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SCREENING AND CHARACTERIZATION OF FUNGAL CELLULASES ISOLATED FROM THE NATIVE ENVIRONMENTAL SOURCE

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Abstract

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc. This study was aimed to screen the cellulytic ability of fungi from native environmental source. Furthermore, optimal condition for enzyme activity and induction of enzyme synthesis were also determined.

Out of 115 fungal cultures isolated from environmental sources including soil, air and infected plant, 78 (67.83%) were found to possess cellulose degrading ability. Cellulytic fungi belonged to *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp., *Alternaria* sp., *Penicillium* sp., and *Rhizopus* sp. Cellulase production by fungi was measured by using enzyme assays.

Highest yield of enzyme was noted at 37°C while maximum activity in the range of pH 4 - 4.8. Kinetics of enzyme production was also studied on selected *Aspergillus* isolates and mostly high enzyme production was observed after 7 days. Cellulase synthesis increased by ~10 folds in the presence of cellulose while it repressed in the presence of glucose.

Introduction

Cellulose is the most abundant component of plant biomass. It is found in nature almost exclusively in plant cell walls, although it is produced by some animals e.g., tunicates and few bacteria (Lynd *et al.*, 2002). Perpetual renewal of plant biomass *via* the process of photosynthesis ensures an inexhaustible supply of such material. Any process which could efficiently and economically convert cellulytic material to glucose would be of immense industrial significance (Walsh, 2002).

Cellulose is totally insoluble in water (Lederberg, 1992). It is a linear, unbranched homopolysaccharide consisting of glucose subunit joined together *via* β 1-4 glycosidic linkages. Individual cellulose molecules (polymer) vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2002). Within the bundles, cellulose molecules can occur in crystalline or paracrystalline (amorphous) structures (Walter, 1998).

Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992). The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa (Alexander, 1961). However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al.*, 2002).

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The initial step in cellulose destruction is the enzymatic hydrolysis of polymers. The enzyme or enzyme-complex involved in the hydrolysis has been given name as cellulases. Cellulase catalyzes the conversion of insoluble cellulose to simple, water-soluble products (Alexander, 1961). Cellulase is a multienzyme system composed of several enzymes with numerous isozymes, which act in synergy (Grassin & Fauquembergue, 1996). The basic enzymatic process for the depolymerization of cellulose requires three types of enzymes: Endoglucanase (EG or CX), hydrolyses internal β-1,4 glucan chain of cellulose at random, primarily within amorphous regions and display low hydrolytic activity toward crystalline cellulose (Walsh, 2002; Grassin & Fauquembergue, 1996); Exoglucanase i.e., exoacting cellobiohydrolases (CBH), removes cellobiose from the non-reducing end of cello-oligosaccharide and of crystalline, amorphous and acid or alkali treated cellulose;. Cellobiase or β-glucosidase (BGL), hydrolyses cellobiose to yield two molecules of glucose which completes the depolymerization of cellulose (Himmel *et al*., 1994).

Cellulases have enormous potential in industrial applications. Glucose produced from cellulosic substrate could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol, butanol, methane, amino acid, single-cell protein etc., (Walsh, 2002). Cellulases have been used for several years in food processing, feed preparation, waste-water treatment, detergent formulation, textile production and in other areas. Additional potential applications include the production of wine, beer and fruit juice. Nevertheless, all these uses are of rather small magnitude compared with cellulase requirements for bioconversion of lignocellulosic biomass to fuel ethanol (Philippidis, 1994).

Considering the importance and application of the cellulases, this study was aimed to screen the indigenous fungal isolates for the cellulytic ability. Furthermore, this study aims to provide better understanding of condition for the production and activity of cellulases by different fungal cultures.

Materials and Methods

All reagents and chemicals used were purchased from Merck/ Oxoid/ BioM.

Isolation of fungi: Isolation was made from different environmental sources including soil, air and infected wheat plants by using enrichment technique. Two methods were used for isolation of fungi from soil. In one method, diluted soil samples were inoculated in a mineral salt medium (Mandels *et al*., 1976) containing Whatman # 1 filter paper strip of 6x1cm, NH_4Cl (0.267%), Na_2HPO_4 (0.535%), $CaCl_2$. $2H_2O$ (0.000006%), $MgSO_4.7H_2O$(0.000006%), $MnSO_4.7H_2O$ (0.0000042%) and $FeSO_4.7H_2O$ (0.0000024%) followed by 1-2 weeks incubation at room temperature. Fungal growth appearing on filter paper was isolated and transferred to Sabouraud dextrose agar (SDA) slants. In another method, soil samples were supplemented with saw dust and synthetic medium (Modified Mandels Medium) containing peptone (0.1%), urea (0.03%), $MnSO_4.H_2O$ (0.0016%), $ZnSO_4.7H_2O$ (0.0014%), $(NH_4)_2SO_4$ (0.14%), $MgSO_4.7H_2O$ (0.03%), $FeSO_4.7H_2O$ (0.05%), $CaCl_2$ (0.01%), $CoCl_2.6H_20$ (0.0029%), KH_2PO_4 (0.2%). This mixture was kept for about one month at room temperature followed by dilution of soil samples and isolation of fungi on SDA, using pour plate method. Isolation of fungi from infected parts i.e., leaf and stem of wheat plant was made by directly scraping the fungal growth and inoculation on SDA; indirectly, by using diluted rhizosphere soil samples of infected

wheat plant, followed by inoculation on SDA. Environmental fungal isolates from air were collected by settle plate method. Each fungal culture which was isolated on SDA slants was then stored at 3 - 4°C in a refrigerator, until needed.

Identification of fungi: Fungal isolates were identified on the basis of routine cultural characteristics and morphological characteristics.

Growth and culture conditions for enzyme production: Each fungal isolate was inoculated in a synthetic medium containing cellulose (ball milled cellulose or Whatman # 1 filter paper) and incubated at 30°C in a shaking water bath (180 rpm) for 7 days. Variation in temperature and time period of incubation was done depending upon the requirement of experiment.

Collection and storage of culture supernatant: Cell-free culture supernatant was prepared after 7 days of incubation which varied depending upon the requirement of experiment by centrifugation at 8000 rpm for 10 minutes. Aliquots of supernatant were dispensed in sterile eppendrof tubes and stored at –20°C.

Enzyme assays

Test tube assay: Initial screening was made by supplementing synthetic agar medium with ball milled cellulose. Agar tubes were allowed to solidify in ice-bath to ensure the uniform distribution of cellulose in the tube. Isolated fungal cultures were inoculated on agar surface and tubes were incubated at room temperature. Vertical zone of clearance from agar surface was measured for cellulytic activity after 7 and 14 days.

Filter paper assay (for total cellulase activity): Cellulase activity was determined by a method of Mandels *et al*., (1976). An aliquot of 0.5 ml of cell-free culture supernatant was transferred to a clean test tube and 1 ml of Sodium citrate buffer (pH 4.8) was added. Whatman #1 filter paper stirp (6 cm ×1 cm) was added to each tube. Tubes were vortexed to coil filter paper in bottom of the tube. Tubes were incubated in a water bath at 50°C for 1 hour followed by an addition of DNS reagent (3 ml). Tubes were then placed in a boiling water bath for 5 minutes and then in an ice-bath, followed by the addition of 15 ml distilled water to each tube. Contents of the tube were mixed and absorbance was noted at 550 nm. Cellulase activity was expressed in term of filter paper unit (FPU) per ml of undiluted culture filtrate. A filter paper unit (FPU) is defined as mg of reducing sugar liberated in one hour under standard assay conditions. Reducing sugar produced in one hour was calculated by comparing A_{550} with that of standard curve.

Carboxymethyl cellulase "CMCase" (endoglucanase) assay: CMCase activity was determined by the method of Mandels *et al*., (1976) as described above with a modification of using 0.5 ml of 1% (w/v) CMC as a substrate instead of filter paper strips. Enzyme activity was determined in terms of International Unit (IU) which is defined as an amount of enzyme that produces one μ mole of glucose per minute.

Cotton assay: Cotton degrading activity was determined by the method of Mandels *et al*., (1976). In a test tube 100 mg of cotton was transferred and 0.1 ml of buffer

concentrate (1.0% Sodium citrate buffer; pH 4.8) added. An aliquot of enzyme (2 ml) was added and mixed by squeezing out air bubbles with spatula. Tubes were incubated at 50°C in water bath for 24 hours followed by addition of 3 ml DNS reagent. Tubes were then placed in boiling water bath for 5 minutes, cooled in ice-bath and 14.4 ml of water was added to the tubes and A_{550} was noted. One unit of enzyme activity is defined as milligram of glucose produced by 1 ml of enzyme preparation under standard assay condition.

Effect of temperature on enzyme production: Fungal isolates were inoculated in the synthetic medium followed by incubation at 30°C and 37°C. Enzyme production was measured after 7 days of incubation by filter paper assay.

Effect of pH on enzyme activity: Effect of pH on enzyme activity was determined by incubating enzyme (culture supernatant) in buffers of different pH for 1 hour during standard filter paper assay. The buffers used were 0.05M Sodium citrate (pH 3.2, pH 4.0 & pH 4.8) and 0.1M Phosphate buffer (pH 6.2 & pH 7.2).

Studies on kinetics of enzyme production: Kinetics of enzyme production was studied for different *Aspergillus* species by growing them in synthetic media supplemented with ball milled cellulose; incubating at 30°C and withdrawing samples of cell-free culture supernatant at 0, 4, 7 and 10 days intervals. Enzyme activity was measured by filter paper assay.

Enzyme induction studies: *Aspergillus niger* was inoculated in a synthetic medium containing glucose as carbon source and grown for 3 days at 30°C in shaking water bath (180 rpm). The fungal growth was then separated by centrifugation at 8000 rpm for 10 minutes, washed with sterile distilled water and divided into two aliquot, one was inoculated in synthetic medium containing glucose as a sole carbon source and second in a synthetic medium containing cellulose (Whatman # 1 filter paper) as a sole carbon source. Both the flasks were incubated at 30°C for 7 days in a shaking water bath followed by determination of enzyme activity in cell-free culture supernatant by filter paper assay.

Results

Isolation and identification of fungi: A total number of 115 isolates of fungi were isolated and identified. Among these, 61 isolates from well aerated and cultivated soil samples belonged to genera *Aspergillus* (42), *Penicillium* (3), *Trichoderma* (3), *Alternaria* (4), *Mucor* (3), *Botrydioplodia* (2), *Microsporium* (1) and *Helminthosporium* (3). Thirty-seven isolates from infected wheat plants or from rhizosphere soil were identified as *Alternaria* (9), *Penicillium* (4), *Aspergillus* (10), *Microsporium* (4), *Rhizopus* (1), *Mucor* (3), *Trichoderma* (1), *Fusarium* (1), *Gliocladium* (3) and *Cladosporium* (1). From air 17 fungal isolates were isolated and identified as species of *Aspergillus* (5), *Alternaria* (1), *Fusarium* (4), *Rhizopus* (3) and *Monilia* (4). Most frequently isolated genus from almost all the environments was *Aspergillus* and *Aspergillus niger* was most predominant species (Table 1).

Table 1. Screening and measurement of cellulase activity and production of endoglucanase by fungal isolates.

Name of Isolates	Number of isolates	Percentage (%)	Zone of Clearance in mm	IFPU		Endoglucanase (IU/ml)	
				Range	Avge	Range	Avge
Aspergillus niger	22	19.13	1.5-7	0.0055-0.33	0.123	0.005-0.415	0.193
Aspergillus flavus	3	2.61	2-10	0.117-0.163	0.137	0.049-0.075	0.06
Aspergillus niveus	1	0.87	18	0.287		0.276	
Aspergillus tereus	6	5.22	1-10	0.069-0.3	0.164	0.031 - 0.337	0.14
Aspergillus fumigatus	1	0.87	10	0.27		0.108	
Aspergillus nidulans	4	3.48	2-7	0.009-0.3	0.188	0.042-0.367	0.16
Aspergillus wentii	1	0.87	0.3	0.081		ND	
Other Aspergilli	19	16.52	0-15	0-0.37	0.123	0 - 0.552	0.132
Penicillium sp.	7	6.09	0-6.0	0-0.139	0.078	0 - 0.075	0.045
Alternaria sp.	14	12.17	0-7.0	0-0.205	0.056	0 - 0.382	0.106
Fusarium sp.	5	4.35	0-11.0	0-0.059	0.023	0 - 0.084	0.059
Rhizopus sp.	4	3.48	0-4.0	0-0.222	0.103	0 - 0.234	0.141
Cladosporium sp.	1	0.87	1	0.052	0.052	ND	
Trichoderma harzianum	1	0.87	1	0.034	0.034	ND	
Trichoderma longibrachiatum	1	0.87	2	0.059	0.059	ND	
Trichoderma pseudokoningii	1	0.87	2	0.059	0.059	ND	
Trichoderma sp.	1	0.87	1	ND		ND	
Botrydioplodia sp.	2	1.739	0	ND		ND	
Gliocladium sp.	3	2.61	0	ND		ND	
Helminthosporium sp.	3	2.61	0	ND		ND	
Mucor sp.	6	5.22	0	ND		ND	
Microsporium sp.	5	4.35	0	ND		ND	
Monilia sp.	4	3.48	0	ND		ND	
Total	**115**	100.019					

Table 2. Degradation of crystalline cellulose by fungal isolates evaluated by using cotton assay.

Isolates	Enzyme activity (U/ml)
Aspergillus niger SJ1	1.24
Aspergillus niger SJ2	0.8
Aspergillus niger SJ3	0.76
Aspergillus niger SJ4	1
Aspergillus sp.	0.96
Aspergillus tereus	0.64

Screening of fungal isolates for cellulytic activity: Screening performed by using test tube assay showed that out of 115 fungal isolates, 78 (67.83%) isolates were cellulase producer. Most of the cellulase producers belonged to *Aspergillus* (52) followed by *Trichoderma* (4), *Alternaria* (10), *Fusarium* (4), *Penicillium* (4), *Rhizopus* (4) and *Cladosporium* (1). However, some isolates of *Aspergillus* (5), *Alternaria* (4), *Fusarium* (1), *Penicillium* (3) and *Rhizopus* (1) and all the isolates belonging to the genus *Botrydoplodia, Gliocladium, Helminthosporium, Mucor, Microsporium* and *Monilia* did not show any cellulytic activity (Table 1).

Measurement of cellulytic activity: Filter paper assay and endoglucanase assay carried out respectively for measurement of total cellulase and endoglucanase activity (Table 1) while cotton assay was performed against 6 *Aspergillus* isolates for their ability to degrade crystalline cellulose (Table 2).

Effect of temperature on enzyme production: Different species of *Aspergillus* when grown in synthetic media showed that majority of them have high degree of enzyme production at 37°C while some *Aspergillus* species showed high degree of enzyme production at 30°C (Fig. 1).

Effect of pH on enzyme activity: Most of the fungal isolates showed maximum activity at pH values ranging between 4-4.8. Differences in decline patterns were noted in all the test strains as the pH value deviate from optimum pH (Fig. 2).

Kinetics of enzyme production: Most of the fungal isolates showed high degree of enzyme production after 7 days which started to decline after 10 days of incubation (Fig. 3).

Induction of enzyme: An induction of 10 fold enzyme activity was observed when *Aspergillus niger* was cultured on synthetic media containing cellulose as a sole carbon source. A low level of enzyme activity was however observed when fungus was grown in a medium containing glucose as a carbon source. Available literature suggests that the production of cellulases is tightly regulated. In most cellulose degraders, readily utilizable carbon sources generally repress cellulase synthesis by a mechanism thought to be similar to catabolite repression (Lederberg, 1992).

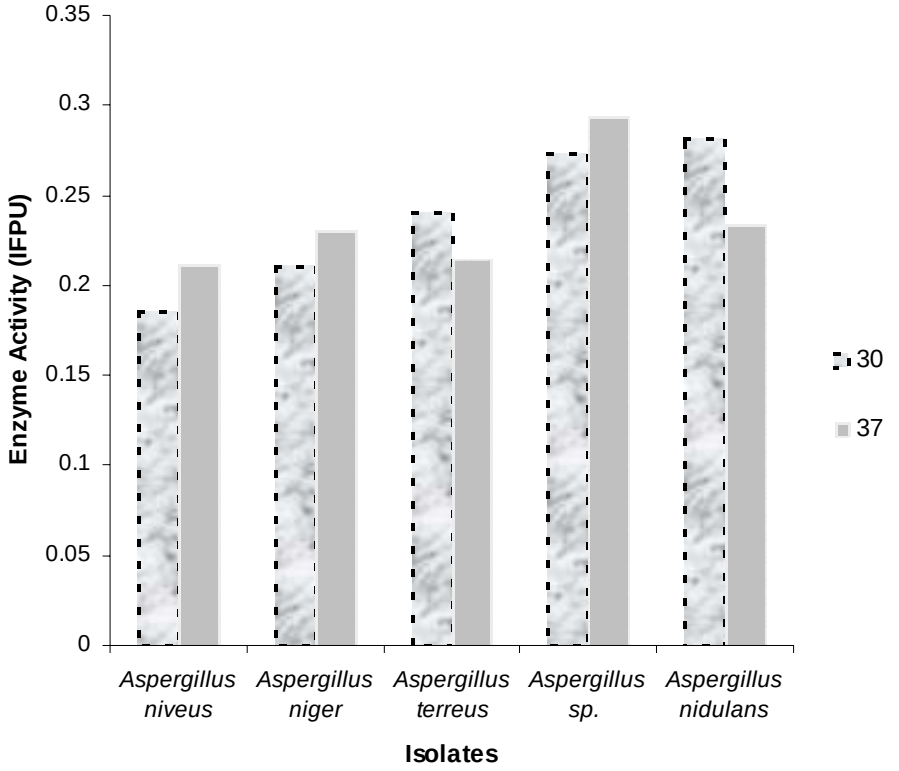

Fig 1. Effect of temperature on enzyme activity.

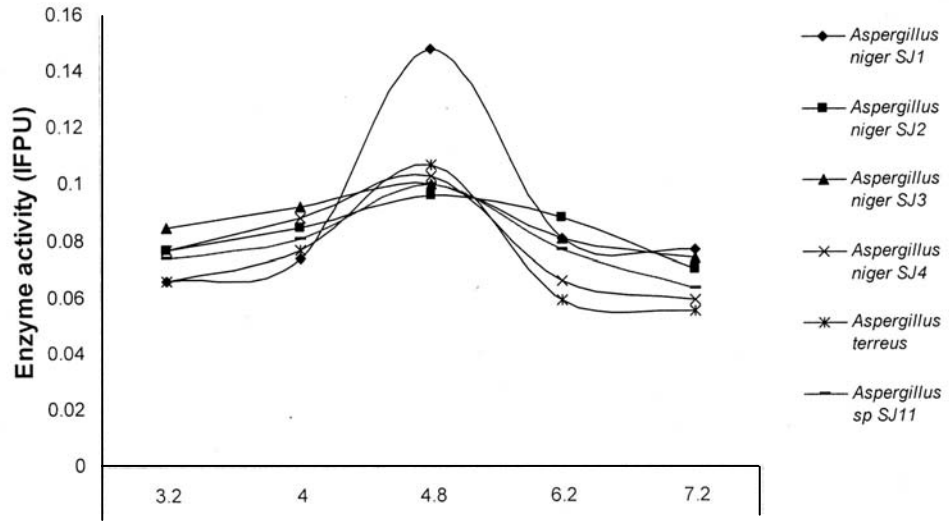

Fig 2. Effect of pH on enzyme activity.

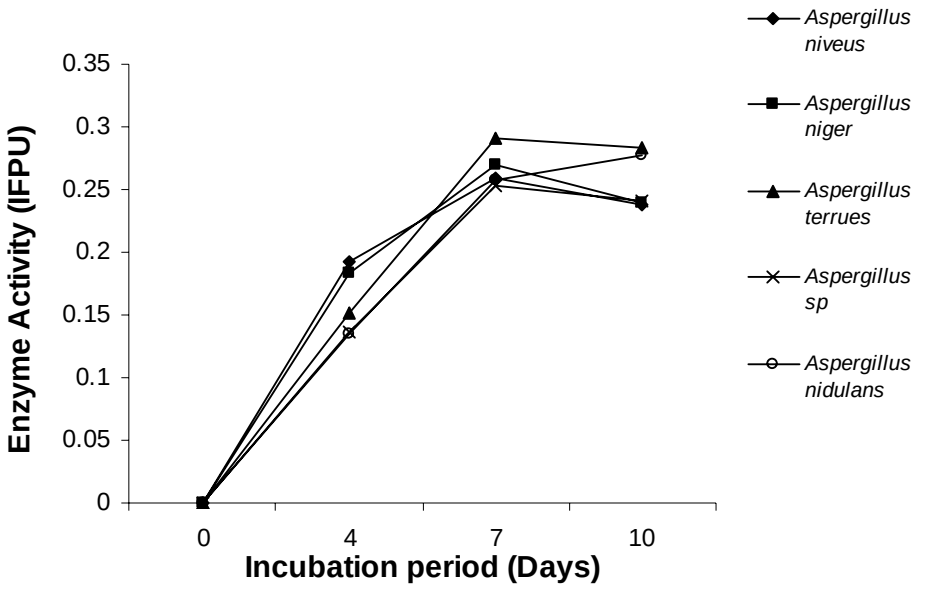

Fig 3. Enzyme production kinetics.

Discussion

Cellulose is world's most abundant organic substance (Ruttloff, 1987) and comprises a major storage form of photosynthesized glucose. It is the major component of biomass energy (Scott *et al*., 1987). Because a large proportion of vegetation added to soil is cellulose therefore, decomposition of cellulose has a special significance in the biological cycle of carbon (Lederberg, 1992). In industry, these enzymes have found novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc., and extraction of valuable components from plants and improvement of nutritional values of animal feed (Wiseman, 1995).

Fungi are well known agents of decomposition of organic matter in general and of cellulosic substrate in particular as reported by Lynd *et al*., (2002). Among the 115 isolated fungal strains we were able to isolate only few strains of *Trichoderma* species, which is the most extensively studied cellulase producer. This may be due to a possibility that the growth of *Trichoderma* was suppressed by rapidly growing fungi like *Aspergillus* and *Rhizopus.* Majority of *Aspergillus*, *Fusarium, Alternaria, Rhizopus, Penicillum* and *Trichoderma* isolates were found to possess cellulytic activity. *Aspergillus* produces wide range of enzymes capable of degrading plant cell wall polysaccharide. A wide range of *Aspergillus* species have been identified to possess all component of cellulase enzyme system (Vries & Visser, 2001) which is in agreement with the present study. The most common and most potent cellulase producers are *Trichoderma ressei, T. koningii, Fusarium* sp., *Aspergillus* and *Penicillium* sp., (Yalpani, 1987). Results obtained during this study suggests that cellulase activity of *Aspergillus* species were found relatively towards the higher side and *Alternaria, Rhizopus, Penicillum* moderate range while *Trichoderma* and *Fusarium* showed low cellulase activity. In addition, all the tested *Aspergillus* isolates showed comparatively good endoglucanase activity as well as good cotton degrading activity.

Sone *et al*., (1999) reported maximum enzyme production by *Aspergillus* species at 37°C. The results of the present study partly confirms the finding that higher enzyme yield was noted at 37°C than at 30°C. Some *Aspergillus* strains however, showed higher enzyme yield at 30°C as reported by Philippidis (1994). Study with kinetics of enzyme production suggested that maximum enzyme activity was noted after 7 days of incubation while its activity decreased on further incubation (Fig. 3). It might be due to enzyme degradation with the availability of glucose which is the product of cellulase catalyzed reaction.

The properties of cellulytic enzyme like those of all proteins are modified by prevailing physical condition such as temperature and pH. Enzyme exhibits its catalytic activity with in these ranges of physical conditions. Beside this, concentration, composition and quality of substrate along with enzyme concentration and reaction time are also important factors that determine the rate of hydrolysis and final yield of the product (Godfrey, 1996; Philippidis, 1994). Enzymes have an optimum pH with in which their activity is maximum and at higher or lower pH values, their activity decreases (Lehinger, 1993). The present study showed that optimum pH for many *Aspergillus* cellulases was found to be near pH 4.8 as also reported by Vries & Visser (2001).

An induction in the enzyme production/activity was noted when fungal isolate (*Aspergillus niger*) was grown on cellulose as a sole carbon source while a very low rate of enzyme activity/production was observed when glucose was used as sole carbon substrate. These results are in agreement with the ones obtained by other workers, where residual enzyme activity was noted when cellulytic fungi were grown in presence of glucose and many fold increase in enzyme yield were reported in the presence of cellulosic substrate (Lederberg, 1992; Lynd *et al*., 2002). The production of cellulase for the utilization of cellulose is induced only in the presence of specific substrate (or product thereof) but suppressed when easily utilizable sugars such as glucose are available (Lynd *et al.*, 2002). Although cellulases are inducible, but there is a low level of constitutive production of these enzymes suggesting that there may be isozymes, some of them remain repressed in absence of inducer and presence of inducer greatly affect the enzyme yield (Yalpani, 1987). However, cellulase production is also influenced by several other factors, such as carbon, nitrogen and phosphorus sources, the ratio of carbon to nitrogen provided, trace elements, pH and aeration rate (Philippidis, 1994).

It is therefore, of great importance for enzymology in general and for applied enzymology like industrial enzymology in particular, to be able to identify and define these optima's, as outside these ranges, enzyme performance is either considerably reduced or completely obliterated (Godfrey, 1996).

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