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ISOLATION AND CHARACTERIZATION OF CELLULASE FROM *ASPERGILLUS NIGER*

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ABSTRACT

Aspergillus niger was isolated from soil sample and the characterization of fungi was determined both by macroscopically and microscopically. The fungal isolates were inoculated on the Czapekdox medium with carboxymethyl cellulose as a sole carbon source for the production of cellulase enzyme the production was about 3 ml/100ml broth. Further optimization study was carried out after partial purification to determine the enzyme activity at different pH and temperature which was found to be 50°C and the optical pH was around 6.0.

Keywords: Cellulase, *Aspergillus niger*, carboxy methyl cellulose, optimization.

INTRODUCTION

Cellulose is the most abundant and renewable biopolymer on earth. Cellulose has been used by man for centuries however its enormous potential as a renewable source of energy was recongnised only after cellulose degrading enzymes or "Cellulases" has been identified (Gokhan et al., 2001). Today these enzymes account for approximately 20% of the world enzyme market mostly from *Aspergillus* and *Trichoderma* species. Apart from these major fungal species certain bacteria and *Actinomycetes* are capable of producing cellulase enzymes (Gokhan et al., 2001). The use of filamentous fungi may have a number of advantages than bacteria and yeast, the most important of which being their capability to be propagated on a wider variety of substrates generally discarded as wastes and it involves comparatively less harvesting operation than bacteria and *Actinomycetes*. The Cellulosic materials include cotton, wood sugarcane molasses and paper materials (Fadal, 2000). This is the major factor responsible for the hydrolysis of cellulosic material. A wide range of fungi and few bacterial species are capable of degrading cellulose by the action of extra cellular enzyme cellulase. In the present study cellulase enzyme was produced by the organism. *Aspergillus niger* using carboxy methyl cellulose as a carbon source and the enzyme optimization study was done using different parameters.

MATERIALS AND METHODS

Isolation of *Aspergillus niger* from soil sample: The soil sample was mixed with sterile distilled water and a series of dilutions were made, from the dilutions 0.5 ml volumes are pipetted on to potato dextrose agar (PDA) and incubated for three days. Fungi were isolated from the mixed isolates from each plate and sub cultured on potato dextrose agar, sub culturing were carried till the pure culture was obtained.

Identification of the organism: Colony Morphology - The colonies are wooly at first white to yellow then turning dark black.

Microscopic appearance: Microscopic appearance was performed by tease mount preparation of Lacto phenol cotton blue staining.

Plate screening methodology: Conidia from one week old PDA plates were suspended in Sabouraud dextrose broth. A small well was created in the middle of the carboxymethyl cellulose agar plates and 40µl of suspension was added to it. The plates were incubated at 28°C for 3 days followed by 18 hours incubation at 50°C. Then the plates were stained with 1% Congo red dye for 0.5-1 hour followed by destaining with 1 M Nacl solution for 15-20 minutes (Habib et al., 2005).

Enzyme Production: Cultivation was carried out in Czapek dox liquid medium containing carboxymethyl cellulose (1%) as a sole carbon source. For growth of the organism a liter Erlenmeyer flask containing 250 ml of growth medium was kept at 28°C on an orbital shaker set at 250 rev / min were used for crude enzyme preparation (Ramasamy et al., 1981).

Crude enzyme preparation: To the culture filtrate (100 ml) ethanol previously chilled to - 25°C was added dropwise at 4°C with continuous stirring to the final concentration at 75%and the solution was let at - 20°C for 24 hours. The resultant precipitate was collected by centrifugation at 10,000 rpm for 20 minutes and dissolved in 12 ml of phosphate buffer and again centrifuged to get the precipitate. The precipitates were suspended in 3 ml of phosphate buffer (Chakraborthy et al., 2000).

Purification of the enzyme by Dialysis: Dialysis bag was activated before loading the sample. One end of the dialysis bag was tightly tied and the pelleted enzymes were transferred inside the bag and the bag was tied tightly to prevent leakage. The bag was suspended in a beaker containing phosphate buffer. The beaker was placed on a magnetic stirrer and kept for overnight. Phosphate buffer was changed in interval of every one hour. Then the enzyme was transferred to eppendroff tube (Ramasamy et al., 1981).

Detection of enzyme activity by double layer plate method:To detect the enzyme activity, bottom layer that contained 15 ml of 0.7% (w/w) agarose and 50 mM potassium phosphate citric buffer was added and allowed to solidify. Then the top layer consisting 5 ml of 0.2 % carboxymethyl cellulose and 0.5% agarose was overlayed. The plates were inoculated with 40µl protein extract and were incubated at 30°C for 20 hours. To detect carboxymethyl cellulose activity, plates were stained with 0.1% of Congo red for 30 min and then rinsed with 1 M Nacl as a destainer (Sadasivam, 1996).

Assay of enzymes by dinitrosalicylic acid method: 1ml of citrate phosphate buffer and 0.5 ml of 1% Carboxymethyl cellulose was taken. To this 0.5 ml of enzyme extract was added. The mixture is incubated at 50°C for 30 minutes. To this 3 ml of dinitrosalicyclic acid reagent was added and mixed well and kept at boiling water bath for 5 mins. The absorbance was taken at 540 nm. To determine the optimum temperature and pH of cellulase performed under different temperature and different pH and the results were obtained (Sadasivam, 1996).

RESULTS AND DISCUSSION

Pure culture of the *Aspergillus niger* was isolated on potato dextrose agar. The enzyme Cellulase was screened by Plate screening methodology using CMC agar plate. A Zone of clearance using Congo red was observed. The diameter of clearing zone was measured about 20 cm. Enzyme was purified from the fifth day culture and extraction was done by ethanol precipitation method. The test result show 3 ml / 100 ml broth was collected and stored as 1 ml aliquot in eppendroff tube. Dialysis was performed and the resultant enzyme extract was used for enzyme activity by double layer plate method [DNS method]. The distinct hydrolysis regions were observed shows the efficacy of the enzyme [Table 1]. Optimization of the enzyme was done by using various parameters such as temperature and pH [Table 2 & Table 3]. From the tabular column table 2 and table 3 (i) the optimum temperature at which the maximum cellulase activity was observed at 50°C (ii) the optimum pH at which the maximum cellulase activity was observed at 6.

Cellulase production has attracted a worldwide attention due to possibility of using their enzyme complex for conversion of abundantly available renewable lignocellulosic material for production of carbohydrate for numerous industrial applications. Although bacteria and actinomycetes have been used for the production of cellulase since it shows activities on wide variety of substrates which are cost effective than other organisms. In the present study an attempt was made to isolate the *Aspergillus niger* from soil sample and their efficiency to produce cellulase enzyme by using Czapek dox liquid medium with carboxymethyl cellulose as a substrate. Similar study was done by (Gokhan et al., 2001) reported that *Aspergillus niger* of Z10 wild type produces enzymes which possesses no ability to bind to hydrolyse insoluble microcrystalline cellulose (Avicel) but were active towards soluble carboxymethyl cellulose. In the present study, cellulase activity was studied on the basis of diameter of clearing zone surrounding the small well on the plate screening medium (carboxymethyl cellulose agar). Similar study was done by (Habib Onsori et al., 2005) ATCC 1004 *Aspergillus niger* strain produces clearing zone of 20 mm whose CM case specific activity was determined to be 2.43 (IU/mg). The enzymatic activity was determined by Dinitrosalicylic (DNS) method at different temperature and different pH but optimum temperature and pH were found to be 50°C and 6 respectively similar studies were reported (Paul L Hurst et al., 1977, Premkumar et al., 2015) that optimum temperature and pH were found to be 45°C and 4 respectively.

Table.1.Estimation of Glucose

No	Volume of working standard	Conc. Of working standard	Volume of DNS (ml)	Volume of Potassium Sodium Tartarate 1ml)	Volume of Distilled water (ml)	Optical density
B	-	-	0.5	1.0	3.5	0.00
S1	0.3	300	0.5	1.0	3.2	0.35
S2	0.6	600	0.5	1.0	2.9	0.70
S3	0.9	900	0.5	1.0	2.6	1.05
S4	1.2	1200	0.5	1.0	2.3	1.40
S5	1.5	1500	0.5	1.0	2.0	1.75

Table.2.Enzymatic Activity at Different Temperature

No	Temperature	Test	Control	OD value	Concentration of Glucose (µg / ml)	Enzyme activity (IU/ml)
1	35°C	0.83	0.52	0.31	255	0.047
2	40°C	0.90	0.57	0.33	277	0.051
3	45°C	1.08	0.63	0.45	375	0.069
4	50°C	1.47	0.83	0.64	540	0.099
5	55°C	0.94	0.53	0.41	345	0.063
6	60°C	0.93	0.76	0.17	135	0.024

Table.3.Enzymatic activity at different ph

No	pH	Test	Control	OD value	Concentration of Glucose (µg / ml)	Enzyme activity (IU/ml)
1	4	0.91	0.35	0.56	480	0.088
2	5	1.10	0.51	0.59	510	0.094
3	6	1.51	0.86	0.65	555	0.102
4	6.6	0.79	0.27	0.52	485	0.089
5	7	0.71	0.23	0.48	405	0.074
6	8	0.35	0.01	0.34	285	0.052

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