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Production of cellulase enzyme by mutant fungal strain *Aspergillus niger* in submerged fermentation

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ABSTRACT

Production of enzymes is a growing field of biotechnology especially related to industrially important enzymes. The present study was carried out to investigate the production of cellulase enzyme from UV mutated *Aspergillus niger* grown on rice bran under submerged fermentation. The fungal strain *Aspergillus niger* was subjected to UV irradiation and the successive mutants showed enhanced cellulase production. Particularly, the mutant fungal strain *Aspergillus niger* $GNUV_3$ was efficient and the activities of carboxy methyl cellulase, filter paperase and β-glucosidase were improved by 4.7, 4.4 and 3.8 – fold, respectively when compared to the wild type strain.

KEYWORDS

Aspergillus niger;
UV mutation;
Rice bran;
Cellulase.

INTRODUCTION

Lignocellulose biomass is the most abundant organic raw material in the world[1]. Lignocelluloses constitute a major portion of agricultural wastes and forest wastes. They are abundant sources of carbohydrates, continually replenished by photosynthetic reduction of carbon dioxide with sunlight energy[2]. Thus they are the most promising feedstock for the production of energy, food and chemicals[3]. The bioconversion of the agro-waste material into fuel has received considerable interest during recent years. Enzymatic hydrolysis of cellulosic biomass is considered as the most efficient and least polluting methods for generating glucose from lignocellulosic, but the production economics of bioethanol is largely depended on cost of cellulases[4]. India is an agricultural country and rice is the one of the most important agricultural crop. With the processing of rice grains a large amount of rice bran is produced which can be used as a substrate for cellulase production.

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 cellulolytic enzymes, namely endoglucanases, exoglucanases, cellobiase, which act synergistically in the conversion of cellulose to glucose[5]. The strains have been mutagenized and genetically modified to obtain an organism capable of producing high levels of cellulases[6]. In the present study the production of cellulolytic enzymes by UV mutated *Aspergillus niger* on rice bran in submerged fermentation is reported.

MATERIALS AND METHODS

Source of *Aspergillus niger*

Aspergillus niger was isolated from soil contaminated with cotton ginning mill effluents by Narasimha,[7] and this strain was cultivated on potato dextrose agar at 28^{0}C for 7days.

Screening of fungal culture for cellulase production

The cellulolytic nature of *Aspergillus niger* isolated from soil was confirmed first through the screening test. To this 1% of CMC was amended with Czapek -Dox agar media and the pH was adjusted to pH 5.The media was poured in sterile petri dishes, after solidification of media a small hole was put on centre of Petri dish aseptically and the culture spores were added to this centre. The plates were incubated for 3days at 30^0 C and 2days at 50^0 C. After incubation the plates were stained with 1% Congo red solution for 15 minutes, after that the Congo red stain was neutralized with 1M Nacl solution. The yellow color zone formation concern the ability of cellulose utilization and enzyme activity of fungal culture.

UV irradiation

The spore suspension of the *Aspergillus niger* was prepared in phosphate buffer and 4 ml quantities of the spore suspension were pipetted aseptically into sterile Petri dishes of 80mm diameter having a flat bottom. The UV light exposer was carried out in a "Dispensing – Cabinet" fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 A^0. The exposure times were 10, 15,30,45,60 and75min, and the distance maintained as 15 and 20 cm. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in phosphate buffer and plated on PDA medium. The plates were incubated for 7days at 28^{0}C and the numbers of colonies in each plate were counted. Each colony was assumed to be formed from a single spore. A total of 10 colonies (designated as GNUV1 to GNUV10) were selected from the plates showing less than 1% survival rate (45, 60 and 75 min UV exposure time) and tested for cellulase production.

Preparation of fungal spore inoculum for cellulase production

The isolated fungal culture was grown on Czapek-Dox agar slants 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 10^6 spores were used for cellulase production.

Substrate

In the present study cheap and locally available lignocellulosic substrate, Rice bran was used for cellulase production. It was sun dried for a period of three weeks and subsequently oven dried slowly at 50^{o}C for 2 days. The dried substrate was chopped into small bits, pulverized to coarse particle sizes and then washed in several changes of hot water in order to remove the residual sugars.[8]

Cellulase production and assay

100 ml of Czapek-Dox medium at pH 5 was distributed into separate Erlenmeyer 250ml conical flask. After sterilization the flask was inoculated with fungal spore suspension. Then the flasks were incubated at 28°C±30°C on a rotary shaker at 180 rpm for 7 days. After incubation the contents of the flask were passed through whatman No.1 filter paper to separate mycelial mat and culture filtrate. The filtrate obtained was used for estimation of extracellular protein content and total cellulase activity such as FPase, endogluconase and β-glucosidase

Filter paper saccharifying activity (FPase)

Filter paper sachharifying activity in the culture filtrates was determined by the method of Ghose,[9]. It is a combined assay for endo β-1,4 glucanase and exo β-1,4glucanase.The standard reaction mixture containing 50mg of Whatman No.1 filter paper strips (1x6cm) as a substrate, suspended in a mixture containing 1ml of 0.05M sodium citrate buffer (pH 4.8) and 0.5ml 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^{0}C in water bath. The reducing sugar content was estimated by Dinitrosalicylic acid method. After incubation, 3ml

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of DNS reagent was added to each test tube and boiled for 5 minutes in a boiling water bath. After boiling, transferred to a cold water bath and 20 ml of distilled water was added. 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[9]. According to this method, one ml of 2% carboxy methyl cellulose as a substrate was added to the mixture containing 1ml of 0.05M sodium citrate buffer (pH 4.8) and 0.5ml of enzyme. This mixture was incubated at 50^{0}C in a water bath for 30minutes. The reducing sugar produced in the reaction was estimated by DNS method. After incubation, 3ml of DNS reagent was added to each test tube and boiled for 5 minutes 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[10]. According to this method 0.2 ml of 5mM ρ-nitrophenyl β-D-gluco pyranoside (PNPG) as a substrate was added to the mixture containing 1.6 ml of 0.05M sodium citrate buffer (pH 4.8) and 0.2ml of enzyme solution. After incubation, for 30 min at 50^{0}C the reaction was stopped by the addition of 4ml of 0.05M 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.

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 was shown in Figure 1.

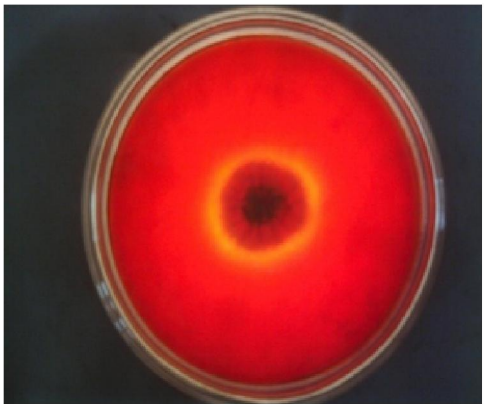

Figure 1 : *Aspergillus niger* showed clear yellow zone of hydrolysis which indicates CMC degradation.

Rice bran, a cheap and locally available lignocellulosic waste was tested to find out whether it could support the production of cellulases by the wild *Aspergillus niger* under submerged fermentation. The FPase, CMCase and β-glucosidase activities of wild strain grown on pea seed husk were 1.6 IU, 1.4 IU and 0.19 IU per ml per min respectively (Figure 2).

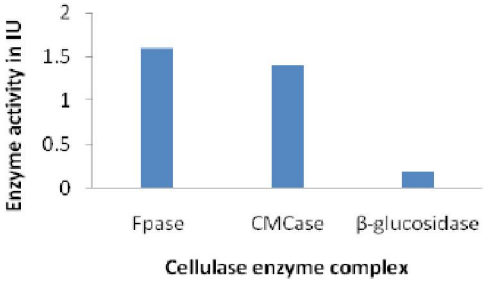

Figure 2 : Cellulolytic activity of wild strain *A.niger* grown on rice bran

***values represented in the figure are the mean of two separately conducted experiments**

- Filter paperase (FPase) is expressed in terms of filter pare 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 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

Further the wild strain *Aspergillus niger* was subjected to UV irradiation and ten mutant fungal strains from $GNUV_1$ to $GNUV_{10}$ were obtained and tested for their total cellulolytic activity shown in Figure 3.

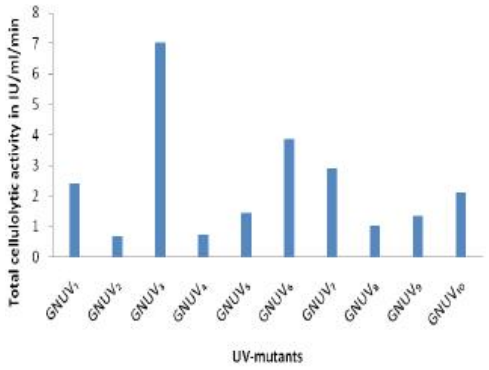

Figure 3 : Total cellulolytic activity of UV-mutants grown on rice bran

***values represented in the figure are the mean of two separately conducted experiments**

- Filter paperase (FPase) is expressed in terms of filter pare 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 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

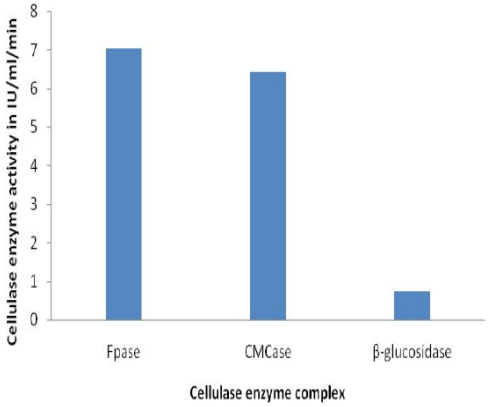

Figure 4 : Cellulase production by UV-mutant *Aspergillus niger* GNUV, grown on rice bran

***values represented in the figure are the mean of two separately conducted experiments**

Among the 10 UV mutants $GNUV_3$ showed maximum total cellulolytic activity (7.05 IU/ml/min) and this strain was selected for cellulase production on rice bran (Figure 4).

- Filter paperase (FPase) is expressed in terms of filter pare 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 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

Damisa,[11] reported that the highest cellulase activities on bagasse, corn cob and corn straw by *Aspergillus niger* AH_3 were 0.067 IU, 0.049 IU and 0.504 IU respectively. In the present study *Aspergillus niger* $GNUV_3$ showed 7.03 IU/ml/min on rice bran under submerged fermentation. Dahot,[12] found that the growth of *Aspergillus fumigatus* on wheat straw produced filterpaperase 0.237 IU/ml and carboxymethyl cellulase 0.674 IU/ml. In another study by Asish,[13] the FPase, CMCase values found to be 0.089U/ml,1.023U/ml respectively when groundnut shells treated with 0.25N HCl. But, in the present study the filter paper activity (7.03 IU) and carboxymethyl cellulase activity (6.04 IU) were obtained. Anita Singh,[14] reported that the FPase and CMCase activities on rice straw by *Aspergillus niger* were 0.96 IU and 0.66 IU respectively. The mutant Trichoderma when grown on wheat bran produced FPase-6.2 IU and β-glucosidase 0.39 IU[15]. In the present study *Aspergillus niger* $GNUV_3$ showed higher FPase-7.03IU, CMCase-6.43IU and β-glucosidase 0.73 IU activities than the previous studies.

CONCLUSION

The UV mutated *Aspegillus niger* showed higher cellulase activities in the present study. The Rice bran is cheaply available lignocellulosic substrate which can reduce the cost of enzyme production.

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