Isolation and Characterization of Cellulolytic Enzymes from *Aspergillus niger*

Dr. Arti Saxena

Associate Professor & Head, Department of Chemistry, A.N.D.N.N.M. College, Kanpur

Abstract **– Under favourable environmental conditions, like moisture, temperature, oxygen, sunlight etc, the spores of various microorganisms including bacteria and fungi present in the atmosphere attack the cellulosic materials and start growing on them. The microorganisms, especially fungi, grow rapidly and produce profuse mycelium, presenting the appearance of mat on the substrate. They may enter the substrate through some weak points or imperfections like cracks, abrasions and other mechanically damaged spots, formed during manufacture or dirting use. The hyphae enter the cellulosic fibre by direct penetration of fibre wall and remain inside the lumen. These hyphae start ramifying inside the lumen that can be observed under the microscope. In order to utilise the cellulosic substrates as food materials, the microorganisms produce cellulolytic enzymes which hydrolyse the cellulose to water soluble products that can be assimilated easily by the cell and take part in metabolism. The cellulolytic enzymes are mainly of two types-- Extracellular Enzymes and Intracellular enzymes. The present work deals with the favourable conditions for maximum isolation of Cellulase Enzymes from *Aspergillus niger* and studying their characteristics.**

Index Terms **– Extracellular, Intracellular , Cellulase, Enzymes.**

I. INTRODUCTION

Cellulases are the enzymes produced chiefly by microorganisms, mainly fungi, that catalyze cellulolysis, i.e., the decomposition of cellulose and of some related polysaccharides. Cellulases breakdown the cellulose molecules into monosaccharides, such as, β-glucose or shorter polysaccharides and oligosaccharides. The specific reaction involved is the hydrolysis of the 1,4-β-D-glycosidic linkages in cellulose, hemicellulose, lichenin and cereal β-D-glycans.

Cellulose is the most abundant organic material in nature and is put to practical uses in every walk of life, such as, cotton textiles, linen goods, paper and pulp products, rubberized articles, leather, rexine and plastic products, such as, ropes, twines, water proof covers and many other articles used in defence services under tropical and sub-tropical conditions.

The first hypothesis explaining cellulose biodegradation in fungi was put forward in the early 1950s by REESE. Corrosion of the metallic surfaces occurs as a result of microbial growth (Gupta et al 1973). Even the electronic components have not been spared from fungal damage. The most severe deterioration in indoor environments is primarily caused by cellulolytic fungi attacking textiles, resulting in loss of fiber strength and actual material failure. Other microbial spoilage can occur as a result of the permanent staining from pigmentation and mycelial penetration by cellulolytic fungi.

The rate of cellulolysis for a given organism is limited by several factors including the nature and amount of enzyme secreted, the sensitivities of enzymes to end product inhibition and the nature of cellulosic substrate. The cellulases synthesized by the cellulolytic fungi consists of three principal types of enzymes, namely, exo-(1,4)- β-glucosidase. The exoglucanases and endoglucanases hydrolyze cellulose to the disaccharide cellobiose, which in turn is hydrolyzed to glucose by β- glucosidases. The β-glucosidase not only produces glucose but also reduces the inhibition of cellobiose degradation, allowing the cellulolytic enzymes to function more efficiently. The result is a rapid measurable shortening of chain of molecules of β- D- glucose that are linked together to form a polymer of 7,000 to 10,000 units. This causes a decrease in the degree of polymerization (Shnabel, 1981; Fahnrich et al., 1981). This shortening of chain leads to a considerable decrease in tensile strength. Fungi, such as *Aspergillus niger*, are potential β- glucosidase sources. Wyk & Mohulatsi (2003) studied the biodegradation of waste paper by cellulase from *Trichoderma viride*.

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 *Aspergillus niger* DMSRDE No.51 was obtained from Defence Materials Stores Research and Development Establishment, 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 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 A. niger as well as the enzyme elaborated by it, is given in Table – 2.

From Table 2 it is evident that the maximum growth of *Aspergillus niger* and the maximum enzyme production by it was observed in Czapek's medium as compared to that in Omeliansky's medium. In 1980 Dholakia and Chhatpar reported the mycelial dry weight of *Aspergillus* sp.1 (isolated from blue color) to be as high as 405 mg/ 100 ml flask without any inhibitor and the corresponding cellulase activity to be equal to 650 units/100 ml. Later Chopra et al. (1989) reported the mycelial mass of three isolates of *Aspergillus niger* without any antibiotic, to be equal to 358, 340, and 360 mgs., respectively. The culture of A. niger grows well in a basal medium containing alkali - treated corn cobs as the sole carbon source (Singh et al. 1990). The spore bearing fungus A. japonicus was found to grow well in 1.5 / malt medium (Sanyal et al., 1988). This organism produced a thick mycelial mat and was also capable of utilizing the sodium salt of carboxymethyl cellulose and jute powder. Several factors play important roles in the successful bioconversion of cellulose that include, nature of cellulose, source of Cellulolytic Enzymes, optimal condition for catalytic activity (like temperature, presence or absence of oxygen) and production of enzymes (Chandra et al.; 2007).

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 *Aspergillus niger* 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.

The results indicate that the maximum activity of the enzyme is observed at 32 °C and at pH 5.3 and a further increase in temperature results in decrease in enzyme activity. Rudick et al. (1979) reported 37 °C as the optimum temperature for maximum enzyme activity of *A. niger*. Rudick and Elbein (1973)

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 2

Suitable medium for growth and elaboration of enzymes by Aspergillus niger

Medium of growth	Cell mass (mg/100 ml)	Enzyme activity (ug/ml)
Czapek's medium	370	710
Modified Omeliansky's medium	340	590

Number of determinations were three in each case.

Table 3

Optimum temperature for enzyme activity

Temperature of Incubation	Enzyme activity (µg/ml) A. niger
25	525
30	680
32	715
35	610
37	564
40	490
45	420
50	380
55	360
60	310
65	170
70	65

Table 4

Optimum pH for enzyme activity

pH	Enzyme activity (ug/ml)
	A. niger
3.0	48
3.4	90
3.8	160
4.2	480
4.6	510
5.0	660
5.3	700
5.5	680
6.0	500
6.8	380
7.0	350
7.5	92

Table 5

Optimum period of incubation for enzyme activity

Period of Incubation (days)	Enzyme activity (ug/ml)
	A. niger
1	325
2	460
3	675
4	710
5	600
6	525
7	520
8	500
9	486
10	450
11	450
12	434

reported that the enzymes elaborated by A. fumigatus have optimum pH of 5.0, whereas the enzyme activity of *A. terreus* was reported to be stable and maximum at pH 4.5-5.0 (Zeltins and Edzins, 1976).

Optimum pH for enzyme activity-

The clear metabolic liquor, obtained from the culture filtrate of A. niger 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.

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--7.5 at the same temperature (Shinichi et al., 1986). Knapp and Legg (1986) described that there is a drop in the enzyme activity produced by *Trichoderma reesei* as pH increases from 3 to 4, followed by a rise to a maximum without pH control. Sharma et al., (1991) found that pH 5.0 was optimum for all the isoenzymes (including cellulases) from species of genus *Aspergillus*.

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 the enzyme elaboration reaches maximum on the 4th day of incubation and then decreases gradually. A similar type of result was obtained by Dholakia and Chhatpar (1980) with *Aspergillus* sp. which was found to be responsible for the spoilage of blue poster color. Ali et al (1991) reported the maximum yield of cellulases from *A. terreus* after 220 h of incubation.

III. CONCLUSION

The cellulases isolated from the fungus *Aspergillus niger* showed maximum enzyme activity at 32 °C and pH 5.3. A further increase in temperature resulted in a decrease in enzyme activity. Studies on optimum period of incubation showed that the enzyme activity reached maximum on 4th day of incubation, decreasing gradually with time.

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