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ORIGINAL RESEARCH ARTICLE

Isolation and Screening of Cellulase Producing Fungi from Forest Waste

Uttam Kumar*[1], Ashwani Tapwal[1], Pinky Kalkal[2], Shiju Varghese[3], Suresh Chandra[1]

[1]*Forest Research Institute, P.O. New Forest, Dehradun, Uttarakhand, India -248006*
[2]*Shri Guru Ram Rai Institute of Technology & Science, Dehradun, Uttarakhand, India -248001*
[3]*Doon (P.G.) College of Agriculture Science & Technology, Dehradun, Uttarakhand, India -248007*

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ABSTRACT

The goal of this study was to isolate and screen the fungal species associated with plant material laying on Forest floor for cellulase activity. Eleven fungal species were isolated and screened for their ability to produce cellulases. Among them, *Trichoderma viride* and *Aspergillus niger* were recorded as cellulase producing species. *T. viride* has exhibited maximum zone of hydrolysis for carboxymethylcellulose and produce higher titers of cellulases. Production of cellulase was analyzed by Dinitrosalicylic acid (DNS) and Carboxymethyl cellulase assay methods. Maximum enzyme production (1.76 U/ml) was achieved at 30°C, pH of 5.0 by *T. viride* and on 5th day of incubation.

Key words: Cellulase, Fungal strains, Forest wastes, DNS, Partial purification.

INTRODUCTION

Forest consistently produces huge amount of organic matter in the form of leaves, twigs, branches, fruits and reproductive parts like flowers, seeds and spores [1]. The soil microflora transform the plant residues into CO_2 and relatively stable humus components [2]. Cellulose is most abundant compound on this planet formed by stoking of glucose units obtained through photosynthesis. The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component. Plant biomass comprises on average 23% lignin, 40% cellulose and 33% hemicellulose by dry weight [3]. The highly ordered structure of cellulose makes it hard to degrade. Biological degradation of cellulosic biomass to fermentable sugars by utilizing cellulase producing microbes is feasible process and offers potential to reduce the use of fossil fuels [4, 5].

Vast uses of cellulose have attracted the interest of researchers in this field. The major industrial applications of cellulase includes in textile industry for bio-polishing of fabrics, producing stonewashed look of denims and in household laundry detergents for improving fabric softness and brightness [6]. Besides, they are also used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, in baking etc. [7]. The cellulases that are used for in industrial applications are obtained from fungal sources [8]. High cost of production and other factors like complexity of cellulose structure, the type and source of cellulose and its production by cellulolytic organisms is major factor which influence the economics of cellulase production [9]. Successful utilization of cellulosic materials as renewable carbon sources can be achieved by developing economically feasible technologies for cellulase production, and enzymatic hydrolysis of complex cellulosic materials to low molecular weight monomers [10]. Therefore, the present study was aimed on isolation and screening of potential cellulase producing native fungal species from natural degrading plant material. A novel methodology of isolation and primary screening of cellulolytic fungi was adopted by exposing carboxymethyl cellulose agar, a selective media for cellulolytic microorganisms and partial purification and enzyme activity has been determined.

MATERIALS AND METHODS

Isolation and identification of fungi

Forest wastes like degrading wood, leaf litter and soil were collected from the forest nearby Forest Research Institute, Dehradun. Pure cultures were

***Corresponding Author:** Uttam Kumar**, Email:** ut.uttam@gmail.com

isolated on potato dextrose agar and maintained at 4°C. Fungal species were identified on the basis of cultural, morphological and microscopic characteristics.

Plate screening for cellulase enzyme activity

Isolated fungal strains were primarily tested for cellulase enzyme activity by culturing on Czapeck-Dox medium supplemented with 1% Carboxymethyl cellulose (CMC). The plates were incubated at 28±2°C for 7 days. After incubation plates were stained with 1% Congo red reagent. The clear zone around the fungal colony was regarded positive for cellulase activity. The fungal species exhibiting cellulase activity were selected for enzyme production.

Enzyme production through submerged state fermentation:

In pre-optimized conditions the selected fungi were inoculated in 100mL fermentation media 0.004- $ZnSO_4.7H_2O$, 10.5- KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.5-$CaCl_2$, 0.13-$FeSO_4.7H_2O$, 0.005-$MnCl_2.4H_2O$, 0.5-K_2HPO_4, 0.5-$NaMoO_4$ and 0.5-$(NH_4)_2SO_4$ [11] and crude enzyme were harvested at the interval of 24 hrs. The fermented biomass samples were filtered and centrifuged at 10,000 rpm for 15 minutes at 10°C temperature in the centrifuge (Remi cooling centrifuge) to remove undesirable biomass. The supernatant was carefully collected and stored aseptically under refrigerated conditions to prevent contamination. The crude enzyme thus obtained was subjected to purification after the enzyme assay [12].

Partial purification of cellulase

The method of De-Moraes *et al.*, (1999) [13] was followed for purification of Carboxymethyl cellulase (CMCase). Different levels of ammonium sulphate (40, 60 and 80%) in citrate buffer (pH 4.8) were used for the enzyme precipitation. The respective amounts were added to 5 mL of crude enzyme solution and placed at 4°C for one to two hours with continuous stirring. These were centrifuged at 15300 x g for 15 minutes in macro centrifuge. The pellets were collected carefully and the supernatant was discarded. The pellets were dissolved in distilled water at a rate of 0.1g/mL. The dissolved pellets were assayed for CMCase activity. The activity of CMCase reflected the best level of $(NH_4)_2SO_4$ concentration (gram) for purification. Partially purified enzyme was confirmed by SDS- PAGE performed using the method of Laemmli (1970) [14], with the stacking and separating gel concentrations were 4% and 12% of polyacrylamide, respectively. After the electrophoresis, the gels were stained with Coomassie brilliant blue R-250 for visualization of protein bands.

Enzyme assay

Carboxymethyl cellulase (CMCase) hydrolyzes Carboxymethyl cellulose to produce free Carboxymethyl glucose units. The free Carboxymethyl glucose units react with 3-5 dinitrosalicylic acid (DNS) reagent to form a colored complex which is detected spectrophotometrically (Thermo-Scientific UV-1) at 540 nm [15].

RESULTS AND DISCUSSIONS

The fungi isolated from different sources like degrading wood, leaf litter and soil collected from forest nearby Forest Research Institute, Dehradun, were identified as *Trichoderma viride, Aspergillus niger, A. fumigatus, A. flavus, Fusarium oxysporum* and species of *Trichoderma, Chaetomium, Curvularia, Penicillium, Alternaria* etc. Among these *Aspergillus niger* and *Trichoderma viride* were recorded from all samples (**Table 1**).

Plate screening for cellulase enzyme activity

The screening of the isolated fungi for the cellulose degrading activity was carried out on CMC medium. The appearance of the clear zone around the colony after the addition of Congo red solution was strong evidence for the secretion of cellulase. Out of 11 fungal species, only two fungi (*A. niger* and *T. viride*) have exhibited considerable activity to degrade the cellulose, highest zone of clearance has been recorded for *T. viride* (24.2 ± 1mm) followed by *A. niger* (16.4 ± 1mm). Therefore, they were selected for further experimentation.

Production of CMCase in submerged state fermentation

For the production of cellulase in submerged state fermentation, the selected fungi were inoculated separately in 100mL fermentation media in 250 mL capacity flask. The fungi utilize the media for its growth and secret various secondary metabolites including cellulases into the culture flask. The enzyme quantity expected to increase with increase in fungal growth and period of incubation. Therefore, the crude extract from media was harvested at the interval of 24 hrs up to 144 hrs. The cultivation time allows maximum growth of microorganism and product formation to a certain degree in a fermentation system. The enzyme activity of crude extract was determined

by using spectrophotometer. The results revealed increasing trend of enzyme activity (U/mL) for both tested fungal isolates up to 120 hours of incubation and then decline. This may be due to increase in concentration of certain toxic wastes and depletion of nutrients in fermentation media which leads to decreased fungal growth and enzymes production. Another reason may be the high viscosity of the medium, which decreases the oxygen supply to the microorganism. High viscosity leads to retard cell division, resulted in low production metabolism and cellulase excretion [16]. The enzyme production was recorded higher by *T. viride* in comparison to *A. niger* (**Fig 1**). After 120 hours of incubation *T. viride* has exhibited 1.76 U/ml enzyme activities in comparison to 0.92 U/mL for *A. niger*. Sun *et al*. (2010) [17] has recorded high enzyme activity on apple pomace by *Trichoderma* sp. at 120 h in SmF. The crude enzyme extracts harvested from SmF generally contains other impurities in addition to cellulase. Therefore, partial purification of the crude extract has been carried out by ammonium sulphate precipitation in different fractionation concentrations. Fungi have many different functions in soils, which include either active roles, such as the degradation of dead plant material, or inactive roles where propagules are present in the soil as a resting stage [18].

Activity of partial purified enzyme

After purification of crude enzyme by ammonium fractionation, the assay of purified enzyme has been carried out by DNS method as described in methodology. The enzyme activity was increased with the increase in fractionation concentration of ammonium sulphate. This may be due to precipitation of more impurities at the higher fractionation concentration. Like crude extract, the partial purified extract also exhibited higher activity for *T. viride* in comparison to *A. niger* (**Fig 2**). It was 1.81 U/mL for *T. viride* and 1.02 U/mL for *A. niger* on 80% fractionation concentration. The production of cellulase in shaking condition becomes higher, because of good oxygen supply, the cultures mix well to the substrates and significant increase in the time of contact and area between the fungal cells and substrates. In the static culture, a layer of mycelium grows at the top of the culture while the substrate remained at the bottom of the flask, which significantly reduced the contact time and area between the fungal cells and substrates. Fungi are well known agents of decomposition of particularly cellulose and xylan containing organic matter. The decomposition of cellulose and xylan is of significance in the biological carbon cycle [19].

Table 1: Fungi isolated from different samples

S. No	Name of Fungi	Sample		
		Leaf litter	Degrading wood	Soil
1	*Trichoderma viride*	+	+	+
2	*Aspergillus niger*	+	+	+
3	*A. fumigatus*	-	+	+
4	*A. flavus*	+	+	-
5	*Fusarium oxysporum*	-	+	-
6	*Trichoderma* sp.	+	+	+
7	*Chaetomium*sp.	-	-	+
8	*Curvularia* sp.	-	+	+
9	*Penicillium* sp.	+	-	+
10	*Alternaria* sp.	+	+	-
11	*Rhizoctonia* sp.	+	-	-

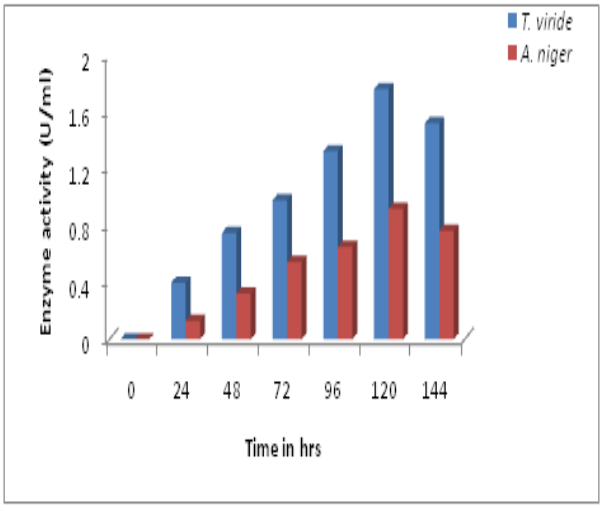

Figure 1: Cellulase production by *T. viride* and *A. niger* at different periods of incubation

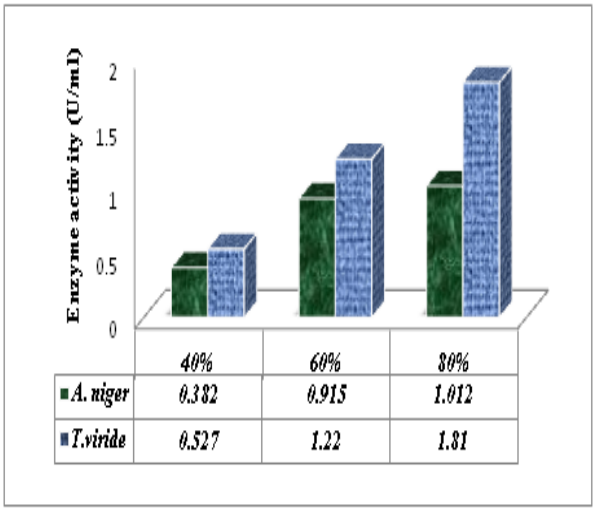

Figure 2: Enzyme activity (U/ml) of partial purified crude extract

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