Isolation and Characterization of Cellulases from an Ascomycetes Fungus

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***Abstract* – The biodegradation of cellulose under the influence of the polyenzymatic cellulase complex is a fairly involved biochemical process. Its effectiveness is dependent primarily on the composition and properties of cellulose preparations, the structure of substrate, the conditions under which reactions are carried out and the concentration of the hydrolytic products, accumulating in the reaction system. Availability of water is the most important parameter for enzymatic activity of the cellulase. Only a few species among the fungi are able to germinate and grow at water activity value of the substrate below 0.8. As such, commercial textile laundering and dry cleaning stuffs report an increase in fungal damage, mainly caused by damp storage condition. The present work deals with the isolation and Characterization of a cellulose - degrading enzyme from ascomycetes fungus, *Neurospora crassa*. This mutant carrying deletions of two genes encoding extracellular beta-glucosidase lacks beta- glucosidase activity, but efficiently induces cellulase gene expression in the presence of cellobiose, cellotriose or cellotetraose as a sole carbon source.**

***Index Terms* – Biodegradation, Ascomycetes, Cellulase.**

I. INTRODUCTION

Among all the organism, fungi are largely responsible for biodegradation of cellulosic materials under favorable conditions of moisture and temperature. The relative humidity, approaching nearly to 80% and temperature ranging between 25- 35 °C constitute the favorable conditions for the growth of fungi. *Neurospora* is a genus of Ascomycetes fungi. The genus name, meaning 'nerve spore' refers to the characteristic striations on the spores that resemble axons. The best-known species in this genus is *Neurospora crassa*. It metabolizes both cellulose and hemicellulose from plant cell walls. Cellobiose functions as an inducer of lignocellulolytic gene expression in *Neurospora crassa*. The ability to induce cellulase gene expression using a common and soluble carbon source simplifies enzyme production and characterization which could be applied to other cellulolytic filamentous fungi (Elizabeth A Znameroski et al. 2012).

Majority of cellulase enzyme complex comprises of three extracellular enzymes - endoglucanase, exoglucanases and β-glucosidase. The most intensively studied cellulolytic fungus is *Trichoderma reesei* (Bisaria & Ghose, 1981).

The production of endo-β-1,4- and exo-β-1,4-glucanases, β- glucosidase and related enzymes by thermotolerant *Aspergillus fumigatus*, the thermophilic *Geosmithia emersonii* and the mesophilic *A. niger*, *Penicillium funiculosum*, *P. ochrochloron* was greatest after growth on CMC. Enzyme activity in culture filtrates of *P. janthinellum* was shown to be independent of the nature of the substrate.

II. EXPERIMENTAL

I.Materials & Method:

Chemicals – All the inorganic chemicals used were of analytical grade, obtained from BDH Laboratories (Bombay, India). Carboxymethyl cellulose was obtained from Loba Chemie Indo Austranal.

Microorganisms – The cellulolytic fungus *Neurospora crassa* was obtained from Chandra Shekhar Azad Agricultural and Technological University, Kanpur. The culture was grown on potato dextrose agar slants containing filter paper strips as cellulosic substrate at a temperature of 30 ± 2 °C for a period of ten days and maintained at 4 °C by subculturing every month.

Culture Conditions & Growth Media – From ten days old cultures, spores' suspension was prepared by adding sterile distilled water to the culture tubes under aseptic conditions. The suspensions were filtered through sterile muslin cloth. Three milli liters of spores' suspension was transferred to a 500 ml Erlenmeyer Flask containing 100 ml of sterile medium adjusted to pH under aseptic conditions and incubated at 30 ± 2 °C on rotatory shaker (200 cycles/min) for a period of ten days.
Various growth media were tried for maximum growth of the organism and the maximum elaboration of the enzyme from the culture.
(Table – 1)

Elaboration of Enzymes – On 7th day of growth of the organism, the mycelia were harvested by filtration through four layers of cheese cloth. The culture filtrates were directly centrifuged at 5,000 rpm for 20 minutes at 4 °C. Simultaneously, the mycelial mat was collected on preweighed Whatman filter paper No.1, washed with distilled water and dried at 70 °C until constant weight was obtained. The mycelial growth was expressed in mgs. dry weight per 100 ml flask. The supernatant (metabolic liquor) obtained was used as a source of crude Extracellular Enzyme preparation.
After removing the supernatant, the mycelial mat thus obtained, was dried between the folds of filter paper. The partially dried mycelia were crushed in a grinder with small amount of distilled water which was diluted to obtain 2.0

% solution of mycelia. This suspension was used for the study of Intracellular Enzymes.

II. Result and Discussion:

The comparative dried cell mass produced by the fungus *N. crassa* as well as the enzyme elaborated by it, is given in Table – 2.

From Table – 2 it is evident that the fungus *Neurospora crassa* showed its maximum growth and maximum elaboration of cellulolytic enzyme in Malt-yeast extract medium. The maximum cellulase activity from two different strains of *N. crassa* was reported in yeast- malt extract- agar medium (Rao et al.1983).

The conditions under which maximum elaboration of Cellulolytic Enzymes takes place were determined by studying the optimum conditions for enzyme activity. The enzymes were characterized for the following properties:

1) Optimum temperature

2) Optimum pH

3) Optimum period of incubation.

Optimum temperature for enzyme activity-

The clear metabolic liquor, obtained from the culture filtrate of *N. crassa* was incubated with 1.0% (w/v) solution of carboxymethyl cellulose (substrate) at different temperatures ranging from 25 to 70 °C. The enzyme activity was assessed by Nelson- Somogyi method as reducing sugars formed at the end of enzyme - substrate reaction. The results obtained are recorded in Table – 3. From the Table it is evident that the maximum activity of enzyme, elaborated by *N. crassa*, is observed at 35 °C.

Optimum pH for enzyme activity-

The clear metabolic liquor, obtained from the culture filtrate of *N. crassa* was incubated with carboxymethyl cellulose as substrate at 35 ± 2 °C. The enzyme activity of the fungus was assessed for reducing sugars formed at different pH levels, ranging from 3.0 - 7.5, using 100 mM citrate - phosphate buffer. The results obtained are recorded in the Table – 4.

From the table it is clear that the enzyme activity was maximum between pH 5.0 to 5.3.

The crude enzyme preparation from *Sporotrichum cellulophilum* had an optimum pH of 5.0-5.5 at 37°C with KC-Floc as substrate, and it was found to be stable in the pH range of 4.0 to 7.5 at the same temperature (Shinichi et al., 1986). The optimum pH for all the isoenzymes (including cellulases) from species of genus *Aspergillus* was found to be 5.0 (Sharma et al.,1991).

Optimum period of incubation for enzyme activity-

The clear metabolic liquor from the culture filtrate of the fungus was incubated at 35 ± 2 °C with 1.0% concentration, maintained at pH 5.0-5.2, 100 mM citrate phosphate buffer, for different periods. After different periods of incubation, the reaction mixture was deactivated and cellulase activity was assayed for the reducing sugars formed at the end of the reaction. The results obtained are given in the Table – 5.

From the Table it is clear that *N. crassa* shows a maximum enzyme elaboration on the 7th day of incubation which goes on decreasing gradually with time. Qingxin et al., (2014) isolated and identified *N. crassa* as a cellulase - producing strain grown on oil palm empty fruit bunch. According to them the fungus started to secrete cellulases into the medium after 24h of cultivation at 30°C and reached its maximal cellulase activity at 240h.

Table 1

Various growth media

(i) Czapek's medium (g/L)

Sodium nitrate	2
Potassium dihydrogen phosphate	1
Potassium chloride	0.5
Ferrous sulphate	0.01
Magnesium sulphate	0.5
Sucrose	30
Distilled water	1 L
Temperature	28± 1°C
1.2% Carboxymethyl cellulose (as substrate)	3.5
pH	5.2

(ii) Modified Omeliansky's medium (g/L) [Verma et al. 1982]

Dipotassium hydrogen phosphate	1.00
Ammonium chloride	1.00
Sodium chloride	Traces
Magnesium sulphate	0.50
Calcium carbonate	2.00
Carboxymethyl cellulose	15.00
Distilled water	1 L
Temperature	30±2°C
pH	6.2 to 6.4

Table I contd...

(iii) **Malt-yeast extract medium**

Yeast extract	3 g
Malt extract	3 g
Peptone	5 g
Alkali treated cellulose powder (ATCP)	10 g
1% D- glucose	
Temperature	28°C
pH	5.0

Cellulose powder was treated with 1N NaOH in a 20% slurry and autoclaved at 15 lb for 20 minutes, washed free of alkali with distilled water.

Table 2

Suitable medium for growth and elaboration of enzymes by Neurospora crassa

Medium of growth	Cell mass (mg/10c ml)	Enzym activity (ug/ml)
Malt - yeast extract medium	58	500
Czapek's medium	50	410
Modified oxeliansky's medium	52	480

Number of determinations wre three in each case.

Table 3

Optimum temperature for enzyme activity

Temperature of Incubation	Enzyme activity (µg/ml) N. crassa
25	400
30	430
32	472
35	490
37	470
40	410
45	320
50	300
55	270
60	110
65	50
70	15

Table 4

Optimum pH for enzyme activity

pH	Enzyme activity (ug/ml)
	N. crassa
3.0	23
3.4	82
3.8	184
4.2	380
4.6	430
5.0	500
5.3	500
5.5	470
6.0	418
6.8	298
7.0	212
7.5	80

Table 5

Optimum period of incubation for enzyme activity

Period of Incubation (days)	Enzyme activity (ug/ml)
	N. crassa
1	30
2	80
3	170
4	300
5	360
6	410
7	500
8	490
9	480
10	480
11	400
12	320

III. CONCLUSION

The cellulolytic enzymes isolated from the fungus *Neurospora crassa* showed maximum enzyme activity at 35 °C and pH 5.0 to 5.3. The fungus shows maximum enzyme elaboration on the 7th day of incubation which goes on decreasing gradually with time.

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