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RESEARCH **P**APER

Cellulytic activity of a thermophilic fungus *Aspergillus fumigatus* isolated from paper industry effluent

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Cellulases are used in numerous industrial applications and for cellulose conversion to value added products. A cellulase producing thermophilic *Aspergillus fumigatus* was isolated from paper industry effluent sample after 72 h incubation on potato dextrose agar at 45°C. In the present study the production of cellulase enzyme by *Aspergillus fumigatus* cultivated on wheat bran: rice straw (1:4) using solid state fermentation (SSF) technique. The ability to produce cellulase under varying conditions of temperature, pH, moisture content, and nitrogen sources was evaluated. The higher cellulase activity was obtained when the fungus was cultivated on substrate (wheat bran: rice straw (1:4)) with moisture content (1:4), pH 5.5, urea as nitrogen source and incubated in environmental chamber at 45^0C for 3 days as it give 1.85FPU/ml.

Key words : Cellulase, Thermophilic, *Aspergillus fumigates*, Paper industry, Solid state fermentation

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INTRODUCTION

Among the eukaryotic organisms, only a few species of fungi have the ability to grow at higher temperature. Such fungi comprises thermophilic and thermotolerant forms, the thermophilic fungi have a growth temperature maximum at or above 40°C (Bergey and Bhatt, 2006) and the thermotolerant forms have a temperature range at or above 55°C. Some species of which are able to grow near or above 100°C in thermal springs, solfatara fields, or hydrothermal vents by any organism. Thermophilic fungi are the chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter where in the warm, humid and aerobic environment provides the basic conditions for their development.

Cellulose is one of the most abundant biological polymers on earth (Karlsson *et al.*, 2002). It has been approximated that 7.2 × 1011 tonnes of cellulose is reserved in plants and that the yearly production of cellulose is 4×10^{10} tonnes (Coughlan, 1985).Cellulose is chemically simple because it contains simple repeating units of glucose, but has a complex structure because of the long chains of glucose subunits joined together by β–1,4–linkages (Lynd *et al.*, 2002). Cellulose is stabilised by some interactions, these stabilising factors are weak individually but collectively form strong bonds. The chains are in layers held jointly by van der Waals forces and hydrogen bonds (intramolecular and intermolecular). Degradation of cellulose is brought by various bacteria, actinomycetes and filamentous fungi by secretion of extra cellular enzyme cellulase but molds have generally higher enzyme activity (Pothiraj *et al.*, 2006). Cellulases are

widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. It is also used in the fermentation of biomass into biofuels.

The cost of enzyme production is reduced by the use of lignocellulosic materials as substrate by solid state fermentation (SSF) (Beg *et al.*, 2000 and Senthilkumar *et al.*, 2005). Lignocellulosic waste such as palm kernel cake, paddy straw, rice husk, sugarcane baggase and sago waste contributes more than 5 million tones of waste per year (Kheng and Omar, 2005).The major agricultural residue in India is straw which is about 60.53 per cent. Rice straw has traditionally been dried and burned in the fields reducing the local air quality considerably, this have directed a world wide attention towards utilization of rice straw for bioethanol. It contains between 25-45 per cent of cellulose, 20- 30 per cent hemicellulose and 10-15 per cent lignin (Sun and Cheng, 2002).So these agricultural residues can be used economically by solid state fermentation(SSF) to produce fungal cellulase enzymes.

Solid state fermentation (SSF) is attractive process to produce microbial enzymes because of its lower operating cost (Chahal *et al.*, 1996). It is a fermentation process occurring in the absence or near-absence of free water and it involves heterogeneous interactions of microbial biomass with moist solid substrate. In SSF, sugars and other nutrients are supplied from the moist substrate matrix, while oxygen is available in the continuous gas phase. The near absence of water in SSF promises a more efficient downstream process. The enzymatic hydrolysis of cellulosic materials to produce fermentable sugars has an enormous potential in meeting global bioenergy demand through the biorefinery concept, since agrifood processes generate millions of tones of waste each year such as sugar cane baggase, wheat straw and rice straw. Current technology for conversion of lignocellulose to ethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by naturally fermented to ethanol by the yeast *Saccharomyces cervisiae*. The large amounts of enzymes required for enzymatic conversion of hemicelluloses and cellulose to fermentable sugars impacts severely on the cost effectiveness of this technology.

The aim of the study was to isolate a fungus capable of hydrolyzing a wide range of native cellulose source on cheap and easily available substrate. A thermophilic *Aspergillus fumigatus* was isolated from paper industry effulent from Ludhiana in Punjab (India).

RESEARCH METHODOLOGY

Isolation of organism :

Sample collected in February, 2010 from paper industry in Ludhiana, Punjab was grown on potato dextrose agar and Rose Bengal Chloramphenicol (RBC) Agar by serial dilution method and pure culture of fungi was obtained by streaking method at 45°C. Confirmation of cellulytic microbes done with the help of Czapek-mineral salt agar medium which contain carboxymethyl cellulose. One of the enzymes required to convert cellulose to glucose is endo-1,4-β-glucanase which can be detected by the hydolysis carboxy methyl cellulose solution (CMC). Each pure culture was streaked on Czapek-mineral salt agar medium which contained CMC (1mg/ml) and incubated at 45^{0}C for 24 hrs. Plates were flooded with an aqueous solution of congo red for 15 minute. After pouring off the congo red solution, plates were flooded with 1M NaCl for 15 minute. After that zones of hydrolysis could be seen as clear areas.

Cellulose source :

Rice straw (Oryz*a Sativa*) and wheat bran was used as cellulose source.

Production of crude enzyme :

The inoculum were prepared by growing the fungus on potato dextrose agar (PDA). The crude enzyme was produced by solid state fermentation and fermentation using Mandel's medium (Mandel *et al.*, 1976). The composition of the Mandel's medium was as follows: (Table A).

Table A : Composition of the mandel's medium

Chemical	Composition
Urea	0.3g
$MgSO_4.7H_2O$	0.3g
$FeSO_4.7H_2O$	0.005g
$MnSO_4.4H_2O$	0.0016g
$ZnSO_4.7H_2O$	0.0014g
$CaCl_2$	0.3g
Peptone	0.75g
Yeast extract	0.25g
$(NH_4)_2SO_4$	1.4g
KH_2PO_4	2.0g
$CaCl_2.6H_2O$	0.020g

All the components of Mandel's medium were dissolved in 100 ml of distilled water in a flask and volume made unto 1000 ml. Substrate rice straw and wheat bran (4:1) was taken with 20 ml of Mandel's medium *i.e.* 1:4 ratio and autoclaved at a pressure of 1.1 kg/cm² for 15 minutes. The flasks were cooled down at room temperature. The flasks were then inoculated with 1ml of spore suspension and incubated for 4 days in case of each culture at the temperature 45^{0}C, two replicates were used for each treatment.

Extraction of crude enzyme :

At the end of fermentation time precipitates were dissolved in 5 ml of 0.1 M citrate buffer having pH 4.8 was added to give total 35 ml/flask *i.e.* the Mendel weber media added in flask should be 7 times to the weight of the substrate (Rajinder, 1992). The flasks were shaked at 110 rpm for 30 min. Then filtered by the whatman's filter paper1 and then centrifuge at 4000rpm for 10 minutes at 4^{0}C temperature to obtain clear filtrate for determining enzyme activity.

Enzyme assay :

Enzyme activities of the partially purified enzymes were expressed in International Units (IU). One IU is defined as one µmol of xylose (for xylanase activity), glucose (for carboxy methyl cellulase activity) equivalents released per minute per ml under the following assay conditions by using xylose and glucose standard curve (Silva *et al.,* 2005). Appropriate dilution factors were used during the estimation of the enzyme activity.

Cellulase activity :

Cellulase was estimated as carboxy methyl cellulase (CMCase) (Ray *et al*., 1993). Carboxy methyl cellulose (1 g) was dissolved in 90 ml of 0.1 M citrate buffer of pH 4.8 and volume was made to 100 ml with distilled water. The solution was stored in a refrigerator.

Effect of various substrates on enzyme production:

The effect of various substrate *viz.*, rice straw, wheat straw, rice bran and wheat straw: rice straw (1:1) on enzyme production by solid state fermentation was examined by adding 10g of each substrate in 500 ml Erlenmeyer flask with 50 ml of Mandel's medium. Cultivation was carried out at 30°C for 96 h in a BOD incubator. The enzyme activities were determined by using the partially purified enzymes. The substrate which yielded maximum enzyme activity was selected for further study.

Effect of different cultural conditions on enzyme production:

Effect of moisture content on enzymes production :

The effect of initial moisture level on enzyme production was tested by varying the substrate to Mandel's medium ratio (w/v) in the range of 1:3, 1:4, 1:5 and 1:6. The flask (500 ml Erlenmeyer flask) containing 10 g of substrate and 40 ml, 50 ml, 60 ml and 70 ml of medium, respectively were inoculated with 2 ml of spore suspension (10^7 spores/ml) and incubated for 96 h at 30°C in a BOD incubator. Moisture was provided by medium itself at pH 5.5.

Effect of initial pH on enzyme production :

To evaluate the effect of initial pH on enzymes production was investigated by adjusting the initial pH of Mandel's medium to 4.5, 5.0, 5.5 and 6. The flasks containing 10 g of substrate and 50 ml of sterile Mandel's medium were inoculated with 2 ml of spore suspension (10^7 spores) and incubated for 96 h at 30°C in a BOD incubator.

Effect of incubation temperature on enzyme production :

The effect of incubation temperature on enzyme production was examined by varying temperature 40, 45, 50 and 55°C.

Effect of inoculum concentration on enzyme production :

To evaluate the effect of inoculum concentration on enzymes production was examined by varying conc. from 1ml, 1.5 ml, 2ml, 2.5ml and 3ml, respectively.

Effect of incubation time on enzyme production :

The effect of incubation time on enzyme production was examined by varying incubation time 24, 48, 72, 96 and 120h.

RESEARCH FINDINGS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Effect of incubation temperature on enzyme production :

Incubation temperature not only influences the growth of micro-organisms but also their biological activities and it is one of the important parameters that determine the success of SSF system. To know the optimum temperature for the production of cellulase enzymes by *Aspergillus fumigatus* on rice straw, a temperature range between 40-55°C was studied and it was observed that gradual increase in enzyme activity from 40 to 45°C but further increase in temperature resulted in decrease in production of enzymes (Fig. 1). Lesser activity of fungal enzymes at high temperature (50-55°C) and at temperature (40-45°C) increased in production of enzymes.

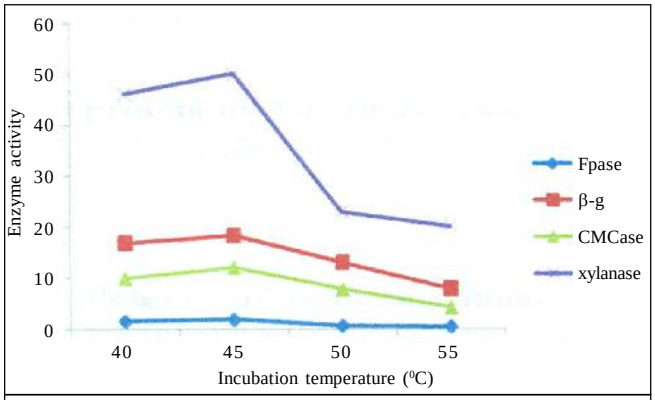

Fig. 1 :Effect of incubation temperature on enzyme production

Effect of initial moisture content on enzymes production :

Moisture is the key element for regulating and optimizing the solid state fermentation process (Laukevics *et al.*, 1984). An intermediate moisture content is required for an efficient solid state fermentation process. Hence, varying amount of Mandel's medium was added to the substrate, to study the effect of moisture content on enzyme production. The substrate to Mandel's medium ratio was varied in the range of 1:3, 1:4, 1:5 and 1:6 (w/v), which was 15, 20, 25 and 30 ml of Mandel's medium / 5g substrate. The results illustrated an increase in the cellulase activities with increase in Mandel's medium level from 15 to 20 ml/ 5g substrate and with further increase in moisture content up to 30 ml/ 5g substrate lead to a decrease in enzyme activities (Fig. 2).

Effect of initial pH on enzyme production :

The results indicate that with increase in pH value

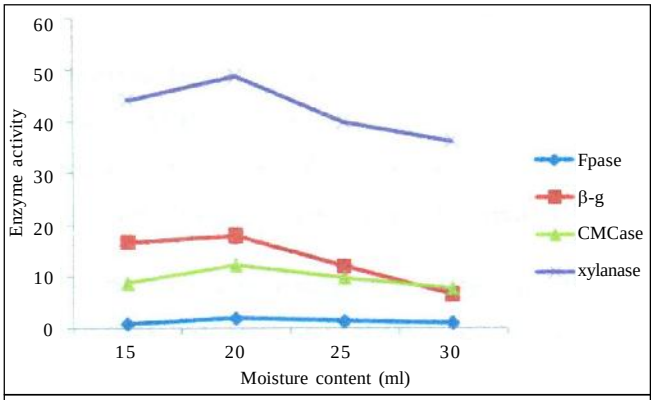

Fig. 2 :Effect of initial moisture content on enzymes production

from 4.5 to 5.5, the activity of cellulase enzymes reached to the maximum followed by a gradual decrease thereafter. Optimum pH for cellulase activity was 5.5 and their activity fell when pH increased from 5.5 to 6.0 (Fig. 3). The increase in enzyme production from 1ml to 2ml, further increase in inoculum concentration showed a fast decline in enzyme activities, which may be due to rapid degradation of the substrate leading to the limitation of the substrate. It was found that the maximum yield of cellulase was observed on 72hrs of incubation. Thus, the optimum incubation time for maximum enzyme production depends on the type of media, growth rate of micro-organism and its enzyme production pattern.

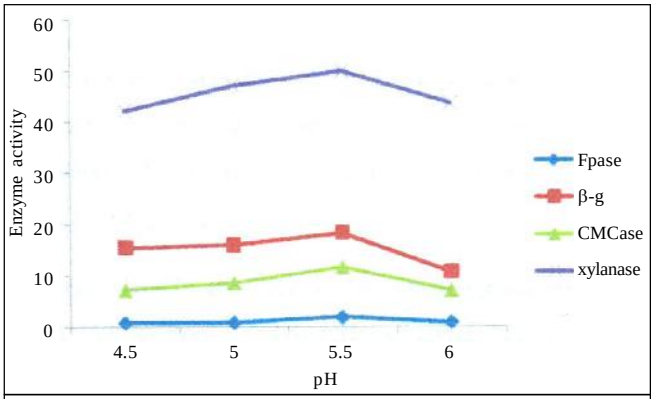

Fig. 3 :Effect of initial pH on enzyme production

Effect of different nitrogen sources on enzyme production:

The effect of nitrogen sources was evaluated by checking enzymes activities with different nitrogen sources such as potassium nitrate, ammonium nitrate and urea dissolved in Mendel's media with 0.2 per

cent concentration at 45°C. It was found that the maximum yield of cellulase was observed on urea after 72 hrs of incubation. And afterwards maximum yield of cellulase was observed on ammonium nitrate after 72hrs of incubation. There was the little bit difference between the ammonium nitrate and urea production of enzyme. And yield of cellulase on potassium nitrate was low (Fig. 4).

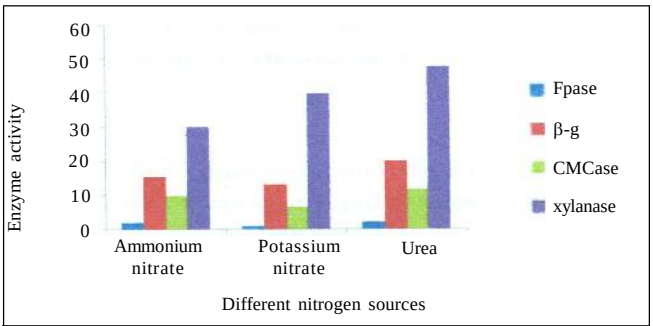

Fig. 4 : Effect of different nitrogen sources on enzyme production

Effect of inoculum concentration on enzyme production :

Proper amount of inoculum is essential for an efficient solid state fermentation. Each flask was inoculated with 1ml, 2ml, 3 ml and 4 ml spore suspension, respectively. The results showed that increase in enzyme production from 1ml to 2ml further increase in inoculum concentration showed a fast decline in enzyme activities, which may be due to rapid degradation of the substrate leading to the limitation of the substrate (Fig. 5).

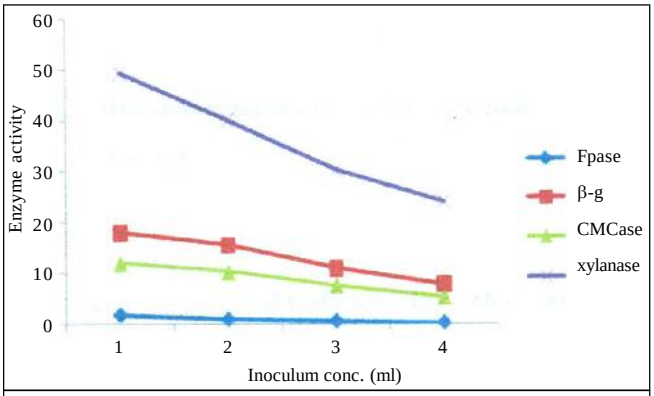

Fig. 5 :Effect of inoculum concentration on enzyme production

Effect of incubation time on enzyme production :

Incubation time plays an important role in substrate utilization and enzyme production. The effect of incubation time was evaluated by checking enzymes activities after 24, 48, 72, 96 and 120hrs of incubation at 45°C. It was found that the maximum yield of cellulase was observed on 72hrs of incubation. Further, increase or decrease in incubation time didn't show any enhancement in the level of enzyme production (Fig. 6). Thus, the optimum incubation time for maximum enzyme production depends on the type of media, growth rate of micro-organism and its enzyme production pattern.

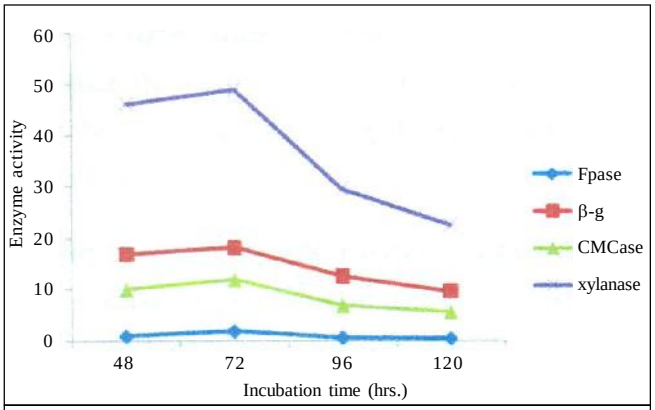

Fig. 6 :Effect of incubation time on enzyme production

Conclusion :

In this study we conclude that solid state fermentation of rice straw is an economical method for the production of cellulase enzymes. The cultivation system can easily be modified and enhance the productivity of cellulase by *Aspergillus fumigates*. All the independent variables *viz.*, inoculum conc., incubation time, pH, temperature and moisture level were found to have statistically significant effect on the production of enzymes. Interaction between five variables was found to contribute to the response at a significant level.

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