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Full Length Research Paper

Corn stover-enhanced cellulase production by *Aspergillus niger* NRRL 567

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The production of extracellular cellulases by *Aspergilus niger* NRRL 567 on corn stover was studied in liquid state fermentation. In this study, three cellulases, exoglucanase (EXG), endoglucanase (EG) and β-glucosidase (BGL) were produced by *A. niger* NRRL 567. The optimal pH, temperature and incubation time for cellulases production was found to be 3.5, 30°C and 96 h, respectively. Maximal cellulases activities were achieved with 4% corn stover, 0.1% molasses and 1% yeast sludge. To our knowledge, this is the first report on production of cellulases by using corn stover as a substrate from *A. niger* NRRL 567.

Key words: Corn stover, yeast sludge, cellulases, *Aspergillus niger.*

INTRODUCTION

The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component (Saleem et al., 2008). Plant biomass comprises an average of 23% lignin, 40% cellulose and 33% hemicellulose by dry weight (Ahmed et al., 2009a). Cellulose is the most abundant renewable natural resource in the world and a potential source for the production of industrial useful materials such as fuels and chemical (Facchini et al., 2010). Its annual biosynthesis by both land plants and marine algae occurs at a rate of 0.85×10^{11} tonnes per annum (Niranjane et al., 2007). Cellulase degradation and its subsequent utilizations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Jamil et al., 2005).

Cellulases and hemicellulases are two important classes of enzymes produced by filamentous fungi and secreted into the cultivation medium (Saadia et al., 2008).

Cellulase enzymes which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three types: endoglucanase (endo-1,4-β-D-gluca-nase, EG, EC 3.2.1.4); exoglucanse (also called as cellobiohydrolase) (exo-1,4- β -D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4- β -D-glucosidase, BG, EC 3.2.1.21) (Ahmed et al., 2009b).

Cellulases are of substantial industrial interest. Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting, brewing and extraction of fruit and vegetable juices and pulp and paper industries (Sipos et al., 2010). In recent years, the interest in cellulases has increased due to many potential applications, for example, in the production of bio-energy and bio-fuel, in the textile industry and pulp and paper industry (Zhou et al., 2008). The most promising technology for conversion of lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzyme (Ahamed and Vermette, 2008). Currently, xylanases and cellulases together with pectinases account for 20% of the world enzyme market (Ahmed et al., 2007). The application of cellulases to the hydrolysis of lignocelluloses biomass in order to further convert the

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released fermentable sugars into ethanol has increased due to not only its environmental benefits, but also the worldwide demand for renewable fuels (de Castro et al., 2010).

Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Saleem et al., 2008). A variety of microorganisms' including bacteria and fungi have the ability to secrete cellulases (Jiang et al., 2011; Gamarra et al., 2010). Many fungal strains secrete higher amounts of cellulases than bacterial ones. Cellulases from *Trichoderma* and *Aspergillus* species have been investigated in detail over the past few decades (Fang et al., 2008; Tao et al., 2010). *Aspergillus* sp. is an important commercial source of cellulases for food textile and pharmaceuticals industries (Naika and Tiku, 2010).

In Pakistan, many cellulosic residues are produced to as much as 50 million tons every year (Irshad et al., 2008) and could be utilized for bulk production of cellulases. The aim of the present study was to improve the cellulase production by *Aspergillus niger* NRRL 567 with corn stover as a main substrate. So, in this study, corn stover was used as a substrate to investigate optimum fermentation conditions for the production of cellulases from *A. niger* NRRL 567.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade unless otherwise stated.

Substrate

Corn stover obtained from local market of Faisalabad, Pakistan, was air dried and stored in an oven at 65°C to a constant weight. Substrate was ground to 2 mm sieve and stored in air tight plastic jars. Analysis of the corn stover was performed following AOAC methods (1990) to find out its nutritive value.

Microorganism

The strain utilized in the present study was *A. niger* NRRL 567 which was a kind gift from Dr. S. W. Peterson, Agricultural Research Service Culture Collection, Northern Regional Research Laboratory U.S., Department of Agriculture, Illinois, U.S.A. The fungus was maintained on agar slants medium which consisted of (g/l); corn stover 20; $CaCl_2.2H_2O$ 0.05; $MgSO_4$, 0.05; KH_2PO_4, 1.5; urea, 3; agar, 20.

Media and culture conditions

The inoculum medium for *A. niger* NRRL 567 consisted of (g/l); corn stover 20; $CaCl_2.2H_2O$ 0.05; $MgSO_4$, 0.05; KH_2PO_4, 1.5; $(NH4)_2SO_4$, 2 at pH 3.5 and grown at 30°C on an orbital shaker working at 120 rpm for 24 h. A 10 ml of liquid culture from the inoculum was transferred to 1000 ml Erlenmeyer flasks containing 250 ml fermentation medium under the same conditions as discussed earlier. The pH of the fermentation medium was adjusted to 3.5 and temperature 30°C for 96 h to optimize different fermentation conditions for cellulases production. Biomass was harvested by centrifugation at 10,000 rpm for 10 min at 4°C. Resulting supernatant was tested for cellulases activity.

Effect of pH on cellulase production

To examine the effect of pH on cellulase production, the experiment was carried out at various pH (3, 3.5, 4.0 and 4.5) at 30°C.

Effect of temperature on cellulase production

To examine the effect of temperature, the fermentation experiment was performed at various temperatures (25, 30, 35, 40°C) at pH 3.5

Effect of incubation time on cellulase production

Effect of various time periods on cellulase production was also investigated from *A. niger* NRRL 567 at pH 3.5 and 30°C.

Effect of substrate concentration on cellulase production

Different corn stover concentrations (1, 2, 3, 4, 5 and 6 w/v %) were tested for cellulase production. Optimum substrate (corn stover) concentration was adopted in subsequent experiments.

Effect of ionic concentration on cellulase production

Different ionic concentrations of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4 were tested to get optimal cellulase production from *A. niger* NRRL 567.

Effect of nitrogen sources on cellulase production

$(NH_4)_2SO_4$ and urea were tried as nitrogen source for the production of cellulase, to find out which is a better nitrogen source.

Effect of cane molasses and yeast sludge on cellulase production

Different concentration of cane molasses (0.025, 0.05, 0.075, 0.1, 0.125 and 0.15 w/v %) was tested to get the maximum cellulase production from *A. niger* NRRL 567. Different concentration of yeast sludge (0.5, 1, 1.5, 2, 2.5 and 3 w/v, %) were tested to get the maximum cellulase production from *A. niger* NRRL 567.

Cellulase assay

Cellulase (EXG, EG and BGL) activities were assayed in reaction mixture (I ml) containing 1% substrate that is, avicel (for EXG) or CMC (for EG) or salicine (for BGL) in 0.05 M acetate buffer, pH 5.0 and appropriately diluted enzyme solution. After incubation at 60°C for 30 min, the reaction was stopped by adding 3 ml dinitorsalycilic

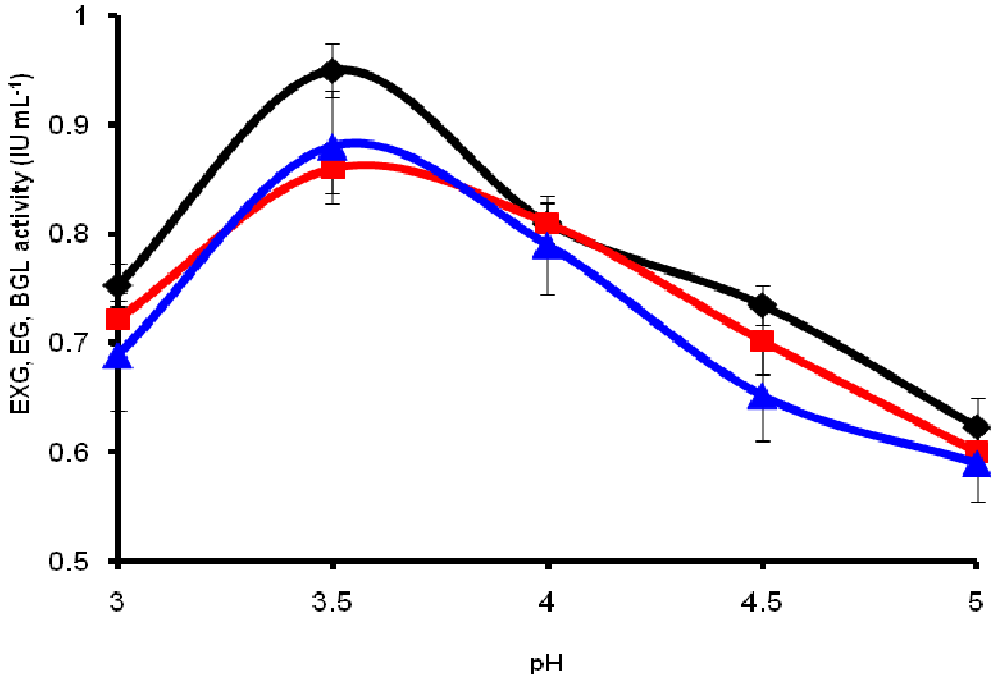

Figure 1. Effect of pH on cellulase production from *A. niger* NRRL 567 at 30°C. (♦) EXG; (■) EG; (▲) BGL.

acid solution (Shamala and Sereekanth, 1985). One unit (I U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of glucose or p-nitrophenol from the appropriate substrates under the standard conditions.

Protein estimation

The protein content of the culture biomass was determined by Lowry method (1951).

RESULTS AND DISCUSSION

Optimal conditions for cellulase production

Initial studies were performed in shake flasks to optimize fermentation conditions for the production of cellulase enzymes. The fermentation temperature was maintained at 30°C and pH 3.5, throughout the fermentation period. The orbital shaker was operated at a speed of 120 rpm. Many microorganisms have been classified as cellulolytic but, only few possess complete cellulase complex capable of efficient depolymerization of crystalline cellulose. In the present study, different fermentation conditions were optimized for cellulases production by *A. niger* NRRL 567 by trying to minimize production cost in order to enhance the commercial viability of cellulase production technology.

Effect of pH on cellulase production

EG, EXG and BGL activities were detected within pH range of 3 to 5 with maximum activites achieved at pH 3.5 (Figure 1). The optimal pH for fungal cellulases varies from species to species though in most cases the optimum pH ranges from 3.0 to 6.0 (Niranjane et al., 2007; Ahmed et al., 2005, 2003).

Effect of temperature on cellulase production

The temperature of the fermentation medium is one of the critical factors that have profound influence on the production of end product. The temperature for cellulases production of *A. niger* was optimized. The production of cellulases by *A. niger* NRRL 567 in fermentation medium at different temperatures (25 to 40°C) was carried out. Maximum EG, EXG and BGL activities were achived at 30°C (Figure 2). Further increase in temperature resulted in decrease in cellulase production.

The optimum temperature for cellulases production was similar to those of other mesophilic fungi such as *Aspergillus japonicas* C03 (Facchini et al., 2010), *Aspergillus glaucas* XC8 (Tao et al., 2010), *A. niger* MS82 (Sohail et al., 2009), *Trichoderma reesei* Rut C30 (Sipos et al., 2010), *Trichoderma viride* strain (Jiang et al., 2011), *Penicillum echinulatum* (Camassola and

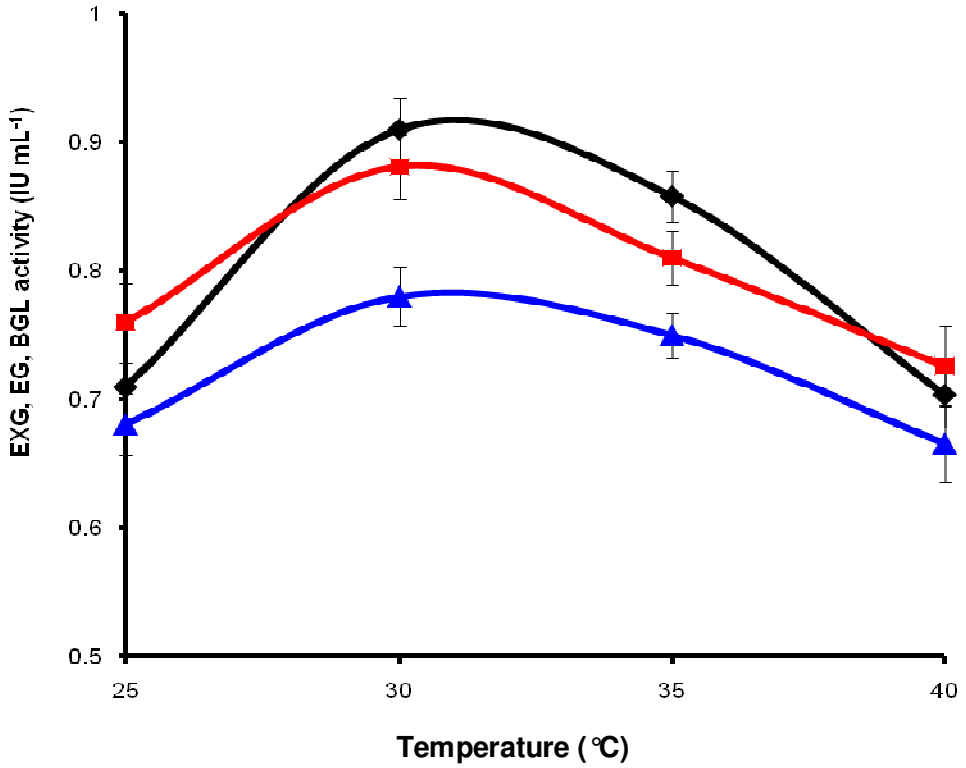

Figure 2. Effect of temperature on cellulase production from *A. niger* NRRL 567 at pH 3.5. (♦) EXG; (■) EG; (▲) BGL.

Dillon, 2007) and *Fusarium oxysporum* (Panagiotu et al., 2003).

Effect of incubation time on cellulase production

Incubation period is considered as one of the most important factor in cellulase production. Time course for cellulase production was investigated in fermentation medium with 4% corn stover as a substrate. Maximum EG, EXG and BGL activities were achieved after 96 h of incubation (Figure 3). Further incubation resulted in decrease in cellulase activities.

The optimum incubation time of 96 h for maximum cellulases production found in this study was similar to those of other fungi such as *Aspergillus terreus* M11 (Gao et al., 2008), *A. niger* (Acharya et al., 2008) and *Aspergillus fumigatus* (Sherief et al., 2010).

However, the optimum incubation time of 96 h for cellulases production observed in this study is shorter than the optimium incubation time of 120 h for cellulases production by *T. harzianum* (Ahmed et al., 2005; Sheikh et al., 2003), *Aspergillus phoenix* (Dedavid et al., 2009), *A. niger* C-6 (Kirchner et al., 2005). Time course which is required to reach maximum level of cellulase activity may be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose (Ogel et al., 2001).

Hence optimum pH 3.5, optimum temperature 30°C and 96 h incubation time were used in all the subsequent experiments.

Effect of corn stover on cellulase production

The fungus produced cellulases extracellularly when grown on corn stover. Among different substrate (corn stover) concentrations, 4% corn stover supported higher cellulases production (Figure 4). Maximum production of EG (0.61 IU ml^{-1}) EXG (1.094 IU ml^{-1}) and BGL (0.67 IU ml^{-1}) were obtained with 4% corn stover concentration. Gradual reduction in cellulase production was observed when corn stover concentration was increased or decreased.

Lignocellulosic biomass, such as corn stover which is very abundant, cheap and easily available (Yang et al., 2006; Sheikh et al., 2003) can be used for economic production of cellulases. Corn stover is recently attracting more and more attention because it is very cheap, abundant and renewable (Zheng et al., 2010).

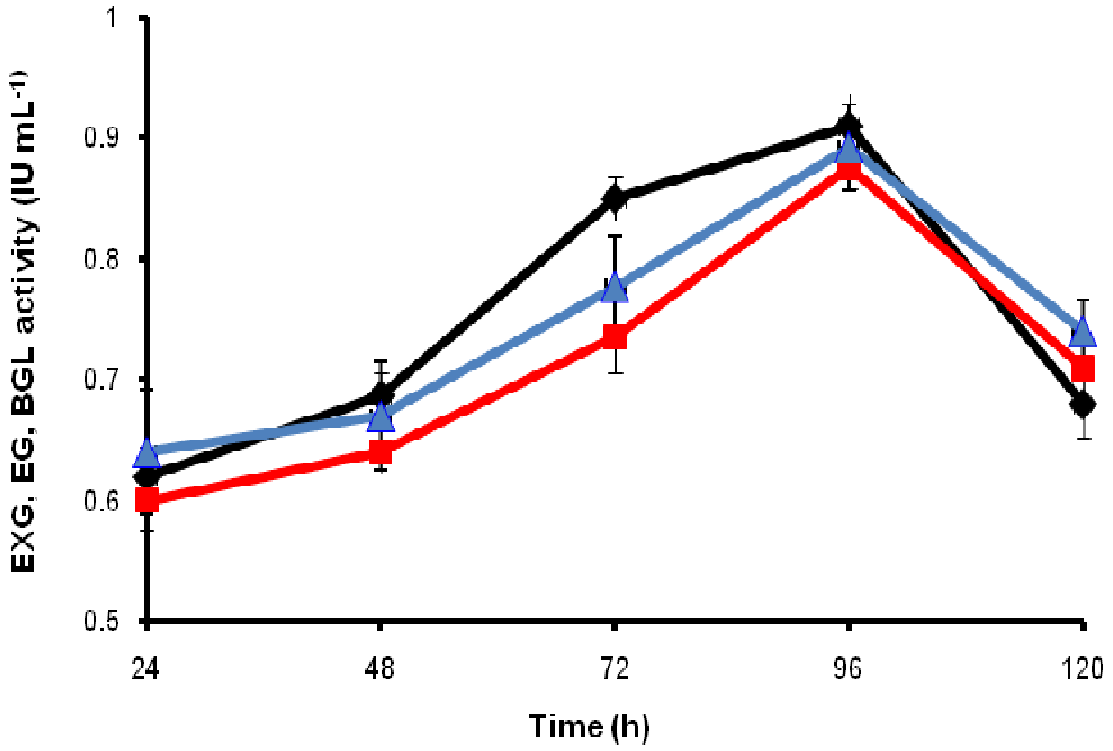

Figure 3. Time course of cellulase production from *A. niger* NRRL 567 at pH 3.5 and 30°. (♦) EXG; (■) EG; (▲) BGL.

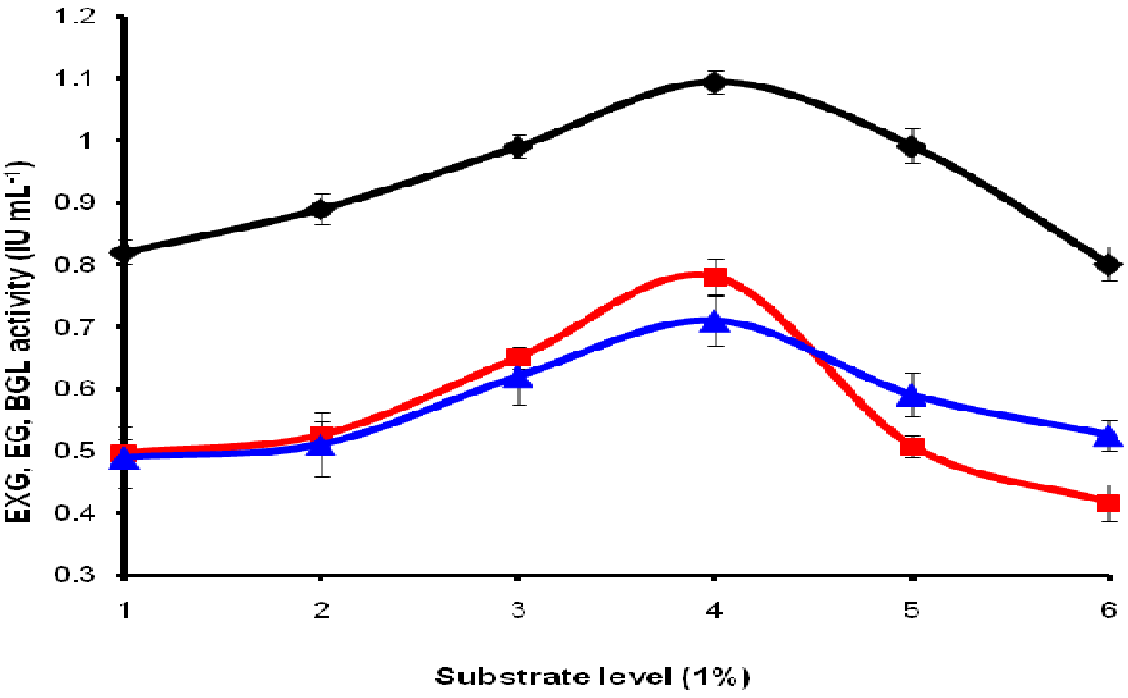

Figure 4. Effect of various susbtrate concentrations on cellulase production from *A. niger* NRRL 567. (♦) EXG; (■) EG; (▲) BGL.

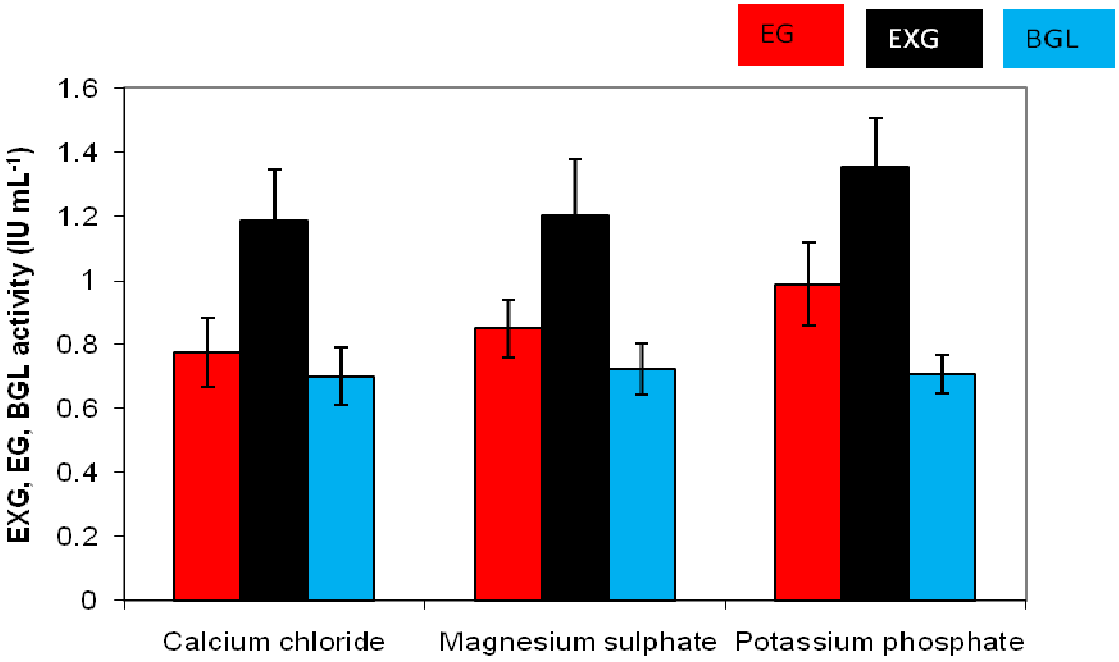

Figure 5. Effect of various levels of $CaCl_2$. $2H_20$, $MgSO_4.7H_2O$ and KH_2PO_4 on cellulase production from *A. niger* NRRL 567.

Hence, it was found in this study that *A. niger* NRRL 567 is able to ferment corn stover which is produced in huge amounts in Pakistan annually.

Effect of ionic concentration on cellulase production

Effect of addition of varying concentration of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and KH_2PO_4 were studied for maximum productivity of cellulases by *A. niger* NRRL 567 in the fermentation medium containing 4% corn stover as as substrate. Economic production of cellulases is required for their production at industrial scale. In this study, we have formulated a medium with optimized ionic concentrations for the enhanced production of cellulases. Maximum activities of EG, EXG and BGL were obtained at 0.02% $CaCl_2.2H_2O$, 0.03% $MgSO_4.7H_2O$ and 0.3% KH_2PO_4 (Figure 5).

Effect of nitrogen sources $(NH_4)_2SO_4$/urea on cellulase production

Replacement of one nitrogen source with another in the medium causes a change in protein synthesis as well as product formation. Generally, the results confirmed that urea; a low cost fertilizer, supported the maximum production of cellulases when compared with $(NH_4)_2SO_4$ (Figure 6).

Effect of supplementation with cane molasses and yeast sludge on cellulase production

In this study, to improve the cellulase production of *A. niger* produced from corn stover, cane molasses and yeast sludge were added to the fermentation medium. The present result demonstrates the potential of cane molasses and yeast sludge along with corn stover as a substrate for cellulases production.

Cultivation of *A. niger* NRRL 567 under previously optimized conditions with molasses (0.025 to 0.15%) showed that, 0.1% molasses gave maximum productivity of EG (1.150 IU ml^{-1}), EXG (1.535 IU ml^{-1}) and BGL (1.560 IU ml^{-1}) by *A. niger NRRL* 567 (Figure 7). Addition of higher concentrations of molasses resulted in lower cellulase production. Yeast sludege, a by-product of brewing industry has attracted the attention of scientists (Sattar et al., 2008; Ali et al., 2009). Among the different yeast sludge concentrations, 1% yeast sludge supported significantly higher cellulase production from *A. niger* NRRL 567 (Figure 8). Furthermore, addition of yeast sludge caused decrease in cellulase production.

Molasses, cheap by-products, are widely available from the sugar industry and consist of water, sucrose (47 to 50%, w/w) which is the disaccharide most easily utilized

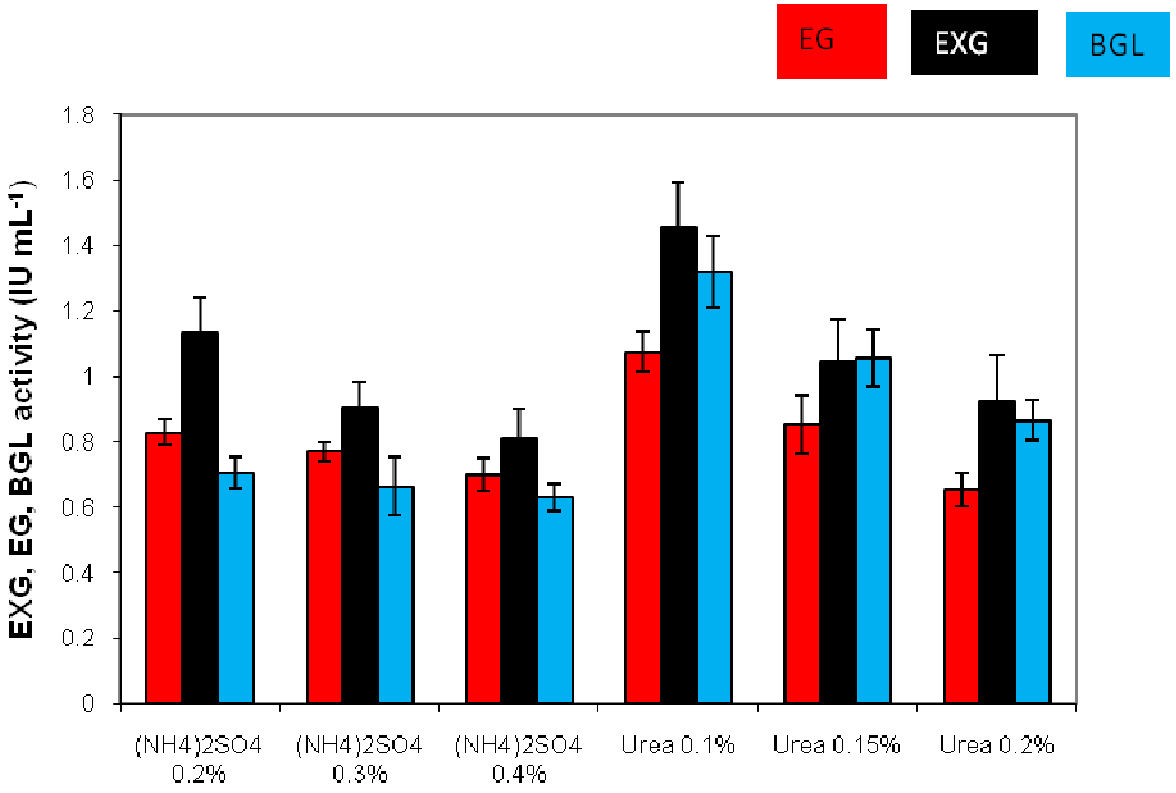

Figure 6. Effect of different concentration of $(NH_4)_2SO_4$/Urea on cellulase production from *A. niger* NRRL 567.

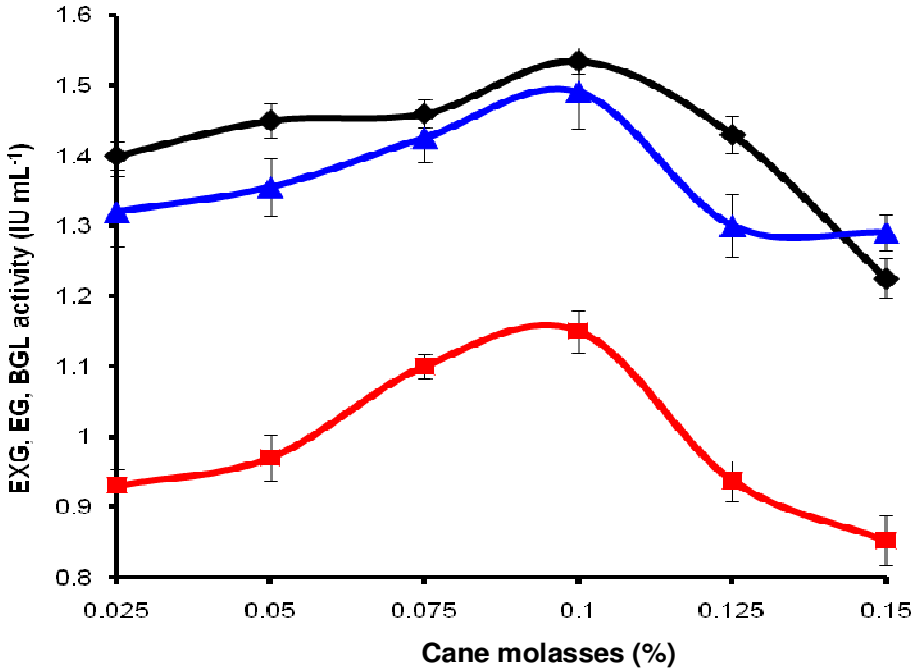

Figure 7. Influence of cane molasses on cellulase production by *A. niger* NRRL 567. (♦) EXG; (■) EG; (▲) BGL.

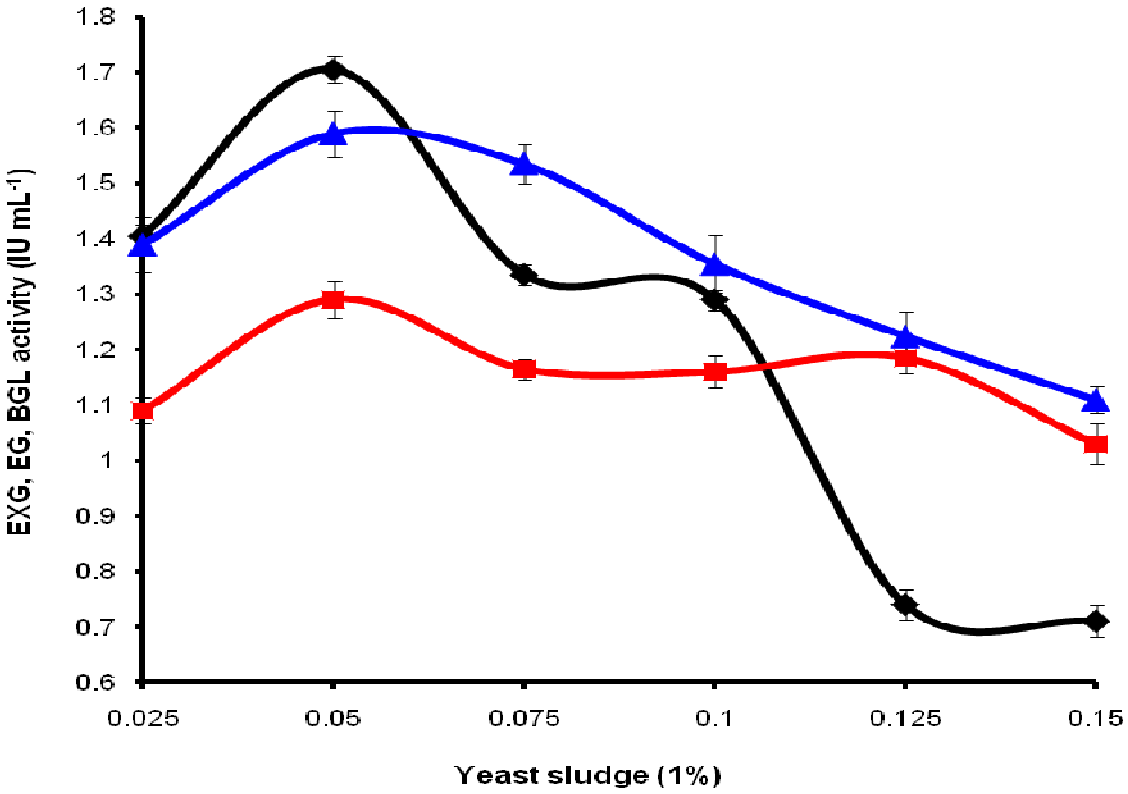

Figure 8. Influence of yeast sludge on cellulase production by *A. niger* NRRL 567. (♦) EXG; (■) EG; (▲) BGL.

by yeast cells, 0.5 to 1% of nitrogen source, proteins, vitamins, amino acids, organic acids and heavy metals (Athar et al., 2009; Ali et al., 2009). Hence, it is a very attractive carbon source for cellulase production from *A. niger* from the economic point of view. One criterion that is crucial in the selection of microbial strains for cellulases production is its ability to grow on cheap substrates. This criterion is satisfied with the results obtained with the current strain of *A. niger* NRRL 567 used in this study, which was found to grow well and produced cellulases on corn stover along with molasses.

Conclusions

The present work showed that, cellulase production by *A. niger* NRRL 567 was increased by culturing this strain in corn stover medium. It was found that, corn stover and molasses can be used to generate cellulases by *A. niger* NRRL 567 without costly pretreatment or nutrient supplementation. Meidum optimization has greatly enhanced the cellulolytic enzyme yield. Corn stover (4% w/v) has shown excellent potential as a substrate for cellulases production. 0.1% molasses and 1% yeast sludge gave higher cellulases production at pH 3.5, 30°C and 72 h of incubation. The present research indicated that, *A. niger* effectivley produced celluases and could be utilized for industrial production of cellulaes. The possibility of using locally available substrates such as corn stover and yeast sludge for cellulases production was promising. The organism is prone to mutagenesis and will be more suitable for its application in future for bulk production of cellulases for paper and pulp industry in Pakistan.

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