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Production of Cellulolytic Enzymes by Coculturing of *Aspergillus ellipticus* and *Aspergillus fumigatus* Grown on Bagasse Under Solid State Fermentation

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ABSTRACT

Production of cellulolytic enzymes on bagasse under solid state fermentation by coculture of *Aspergillus ellipticus* and *Aspergillus fumigatus* was studied. Cocultivation of *A. ellipticus* and *A. fumigatus* showed improved hydrolytic and β-glucosidase activities as compared to the occasions when they were used separately. Various pretreatment methods were used to make cellulose accessible to enzymatic attack. Best results were obtained through pretreatment with 2% (w/v) calcium hydroxide. Maximum enzyme production was obtained after 8 d of fermentation process.

Index Entries: Pretreatment; coculture; *Aspergillus* spp.; solid-state fermentation.

INTRODUCTION

Bioconversion of cellulosic materials to sugars that could be used as sources of food, fuel, and chemicals is of potential importance in view of the increasing pressure on the existing food and energy sources *(1)*. It is estimated that the photosynthetic process produces 1.5×10^{11} tons of dry material annually with respect to carbon, of which about 50% is cellulose *(2)*. This plant polysaccharide can be used as an energy source by numerous microorganisms, including fungi and bacteria. Biomass in the form of lignocellulosic residues provide a mean of harnessing and storing solar energy and, hence, represents an important energy and material resource *(2)*.

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Enzymatic degradation of cellulose requires the synergistic action of at least three enzymes, endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). *Trichoderma reesei* is generally considered one of the best sources of the first two enzymes. However, it is a low producer of β-glucosidase *(3)*. The hydrolytic action of the cellulase complex produced by *Trichoderma* is greatly enhanced by supplementing β-glucusidase *(4)*. Many species of *Aspergillus* show high degree of β-glucosidase activity *(5)*, and the β-glucosidase produced by *Aspergillus* possesses a high degree of synergism with *Trichoderma* cellulase *(4,6)*.

The molecular properties of the *Trichoderma* cellulases, the cloning of their genes, and their applications have been the subject of recent work *(7–9)*. The availability of cloned cellulase genes and those of molecular biological methods for *Trichoderma reesei* has recently catalyzed some progress in understanding the regulation of cellulase biosynthesis and its application on remediation of β-glucosidase deficiency of *Trichoderma reesei (8,9)*. The genetic improvement of *Trichoderma reesei* for cellulase production has been investigated in several laboratories and hyperproducing mutant strains have been obtained *(10–12)*. However, production of cellulase enzymes is still too expensive. There is the need to improve the economics of current cellulose technology. Therefore, screening was carried out to look for a new potential source of cellulase and β-glucosidase.

We isolated in our screening program, *Aspergillus ellipticus* and *Aspergillus fumigatus* from decayed wood showing good activities of β-glucosidase, endoglucanase, and cellobiohydrolase. Enzyme ratios were different in these two strains. It has been documented that cellulases from different cellulolytic microorganisms can act synergistically *(13)*. This cross-synergism offers the potential for engineering cellulase mixtures with higher activities than are normally present in crude enzyme extracts *(14,15)*.

We tested the production of cellulolytic enzymes using sugarcane bagasse as a cellulosic substrate by coculture of our two best cellulase producers: *Aspergillus ellipticus* and *Aspergillus fumigatus*, under solid-state fermentation. Solid-state fermentation requires no complex controls and has many advantages over submerged fermentation *(16)*. A more concentrated enzyme can be obtained by solid-state fermentation than in submerged fermentation, which uses much more liquid. Various pretreatments were used to make the sugarcane bagasse accessible to the enzymatic attack.

MATERIALS AND METHODS

All chemicals used were of analytical grade. Twenty microbial cultures were isolated locally which exhibited cellulolytic activities, of which *A. ellipticus* and *A. fumigatus* showed the highest activity. The cultures were maintained on potato dextrose agar (PDA) at 26 ± 2°C and stored at 4°C. They were subcultured once a month.

Bagasse was milled and sieved (50-mesh size) and then subjected to different pretreatments before being used as a substrate. Bagasse (50 mesh size) was treated with 1*M* NaOH in a 5% (w/v) slurry for 18 h at room temperature, 5*M* NaOH in a 5% (w/v) slurry for 18 h at room temperature, 1*N* HCl and 5*N* HCl in a 5% (w/v) slurry at room temperature for 18 h, 2% $Ca(OH)_2$ in a 5% (w/v) slurry at room temperature for 18 h, 70% H_2SO_4 in a 5% (w/v) slurry at room temperature for 18 h, steam treated 5% (w/v) slurry at 121°C for 2 h and untreated as such (50-mesh size). Bagasse was washed thoroughly after each pretreatment repeatedly with water to neutral pH and dried overnight at 60°C and reground to 50-mesh size.

Bagasse (5 g) was placed in 250-mL Erlenmeyer flask having 25 mL of fermentation medium. The medium contained the following ingredients (g/L) 0.3, Urea; 1.4, $(NH_4)_2SO_4$; 2.0, KH_2PO_4; 0.3, $CaCl_2$; 0.3, $MgSO_4 \cdot 7H_2O$; 1.0, proteose peptone and (mg/L) 5.0 mg, $FeSO_4$ $7H_2O$; 1.6 mg, $MnSO_4$ $7H_2O$; 1.4 mg; $ZnSO_4$ $7H_2O$; and 2.0 mg $CoCl_2$ and Tween-80 1 mL/25 mL of medium. Medium was sterilized at 121°C for 15 min.

Inoculum of 5×10^8 spores/mL was prepared by harvesting spores from one week old PDA slants of *A. ellipticus* and *A. fumigatus* in the proportion of 1:1 in sterile distilled water containing a small amount of Tween 80. Inoculum was added to the medium of fermentation and was incubated at 26 ± 1°C. Fermentation for the production of cellulolytic enzymes was continued for 10 d. Flasks were removed at regular intervals and the contents were transferred to muslin cloth and squeezed. Liquid extract obtained was centrifuged and the supernatant was analyzed for the enzyme activities.

Enzyme assays of exoglucanase (cotton activity) (EC 3.2.1.91) and endoglucanase (carboxymethylcellulase activity) (EC 3.2.1.4) in the culture filtrate was determined according to Mandels *(17)*. Enzyme assay of β-glucosidase (*p*-nitrophenyl β-D-glucosidase) (EC 3.2.1.21) was determined according to Kubicek *(18)*.

One unit of endoglucanase and filter paper unit was defined as the μmol of glucose equivalent liberated per mL per minute of culture filtrate under assay conditions. The unit of exoglucanase was expressed as mg of glucose liberated per mL of culture filtrate per 24 h and one unit of β-glucosidase is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol/mL/min. Soluble protein was measured by Folin-Lowry method *(19)*. Reducing sugar estimated as glucose using dinitrosalyclic acid *(20)*.

All experiments were carried out in quadruplicate and data given in Table 1 are with standard deviation and are characteristic of other results.

RESULTS AND DISCUSSION

Bagasse is an excellent cellulosic renewable resource, which can be exploited to produce monomeric sugars using cellulase enzyme. However, cellulose is available in a complex form. It is associated with lignin and

Table 1
Enzyme Activities Obtained by Solid-State Fermentation
With Cocultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus*
on Bagasse Under Different Pretreatments on D 8

PRETREATMENTS	EXO-GLUCANASE	ENDO-GLUCANASE	FPU	β - GLUCOSIDASE	PROTEIN
	Units / g Substrate				mg / ml
Untreated	14.11 ± 0.35	14.55 ±0.30	4.38 ±0.10	21.66 ±0.30	7.60 ±0.50
2 % $Ca(OH)_2$	17.73 ±0.60	20.12 ±0.85	5.56 ±0.30	68.73 ±0.90	7.32 ±0.30
1 M NaOH	16.83 ±0.40	15.37 ±0.30	4.56 ±0.25	24.30 ±0.80	8.40 ±0.80
Steam treated	14.77 ±0.85	15.23 ±0.25	4.47 ±0.40	23.30 ±0.70	5.73 ±0.30
5 M NaOH	7.41 ±0.40	11.47 ±0.65	3.75 ±0.40	21.10 ±0.75	7.47 ±0.50
1 N HCl	7.30 ±0.75	10.50 ±0.20	3.70 ±0.20	24.25 ±0.40	5.49 ±0.40
5 N HCl	7.45 ±0.20	12.25 ±0.70	2.54 ±0.10	20.18 ±0.60	5.73 ±0.60
70 % H_2SO_4	1.87 ±0.65	0.546 ±0.55	0.572 ±0.05	3.30 ±0.65	0.21 ±0.04

other components. Recent advances in fermentation technology have dramatically increased the yield of cellulase produced by fungi such as *Trichoderma reesei (21)*. The use of this enzyme under practical hydrolysis conditions results in an accumulation of cellobiose, a strong competitive inhibitor of exoglucanase. This results in a decrease in the rate of sugar production. Therefore, attempts have been made to increase the hydrolytic ability of cellulase complex by cocultivation under solid-state fermentation. Here we describe the use of our recent natural isolates showing synergism of the mixed—culture fermentation with improved enzyme yields.

Cocultivation of *A. ellipticus* and *A. fumigatus* increased the production of endo- and exoglucanase in addition to β-glucosidase as compared to when they were used alone as seen in Fig. 1. There appears to be a good compatibility between *A. ellipticus* and *A. fumigatus*. This offers the possibility of eliminating costly fermentation and recovery steps traditionally used to produce β-glucosidase. In mixed culture, cellobiose accumulation is avoided thus inhibition caused by cellobiose on endo- and exoglucanase

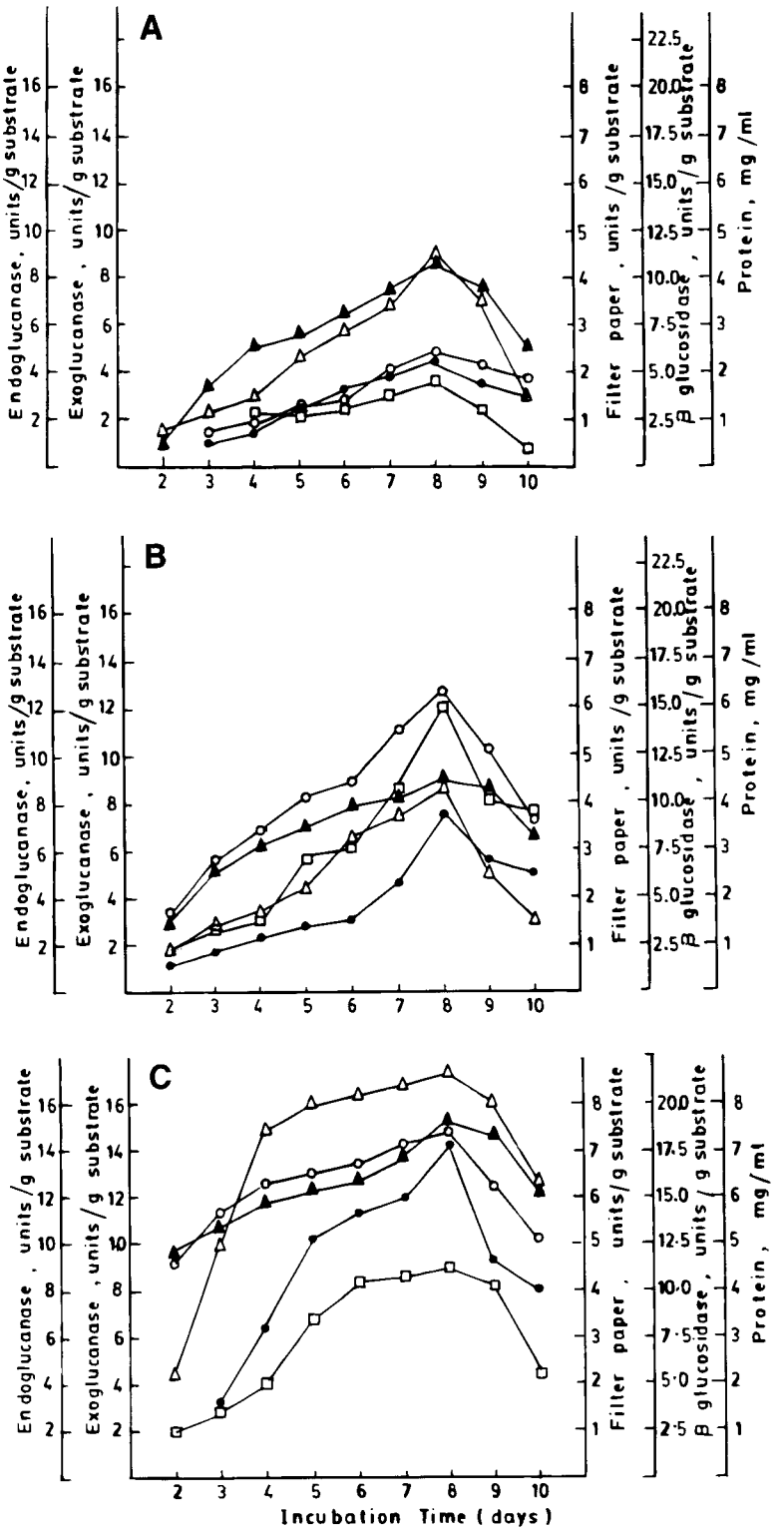

Fig. 1. Enzyme activities obtained by solid state fermentation with *Aspergillus ellipticus* **(A)**, *Aspergillus fumigatus* **(B)**, and with cocultivation of *A. ellipticus* and *A. fumigatus* **(C)** on bagasse. Symbols: exoglucanase (●); endoglucanase (○); β-Glucosidase (△); filter paper unit (□); protein (▲).

is eliminated by high activity of β-glucosidase produced by *A. ellipticus* in coculture. This demonstrates the enhanced hydrolytic potential of cellulase produced by mixed cultivation of *A. ellipticus* and *A. fumigatus*.

Increase in enzyme activities with increase in time is evident, reaching the maximum on the d 8 of the fermentation process under solid-state fermentation. However, further incubation did not improve enzyme production.

Table 1 shows the data of cellulase production by coculturing of *A. ellipticus* and *A. fumigatus* on bagasse under solid-state fermentation using various pretreatments. Lignin, which is a main obstacle to enzymatic degradation, forms the major component of bagasse and delignification of bagasse can improve enzyme production. The susceptibility of cellulose or cellulosic material as a substrate for bioconversion process is determined by its accessibility to cellulase enzymes *(22)*. In order to improve the production of cellulolytic enzymes various pretreatments were applied to the lignocellulosic substrate (bagasse).

Various pretreatments like alkali treatments with 2% $Ca(OH)_2$, 1*M* and 5*M* NaOH, acid hydrolysis using 1*N* and 5*N* HCl, 70% H_2SO_4, steam treatment were applied to bagasse powder. During the alkali treatment, it was observed that bagasse suffered a weight loss. This may be due to the partial solubilization of bagasse components *(23)*. In our investigation, production of cellulase was maximum when 2% $Ca(OH)_2$ treated bagasse was used. Production of the exoglucanase, endoglucanase and β-glucosidase was increased by almost 1.6-, 1.3-, and more than 4-fold, respectively. Among the treatments examined, 2% $Ca(OH)_2$ treatment was found to be best, followed by 1*M* NaOH treatment (Table 1). Mild alkali treatment improved the susceptibility of the substrate by alteration in its structure and thus resulted in higher production of the cellulolytic enzymes *(22)*. This treatment is also responsible for delignification as a result of solubilization of lignin; lignin would otherwise be a major obstacle to enzymatic degradation. However, it is not clear why treatment with $Ca(OH)_2$ is a better method over other alkali treatment.

Steam-treated bagasse also improved the production of the celluloytic enzymes indicating that in moist conditions and at high temperature, the structure of the bagasse is modified physically for imparting better accessibility of cellulose to microbial attack.

As in other cellulosic materials, it is necessary to carry out adequate pretreatment of the sugarcane bagasse, otherwise the enzymatic hydrolysis will not be significant *(23–25)*. But these pretreatments would lead to an overall price increase for the process, due to the employment of a large amount of energy or expensive chemicals. The alkali pretreatment with NaOH is the most effective at present, and it has been employed by others *(24)*. However, $Ca(OH)_2$ pretreatment is as effective as that of NaOH, moreover, $Ca(OH)_2$ pretreatment is more economical. Similar observation have been reported by Ellenrider and Castillo *(23)*.

The compositional changes in the treated bagasse can be advantageous or disadvantageous from the point of view of saccharification. The solubilization of lignin frees cellulose for enzymatic attack. However, the soluble lignin derivatives may have toxic effects during the treatment *(26)*.

Treatment with 5*M* NaOH, 1*N*, and 5*N* HCl, and 70% H_2SO_4 showed detrimental effects on the enzyme production. This may be due to the accumulation of toxic products during treatment. This results in poor production of enzymes.

Improved yields of cellulase and β-glucosidase and its high hydrolytic potential is attributed to the synergistic activities by cellulase enzymes produced by mixed cultivation of *A. ellipticus* and *A. fumigatus*. These species are natural isolates and further research is needed to increase the levels of enzyme activities.

A more concentrated enzyme is obtained by solid-state fermentation. In submerged cultures, the enzyme is diluted in the large bulk of the liquid medium *(15)*. Solid-state fermentation is therefore, a better method for getting a more concentrated enzyme, which is easier to purify, rather than a large quantity of diluted enzyme.

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