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Evaluation of Fungal Endophytes for Cellulolytic Enzyme Production Isolated from Medicinal Plants of Tumakuru, Karnataka

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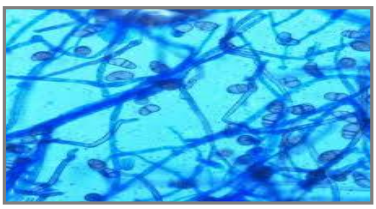
ABSTRACT

Cellulose is a polysaccharide composed of several glucose units linked together by chemical bonds. Cellulases, such as endoglucanases, beta-glucosidase and exoglucanases break the chemical bonds between the glucose units. Fungi, including the endophytic species, can be great cellulase producers. This study aimed to evaluate cellulase production by four endophytic fungi isolated from medicinal plants locally available around Tumakuru region. The isolation of endophytic fungi was carried from leaf, stem and roots tissues using Potato dextrose agar(PDA) 2.5% supplemented with the antibiotic streptomycin sulphate (100 mg L^{-1}). The fungal isolates were identified based on colony morphology and microscopic features as Alternaria from the leaf of Ocimum basilicum and Fusarium on root tissue and Ocimum sanctum leaf showed Fusarium and root isolate was Penicillium and no isolate from stem was observed. Finally leaf of Leucas aspera showed Alternaria and root isolate showed Curvularia. The cellulolytic capacity of the fungi was screened on solid agar with cellulose as the substrate using Congo red as an indicator dye. The most potent fungus that degrades cellulose was Peniciliumsps isolated from root of the Ocimum basilicum followed by Curvulariasps isolated from root of Leucas aspera. Quantitative estimation was carried out by DNS method. Maximum cellulase activity was by Curvulariasps, followed by Pencilliumsps, Alternariasps showing moderate activity and Fusariumsps showed low activity. The results from the present study reveals that Peniciliumsps and Curvulariasps are extremely potent producers of cellulases and can thus be used for eco-friendly and economic hydrolysis of biomass for biofuel production.

Graphical Abstract



Microscopic view of the endophytic fungi.

Keywords: Cellulose, Endophytic fungi, *Ocimum basilicum*,*Ocimum sanctum*, *Leucasaspera*, Cellulolytic activity.

INTRODUCTION

Cellulose constitutes bulk of the plant cell wall materials and the most abundant and renewable non-fossil carbon source on earth. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited and inexpensive which can be used for the production of a greener energy [**1**]. But there is depletion of fossil fuels reserves at an alarming rate and has created lot of problems for civilized world. Therefore there is obvious need to replace and utilize renewable resources for the production of a greener energy which can meet the high energy demand of the world. The cellulose is initially hydrolyzed into smaller sugar subunits like monomers and dimers. These sugar subunits can be further hydrolyzed to form biofuels or bioethanol [**2, 3**]. Cellulases which can hydrolyze these cellulose are multi-enzyme system that consist of three major components: (1) endo-*β-1,4-glucanases* (EC 3.2.1.4), (2) *exo-β-1,4-glucanases* (EC 3.2.1.91), and (3) *β-glucosidases* (EC 3.2.1.21). These three components work together synergistically to hydrolyze cellulose into the sugar. Endoglucanases hydrolyzed internal β-1,4 linkages cellulose to create new reducing and non-reducing ends. Currently, cellulases are the third most industrially produced enzymes worldwide because of their applications, for example in cotton processing, paper recycling, and the extraction of juices, as enzymatic detergents and as animal food additives [**4**]. Endophytic fungi are known to be associated with most of the plant species studied [**5, 6**]. Endophytic fungi are known to produce metabolites that help the host plant to tolerate biotic and abiotic stress, increase growth rate and extent of reproduction [**7, 8**]. The chemical constituents and the medicinal property of the plant may also be due to the interactions with its endophytes [**9**]. Bioprospecting of such fungi for useful compounds is essential in biotechnology. Hence, this study was conducted to evaluate potent cellulolytic of endophytic fungi isolated from medicinal plants of local regions around Tumakuru, Karnataka.

MATERIALS AND METHODS

Sample collection and selective isolation of endophytic fungi: The fresh and healthy medicinal plants such as *Ocimum basilicum, Ocimum sanctum* and *Leucas aspera* were collected from the botanical garden of University College of Science, Tumakuru campus. Each sample was tagged and placed in separate polythene bags and processed within 24 h of collection. Fresh plant materials were used for isolation of endophytic fungi to reduce the chance of contamination**.** The healthy plant samples collected were cut into small pieces approximately 0.5 cm diameter using surgical blade and surface sterilized by the modified method of Arunachalam and Gayathri [**10**]. The procedure for sample pre-treatment is shown below:

Fresh plant leaves

↓

Washed with running tap water

↓

Whished with 70% ethanol for 2-3 min

↓

Washed with 2% sodium hypochlorite containing 0.1%Tween 20 for 20 seconds

↓

Washed with distilled water for 2 min

↓

Washed with sterile distilled water five times

The surface sterilized leaf, stem and root segments were evenly spaced in petri dishes containing potato dextrose agar (2.5%) supplemented with the antibiotic streptomycin sulphate (100 mg L^{-1}) in order to prevent bacterial contamination for isolation of fungus. The petri dishes were incubated at room temperature for 3 days and monitored every day to check the growth of endophytic colonies from the leaf, stem and root segments, after the incubation, the colonies were sub cultured into the new respective medium and stored at 4ºC. The morphological identification of the isolates was done based on the fungal colony morphology and characteristics of the reproductive structures and spores [**11**]. All fungal mounts were made on microscopic glass slides using lacto phenol cotton blue stain.

Plate based assay for Cellulase enzymes: The four endophytic fungal isolates isolated from the respective medicinal plants were tested for Production of cellulase using 1% cellulose, as carbon source. An agar diffusion method was used as a qualitative assay method. After incubation of 72 h at 25±2°C, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1M NaCl for 15 minutes. The diameter of the clear zone was measured. Appearance of yellow areas around the fungal colony in an otherwise red medium indicated the cellulose activity [**12**].

Estimation of the extracellular enzyme in liquid culture medium: For quantitative estimation, samples were grown in Potato dextrose broth (100 mL) for 4 days. Samples (10 mL) were taken from the flasks after 24, 48, 72 and 96 h. Fungal cells were removed by filtration and filtrate was used for the enzyme assay. Enzyme production in the broth culture was determined by the 3-5, dinitrosalicylic acid (DNS) method of Miller (1959) [**13**].

RESULTS AND DISCUSSION

Endophytic fungi residing in plants are unstudied and are being considered as potential source of novel bioactive products used in medicine, agriculture and industry. In the present study four colonies were isolated from leaves and roots and no isolate from the stem was observed (Figure1).

Figure 1.Cultural morphology of fungal endophyte

Isolation and identification of fungal endophytes: Potato Dextrose Agar medium (2.5%) supplemented with the antibiotic streptomycin sulphate (100 mg L^{-1}) was used to observe the morphology of the isolates. The fungal isolates were identified based on colony morphology and

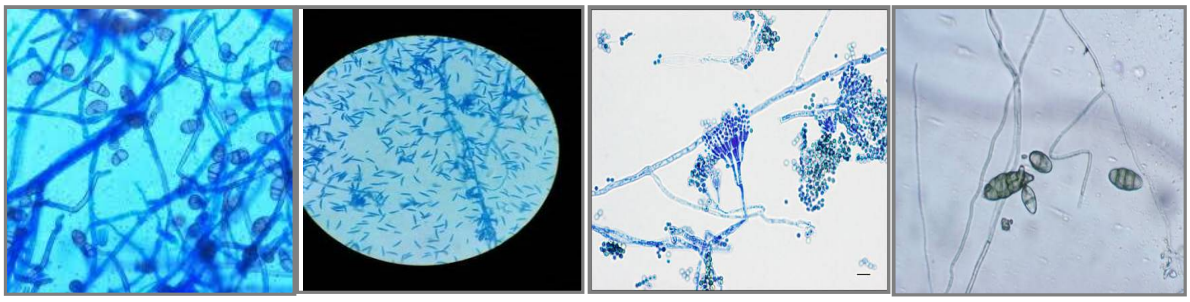


Figure 2. Microscopic view of the endophytic fungi.

microscopic features as *Alternaria* from the leaf of *Ocimum basilicum and Fusarium* on root tissue and *Ocimum sanctum* leaf showed *Fusarium* and root isolate was *Penicillium* and no isolate from stem was observed. Finally leaf of *Leucas aspera* showed *Alternaria* and root isolate showed *Curvularia* (Figure 2).

Cellulolytic activities: Fungal endophytes were screened for their production of extracellular enzymes. Cellulolytic activity was detected in all fungal isolates. The results of plate assay of endophytic isolates revealed that the most potent fungi that degrade cellulose was *Penicilium* sps isolated from root of the *Ocimum basilicum* followed by *Curvularia* sps isolated from root of *Leucas aspera*. The rest of the isolates were unable to produce this enzyme (Figure 3 and Table 1)

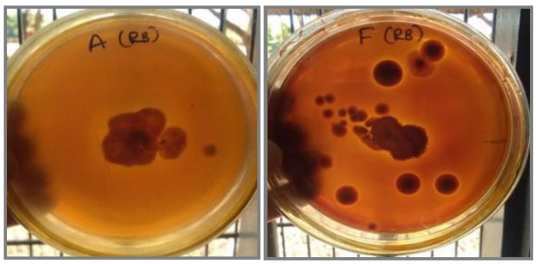


Figure 3. Fungal isolates showing cellulase production.

Table 1. Colony diameter and zone diameter after 72 h and Cellulase activity

Isolate name	Colony diameter (cm)	Zone Diameter (cm)	Enzyme Activity (IU mL^{-1})
Fusarium	2.2	0.3	0.12
Pencillium	3.5	2.8	0.5
Curvularia	3.0	2.5	0.6
Alternaria	2.8	0.6	0.25

Quantification studies revealed that Maximum cellulase activity was by *Curvularia* sps, followed by *Pencillium* sps, *Alternaria* sps showing moderate activity and *Fusarium sps* showed low activity. The results from the present study reveal that *Curvularia* sps and *Pencillium sps* are extremely potent producers of cellulose Similarly *Fusarium solani* and *Talaromyce* sps. Isolated in this study is an excellent source for extracellular cellulases [**14**]. Similar results were obtained such as strong cellulase activity was found with *Penicillium* sp 51 [**15**].

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APPLICATION

Fungal endophytes are known to produce cell wall degrading enzymes including cellulases, which play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded in unit of glucose [**16**]. In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol [**17**], organic acids [**18**], detergents and other chemicals [**19**].

CONCLUSION

In present study, four different endophytic fungal isolates were screened qualitatively and quantitatively for the presence of extracellular enzyme such as Cellulase which has shown positive for cellulose degradation are *Penicillium, Curvularia, Alteranaria* and *Fusarium*. Based on the results

presented, it can be clearly seen that fungal endophytes isolated from medicinal plants may be potent producers of cellulases and can thus be used for eco-friendly and economic hydrolysis of biomass for biofuel production. Further, characterization and optimization of the culture condition is under progress.

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