum*, *Magnolia champaca*, *Datura stramonium*, *Piper betle*, *Phyllanthus emblica* collected from the in K.T.H.M college,India. The Endophytic fungi were subcultured on PDA medium.

Screening of fungal extracellular enzymes

Screening was done by two methods, qualitative method i.e. agar plate method and by quantitative method i.e. liquid culture method. The functional role of extracellular enzymes by fungal endophytes was assessed by growing them on PDA for 6-7 days, incubated at 25^{o}C and placing 5 mm mycelial plugs on the solid media. After incubation, at room temperature, the zone of enzyme activity surrounding the fungal colony was measured [9]. Procedure followed for the qualitative and quantitative estimation of amylolytic, Proteolytic, Cellulolytic and Lipase activity is given below.

Amylolytic Activity

In qualitative method, amylase activity was assessed by isolates were inoculated in nutrient agar with 1% starch at pH 6.0. After incubation, the plates were treated with iodine. A clear zone around the active colonies indicated amylolytic activity[12] Whereas, in quantitative method, the fungi were grown on minimal broth with 1% starch after incubation, a reaction mixture containing 1 ml of enzyme broth (filtrate) and 0.5 ml of 1% starch was incubated for 30 minutes at 37^{o}C. The reaction was stopped by using Dinitrosalicylic acid as

color reagent. The reaction mixture was diluted to 5 ml with distilled water. The reddish brown color was observed which was measured spectrometrically at 540 nm. Amount of sugars produced were read off from a standard curve obtained by recording the absorbance of concentration of maltose standard solution[5]. One unit of amylase was defined as the amount of enzyme that releases 1µmol of reducing sugar as maltose per min under the assay conditions[13].

Proteolytic Activity

To determine protease activity qualitatively, the fungi were grown on Glucose Yeast extract Peptone Agar (GYP) medium (glucose-1g, yeast extract -0.1g, peptone-0.5g, agar -16g, distilled water-1L) containing 0.4% gelatin at pH-6. After 3-5 days of fungal colony growth, the plates were treated with saturated ammonium sulphate and the clear zone were observed around the active colonies [5,9]. For Quantitative assay, protease activity was measured by degradation of casein,1ml of filtrate was added to 1ml of 1% (w/v) casein (pH-7.5) and incubated for 1hour at 45^{o}C. The reaction stopped by adding protein precipitating agent, 3ml of 0.5M Trichloroacetic acid (TCA). Solutions were centrifuged at speed of 5000 rpm for 30 minutes and absorption of filtrate was measured at 275nm. One enzymatic unit represents the quantity of enzyme which liberates 1µg of tyrosine under enzyme assay condition[15].

Cellulolytic Activity:

For qualitative determination of cellulolytic activity, Yeast Extract Peptone Agar medium containing 0.5% Carboxy-methylcellulose(CMC) was used. After 3-5 days of fungal colony growth, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1M NaCl for 15 minutes. Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulose activity [9, 16]. For quantitative estimation, samples were grown in Czapek's Dox broth for 4 days. Samples (10ml) were taken from the flasks after 24, 48, 72 and 96 hours. Fungal cells were removed by filtration and filtrate was used for the enzyme assay. The reaction mixture, made up of 1ml filtrate, 1ml of citrate acetate buffer 0.5 M (pH 5) and 2.5 ml of 1% carboxymethylcellulose (CMC) as a substrate was incubated for 1 hr. at 37^{o}C. This reaction mixture served as sample for determining the presence of glucose by DNS (Dinitrosalicylic acid) method. One unit of cellulose was defined as 1.0mg of glucose released from 1% CMC hour^{-1} at 37^{o}C and pH5.[14,17].

Lipase Activity

For lipase activity, the fungi were grown on Peptone Agar medium (peptone 10g, NaCl 5g, $CaCl_2$. $2H_2O$ 0.1g, agar- 16g, distilled water-1L; pH 6.0) supplemented with separately sterilized Tween 20 and 1% added to the medium. At the end of the incubation period, a clear zone formed around the active colony indicates lipase activity[11,12,16]. For quantitative determination, samples were grown in 250ml flasks containing 100ml of autoclaved medium. Autoclaved medium contained $(NH_4)_2SO_4$-3g, $MgSO_4$- 0.7g, Nacl- 0.5g, $Ca(NO_3)_2$-0.4g, KH_2PO_4 -1.0g, glucose-5.0g, yeast extract-1.0g at pH5. Samples were kept on shaker at 30^{o}C and 110 rpm for 4 days. Olive oil was sterilized separately in dry oven and 1ml of it was added per 100 ml of sterile medium. 10ml of samples were taken after every 25 hrs for 4 days and samples were centrifuged at 5000 rpm for 20 min and supernatant was used for determining lipase activity. For this, 1.3 ml of olive oil, 1ml of phosphate buffer (0.066M at pH 7), and 3 ml of supernatant

and 1.5 ml of distilled water were shaken for 3 minutes, then placed in an incubator shaker at 30°C and at 150 rpm for 5hrs. After incubation, 15 ml of ethanol was added to the reaction mixture, then 12.5 ml of diethyl ether was added to destroy the emulsion. The mixture obtained was titrated against 0.1N NaOH solution using Thymolphthalein as an indicator. One unit of lipase was defined as the amount of enzyme required to decrease the O.D. (optical density) value by 0.001 units per hour per mL of the liquid culture media containing the enzyme substrate under the assay conditions[14]. One unit of lipase activity was defined as the amount of enzyme that liberated 1µmol fatty acid min-1 at 30 °C and pH 7 under the assay conditions.

Table no 1:- Isolation of endophytes from medicinal plants on PDA media.

Sr.no.	Plant	Color of mycelia	Genus Name
1	*Azadirachta indica* (Neem)	Orange Green	*Cladosporium sp.* *Curvularia sp.*
2	*Citrus limon* (lemon)	Black	*Rhizoctonia sp.*
3	*Gossypium hirsutum* (Cotton)	Black White	*Aspergillus sp.* *Fuzarium sp.*
4	*Magnolia champaca* (Son chafa)	Black	*Chaetomium sp.*
5	*Datura stramonium* (Datura)	Orange	*Biosporus sp.*
6	*Piper betle* (Paan)	Orange	*Colletotrichum sp.*
7	*Phyllanthus emblica* (Amla)	White	*Cladosporium sp.*

Lipase activity = Vol. of NaoH consumed (mL) × Molarity of NaoH/ Vol. of Lipase (mL) × Reaction Time (min)[18].

Result and Discussion

Isolation and Screening of Fungal cultures for Extracellular enzyme production

The nine different endophytic fungi were isolated from seven different medicinal plants. All isolates were subcultured routinely and maintained in the department. Each isolate was able to produce one or the other extracellular enzymes (Table 1). None of the strain was able to produce all four enzymes.

Nine isolates were screened for amylase production, out of which three endophytes were able to produce extracellular amylase. These three isolates were *Aspergillus sp., Rhizoctonia sp., Chaetomium sp.,* from the medicinal plants *Gossypium hirsutum* , *Citrus limon* , *Magnolia champaca* respectively (table 2). Amongst the positive isolates *Rhizoctonia sp.* (4.1U/ml) (isolated from *Citrus limon* plant) was found to be potential amylase producer with maximum amylase activity (Table 2). Other isolates *Aspergillus sp., Fuzarium sp., Curvularia sp., Colletotrichum sp., Biosporus sp.* showed moderate to low amylase activity while *Cladosporium sp.* was weak producers of the enzyme (table 2). Amirita *et al.* [2] in a study reported that out of eleven, eight isolates showed positive amylolytic activity from the medicinal plants such as *Adhatoda vasica, Costus igneus, Coleus aromaticus* and *Lawsonia inerims* also Amirita *et al.*[2] hypothesize the amylase of fungal origin are more stable than bacterial amylase enzyme. The amylolytic potential of these endophytes may help them to degrade starch which is available when the plant senesces.

Among the tested organisms, maximum extracellular protease activity was observed in *Biosporus sp.*(11U/ml)(Table2) other

isolates of *Cladosporium sp., Rhizoctonia sp., Aspergillus sp.* from *Phyllanthus emblica* , *Citrus limon* , *Gossypium hirsutum* respectively exhibited moderate protease activity. The protease activity was observed in *Biosporus sp., Aspergillus sp., Cladosporium sp.,Curvularia sp., Rhizoctonia sp.,Chaetomium sp., Cladosporium sp.*indicated by formation of clear zone around the colony because of degradation of gelatin,while other two isolates *Colletotrichum sp., Fuzarium sp.* from the medicinal plants *Citrus limon* and *Gossypium hirsutum* respectively indicated negative results. Similar result was reported by Pavithra *et al.*[20] where out 20 fungal isolates from *Ocimum sanctum* plant 50% of fungal isolates were found positive for protease. Proteases are used in clinical applications especially in the treatments like Diabetes. Cellulolytic activity was prominent in *Colletotrichum sp.* from *Piper betle* medicinal plant. Out of nine, only four endophytic fungal isolates *Biosporus sp.,Aspergillus sp., Colletotrichum sp.,Cladosporium sp.* showed positive results while other five isolates was negative for cellulase. For quantitative estimation, *Rhizoctonia sp.*(0.3U/ml) produced maximum cellulase activity while other isolates such as *Biosporus sp.,Chaetomium sp.,Fusarium sp.* exhibited moderate to low activity. *Cladosporium sp.* fungal isolates from *Azadirachta indica* did not showed cellulolytic activity. Hameed *et al.*[14] reported highest cellulase activity in cultures of *Fusarium sp., M,phaseolina, V. albo-atrum ,R.solani, M.phaseolina* and *F.graminearum* Bhagobaty *et al.*[13] reported highest cellulase production by the endophytic *Mortierella hyalina* (RS07OS) isolated from *Osbeckia stellata*.

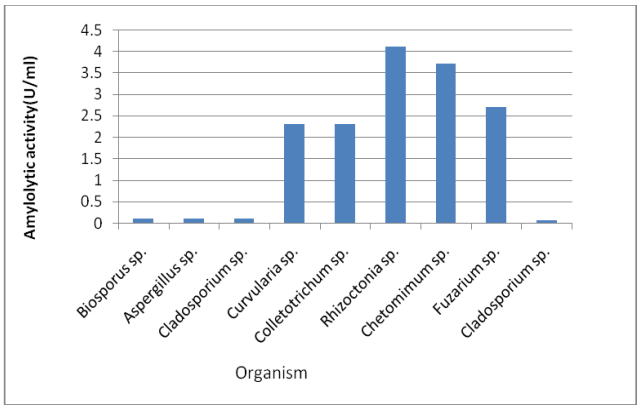
Out of nine endophytic fungal isolates only one showed positive results for extracellular lipase production The isolates, *Cladosporium sp.* of *Phyllanthus emblica* medicinal plant was the maximum producer of lipase activity(900 arbitrary units).

The production of extra-cellular enzymes was greater in the liquid medium as compared to the plate based assays. Enzymes that were not detected in plate based assays for some particular isolates were found positive in liquid culture conditions.

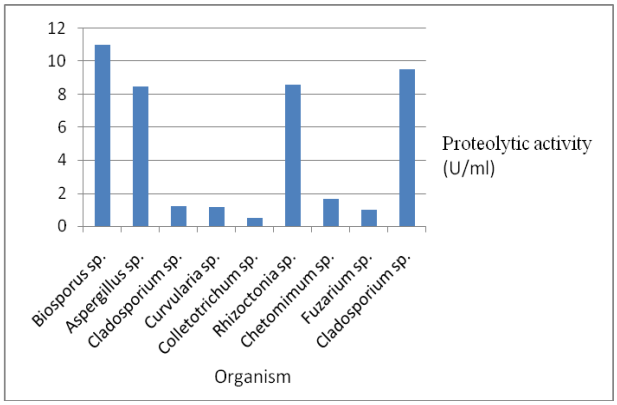
Table 2. Qualitative and Quantitative enzyme activities evaluation of isolates by using different cultures

Endophytes	**Amylase (Qualitative)**	**Amylase (U/ml)**	**Protease (Qualitative)**	**Protease U/ml**	**Cellulase (Qualitative)**	**Cellulase (U/ml)**	**Lipase (Qualitative)**	**Lipase (arbitrary units)**
Biosporus sp.	_	01	+	**11**	+	0.1	_	002
Aspergillus sp.	+	0.1	+	8.5	+	0.05	_	005
Cladosporium sp.	_	0.1	+	1.2	_	0.25	_	013
Curvularia sp.	_	2.3	+	1.17	_	0.25	_	007
Colletotrichum sp.	_	2.3	_	0.5	+	0.1	_	010
Rhizoctonia sp.	+	**4.1**	+	8.6	_	**0.3**	_	003
Chaetomium sp.	+	3.7	+	1.65	_	0.005	_	008
Fuzarium sp.	_	2.7	_	1.0	_	0.1	_	005
Cladosporium sp.	_	0.005	+	9.5	+	0.25	+	**900**

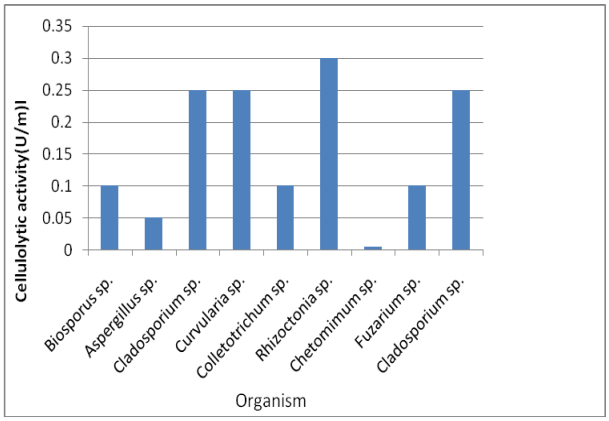
+ = Indicates the presence, - = Indicates the absence



Graph 1: Amylolytic activity shown by different endophytic fungi.



Graph 2: Proteolytic activity shown by different endophytic fungi.



Graph 3: Cellulolytic activity shown by different endophytic fungi.

In present study, nine different endophytic fungal isolates were screened qualitatively and quantitatively for the presence of extracellular enzymes such as Amylase, Cellulase, Protease and Lipase, which has grown on specific mediums. The order of enzymes activites found in this study for the isolated microorganisms is proteolytic > amylolytic > cellulolytic > lipolytic. Based on the results presented, it can be clearly seen that endophytes isolated from medicinal plants may be beneficial to the host.

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