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Production, Partial Purification and Characterization of Cellulases by *Aspergillus niger* Using Corncob as a Substrate

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ABSTRACT

Aspergillus niger was screened for cellulase production in minimal agar media supplemented with 1% CMC. *Aspergillus niger* was studied for its growth parameters i.e. growth kinetics, effect of temperature, effect of pH on growth. Cellulase was produced using corncob as a substrate employing solid state fermentation. Crude extract obtained was partially purified by ammonium sulphate fermentation upto 70 % saturation and later dialysis was performed. Purified enzyme was characterized for the effect of temperature and pH. Purified enzyme had an activity of 0.009 U/ml/min and was stable in a temperature range of 22°C to 37 °C, it was also stable in a pH range of 5.9 to 7.0.

Key words: *Aspergillus niger*, CMCase activity, Corncob, Partial Purification, Dialysis, Solid State Fermentation.

INTRODUCTION

Cellulases (EC 3.2.1.4.) are enzymes belonging to the class 3 of enzyme nomenclature and are known to be cellulose hydrolyzers. Cellulases have been reported to be a major outcome from sources like fungi, bacteria, and protozoans. Cellulases have been divided into five types based on their mode of

hydrolysis **a) Endocellulase** which breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains ^[1] **b) Exocellulase** which cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or

disaccharides such as cellobiose. c) **Cellobiase** or beta glucosidase hydrolyses the exocellulase product into individual monosaccharides [2] d) **Oxidative cellulases** depolymerize cellulose by radical reactions, as for instance. e) **Cellulose phosphorylases** which depolymerize cellulose using phosphates instead of water.

Cellulases are finding applications in coffee processing, textile industry and in laundry detergents, pulp and paper industry, pharmaceutical industry, biofuel industry etc [3].

Because of the increased demand of cellulases there is a price hike and it is the need of the hour to search for such techniques or substrates which could help reducing the cost of enzyme production. Majorly the increased cost of production is because of cost of substrates and fermentation procedure. The present investigation also focuses on the same and that's why we are using corncob as a substrate which is available at nearly zero cost. We are also trying to use solid state fermentation which is a bit cheaper in comparison to submerged fermentation [4, 5].

MATERIALS AND METHODS

Microorganism

Aspergillus niger strain available at MRD LifeSciences was subcultured on potato dextrose agar plates by point inoculation.

Screening of *Aspergillus niger* for cellulase production

Aspergillus niger was screened for its cellulase producing abilities by the method given in [6] on CMC agar plates in which it was inoculated at the centre of CMC agar media containing 1% CMC by point inoculation and incubated at 28°C for 48hours. After the completion of incubation period the plate was flooded with 0.1% Congo red solution and washed with 1M NaCl for 15–20 min. Plates were observed for zone of cellulose hydrolysis.

Study of growth parameters

a) Growth Kinetics

Aspergillus niger was studied for its growth pattern wherein 100ml PBD was prepared and divided into two flasks containing 80ml and 20ml media respectively. Both the flasks were autoclaved, cooled to room temperature and the flask containing 80ml media was inoculated with *Aspergillus niger*.

Absorbance was read after every 24 hours at 600 nm against uninoculated blank.

b) Effect of temperature on growth of *Aspergillus niger*

In order to have an idea of the optimum temperature for the growth of *Aspergillus niger*, it was inoculated on four sterile PDA plates by point inoculation, and incubated at various temperatures as 28°C, 37°C, 18°C and 50°C for 48 hours. After incubation the growth of isolate was quantified based on visual identification.

c) Effect of pH on growth of *Aspergillus niger*

In order to have an idea of the optimum pH for the growth of *Aspergillus niger*, it was inoculated in four flasks containing 20 ml of PDB each maintained at different pH i.e pH 5.2, 5.6, 5.9 and 6.2 respectively. All the four flasks were incubated in shaker incubator at 120 rpm at 28 °C for 48 hours. After that growth of fungal isolate was studied by reading the absorbance of the flasks at 600 nm against uninoculated PDA.

Production of Cellulases by Solid state fermentation using corncob as substrate

For production of cellulases 20 gm of corncob was dried and cut to small pieces, dried sample was treated with 0.1 %

H₂SO₄. Treated sample was taken in 250 ml flask and moistened with 50 ml of Mineral salt media i.e MSM comprising the following in g/l (0.8 g NaCl , 0.8 g KCl , 0.1 g CaCl₂ , 2.0 g Na₂HPO₄ , 0.2g MgSO₄ , 0.1 g FeSO₄, 8.0 g Glucose, 2.0 g NH₄Cl pH 6.2). Flask was autoclaved, cooled to room temperature, and inoculated with 1 ml of 48 hour grown broth culture of *Aspergillus niger*. Flask was incubated in shaker incubator (120 rpm) at 28°C for 120 hours.

Extraction of crude enzyme

For extraction of crude enzyme 100 ml of 100mM Tris buffer was added to the fermented media and shaking the flask vigorously at 150 rpm for 1 hour. The resultant was filtered by the help of masculine cloth, later by Whatman's filter paper No.1 and centrifuged at 8000 rpm for 5 minutes at 4°C. After centrifugation supernatant was collected and treated as crude enzyme.

Partial purification

Crude enzyme was partially purified by ammonium sulphate precipitation upto 70 % saturation and later the precipitated enzyme was dialyzed in order to remove salts if present.

Protein estimation in crude and purified enzyme

Amount of protein in crude and purified enzyme was determined by Lowry's method [7] of protein estimation, in which 0.5ml of crude enzyme, 0.5ml of distilled water was reacted with 5ml of Lowry's reagent C and 0.5ml of Reagent D and the absorbance was read at 660nm. Absorbance was compared with the standard graph prepared by reacting known concentration of protein ranging from 0.02 mg/ml to 0.20mg/ml with the Lowry's reagents and plotting a graph between concentration of protein BSA (X axis) and OD at 660 nm (Y axis).

Enzyme assay in crude and purified enzyme

CMCase activity in crude and purified enzyme was determined by DNS method of [8] in which 0.1ml of enzyme was reacted with the substrate (1% CMC in Sodium phosphate buffer) for 15 minutes at 28 °C and the reaction was stopped by adding DNS reagent and the test tubes were boiled for 15 minutes and absorbance was read at 540nm. Absorbance was compared with the standard graph plotted by reacting known concentration of glucose (0.05 to

0.5mg/ml) with DNS reagent and plotting a graph between concentration of glucose (X axis) and OD at 540nm (Y axis). One unit CMCase activity was defined as amount of enzyme that releases 1 micromoles of glucose per minute under standard reaction conditions.

Characterization of purified enzyme

Effect of temperature on enzyme activity

Purified enzyme was characterized for the effect of temperature by incubating the enzyme substrate mixture at different temperatures i.e 22°C, 28°C, 37°C and 50°C. After that enzyme activity was determined by DNS method as described earlier.

Effect of pH on enzyme activity

Substrate 1% CMC was prepared in sodium phosphate buffer of various pH 5.2, 5.6, 5.9 and 6.2 and reacted with enzyme under standard conditions using DNS method as described earlier.

RESULTS

Screening of *Aspergillus niger* for cellulase production

Aspergillus niger was screened for their cellulase producing potential and it was

found to be positive in screening as it showed good cellulose hydrolysis zone after staining with 0.1 % congo red and destaining with 1M NaCl.

Study of growth parameters of *Aspergillus niger*

Growth parameters including growth curve, pH and temperature were studied in order to have a proper idea of the stationary phase, optimum temperature and pH of the isolate so that this environment could be provided during fermentation procedure.

a) Growth Kinetics

Table 1 and Figure 1 show the growth kinetics statistics of *Aspergillus niger*, it can be seen that stationary phase reached between day 4-5.

b) Effect of temperature on growth of *Aspergillus niger*

Table 2 shows the effect of temperature and it can be seen that *Aspergillus niger* showed maximum growth at 28 °C.

c) Effect of pH on growth of *Aspergillus niger*

Table 3 shows that pH 6.2 is the optimum pH for the growth of *Aspergillus niger*.

Protein estimation in crude and purified enzyme

Table 1: Growth Kinetics

Protein concentration in crude and purified enzyme was done by Lowry's method of protein estimation. **Table 4** shows concentration of protein in crude and purified enzyme.

Enzyme assay in crude and purified enzyme

Enzyme assay was done by DNS method and the data of enzyme activity has been given below in **Table 5**.

Characterization of purified enzyme

Purified enzyme was characterized for the effect of temperature and pH on enzyme activity, detailed results are discussed below.

Effect of temperature on enzyme activity

Purified enzyme was characterized for the effect of temperature and it was found to be stable between 22 °C to 37 °C. **Table 6 and Figure 3** shows the data of the same.

Effect of pH on enzyme activity

Purified enzyme was characterized for the effect of pH and it was found to be stable between a pH range of 5.9 to 7.0. **Table 7 and Figure 4** shows the results of the same.

S. No.	TIME (IN DAYS)	O.D AT 600nm
1.	0	0.0
2.	1	0.01
3.	2	0.02
4.	3	0.05
5.	4	0.07
6.	5	0.07
7.	6	0.06
8.	7	0.05
9.	8	0.04

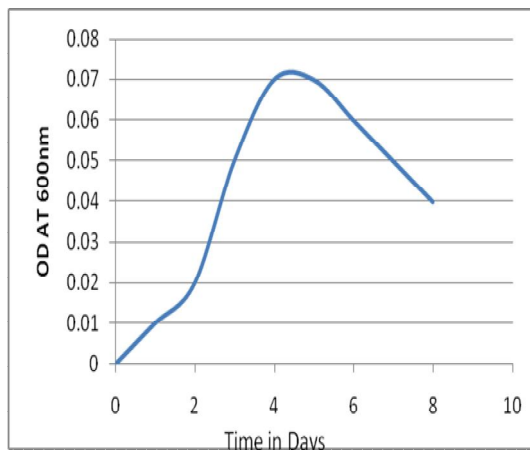


Figure 1: Growth Kinetics

Table 2: Effect of temperature on growth

S. No.	INCUBATION TEMPERATURE (°C)	REMARKS
1.	22	-
2.	28	+++
3.	37	++
4.	50	-

Table 3: Effect of pH on growth

S. No.	pH OF MEDIA	OD AT 600nm
1.	5.0	0.17
2.	5.6	0.13
3.	5.9	0.12
4.	6.2	0.21

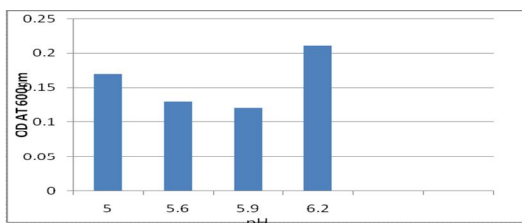


Figure 2: Effect of pH

Table 4: Protein estimation in crude and purified enzyme

S. No.	ENZYME (IN ml)	DISTILLED WATER (IN ml)	REAGENT C (IN ml)	INCUBATED AT ROOM TEMP. FOR 15 MINUTES	REAGENT D (IN ml)	INCUBATED FOR 30 MINUTES IN DARK	O.D.	CONC. OF PROTEIN (mg/ml)
BLANK	0.0	1	5		0.5		0.0	0.0
CRUDE ENZYME	0.5	0.5	5		0.5		0.37	0.125
PURE ENZYME	0.5	0.5	5		0.5		0.15	0.05

Table 5: Enzyme assay of crude and purified enzymes

S No.	ENZYME (in ml)	1% STARCH (in ml)	INCUBATED AT 37°C FOR 15 MINUTES	DNS (in ml)	BOIL FOR 15 MINUTES AT 100°C	O.D AT 540 nm	Enzyme activity (U/ml/min)
BLANK	0	0		1		0.0	0.0
CRUDE ENZYME	0.5	0.5		1		0.76	0.027
PURE ENZYME	0.5	0.5		1		0.26	0.009

Table 6: Effect of temperature on enzyme activity

S. No.	Incubation temperature	OD AT 540nm	Enzyme activity (U/ml/min)
1.	22 °C	0.29	0.01
2.	28 °C	0.29	0.01
3.	37 °C	0.26	0.009
4.	50 °C	0.00	0.00

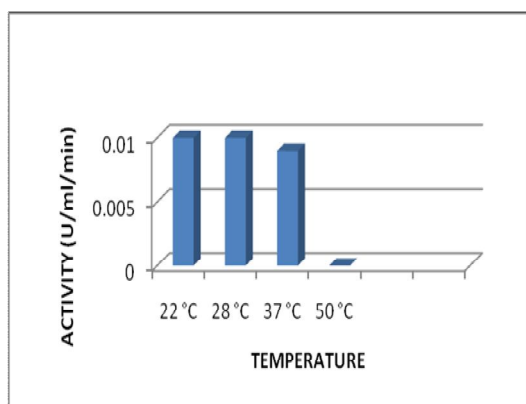


Figure 3: Effect of temperature on enzyme activity

Table 7: Effect of pH on enzyme activity

S. No.	pH	OD AT 540nm	Enzyme activity (U/ml/min)
1.	5.9	0.14	0.005
2.	6.2	0.14	0.005
3.	6.5	0.17	0.006
4.	7.0	0.14	0.005

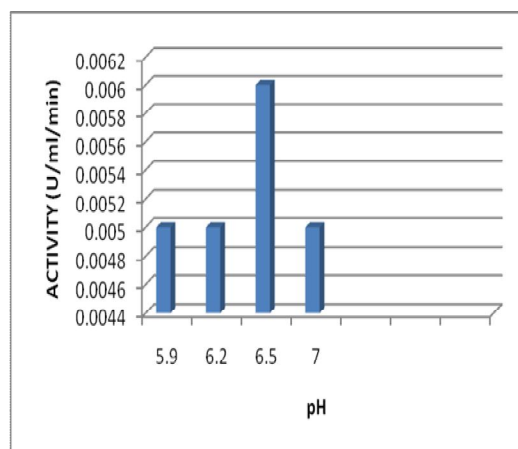


Figure 4: Effect of pH on enzyme activity

DISCUSSION

Aspergillus niger was screened in minimal agar media supplemented with 1 % CMC further the plates were stained with 0.1% congo red and destained with 1M NaCl as given in [6].

Growth parameters of *Aspergillus niger* were studied and it was found that stationary phase was obtained on 4th -5th day, optimum growth was found at 28 °C and pH 6.2.

Solid state fermentation was used to for production of cellulase and corncob was

used as substrate, solid state fermentation has been used earlier for production of cellulases by [9].

Crude enzyme was purified by salt precipitation and dialysis, purified enzyme was characterized for effect of temperature and pH as done earlier by [10]. Purified enzyme was found to be stable between 22 °C to 37 °C and pH 5.9 to 7.0.

CONCLUSION

Based on the research work it can be said that *Aspergillus niger* can be a good source for production of cellulases using corncob as a substrate. Corncob which is available at nearly zero cost can be a good and low cost substrate for production at lower costs which is the need of the time to control the increasing prices of cellulases. Enzyme was stable at a range of temperature and pH thus giving indications of its varied applications.

Future prospects of the present study includes the optimization of some other pretreatment procedures for optimum production of cellulases. Further purification of cellulase enzyme by the help of further sophisticated procedures like ion exchange chromatography and

affinity chromatography etc. And also further characterization of purified enzyme for effect of activators, inhibitors and substrate concentration.

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