Insight Biotechnology 1 (2): 17-22, 2011
ISSN 2040-8331
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Utilization of Pea Seed Husk as a Substrate for Cellulase Production by Mutant *Aspergillus niger*

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Abstract: **Background:** The main aim of this study was to produce higher titers of cellulase enzyme from cellulolytic mutant fungi *Aspergillus niger* isolated from soil contaminated with cotton ginning mill effluents. The fungal strain was subjected to chemical mutations with Ethyl Methane Sulfonate (EMS) and the best mutant strain ($GNEM_7$) was selected for cellulase production. A locally chiefly available lignocellulosic agricultural waste, pea seed husk, was used as substrate for fungal cellulase. Further this substrate was treated with various chemicals like acid, alkali and hydrogen peroxides for depolymerization of cellulose from lignocelluloses, serves as main substrate for cellulase. **Results:** Enhanced production of cellulase enzyme was obtained when the substrate pea seed husk pretreated with 1N HCl, NaOH and 10% hydrogen peroxide and the cellulase activities such as CMCase (18.03 IU), FPase (19.73 IU) and β-glucosidase (1.760 IU) were 15, 14 and 10 folds respectively by mutant fungal culture, *A. niger*. **Conclusion:** A significant enhancement in cellulase production was obtained by EMS mutant *Aspergillus niger* ($GNEM_7$) on chemically pretreated lignocellulosic substrate pea seed husk. Among the chemicals used in the present study hydrogen peroxide serves a good chemical agent for pretreatment of lignocelluloses.

Key words: Pea seed husk, *Aspergillus niger* (GNEM7), chemical pretreatment, submerged fermentation, cellulases

INTRODUCTION

Long-term economic and environmental concerns have resulted in a great amount of research in past couple of decades on renewable source of liquid fuels to replace fossil fuels. The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages (Kuhad *et al*., 1997). Important distinguishing features of cellulose biomass among potential feeds for biological processing include low purchasable price, potential for supply on very large scale, recalcitrance to reaction and heterogeneous composition. Cellulosic waste may be converted to products of commercial interest such as glucose, soluble sugars, alcohol, single cell proteins (Ojumu *et al*., 2002). The key element in bioconversion process of lignocellulosics to these useful products is the hydrolytic enzymes mainly cellulases (Immanuel *et al*., 2007). The saccharification process of cellulose waste relies on participation of cellulolytic organisms and their cellulase enzymes (Lynd *et al*., 2002). In addition, cellulolytic enzymes have been exploited for commercial application in a variety of industries. However, the saccharification process has not yet reached to the level of commercialization due to many factors-complexity of cellulose structure, production of celluloses in low amounts by cellulolytic organisms due to carbon repression, high cost of cellulase production and poor yields of glucose (Gregg and Saddler, 1996). In order to enhance the rate of saccharification, it has become necessary to search for highly efficient cellulolytic organisms with secretion of higher titers of cellulase. Members of fungal genus *Trichoderma* and *Aspergillus* have been extensively studied, particularly due to their ability to secrete cellulose degrading enzymes. The strains that have been mutagenized and genetically modified to obtain an organism capable of producing high levels of cellulases (Vu *et al*., 2009). The use of different mutagenic agents for strain improvement was demonstrated by Parekh *et al.* (2000). Simultaneous treatment with NTG and Ethidium bromide improved Fpase and CMCase activities than wild type fungi (Chand *et al*., 2005).

The purpose of this study was to screen fungal culture for producing high levels of cellulase enzyme by chemical mutations and depolymerization of

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lignocellulosics by pretreatment with various chemical agents for cellulase. In this study pea seed husk which is a cheap and locally available lignocellulosic waste was tested as a novel substrate for cellulase production by Mutant *Aspergillus niger*.

MATERIALS AND METHODS

Microorganism: *Aspergillus niger* was isolated from the cotton industry effluent soil collected from Nandyal, Andhra Pradesh, India (Narasimha *et al*., 1999). This strain was cultivated on potato dextrose agar medium at 28°C for 7 days.

Screening of *Aspergillus niger* for cellulase production: The cellulolytic nature of *Aspergillus niger* was confirmed first through the screening test. To this 1% of CMC was amended with Czapeck-Dox agar media and the pH was adjusted to 5. The media was poured into sterile Petri dishes, after solidification of media a small hole was made on the centre of Petri dish aseptically and the culture spores were added to this centre. The plates were incubated for 3 days at 30°C and 2 days at 50°C. After incubation the plates were stained with 1% Congo red solution for 15 min, after that the Congo red stain was neutralized with 1 M NaCl solution. The yellow color zone formation concern the ability of cellulose utilization and enzyme activity of fungal culture.

Mutation studies: The spore suspension of the *Aspergillus niger* was used for EMS chemical treatment. 4mL of spore suspension was added to 12 mL of EMS solution (4 mg mL^{-1}) and the reaction was allowed to proceed. Two milliliter of this solution was taken at intervals of 30, 60, 90,120,150,180 and 210 min and centrifuged immediately for 10 min at 5000 rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and resuspended in 10 mL of sterile phosphate buffer. The samples were serially diluted in the same buffer and plated over Czapeck-Dox agar medium. A total 10 colonies (designated as GNEM-1 to GNEM-10) were selected from the plates showing less than 1% survival rate (60 and 90 min EMS treated spore suspension) and tested for cellulase production.

Preparation of fungal spore inoculum for cellulase production: The mutant fungal culture was grown on Czapeck-Dox agar slants and they were incubated at room temperature for 7 days. After incubation 3 mL of sterile distilled water was added for each slant. Fungal spore concentration was determined by haemocytometer. Inoculum density was 2×10^6 spores were used for cellulase production.

Substrate: The substrate Pea seed husk was collected from local agricultural fields and it was pretreated with sodium hydroxide, hydrochloric acid and hydrogen peroxide and exposed to cellulolytic attack.

Preparation of substrates: Pea seed husk was sun dried for a period of three weeks and subsequently oven dried slowly at 50°C for 2 days. The dried substrate was chopped into bits, pulverized into coarse particle sizes and then washed in several changes of hot water in order to remove the residual sugars (Rezende *et al*., 2002).

Acid treatment (HCl): In this process the pea seed husk was soaked in 1N HCl in the ratio 1:10 (substrate: solution) for 60 min at room temperature and then washed with double distilled water for removal of chemicals and autoclaved at 121°C for one hour. The treated substrate was filtered for free of fibers and neutralized by washing with dilute aqueous sodium hydroxide. Then the treated substrate was washed with double distilled water until the filtrate becomes neutral. The substrates were dried at 60°C for 12h on hot air oven and used for enzyme assay.

Alkali treatment (NaOH): Pea seed husk was soaked in 1 N NaOH solution in the ratio 1:10 (substrate: solution) for 60 min at room temperature. After that, the substrate was subjected to washing with double distilled water for free of chemicals and autoclaved at 121°C for one hour. Then the treated substrate was washed with double distilled water until the wash water turned to become neutral pH and dried at 60°C for 12 h on hot air oven.

Hydrogen peroxide treatment (H_2O_2): Hydrogen peroxide (H_2O_2) at concentrations of 5 and 10% were used to pretreat the pea seed husk. The pH of the suspension was adjusted to 11.5 with 0.1 N NaOH and stirred gently at room temperature 25°C for 20 h. The contents were filtered and washed with distilled water until pH returns to neutral. The treated sample was dried at 110°C for overnight.

Filter paper saccharifying activity (Fpase): Filter paper sachharifying activity in the culture filtrates was determined by the method of Ghose (1987). It is a combined assay for endo β-1,4 glucanase and exo β-1,4glucanase.The standard reaction mixture containing 50 mg of What man No.1 filter paper strips (1×6 cm) as a substrate, suspended in a mixture containing 1 mL of 0.05 M sodium citrate buffer (pH 4.8) and 0.5 mL of enzyme source. The enzyme control was also prepared simultaneously by adding distilled water instead of enzyme. This mixture was incubated for one hour at 50°C

in water bath. The reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959). After incubation; 3 mL of DNS reagent was added to each test tube and boiled for 5 min in a boiling water bath. After boiling, transferred to a cold water bath. Added 20 mL of distilled water. Mixed completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion. The color developed in the test tubes was read at 540 nm in a spectrophotometer. The enzyme activity was expressed in filter paper units. Filter paper units were defined as the amount of enzyme releasing μ moles of reducing sugar from filter paper per minute per mL.

Endoglucanase assay (CMCase): Endoglucanase activity of fungal culture was quantified by Carboxy methyl cellulase method (Ghose, 1987). According to this method, 1 mL of 2% carboxy methyl cellulose as a substrate was added to the mixture containing 1 mL of 0.05 M sodium citrate buffer (pH 4.8) and 0.5 mL of enzyme. This mixture was incubated at 50°C in a water bath for 30 min. The reducing sugar produced in the reaction was estimated by DNS method. After incubation, 3 mL of DNS reagent was added to each test tube and boiled for 5 min in a boiling water bath. After boiling, transfer to a cold water bath and added 20 mL of distilled water. Mixed completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion. The color developed in the test tubes was read at 540 nm in a spectrophotometer. The enzyme activity was expressed in terms of CMC units. CMC units were defined as the amount of enzyme releasing μ moles of reducing sugar from the substrate per minute per mL.

β-glucosidase assay: β-glucosidase activity in the culture filtrates was determined by the method of Herr (1979). According to this method, 0.2 mL of 5 mM ρ-nitro phenyl β-D-glucopyranoside (PNPG) as a substrate was added to the mixture containing 1.6 mL of 0.05M sodium citrate buffer (pH 4.8) and 0.2 mL of enzyme solution. After incubation, for 30 min at 50°C the reaction was stopped by the addition of 4 mL of 0.05 M NaOH-glycine buffer (pH-10.6) and the yellow colored para-nitro phenyl was measured at 420 nm in spectrophotometer. One unit of β-glucosidase activity is defined as that releasing μ mole of PNP from PNPG per minute per mL.

Protein estimation: Protein content in culture filtrate was determined by Lowry's method (Lowry *et al.*, 1951). The culture filtrate with appropriate dilution was mixed with 5 mL of alkaline solution. After 10 minu, 0.5 mL of folin-ciocalteau reagent was added. After 30 min of incubation in a dark place the colour developed was measured by spectrophotometer at 660 nm.

RESULTS AND DISCUSSION

The fungal strain *Aspergillus niger* was screened for cellulase enzyme production. The formation of clear yellow zone of hydrolysis concerns its ability for cellulase production (Fig. 1).

Pea seed husk, a cheap and locally available lignocellulosic waste was tested to find out whether it could support the production of cellulases by *Aspergillus niger* under submerged fermentation. The Fpase, CMCase and β-glucosidase activities of wild strain grown on pea seed husk were 3.2 IU, 2.8 and 0.4 per mL per min, respectively (Fig. 2).

- Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the filtrate that releasing 1μmole of reducing sugar from filter paper/mL/min
- Carboxymethyl cellulose (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 μmole of reducing sugar from carboxymethyl cellulose/mL/min

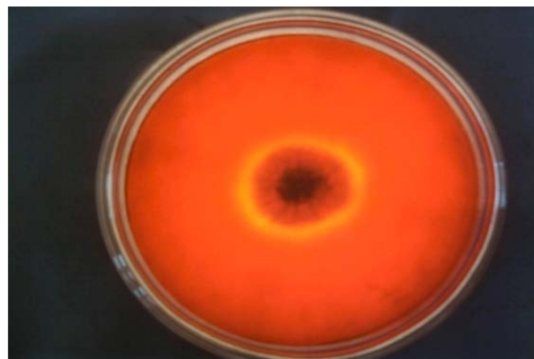

Fig. 1: *Aspergillus niger* showed clear yellow zone of hydrolysis which indicates CMC degradation

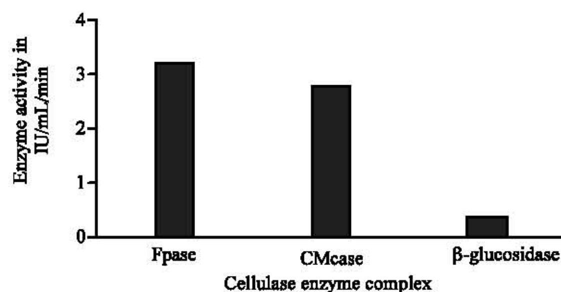

Fig. 2: Cellulolytic activity of wild strain *A. niger* grown on pea seed husk. Values represented are the Mean of two separately conducted experiments

- One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1 μmole of p-nitro phenol/mL/min

The wild strain *Aspergillus niger* was subjected to ethyl methane sulfonate mutations and ten mutant strains from $GNEM_1$ to $GNEM_{10}$ were tested for total cellulase production (Fig. 3). Among the 10 EMS mutants $GNEM_7$ showed maximum total cellulolytic activity (6.91 IU/mL/min) and this strain was selected for cellulase production on pre-treated pea seed husk.

In order to obtain a high amount of fermentable sugars various chemical pretreatments have been used in the present study. The combined effect of acid (1 N HCl), alkali (1 N NaOH) and oxidation (5 and 10% H_2O_2) pre-treatment methods was examined. The cellulolytic activities of $GNEM_7$ on pea seed husk treated with different chemicals was determined (Fig. 4) and it showed maximum cellulase activities (FPase-19.73 IU, CMCase-18.09 IU and β-glucosidase-1.73 IU) on 10% H_2O_2 pre-treated pea seed husk than that of the remaining pre-treatments.

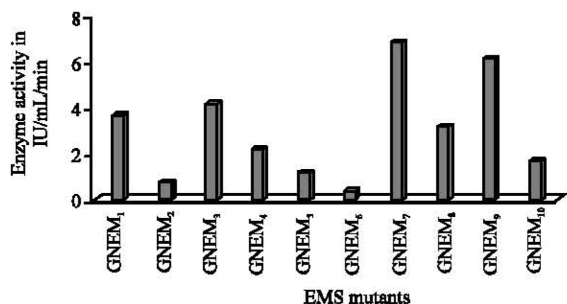

Fig. 3: Total cellulolytic activity of ethyl methane sulfonate mutants grown on pea seed husk. Values represented are the Mean of two separately conducted experiments

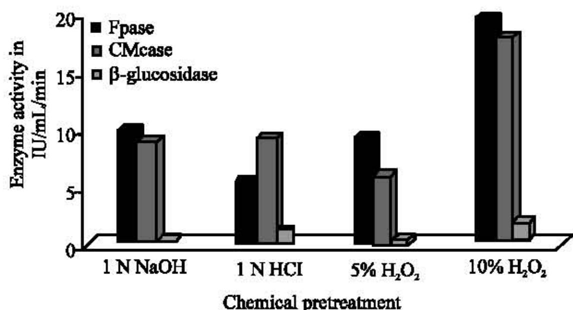

Fig. 4: Cellulase activities of EMS mutant (GNEM7) on chemically pretreated pea seed husk. Values represented are the Mean of two separately conducted experiments

Maximum reducing sugar concentration 1.6 mg mL^{-1} (Fig. 5) and protein concentration 2700 μg mL^{-1} (Fig. 6) were obtained when pea seed husk treated with 10% hydrogen peroxide.

Damisa *et al.* (2008) reported that the highest cellulase activities on bagasse, corn cob and corn straw pre-treated with 2 M NaOH by *Aspergillus niger* AH_3 were 0.067, 0.049 and 0.504 IU, respectively. Another study by Fatma *et al.* (2010) reported that the maximum cellulase activity on rice straw treated with 1% NaOH using *Trichoderma reesei* F418 was 11.17 IU g^{-1}. Similarly, in the present study, *Aspergillus Niger* $GNEM_7$ showed 9.93 and 19.73 IU of cellulase activities on pea seed husk pre-treated with 1 N NaOH and 10% H_2O_2, respectively.

The total cellulolytic activity of *Aspergillus niger* GNEM7 on I N Hcl pretreated pea seed husk was 9.13 IU/mL/min. Similar results were made with *Aspergillus fumigatus* on Hcl pretreated wheat straw, values were found to be 0.237 and 0.674 IU mL^{-1} (Dahot and Hanif, 1996). Similarly, the FPase, CMCase values found to be 0.089 and 1.023 U mL^{-1}, respectively when groundnut shells treated with 0.25 N HCl (Vyas *et al.*, 2005).

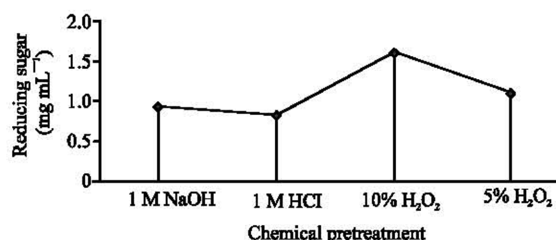

Fig. 5: Reducing sugar concentration in pea seed husk treated with different chemicals. Values represented are the Mean of two separately conductedexperiments

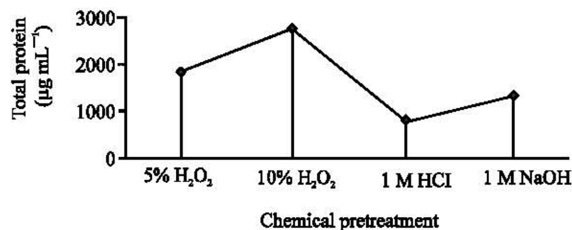

Fig. 6: Total protein concentration in pea seed husk treated with different chemicals. Values represented are the Mean of two separately conducted experiments

In the present study the cellulase activities, FPase (19.73 IU), CMCase (18.09 IU) activities were higher on 10% hydrogen peroxide pretreated pea seed husk. A similar observation was made by Haq *et al.* (2008), higher enzyme activities Fpase (5.82), CMCase (10.54), β-glucosidase (9.85 U mL^{-1}) were obtained when sawdust treated with 5% hydrogen peroxide. Singh *et al.* (2009) reported that the FPase and CMCase activities on rice straw by *Aspergillus niger* were 0.96 and 0 .66 IU, respectively. The mutant Trichoderma when grown on wheat bran produced FPase-6.2 IU and β-glucosidase 0.39 IU (Jun *et al.*, 2009). In the present study *Aspergillus niger* GNEM7 showed higher Fpase-19.73 IU, CMCase-18.09 IU and β-glucosidase-1.73 IU activities than the previous studies.

CONCLUSION

The production of cellulases on pea seed husk under submerged fermentation was studied by mutant *Aspergillus niger*. Pea seed husk is a cheap and locally available residue was used as a novel substrate for cellulase enzyme production and reduces the cost of enzyme production. The enzyme activities obtained on this substrate were maximum than the other lignocelluloses studied earlier.

ACKNOWLEDGMENTS

We are grateful to University Grants Commission (UGC), New Delhi for providing financial support to carry out this work.

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