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Production and Optimization of Endoglucanase by *Aspergillus sp.*, *Trichoderma sp.* and *Penicillium sp.*

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A B S T R A C T

Endoglucanase is one of the most important industrial enzymes. In the present study, three different fungi *Aspergillus sp.*, *Trichoderma sp.* and *Penicillium sp.* were isolation for endoglucanase production from soil samples on PDA plate. All three fungus processed by submerged fermentation and solid state fermentation for its enzymatic activity. *Aspergillus sp*., showed highest enzymatic activity in both type of fermentation. In solid state fermentation using sawdust, corncob and wheat bran substrate, all three fungus give highest enzyme activity in corncob substrate as compared to sawdust and wheat bran substrate. The optimization of endoglucanase activity of *Aspergillus sp.*, *Trichoderma sp.* and *Penicillium sp.* with different parameter like pH, temperature, carbon source, nitrogen source, CMC concentration and incubation period. *Aspergillus sp.* give maximum endo-ß-glucanase activity at pH 3, 28^{o}C temperature, corn starch as a carbon source, sodium nitrate as a nitrogen source, 1.5% CMC concentration and 6 days incubation period.

Introduction

Endoglucanase is one of the most important industrial enzymes. Approximately 75% of industrial enzyme is used for hydrolysis and depolymerization of complex natural substance (Kirk *et al*., 2002). Microbial enzymes have two enormous advantages of being able to be produced in large quantities by established fermentation technique. Also, it is infinitely easier to improve the productivity of a microbial system compared with plant or animal and microbial enzyme has various applications in different sectors (Periasamy *et al.*, 2013). Successful utilization of cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies for cellulose production. A cellulosic enzyme system consists of three major components: endo-ß-glucanase, exo-ß-glucanase and ß-glucosidase (Bhat, 2000). Most fungal cellulases have a two-domain structure, with one catalytic domain and one cellulose binding domain that are connected by a flexible linker. This structure is adaption for working on an insoluble substrate, and it allows the enzyme to diffuse two-

dimensionally on a surface in a caterpillar way. However, there are also cellulases (mostly endoglucanases) that lack cellulose binding domains. These enzymes might have a swelling function (Saddler *et al.,* 1995). Most cellulases studied have similar pH optima, solubility and amino acid composition (Schulein, 1997). Thermal stability and exact substrate specificity may vary. This study focused on isolation of a high endoglucanase producing fungi from soil samples and improvement of enzyme productivity by supplementation with nitrogen and carbon sources, pH and optimization.

Materials and Methods

Isolation and identification of fungi

Collect soil samples in sterile bag and diluted in distilled water. Diluted soil samples (10^{-4}) were spread on PDA plates and incubated at 28˚C for 5 days.PDA plates were observed for the morphology of fungi (Table 1). Alactophenol cotton blue stain was used for the identification of fungi (Aneja, 2001).Cultivated fungus of PDA plate was grown on CMC agar plate and Incubated at 28˚C for 5 days was stained with congored (1%) for 15 min and wash with 1M NaCl according to the method of cellulolytic activity of fungus (Hankin and Anagnostaksis, 1975).Pure culture of fungi (*Aspergillus sp., Trichoderma sp. and Penicillium sp*.) was preserve on PDA slant at 4˚C (Fig. 3).

Growth condition and enzyme production

Submerged fermentation

Take the spore suspension into CMC broth in flask and incubated in shaker at 120 rpm, 28˚C for 5 days. After incubation filter it with muslin cloth, collect the filtrate and centrifuge at 10,000 rpm, 4˚C for 15 min. Collect the supernatant and used as crude enzyme.

Solid state fermentation

Three substrates (saw dust, corn cob, and wheat bran) were collected from the local market. Substrates were crushed with the help of grinder, sieve and autoclave it at 121˚C for 15 min. For fermentation 10-15 ml 5 days old broth culture of fungi mix with saw dust, corn cob, and wheat bran substrates in different flask and incubate at 28˚C for 5 days in static condition. After incubation add 10-15 ml of phosphate buffer (0.1M, pH 5) in each flask and mix it properly. Then filter through muslin cloth. Collect the filtrate and centrifuge at 10,000 rpm, 4˚C for 15 min. collect the supernatant and used as the crude enzyme preparation.

Enzyme assay

Enzyme assay was done by DNS or DNSA method (3,5-Dinitrosalicylic acid)(miller 1959).A reaction mixture composed of 1 ml of crude enzyme, 0.5 ml CMC (0.5%),0.5 ml citrate buffer(0.05M) was incubated at 40˚C in water bath for 15 min. The reaction was terminated by adding 3 ml3,5-Dinitrosalicylic acid (Sigma Aldrich, USA) and optical densities were measured at 540 nm by using spectrophotometer against a blank containing all the reagents. Results were interpreted in term of enzyme activity in which one unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 µmol glucose per minute under the above condition.

$$\text{Enzyme Activity} = \frac{\text{Amount of sugar released (Conc)}}{\text{Mol.weight of glucose} \times \text{Vol. of enzyme (ml)} \times \text{Incubation time (min)}}$$

Factor affecting endoglucanase production

The effect of carbon and nitrogen sources on growth and endo-ß-glucanase production was investigated by inoculating spore suspension in CMC fermentation medium supplemented with different nitrogen sources(peptone, beef extract, ammonium nitrate, sodium nitrate) and carbon sources(glucose, sucrose, mannitol, corn starch) and incubate for 5 days. After incubation, endoglucanase activity analyze by enzyme assayed. To study the effect of pH(3, 5, 7, 9), Temperature(28°C, 37°C, 50°C), Incubation Period(up to 8 days)and CMC concentration (1.0%, 1.5%,2.0%) was prepared and inoculated. Examine the various factor effects on microbial growth and enzyme activity.

Results and Discussion

Isolation and identification of fungi

Three fungus *Aspergillus sp., Trichoderma sp. and Penicillium sp.* were isolated from the soil sample and identified using lactophenol cotton blue staining method (Fig. 1). All three fungi produce a zone of hydrolysis (Fig. 2) around the fungal colonies when flooded with congo red stain. Aneja (2001) also observes similar result. A zone of hydrolysis around the fungal colonies indicates all three fungi produce endoglucanase enzyme. These methods are rapid and efficient for bacteria and fungi (Ramesh *et al.*, 2008).

Growth condition and enzyme production

Submerged fermentation

In Submerged fermentation *Aspergillus sp*. (0.124 Units/ml) gives highest enzyme activity followed by *Trichoderma sp*. (0.118 Units/ml) and *Penicillium sp.* (0.118 Units/ml). *Penicillium sp.* gives the highest specific activity (1.9 µmol/ml/mg of protein) compared to *Aspergillus sp*.(1.0 µmol/ml/mg of protein) and *Trichoderma sp*. (1.84 µmol/ml/mg of protein) (Table 2). *Aspergillus niger* produce significant cellulase activity in media containing cellulose and CMC as sole carbon sources in submerged fermentation (Gautam *et al.*, 2010).

Solid state fermentation

All three fungus gives highest enzyme activity in corn cob followed by wheat bran and saw dust substrate (Table 3). In contrast to present finding (Abo-state *et al.*, 2010) shown highest cellulases were produced using agriculture wastes in the order Wheat straw> Wheat bran> Rice straw> Corn cob. In present study highest enzyme activity observe in solid state fermentation as compare to submerged fermentation. Present finding was in agreement with Gautam *et al.*, (2011) which observe solid state fermentation gives higher enzyme activity and the lowest chances of bacterial contamination, so solid state fermentation is better than submerged fermentation.

Optimization of endoglucanase production

Effect of pH

For *Aspergillus sp. and Penicillium sp.* maximum enzyme activity (0.325 µmol/ml/min and 0.251µmol/ml/min respectively) at pH 3 and *Trichoderma sp.* maximum enzyme activity (0.116µmol/ml/min) at pH 7 (Table 4), earlier studies reported maximum enzyme activity*of Aspergillus niger*and *Penicillium chrysogenum* at 5 pH (Jayant *et al.*, 2011), *Trichoderma sp.* at 6.5 pH (Gautam *et al.*, 2011) and *Aspergillus niger* at 4.0 - 4.5pH (Acharya *et al.*, 2008).

Effect of temperature

Aspergillus sp. showed maximum enzyme activity of endoglucanase (0.055µmol/ml/min) at 28°C and above 28°C

decrease its enzyme activity. However, *Trichoderma and Penicillium sp* give maximum enzyme activity (0.055µmol/ml/min and 0.096 µmol/ml/min respectively) at 50°C (Table 5).

Similar to present finding Gautam*et al.*(2011) observed *Trichodermasp*. give maximum enzyme activity of endoglucanase at 45˚C and Acharya *et al.*, (2008) found *Aspergillus niger* give maximum activity at 28˚C.

Effect of incubation period

The cellulase activity of endoglucanase was measured at regular intervals. However, the maximum activity was obtained on 5th and 6th days of incubation. *Aspergillus sp.* gives maximum enzyme activity (0.140µmol/ml/min) on 6th day. *Trichoderma* and *Penicillium sp*. gives maximum enzyme activity (0.111 µmol/ml/min and 0.092µmol/ml/min respectively) on 5th day (Table 6).

Table.1 Colony characteristic of fungi

Species	Colour	Size	Margin	Appearance
Aspergillus	Black	Big	Round	Powdery
Trichoderma	Greenish blue	Medium	Spread	Cottony
Penicillium	Green	Big	Round	Powdery

Table.2 Enzyme activity in SmF

Species	Enzyme Activity (µmol/ml/min)	Specific activity (µmol/ml/mg of protein)
Aspergillus	0.124	1.0
Trichoderma	0.118	1.84
Penicillum	0.103	1.9

Table.3 Enzyme activity of SSF

Species	Enzyme activity(µmol/ml/min)		
	Substrates		
	Saw dust	Corn cob	Wheat bran
Aspergillus	0.066	1.42	0.290
Trichoderma	0.067	0.859	0.301
Penicillum	0.087	1.155	0.307

Table.4 Effect of pH

Species	*Enzyme activity(µmol/ml/min)*			
	pH 3	*pH 5*	*pH 7*	*pH 9*
Aspergillus	*0.325*	*0.005*	*0.029*	*0.037*
Trichoderma	*0.098*	*0.029*	*0.116*	*0.037*
Penicillum	*0.251*	*0.061*	*0.048*	*0.011*

Table.5 Effect of temperature

Species	*Enzyme activity(µmol/ml/min)*		
	28˚C	*37˚C*	*50˚C*
Aspergillus	*0.055*	*0.029*	*0.024*
Trichoderma	*0.037*	*0.038*	*0.055*
Penicillium	*0.029*	*0.092*	*0.096*

Table.6 Effect of incubation period

Species	*Enzyme activity(µmol/ml/min)*						
	Days						
	2	*3*	*4*	*5*	*6*	*7*	*8*
Aspergillus	*0.005*	*0.029*	*0.048*	*0.124*	*0.140*	*0.111*	*0.116*
Trichoderma	*0.011*	*0.042*	*0.061*	*0.111*	*0.092*	*0.087*	*0.085*
Penicillium	*0.005*	*0.029*	*0.037*	*0.092*	*0.087*	*0.074*	*0.079*

Table.7 Effect of carbon sources

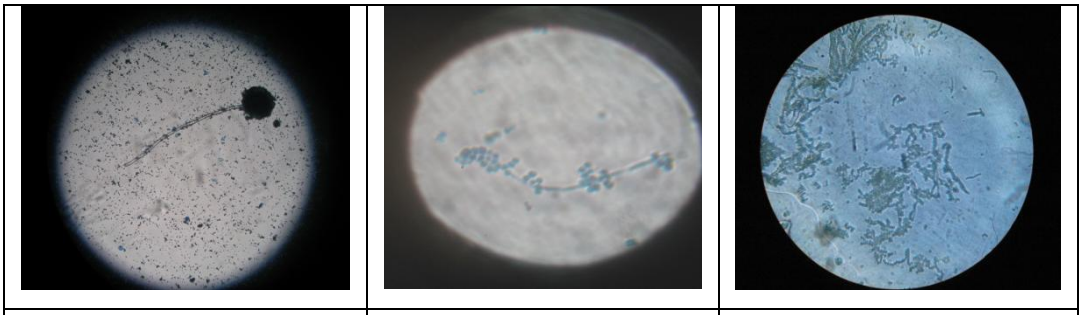
Species	**Enzyme activity(µmol/ml/min)**			
	Glucose	**Sucrose**	**Mannitol**	**Corn starch**
Aspergillus	0.029	0.037	0.024	0.066
Trichoderma	0.011	0.037	0.024	0.074
Penicillium	0.010	0.005	0.055	0.048

Table.8 Effect of nitrogen sources

Species	**Enzyme activity(µmol/ml/min)**			
	Ammonium Nitrate	**Peptone**	**Beef extract**	**Sodium nitrate**
Aspergillus	0.005	0.048	0.010	0.079
Trichoderma	0.018	0.055	0.037	0.074
Penicillium	0.111	0.036	0.066	0.042

Table.9 Effect of concentration of CMC

Species	**Enzyme activity(µmol/ml/min)**		
	1.0%	**1.5%**	**2.0%**
Aspergillus	0.122	0.153	0.129
Trichoderma	0.140	0.185	0.370
Penicillium	0.135	0.214	0.338

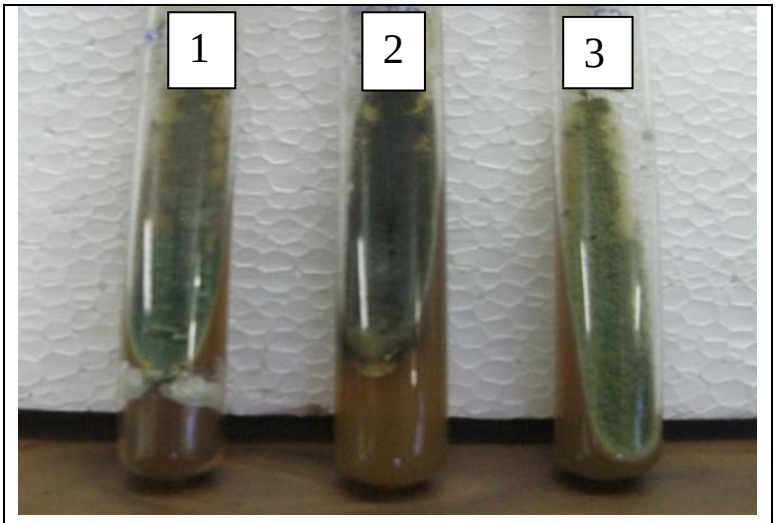


Aspergillus sp.	*Trichoderma sp.*	*Penicillum sp.*

Fig.1 Identification of fungi

Aspergillus sp.	*Trichoderma sp.*	*Penicillum sp.*

Fig.2 Zone of hydrolysis



1. Trichoderma sp.,2. Aspergillus sp., 3. Penicillum sp.

Fig.3 Pure culture of fungi(PDA slant)

Effect of carbon sources

Aspergillus and Trichoderma sp. gives maximum enzyme activity of endoglucanase (0.066µmol/ml/min and 0.074 µmol/ml/min respectively) in corn starch and *Penicillium sp*. gives maximum enzyme activity (0.055µmol/ml/min) in mannitol (Table 7). Gautam *et al*., (2011) observed that *Trichoderma sp*. gives maximum enzyme activity in sucrose as a carbon sources.

Effect of nitrogen sources

Aspergillus and Trichoderma sp. gives maximum enzyme activity of endoglucanase (0.079µmol/ml/min and 0.074µmol/ml/min respectively) in sodium nitrate compared to other nitrogen sources. While *Penicillium sp*. gives maximum enzyme activity of endoglucanase (0.111 µmol/ml/min) in ammonium nitrate (Table 8). In contrast to present finding peptone and yeast extract (1.0% (w/v) was best nitrogen sources for the production of endo-ß-glucanase by *A. niger* and *Trichoderma sp.* (Gautam *et al.*, 2011).

Effect of concentration of CMC (%)

Aspergillus sp. gives maximum enzyme activity (0.153 µmol/ml/min) at 1.5% CMC concentration. *Trichoderma and Penicillium sp*. gives the maximum enzyme activity (0.370 µmol/ml/min and 0.338 µmol/ml/min respectively) at 2% CMC concentration (Table 9). Jahangeer *et al.*, (2005) observed maximum enzyme activity of endoglucanase by *Trichoderma sp*. at 1% CMC concentration and Gautam *et al.*, (2011) observed maximum enzyme activity of *Aspergillus niger* at 1.0% CMC.

In conclusion, in submerged fermentation, the optimum parameter namely, temp, pH, incubation period for *Aspergillus sp*. (28˚C, pH-3 and 6 days), *Trichoderma sp*. (50˚C, pH-7 and 5 days) and *Penicillium sp*. (50˚C, pH-3 and 5 days) were observed. In solid state fermentation, corn cob is the best substrate for endo-ß-glucanase production compared to wheat bran and saw dust. *Aspergillus sp*. gives the highest enzyme activity in solid state fermentation as well as submerged fermentation compared to *Trichoderma sp.* and *Penicillium sp.* higher activity was observed in solid state fermentation compared to submerged fermentation. So, solid state fermentation is better than submerged fermentation.

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