Induction of chemical mutations in Aspergillus niger to enhance cellulase production

M. REDDI PRADEEP, A. JANARDHAN, A. PRAVEEN KUMAR and G. NARASIMHA*

Applied Microbiology Laboratory, Department of Virology, Sri Venkateswara University, Tirupati-517502, AP, and India.

*Address of corresponding author

Dr. G. Narasimha, Ph.D., Applied Microbiology Laboratory, Department of Virology, Sri Venkateswara University, Tirupati – 517502, Andhra Pradesh, India.
Mobile: +91-9700170539
E-mail: gnsimha123@rediffmail.com

Received 01 August 2012; accepted 14 August 2012

Abstract
The fungal strain, Aspergillus niger was subjected to mutations involving treatment of Ethidium bromide (EtBr) for the production of cellulases. After mutagenesis, ten mutant strains (GNEB₁ to GNEB₁₀) were selected and their cellulase activities were assayed. Five of them (GNEB₁, GNEB₄, GNEB₅, GNEB₈, GNEB₉) had significantly stronger ability to produce enzymes than that of normal wild type, and they were also very stable for a long period up to 8 generations to produce cellulase. Mutant Strain GNEB₁ exhibited maximum Activities of cellulases, Filter paperase (FPase - 3.91 IU/ml), Carboxymethyl cellulase (CMCase - 3.15 IU/ml) and β-glucosidase (0.87 IU/ml). These levels were, respectively, 2.3, 2.1 and 4.5 fold higher than those in parent strain (FPase - 1.68 IU/ml, CMCase - 1.48 IU/ml and β-glucosidase - 0.87 IU/ml). The extracellular soluble protein content was also improved in the culture filtrate. The waste lignocellulosic material, ground nut shells was used as low-cost carbon source for cellulase production by mutant Aspergillus niger under submerged fermentation to make the process economically viable.

© 2011 Universal Research Publications. All rights reserved

Keywords: Aspergillus niger, Mutation, Ethidium bromide, Ground nutshells, cellulases.

INTRODUCTION
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 [1]. 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, and single cell proteins [2]. The key element in bioconversion process of lignocellulosics to these useful products is the hydrolytic enzymes mainly cellulases[3]. The saccharification process of cellulose waste relies on participation of cellulytic organisms and their cellulase enzymes [4]. As the production of cellulase enzyme is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically viable. Members of the fungal genus Trichoderma and Aspergillus have been extensively studied, particularly due to their ability to secrete cellulose degrading enzymes. These strains produce extracellular cellulytic enzymes, namely endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21) which acts synergistically in the conversion of cellulose to glucose. The strains have been mutagenized and genetically modified to obtain an organism capable of producing high levels of cellulases [5]. Much of the knowledge of enzymatic de-polymerization of cellulosic material has come from Trichoderma cellulase system. Species of Trichoderma can produce substantial amounts of endoglucanase and exoglucanase but very low levels of β-glucosidase [6]; therefore, attention has recently been diverted to other micro-organisms including the members of genus Aspergillus and it has widely been exploited as it possesses all the three essential components of cellulase system [7]. Ground nut shells, the agricultural byproduct, is a cheaply available resource in India, and has potential as an industrial fermentation substrate. In the current work, ground nut shells was used a substrate in order to reduce the cost of cellulase production. The aim of this study was to obtain high levels of extracellular cellulases by mutating
The values represented in the figure are average of three independent experiments.

- Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the filtrate that releasing one micro mole 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 1µmole of reducing sugar from carboxymethyl cellulose/ml/min.
- One unit of β-glucosidase activity is defined as the amount of enzyme liberating 1µmole of p-nitro phenol/ml/min.

The Aspergillus niger using chemical mutagen Ethidium bromide.

MATERIALS AND METHODS

Chemicals

Ethidium bromide (EtBr) and p-nitro phenol β-D-glucojynoside (pNPG) were procured from Sigma Chemical Co. USA.

Microorganism and culture conditions

Aspergillus niger used in the study was isolated from soil contaminated with effluents of cotton ginning mill [8]. The organism was maintained on potato dextrose agar at 28°C and sub cultured after every 15 days.

Fungal strain improvement by mutagenesis

The organism grown on potato dextrose agar (PDA) slants was scraped off into sterile phosphate buffer (0.02M and pH 7.0) containing Tween-80 (1:5000) to give uniform suspension. The suspension was transferred into a sterile conical flask and thoroughly shaken for 30 minutes on a rotary shaker to break the spore chains. The spore suspension was then filtered through a thin sterile cotton cloth to remove vegetative mycelium from the suspension. This spore suspension was used for EtBr treatment. 4ml of spore suspension was added to 2ml of EtBr solution (10µl/ml) and the reaction was allowed to proceed. 1ml of this solution was taken after 24 hrs and centrifuged immediately for 10 min at 5000rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and resuspended in 10ml of sterile phosphate buffer. The samples were serially diluted in the same buffer and plated over modified Czapeck-Dox agar medium. A total of 10 colonies (designated as GNEB1 to GNEB10) were selected from the plates showing less than 1% survival rate.

Hereditary stability studies of mutants

The mutants obtained by the chemical mutation were studied for their stability for cellulase production for 8 generations. The mutants after every fermentation were inoculated on the ground nut shell fermentation medium and used for inoculating next fermentation.

Substrate

Ground nut waste shells were cheap and locally available source of cellulose. It 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 small bits, pulverized into coarse particle sizes and then washed in several changes of hot water in order to remove the residual sugars.

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 3ml of sterile distilled water was added for each slant. Fungal spore concentration was determined by haemocytometer. Inoculum density was 2 X 10⁶ spores were used for cellulase production.

Total Protein determination

Total Protein content after pretreatment and fermentation was determined according to Lowry method [9].

Enzyme assays

FPase activity (filter paper activity) and CMCase activity (carboxy methyl cellulase activity) in the culture filtrate was determined by Ghose method [10]. β-glucosidase activity was assayed by using the Herr method [11]. Units (IU) of FPase and CMCase were defined as the one micro mole of glucose liberated per minute per milliliter under assay conditions. One unit of β-glucosidase was defined as the amount of enzyme liberating one micro mole of p-nitro phenol per minute per milliliter.

![Fig.1 Cellulase activity of mutants of Aspergillus niger.](image)

RESULTS AND DISCUSSION

Mutagensis is an easy tool to achieve genetic modification of an organism. In the present study, mutagensis was carried out by exposing the fungal culture Aspergillus niger to a potent chemical mutagen Ethidium bromide. A total of 10 strains (designated as GNEB1 to GNEB10) were selected from the plates showing less than 1% survival rate. The results presented in Fig.1 indicated the production of Cellulase enzyme complex by Ethidium bromide mutants. Five of the ten mutant strains (GNEB1, GNEB4, GNEB5, GNEB6 and GNEB7) secreted significantly more enzymes than the parent strain. Mutant, GNEB5 proved to be the most promising extracellular cellulase producer and showed maximum filter paper activity (3.91 IU), CMCase activity (3.15 IU) and β-glucosidase activity (0.87 IU) which were 80%, 112% and 81% higher than the parent strain activities, respectively. These levels were, respectively, 2.3, 2.1 and 4.5 fold higher than those in parent strain (FPase-1.68 IU/ml, CMCase-1.48 IU/ml and β-glucosidase-0.87 IU/ml). The FPase and CMCase production increased 2.0 and 1.3, respectively, using successive treatments of different mutagens such as EtBr and MNNG followed by UV to the spores of Trichoderma[12]. Sequential mutagenesis of Pencilliumchinulaudum with UV, EMS and H₂O₂ improved 1.5 fold cellulase productivity [13].
The five mutants selected in the mutation of Ethidium bromide (GNEB1, GNEB3, GNEB4, GNEB5, and GNEB8) were found to be stable for cellulase production for a long period of 8 generations (Table 1).

**Table 1.** Total cellulolytic activities of mutants of *Aspergillus niger*.

<table>
<thead>
<tr>
<th>EtBr mutants</th>
<th>Total cellulolytic activity in IU/ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNEB1</td>
<td>3.88</td>
</tr>
<tr>
<td>GNEB4</td>
<td>1.54</td>
</tr>
<tr>
<td>GNEB5</td>
<td>2.11</td>
</tr>
<tr>
<td>GNEB8</td>
<td>1.83</td>
</tr>
<tr>
<td>GNEB9</td>
<td>1.69</td>
</tr>
</tbody>
</table>

The values represented in the table are average of three independent experiments.

The cellulase production was carried out under submerged fermentation using a cheap and locally available lignocellulosic residue, ground nut shells. The highest cellulase activities on bagasse, corn cob and corn straw pretreated with 2M NaOH by *Aspergillus niger* AH3 were 0.067 IU, 0.049 IU and 0.504 IU respectively [14]. In another study, the FPase and CMCase activities on rice straw by *Aspergillus niger* were 0.96 IU and 0.66 IU respectively [15]. The mutant *Trichoderma* when grown on wheat bran produced FPase-6.2 IU and β-glucosidase 0.39 IU [16]. But, in the present study the enzyme activities were much improved when the Ethidium bromide mutant grown on ground nut waste.

Figure 2. shows the total protein concentration obtained on the 7th day of fermentation. Mutant strain GNEB3 showed highest protein concentration of 2.23 mg/ml, which was more than that of the parent strain (0.60 mg/ml).

**Fig.2.** Protein content in the culture filtrate of mutant *Aspergillus niger*

The values represented in the figure are average of three independent experiments.

**CONCLUSION**

The present study clearly indicates that the cellulase production in *Aspergillus niger* by chemical mutagenesis, resulting in 2 to 4.5 fold increase in cellulase production. The fermentation was carried out under submerged fermentation using cheap and locally available lignocellulosic waste, ground nut shells to make the process economically viable.

**ACKNOWLEDGEMENTS:**

Authors are grateful to University Grants Commission [UGC], New Delhi for providing financial support to carry out this work.

**REFERENCES**

Source of support: Nil; Conflict of interest: None declared