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Research Article

PRODUCTION OF EXO AND ENDO- 1,4-β-D-GLUCANASESBY PURPUREOCILLIUMLILACINUM ISOLATED FROM FOREST SOIL

Srilakshmi A*., Narasimha G and Saigopal D.V.R

Department of Virology, Sri Venkateswara University, Tirupati, A.P, India-517502

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ABSTRACT

Microorganisms, especially fungi are the leading producers of an array of industrial enzymes. Microbial cellulases are the enzymes that are sold in- large volumes due to their enormous applications in biochemical industry. Selection of the right organism plays a predominant role in high yield of desirable enzymes. Hence an attempt was made in the current study to isolate and screen novel fungi from forest soil for the optimal production of exo and endo- 1,4-β-D-glucanases (FPase and CMCase). In this study eight fungal cultures were isolated and screened, out of these four cultures were found to exhibit cellulolytic activity. The most potent isolate which exhibited maximum cellulolytic activity was identified as *Purpureocilliumlilacinum* based on microscopic observation and molecular characterization. The activities of cellulases were determined by Filter paper assay (FPA), Carboxy-methyl cellulase (CMCase) assay. A significant FPase and CM Case activities was shown by *Purpureocilliumlilacinum* grown on Czapex-Dox medium.

INTRODUCTION

Forests have a significant role in the carbon cycle within the biosphere and degradation of cellulose, the most abundant polysaccharide in the plant litter is especially important in this respect. Plant litter deposited in the forest soils represents a major source of organic carbon (Berg *et al*., 2001). Cellulose is a linear glucose polymer consisting of glucose units joined by a β-1, 4-D-glycosidic bonds (Gielkens *et al*., 1999; Han *et al*., 1995). Glucose production from cellulose is the bottleneck for developing bioprocesses, and enzymatic hydrolysis is preferred than thermo or chemical processes in terms of environmental safety at moderate temperatures and pH values (Hamelineck *et al*., 2005). Thus, the enzymatic hydrolysis of lignocellulosic wastes remains as a promising strategy for efficient utilization of this renewable resource and the production of desired metabolites from the fermentation of resulting reducing sugars. Cellulose hydrolysis is achieved with the aid of cellulase enzyme complex which includes, endoglucanase also called carboxymethylcellulase, exoglucanase and β-glucosidase (Iqbal *et al*., 2011). Although many microbes are capable of utilizing cellulose, *in vitro* a few of them yields notable quantities of cell-free bioactive compounds and aids the completely hydrolysis of crystalline cellulose (Saraswathi *et al*., 2012). Various microorganisms including *Aspergillus* sp., *Trichoderma*sp., *Schizophyllum* sp. *Chrysosporium* sp., *Fusarium* sp., *Sclerotium* sp., *Phanerochaete* sp., and *Bacillus* sp. produces cellulases (Duff and Murray, 1996; Toyama and Ogawa,1975; Selby and Maitland, 1967; Wood and Phillips, 1969; Sternberg, 1976; Fan *et al*., 1987). Fungal cellulase systems comprises of a number of exocellulases and endocellulases with varying properties and functions in the hydrolysis of crystalline cellulose (Irwin *et al*., 2000). Degradation of cellulose is 10 times faster in the fungi dominated forest soil than in bacteria dominated soil (Stursova *et al*., 2012).

The major industrial applications of cellulases are in the textile industry, starch processing, grain alcohol fermentation, paper and pulp, malting and brewing, leather and also used to improve digestibility in ruminants (Sakthivel *et al*., 2010; Yano *et al*., 2012; Shaikh *et al*., 2013). Moreover, there are progressing markets for produced cellulases in the field of detergent industry and saccharification of agriculture wastes for bioethanol technology (Camassola and Dillon *et al*., 2009, Vu *et al*., 2011). In the field of cellulase research, circumventing the high cost of cellulase production remains the top priority. Therefore, there has been much research focused on obtaining novel microorganisms producing cellulase enzymes with higher specific activities and greater efficiency. In view of biotechnological importance of cellulases, an attempt was made in the present study to isolate prominent cellulolytic fungal culture from forest soil for optimal cellulase production.

*Corresponding author: **Srilakshmi A**
Department of Virology, Sri Venkateswara University, Tirupati, A.P, India-517502

MATERIALS AND METHODS

Isolation of fungal cultures for cellulolytic activity

The fungal cultures used in the present study were isolated from soil contaminated with forest litter collected from Talakona forest area, Chittoor District, Andhra Pradesh, India. Serial dilution method was followed in which one gram of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to 10^{-6} and 0.1ml of soil suspension was spread on to the sterilized Czapek-Dox agar medium with following composition (g/L): sucrose – 30, $NaNO_3$ – 2,K_2HPO_4 - 1, $MgSO_4$ – 0.05, KCl – 0.5, $FeSO_4$– 0.01, Agar Agar – 15.0. pH of the medium was adjusted to 5.0. After autoclaving at 121 °C and15 lbs pressure, 20 ml of sterile medium was transferred to sterile Petri plates and allowed to solidify. After solidification of the medium 0.1 ml of soil suspension was spread with the help of spreader and incubated at 28°C for 7 days. The fungal cultures grown on the medium were maintained on the Potato Dextrose agar slants at 4 °C for further studies.

Fungal spore inoculum preparation

The fungal spore inoculum was prepared for cellulase production from 7 days old culture slants. Sterile distilled water (2ml) was added to each fungal agar slant and shaken vigorously for preparing uniform suspension. Inoculum density was adjusted to 2×10^{6} spores of each organism using haemocytometer. The same inoculum size was used throughout the study.

Screening of fungal isolates for cellulolytic activity

The isolated fungal cultures were screened for cellulolytic activity according to the method of Teather & Wood (1982). 100µl of fungal spore suspension of each organism was inoculated in to the wells at the centre point of the petri plate containing medium Czapek-Dox agar medium supplemented with 1% carboxy-methyl cellulose (CMC). The plates were incubated at 28^{o}C for 3 days and at 50^{o}C for 18 hours after which they were stained with 1% Congo red stain for 15 min on rotary shaker at 50 rev/min. Excess dye were removed by washing with 1MNaCl. The production of extracellular cellulases by the organisms was indicated by a zone of clearance around the colony.

Identification of fungal culture

The potent cellulase producing fungal strain (isolate 1) was identified based on, microscopic observations and by the amplification of 18s rDNA based molecular technique. The 18S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score fifteen sequenceswere selected and aligned using multiple alignment software program Clustal W. The phylogenetic tree was constructed using MEGA 5.

Cellulases production

The fungal culture was grown on Czapex – dox and Mandels-Weber media (1969). These media were amended with 1% cellulose. The pH of the medium was adjusted to 5.0 prior to sterilization. Fungal spores (2×10^{6}) was dispersed into four separate 250 ml Erlenmeyer flask that contained 100 ml of the above mentioned medium. The flasks were incubated on rotary shaker (Scigenics biotech, ORBITEK) at 28°Cfor 3, 5, 7 and 9 days .The culture filtrates were filtered through Whatman No1 filter paper and centrifuged at 11000 x g for 10 min. The filtrate thus obtained was used as enzyme source for the further quantitative analysis.

Extracellular protein content

After appropriate incubation period (3, 5, 7 and 9 days) the contents of the flasks were aseptically passed through Whatman No.1 filter paper to separate mycelial mat from culture filtrates. An aliquot of this culture filtrates was used for estimation of extracellular protein content according to the method of Lowry *et al*. (1951). Bovine serum albumin was used as protein standard. Suitable aliquots of filtrates were mixed with 5 ml of alkaline solution. After 30 min, 0.5 ml of appropriately diluted Folin-Ciocalteau reagent was added. The color developed was read at 550 nm by using the spectrophotometer (Thermo scientific)

Determination of fungal biomass

After appropriate time intervals the contents of the flasks were aseptically passed through pre-weighed Whatman No.1filter paper to separate mycelial mat from culture filtrates. The filter papers along with mycelial mat were dried at 70 °C in an oven until constant weight and the weight was recorded. Difference between the weight of the filter paper bearing mycelial mat and weight of pre-weighed filter paper represented fungal biomass, which was expressed in terms of dry weight of mycelial mat (g/100 ml).

Cellulase assays

Filter paper activity (FPA) Filter paper activity of the culture filtrates was analyzed according to the method of Ghosh (1987). Whatman filter paper strips containing 50 mg weight was suspended in 1.0 ml of 0.05M sodium citrate buffer (pH 4.8) at 50 °C in a water bath. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated for 60 minutes at 50 °C. After incubation, the liberated reducing sugar was estimated by the addition of 3, 5-dinitrosalicylic acid (DNS) (Miller 1959). After cooling, the color developed in tubes was read at 540 nm by using the spectrophotometer (Thermo scientific). Appropriate control without enzyme was simultaneously run. Activity of cellulases was expressed in filter paper units. One filter paper unit (FPU) was defined as the amount of enzyme releasing one micro mole of reducing sugar from filter paper /ml /min.

Endoglucanase assay Activity of endoglucanase in the culture filtrates was quantified by carboxy-methyl cellulase method (Ghosh 1987). The reaction mixture with 1.0ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50 °C in water bath for one hour. Appropriate control without enzyme was simultaneously run. The reducing sugar produced in the reaction mixture was determined by Dinitro-salicylic acid (DNS) method (Miller 1959).3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm by using the

spectrophotometer (Thermo scientific). One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing one micromole of reducing sugar /ml /min.

RESULTS

Isolation and screening of fungal cultures from forest soil

A total of eight fungal cultures were isolated from forest soil of which only four cultures exhibited cellulase production. The cellulolytic potency of the fungal cultures was tested on CMC agar plates. The appearance of clear yellow zone around the fungal cultures is an indication of cellulase producing ability of the fungal cultures (Fig.1). Maximum CMC hydrolyzing zone of 35 mm was shown by fungal isolate-1 where as isolates 2, 3 and 4 exhibited 28 mm, 8mm and 8mm respectively. In this study as maximum zone diameter was exhibited by fungal isolate 1, it was considered as prominent and efficient candidate for cellulase production.

Screening of fungal cultures for cellulolytic activity

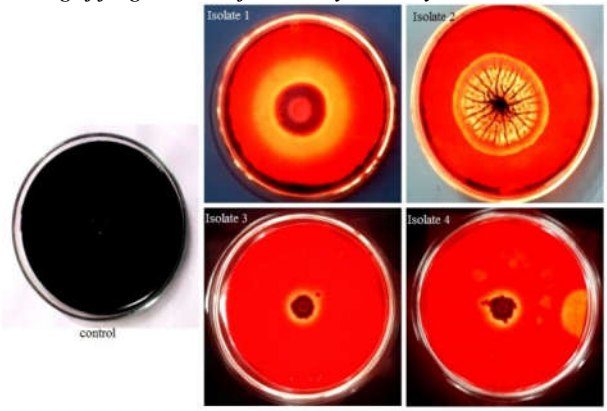


Fig.1. clear yellow zone around fungal cultures

Identification of fungal isolate

The potent cellulolytic fungi was identified based on macroscopic, microscopic observations (Fig.2, 3and 4) and by amplification of 18s rRNA gene using BLAST and the ITS sequence was deposited in NCBI gene bank under accession number KT387301.The phylogenetic tree (Fig.5) was inferred by neighbour-joining method. The results confirmed that 18s rDNA of fungal culture have 100% matching with *Purpureocilliumlilacinum.*

Fig.2. Macroscopic observation (colony morphology on Czapek-Dox aga medium)

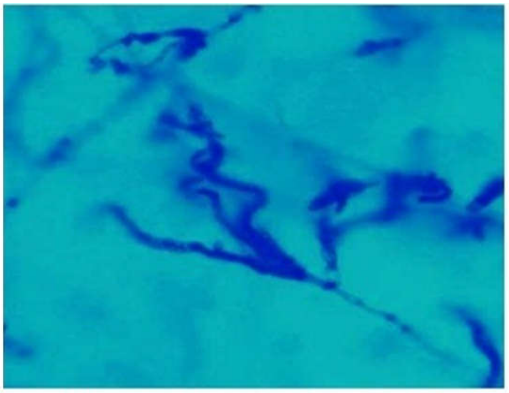


Fig.3 Microscopic image of *Purpureocilliumlilacinum*

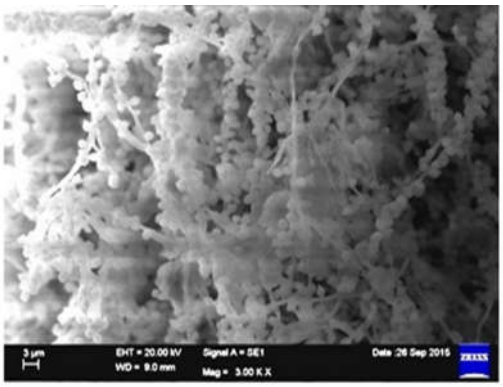


Fig.4 Scanning electron microscope image of *Purpureocilliumlilacinum*

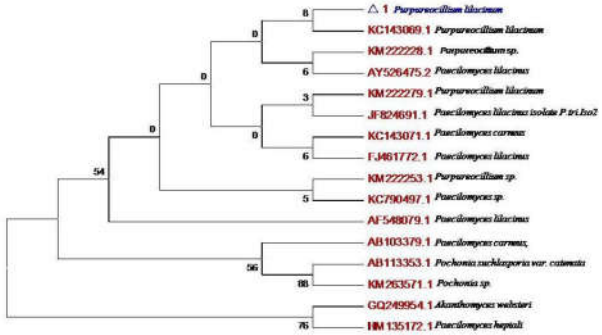
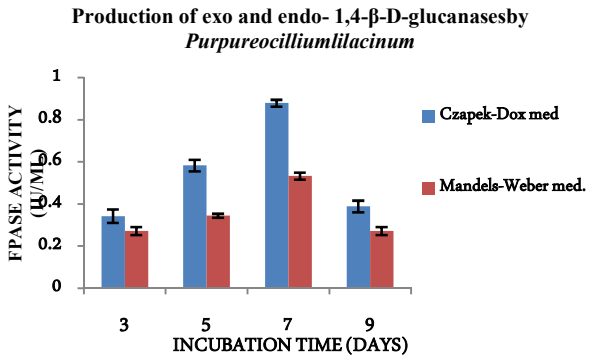


Fig.5 Phylogenetic tree of *purpureocilliumlilacinum*

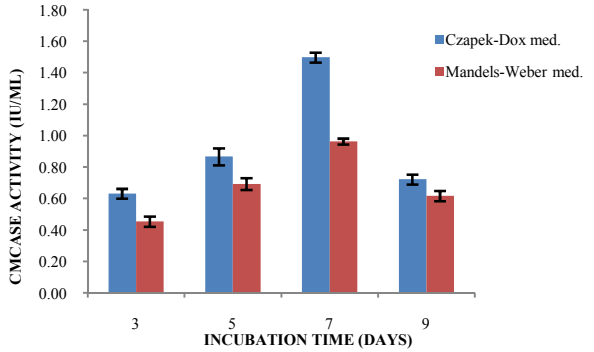
Cellulase production

The cellulase generating ability of *Purpureocilliumlilacinum* was tested through submerged fermentation on Czapex – Dox and Mandels –Weber media (Fig.6 and 7). Cellulose depolymerising ability, extracellular protein content and fungal biomass increased with increase in incubation time reached maximum at 7th day interval and declined thereafter. Of the two media used in the current study Czapex – Dox medium was proved to be efficient in producing higher cellulolytic activity as well as protein content and fungal biomass. This fungus exhibited higher FPase (0.87 IU/ml), CMCase (1.50 IU/ml) on Czapex – Dox medium whereas lower FPase (0.53 IU/ml), CMCase (0.96 IU/ml) was recorded with Mandels-Weber medium. Similarly extracellular protein content (1.49 mg/ml) and fungal biomass (1.82 g/100ml) (Fig.8 and 9) as also recorded as higher in Czapex – Dox medium when compared to Mandels-Weber medium in which lower protein content (1.06 mg/ml) and fungal biomass (1.38 g/100ml) was noticed.



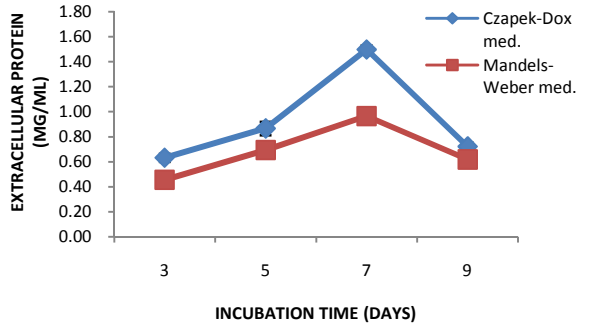
The values represented in the figure are mean of triplicates*

Fig.6 Production of exo- 1,4-β-D-glucanaseby *Purpureocilliumlilacinum* grown on Czapek-Dox and Mandels-Weber media amended with 1% cellulose



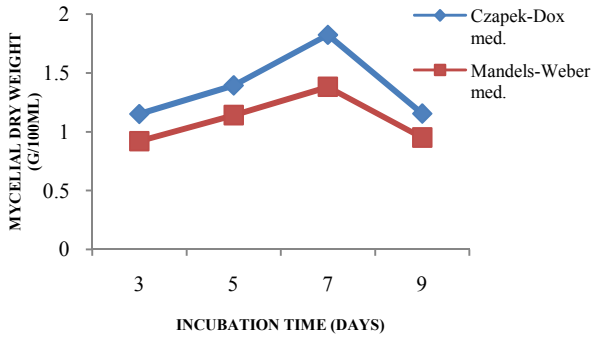
The values represented in the figure are mean of triplicates*

Fig.7 production of endo- 1,4-β-D-glucanaseby *Purpureocilliumlilacinum* grown on Czapek-Dox and Mandels-Weber media amended with 1% cellulose



The values represented in the figure are mean of triplicates*

Fig.8. Production of extracellular protein by *Purpureocilliumlilacinum* grown on Czapek-Dox and Mandels-Weber media amended with 1% cellulose



The values represented in the figure are mean of triplicates*

Fig.9. production of biomass by *Purpureocilliumlilacinum* grown on Czapek-Dox and Mandels-Weber media amended with 1% cellulose

DISCUSSION

In the present investigation maximum zone of hydrolysis (35mm) was exhibited by *purpureocilliumlilacinum* on CMC (Carboxy-methyl cellulose as substrate) agar plates. Similar reports with other fungal cultures has been reported by Naveen kumar *et al*., (2013), 16 ± 0.6 mm by *Aspergillusniger,* (areca nut husk waste), and Lekh Ram *et al*., (2014)., 7mm of colony diameter by PISS- 3 (paper industry soil sample) respectively. Higherexo and endo-β-1,4-glucanaseactivity, extracellular protein, fungal biomass was detected on Czapek-Dox medium amended with 1% cellulose. Similarly Narasimha *et al*., (2006) reported maximum cellulase activity (1.7 FPU/ml), protein content (200 ug/ml) and biomass (650 mg/flask) on Czapek-dox medium by *Aspergillusniger*. Similar observations with other fungi *Humicolafuscoatra*and PISS-3 were made by Rajendran *et al*., (1994) and Lekh Ram *et al.,* (2014) respectively.

CONCLUSION

Fungi are considered as the prominent decomposers of dead plant biomass in terrestrial forest soils. The fungi harboring plant litter play a superior role in decomposing organic matter in forest soil. The use of fungi have a number of advantages than bacteria and yeast, the most important of which being their capability to utilise a wider variety of cellulosic substrates generated from forests and agriculture. Presently our studies investigated the efficacy of *Purpureocilliumlilacinum* isolated from forest soil for the production of extracellular cellulases. Out of two media used Czapek – Dox medium was proved to be efficient in supporting the production of exo and endo- 1,4-β-D-glucanases compared to Mandels and Weber medium at all incubation days. To our knowledge this may the first report on exo and endo- 1,4-β-D-glucanases production bythe fungal culture *Purpureocilliumlilacinum*..Thus we conclude that *purpureocilliumlilacinum* isolated from forest soil in the present investigation has industrially important applications and the culture parameters are needed to be optimized for the efficient cellulase production.

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