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The Production of cellulase by liquid state fermentation of five different mycoflora and optimization of using saw dust

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ABSTRACT

Meghamalai forest soils are found to be rich in cellulolytic organisms. The physicochemical parameters such as temperature, humidity, soil pH and organic matter present in soil with the growth of microbes. From the soil sample (Grazed, disturbed and undisturbed soil) different type of fungus were isolated and identified. They are Aspergillus niger, Curvularia lunata, C. geniculata, Penicillium lanosum and Neurospora crassa. Among the fungi, the cellulolytic activity the fungal species were inoculated onto CMC with congo red medium and showed clear zones around the colonies. The 2% substrate sawdust which was suitable lignocellulosic biowaste and major carbon source for cellulose production. The environmental conditions such as temperature, humidity, pH, organic carbon and organic matter were produce the potential of the rate and extent of cellulose utilization, so optimizations of conditions were studied for all five fungal strains. The cellulase activity was assayed by Dinitrosalicylic (DNS) and Filter Paper assay (FDA) method, in which FAD showed increased maximum enzyme activity. The partial purification of cellulase enzyme was carried out by salt precipitation. The salts present in concentrated protein solution were removed by dialysis. After dialysis the samples were assayed for cellulase activity and also for protein estimation.

INTRODUCTION

Fungi are able to break down resistant materials such as cellulose, gums and lignin. They dominate in acidic, sandy soils and in fresh organic matter. In soils, cellulose is available primarily in the form of litter (dead plant material) that is relatively recalcitrant due to the high lignin content of terrestrial plants. A lack of fixed nitrogen and other nutrients may produce to limit microbial growth, and the low moisture content of soils often favourable the growth of fungi as the dominant cellulolytic biota[1].

Cellulose is the most abundant renewable organic resource. It comprises approximately 45% of dry wood weight. Cellulose-rich plant biomass is one of the sustainable source of fuel, animal feed and feed stock for chemical synthesis [2]. The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages[3,4]. The conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce the environmental pollution[5,6]. The enzymes involved in cellulose degradation are cellobiohydrolase, endoglucanase or carboxymethylcellulase (CMCase) and β-glucosidases[2].

MATERIALS AND METHODS

Collection of soil samples

The soil samples were collected from three different sites (grazing, disturbed, undisturbed) of Meghamalai forest.

Isolation of cellulolytic fungi

The samples was first inoculated on potato dextrose agar (PDA) and later transferred into selective media of Czapek-Dox medium. Cellulolytic fungi creates a clearing zone around the colony of the agar[7]. For identification 'A Manual of Soil Fungi' by Gilman[8] was followed.

Collection of biowaste-substrate (saw dust)

The raw substrates were air dried individually to reduce the moisture content to make them more susceptible for crushing. The crushed substrates were then sieved individually to get powder form. Then the substrates were soaked individually in 1% sodium hydroxide solution (NaOH) in the ration 1:10 (substrate : solution) for two hours at room temperature. After which, they were washed for free of chemicals and autoclaved at 121°C for one hour. The treated substrate were then filtered and washed with distilled water until the wash water become neutral[9].

Inoculum preparation

The isolated culture were maintained as stock culture in potato dextrose (PDA) agar slants. They were grown at 30°C for 5 days and stored at 4°C for regular sub culturing. Inoculum was prepared for each culture using Czapek-Dox broth in Erlenmeyer flasks. The inoculum was kept in shaker (200 rpm) at 30°C for 24 hours before it was used for the fermentation process.

Fermentation process

To 100 ml of the optimized culture medium, a piece of mycelia from respective species was inoculated under controlled conditions. Then it was kept in shaker (200 rpm) at 35°C for a day[9]. Simultaneously, separate media were prepared for saw dust substrates.

Determination of reducing sugar and cellulose activity

Dinitrosalicylic method (DNS)

The total amount of reducing sugars in 1.0 ml supernatant was determined by modified Dinotro salicylic method (DNS)[10]. It is an alternative to Nelson-Somogyi method. It is simple sensitive and adaptable during handling of a large number of samples at a time.

Filter paper assay method (FDA)
Cellulase activity was determined by Filter paper activity method of Stephen *et al.* (2003)[11]. Filter paper activity is a combined assay for Endo β-4, 4-glucanase (Cx) and Exo β-4, 4 glucanase (C1) cellulase.

Estimation of protein
The measurement of specific enzyme activity, the protein concentration of a culture filtrate was determined[12].

Effect of pH on the production of cellulase
The optimized media were prepared using the substrates and the pH was set at different level such as 5, 6, 7 and 8 respectively by adding 1% NaOH and concentrated HCl. Then the media were autoclaved. Later they were inoculated with a piece of mycelila and were placed in a shaker (200 rpm) at 30°C for 5 days. Three replicates were maintained. The enzyme solution obtained from the experiment was individually optimized based on DNS and FPA method.

Effect of temperature on the production of cellulase
The optimized media were prepared using the substrates and autoclaved. Later inoculated with a piece of mycelia and was set at different temperature like 20, 30, 40 and 50°C respectively. The effect of temperature on the production of cellulolytic enzyme was determined by growing the organisms at the above temperatures. Three replicates were maintained, the enzyme solution obtained from the experiment was individually optimized based on DNS and FPA method.

Partial purification of cellulase
Enzyme source preparation
The fungal cultures were grown in optimized media (300 ml) at 30°C for 24 hours individually. The cultures were filtered through Whatman No.1 filter paper.

Salt precipitation of cellulase
Ammonium sulfate was readily obtained pure and was inexpensive, good solubility, stabilizes protein and prevents proteolysis.

Dialysis
Dialysis is commonly used for removing salts from the proteins. The presence of salts in proteins interferes in many ways. Special semipermeable called dialysis tubes have the property to allow compounds with small molecular weight, like proteins which are held back. The protein solution to be desalted is taken inside a dialysis bag and the two ends are secured tightly to prevent leakage. The bag is now suspended in a large vessel containing about 100 fold excess of water of preferably dilute buffer (0.2 M of sodium acetate buffer, pH 5.5) and the contents are kept stirred in cold. Salt molecules pass freely and get diluted by the large volume of fluid in the external medium. Repeated changes of the dialysis fluid help in reducing the salt concentration inside the bag to negligible levels.

Water being freely permeable, would have entered the bag and diluted the proteins also. There are several methods of concentrating this solution like lyophilizatioon and ultrafiltration under vacuum. But a very simple procedure is to carefully bury the dialysis bag with solution in a jar

containing sucrose and kept it in a refrigerator. Water will more out and get absorbed by sucrose. Sucrose being impermeable cannot get inside the bag. The bag now will contain desalted protein.

Statistical analysis
The results obtained in the present were subjected to standard deviation (SD) [13].

Results and Discussion

The fungal work have been done in the fungus like *Trichoderma* sp. [14,15,16]; *Penicillium* sp[14,15,16,17,18]; *Sporotrichium* sp. [19]; *Aspergillus* sp.[20]. In the present study of five fungal species were isolated and identified. They are *Aspergillus niger, Curvulara lunata, Neurospora crassa, Curvularia geniculata* and *Penicillium lanosum*. For testing the cellulolytic activity the fungal species were inoculated into carboxymethylcellulose with congo red agar. They formed clear zones adjacent to microbial growth. Such zones resulted from the action of cellulases on suspended cellulose particles.

Enzyme production at varying pH
In the DNS method, cellulase enzyme production by these organisms was maximum (0.039, 0.034, 0.027, 0.029, 0.032 and 0.041, 0.036, 0.030, 0.030, 0.034 U/ml) at pH 5 and 6 with saw dust as substrate. In the filter paper assay (FPA) method by using saw dust as substrate, high level of enzyme production was obtained at pH 5 and 6 (0.058, 0.045, 0.052, 0.040, 0.055 and 0.060, 0.049, 0.056, 0.046, 0.058 U/ml) (Table 1). The pH was observed that the enzyme activity has a broad pH range between 3.0 and 9.0[21], the activity occurred between pH 4.0 and 7.0, with pH 5.0 being close to optimal for stability [22]; the saw dust is used as substrates, the production of enzyme was optimum at pH 6[23]. Also, this was reported[24,25 and 26].

Enzyme production at varying temperature
The effect of temperature on production of enzymes, the DNS method by using saw dust as substrate showed that high level of production was obtained at 30-40°C that is 0.085, 0.045, 0.068, 0.064, 0.068 to 0.090, 0.070, 0.070, 0.078, 0.085 U/ml with *Aspergillus niger, Curvularia geniculata, C. lunata, Neurospora crassa* and *Penicillium lanosum* respectively. In filter paper assay method, the saw dust used as substrate, high level of enzyme production was obtained at 30-40°C (*A. niger* – 0.088 and 0.095 U/ml, *C. geniculata* – 0.061 and 0.082 U/ml, *C. lunata* – 0.062 and 0.072 U/ml, *N. crassa* – 0.070 and 0.080 U/ml, *P. lanosum* – 0.081 and 0.092 U/ml) respectively (Table 2). Also reported that optimum temperature was observed around 40°C[21]; temperature optima for activity ranged between 45 and 65°C, with the stability optimum between 40 and 50°C[22, 27,28,29 and 24].

Partial purification of cellulase
Ojumu *et al.* [30] studied that saw dust were suitable legnocellulosic biowastes for the production of cellulase enzyme, when compared to that of baggase (or) corncob. In the present investigation of partial purifications were done for all cellulolytic organisms by salt precipitation and dialysis. They showed different results in enzyme activity and protein determination (Table 3). *Aspergillus niger, Curvularia luunata, Curvularia geniculata, Penicillium lanosum* and *Neurospora crassa* with enzyme activities was 0.90, 0.80, 0.81, 0.82 and 0.86 U/ml respectively. However, the *Aspergillus niger* maximum precipitation with enzyme activities.

Likewise the above fungus with total protein 0.98, 0.87, 0.85, 0.90 and 0.88 mg/ml was analysed respectively. *Aspergillus niger* was maximum utilized the protein content by the partial precipitation and dialysis.

Table 1. The effect of pH on the production of enzyme

Cellulolytic organism	pH	Enzyme activity (U/ml)	
		DNS	FPA
Aspergillus niger	5	0.039 ± 0.003	0.058 ± 0.006
	6	0.041 ± 0.002	0.060 ± 0.005
	7	0.038 ± 0.007	0.051 ± 0.007
	8	0.020 ± 0.002	0.041 ± 0.007
Curvularia geniculata	5	0.034 ± 0.004	0.045 ± 0.008
	6	0.036 ± 0.005	0.049 ± 0.003
	7	0.028 ± 0.002	0.032 ± 0.007
	8	0.026 ± 0.004	0.026 ± 0.005
Curvularia lunata	5	0.027 ± 0.002	0.052 ± 0.006
	6	0.030 ± 0.004	0.056 ± 0.006
	7	0.019 ± 0.005	0.046 ± 0.003
	8	0.018 ± 0.004	0.027 ± 0.005
Neurospora crassa	5	0.032 ± 0.005	0.040 ± 0.005
	6	0.034 ± 0.008	0.046 ± 0.005
	7	0.028 ± 0.002	0.035 ± 0.005
	8	0.025 ± 0.004	0.021 ± 0.003
Penicillium lanosum	5	0.032 ± 0.005	0.055 ± 0.003
	6	0.034 ± 0.008	0.058 ± 0.002
	7	0.028 ± 0.002	0.049 ± 0.005
	8	0.025 ± 0.004	0.042 ± 0.006

Table 2. The effect of temperature on the production of enzyme by cellulolytic fungus

Cellulolytic organism	Temperature (°C)	Cellulase (U/ml)	
		DNS	FPA
Aspergillus niger	20	0.077 ± 0.008	0.067 ± 0.004
	30	0.085 ± 0.004	0.088 ± 0.004
	40	0.090 ± 0.010	0.095 ± 0.003
	50	0.049 ± 0.009	0.048 ± 0.004
Curvularia geniculata	20	0.059 ± 0.002	0.050 ± 0.002
	30	0.068 ± 0.004	0.081 ± 0.004
	40	0.085 ± 0.005	0.091 ± 0.006
	50	0.038 ± 0.004	0.042 ± 0.005
Curvularia lunata	20	0.039 ± 0.002	0.055 ± 0.009
	30	0.045 ± 0.004	0.061 ± 0.005
	40	0.070 ± 0.002	0.082 ± 0.007
	50	0.032 ± 0.002	0.039 ± 0.004
Neurospora crassa	20	0.053 ± 0.007	0.058 ± 0.002
	30	0.068 ± 0.004	0.062 ± 0.007
	40	0.070 ± 0.002	0.072 ± 0.004
	50	0.035 ± 0.002	0.039 ± 0.002
Penicillium lanosum	20	0.054 ± 0.002	0.057 ± 0.005
	30	0.064 ± 0.003	0.070 ± 0.007
	40	0.078 ± 0.004	0.080 ± 0.004
	50	0.038 ± 0.004	0.049 ± 0.003

Table 3. Partial purification of cellulase enzyme production at different fungal isolates from soil

S.No.	Fungal isolates	Purification methods	Enzyme activity (U/ml)	Total protein (mg/ml)
1.	*Aspergillus niger*	Crude enzyme	0.78	0.82
		Dialysis	0.90	0.98
2.	*Curvularia geniculata*	Crude enzyme	0.65	0.70
		Dialysis	0.81	0.85
3.	*Curvularia lunata*	Crude enzyme	0.69	0.72
		Dialysis	0.80	0.87
4.	*Neurospora crassa*	Crude enzyme	0.73	0.78
		Dialysis	0.82	0.72
5.	*Penicillium lanosum*	Crude enzyme	0.77	0.83
		Dialysis	0.86	0.90

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