

# Production of Cellulases and Hemicellulases from Cellulytic Fungal Cultures in Submerged Fermentation Using Agricultural Wastes

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## Abstract

Untreated, Chemically treated and enzyme treated (Saccharified) sugarcane bagasse, wheat straw and rice straw were evaluated in submerged fermentation for the production of cellulases and hemicellulase by monocultures and mixed cultures of *Aspergillus niger* and *Trichoderma viride* in submerged fermentation. Maximum CMCase activity of  $6.8 \pm 0.5$  U/mg and xylanase activity of  $105 \pm 2.8$  U/mg was obtained in enzyme treated sugarcane bagasse and pretreated wheat straw with mixed culture respectively. However, monocultures of *Aspergillus niger* and *Trichoderma viride* showed less enzyme production as compared to mixed cultures in rest of the experimentation.

**Keywords:** CMCase; Xylanase; Co-culture; *Aspergillus niger*; *Trichoderma viride*; Agricultural wastes.

## 1. Introduction

Lignocelluloses are major components of biomasses which come from different industries, forestry, agriculture and municipalities. The biodegradation of this lignocellulosic biomass is limited by several factors like crystallinity of cellulose, available surface area, and lignin content. Among these lignocellulosic biomasses agricultural wastes are more important which can be converted into products that are of commercial interest such as ethanol, glucose, and single cell protein [1]. Hydrolysis of these materials is the first step for either digestion to biogas or fermentation to ethanol [2]. Pre-treatment is a potential techniques which is used to degrade these lignocellulosic biomasses. The techniques involves the use of acid and alkaline pretreatments, liquid hot water treatment, pH controlled hot water and flow-through liquid hot water treatments [3]. Pretreatment of cellulose opens up the structure and removes secondary interaction (lignin and hemicellulose) between glucose chains [4,5].

For conversion of these agricultural products into fuels like ethanol, pretreatment is the first step to make the substrates greater exposure to enzymes.

Second step is the hydrolysis of these pretreated substrates by the use of cellulases. These cellulases are produced by a variety of microorganisms. Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* [5-7]. Among all these fungal genera, *Trichoderma* and *Aspergillus* has been most extensively used for cellulase production [8].

In the whole process of converting lignocellulosic biomass into ethanol, the most critical part is the enzyme production cost. Mostly cellulases are produced widely by submerged culture processes yielding high cost enzyme production which ultimately stuck the industrial application of cellulose bioconversion [9]. Since the production of cellulase enzyme is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically viable. The final target of the whole research is to produce economically acceptable enzymatic conversion of cellulosic biomass to glucose for fermentation to ethanol or other products.

In this paper we reported here the production of cellulases and hemicellulases from untreated, pretreated and enzyme treated (saccharified) bagasse, wheat straw and rice straw by pure culture and mixed cultures of *Aspergillus niger* and *Trichoderma viride* in submerged fermentation.

## 2. Materials and Methods

### 2.1 Fungal Cultures

Fungal strains of *Aspergillus niger* and *Trichoderma viride* were obtained from Microbiology laboratory of Food & Biotechnology Research Center (FBRC), Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories complex, Ferozpur road Lahore, Lahore Pakistan. These strains were cultured and maintained on potato dextrose agar (PDA) slants and stored at 4°C for further use.

### 2.2 Substrates

In this investigation three different substrates like sugarcane bagasse, wheat straw and rice straw

were used. These substrates were used in three different conditions which are as follows;

### 2.2.1 Untreated Substrates

In this condition, the substrates were used in untreated forms (without any treatment)

### 2.2.2 Chemically Pretreated Substrates

In this condition, all the three substrates were treated with 5% (v/v)  $H_2O_2$  + 3% (w/v) NaOH with substrate to liquid ratio of 1:10 followed by heating at 130°C, 30psi for 60min. After heating the material was filtered and washed several times up to neutrality [10].

### 2.2.3 Enzyme Treated (Saccharified) Substrates

In this condition the chemically pretreated samples were treated with commercial cellulase enzyme for 8hr at 50°C [10]. After the completion of the reaction material was filtered and the residues were washed and used as a substrate for the production of enzymes by tested fungi.

## 2.3 Enzyme production

For enzyme production, Vogel's media was used for the growth of *Aspergillus niger* and *Trichoderma viridi* in submerged fermentation. In 250ml conical flask 25 ml of the media with 2% substrates were sterilized at 121°C, 15psi for 15 min. After sterilization, the media was allowed to cooled and inoculated with 2% of spore suspension of tested fungi and incubated at 30±1 °C with the agitation speed of 120rpm. After termination of fermentation period the culture filtrate was centrifuged at 8000 x g for 10 min at 4°C to remove unwanted particles and spores. The supernatants obtained after centrifugation were used as the crude extracellular enzyme source.

## 2.4 Analytical Methods

Enzyme activities (CMCase and Xylanase), glucose and total proteins were estimated as described earlier [11]. 500 µL of the enzyme sample along with 500 µL of 1% (w/v) CMC in 50 mM acetate buffer pH 5 was incubated, in a water bath at 50°C, for 30 min. After incubation 1.5 mL of DNS was added and boiled for 5 minutes and absorbance was taken spectrophotometrically at 550nm. The reducing ends liberated were then measured with DNS. Xylanase activity in the culture filtrate was measured by adding 0.5 ml of 1% birch wood xylan prepared in 0.05 M sodium citrate buffer pH (5.0), and 0.5 ml of appropriately diluted enzyme were incubated at 50°C for 30 min. The reaction was terminated by adding 1.5ml of DNS [12]. After this the test tubes containing reaction mixtures were boiled for 5 minutes and absorbance was taken spectrophotometrically at 550nm. One unit (U) of xylanase and CMCase was defined as the amount of enzyme releasing 1 micromole of xylose or glucose per minute under the assays conditions. Total protein in the culture filtrate was determined

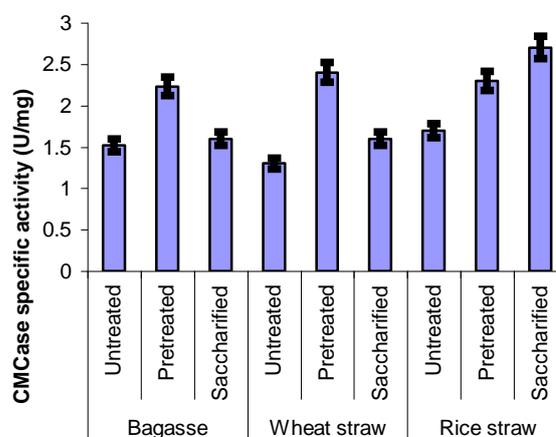
by the method as described by Lowery [13] using BSA as standard.

## 2.5 Statistical Analysis

Data was statistically analyzed by ANOVA using Microsoft Excel program with  $P < 0.05$ .

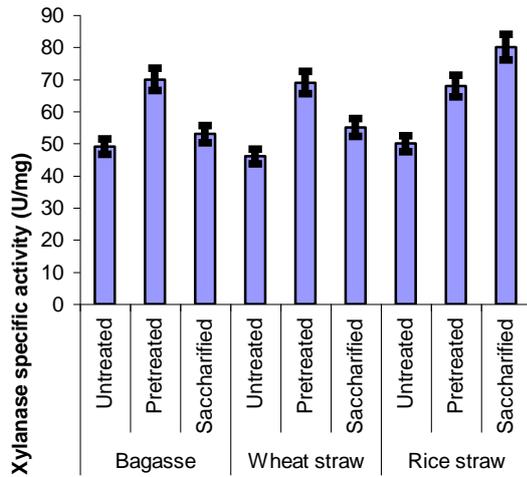
## 3. Results and Discussion

In the present investigation, different experiments were conducted to check the enzyme production by mono cultures and co-cultures using different condition of substrates i.e. untreated, chemically treated and enzyme treated. When mono cultures were grown on untreated substrates enzyme production was lowered due to the presence of hard covering (lignin) on substrates which inhibit the microbial attack. When the substrates were subjected to chemical treatment, the enzyme production was increased as compared to untreated substrates. This chemical treatment ruptures the upper covering and provides a greater surface area for microbial attack. Figure 1 and 2 explains the results of CMCase and xylanase specific activities produced by *Aspergillus niger* in submerged fermentation. Results indicated that pretreated substrates (Bagasse and wheat straw) showed highest enzyme activities (CMCase and Xylanase) as compared to untreated and enzyme treated substrates. But in case of rice straw enzyme production (CMCase specific activity  $2.7 \pm 0.3$  U/mg, xylanase specific activity  $80 \pm 2.3$  U/mg) was found maximum on saccharified (enzyme treated) conditions. In saccharified wheat straw and bagasse enzyme production was reduced which might be due to the accumulation of reducing sugars which inhibits the cell biomass [14]. Wheat straw and bagasse are the best inducer of cellulytic enzyme like  $\beta$ -glucosidase [15]. Glucose production was higher in saccharified substrates (Figure 3) as compared to untreated and pretreated substrates.

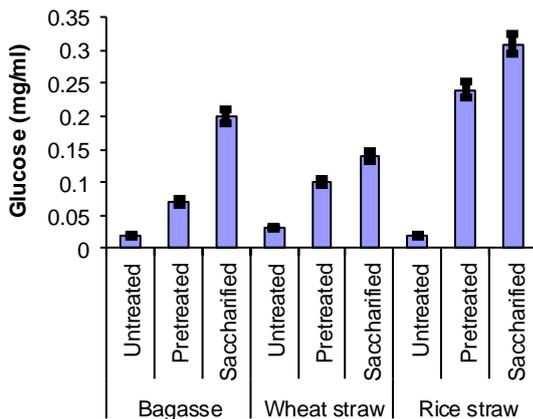


**Figure 1.** CMCase specific activity of *Aspergillus niger* grown on different substrates for 96h of fermentation period at 30°C with agitation of 120rpm. The error bars

represent the standard deviation of the means based on three replicates.



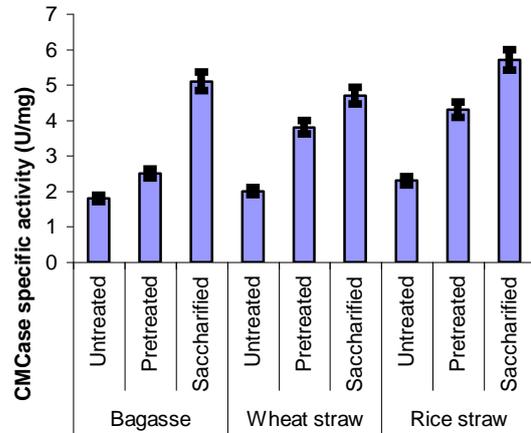
**Figure 2.** Xylanase activity of *Aspergillus niger* grown on different substrates for 96h of fermentation period at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.



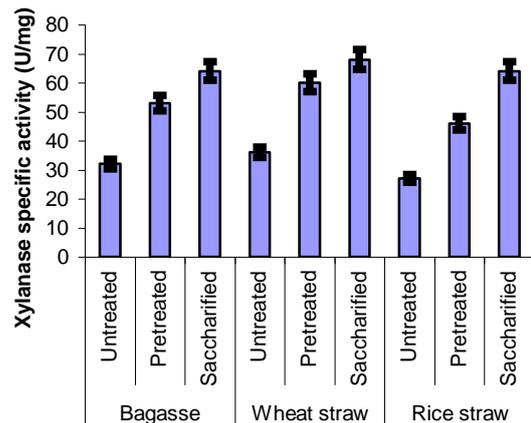
**Figure 3.** Glucose production from *Aspergillus niger* grown on different substrates for 96h of fermentation period at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

When *Trichoderma viride* was grown as monoculture on various substrates like wheat straw, bagasse and rice straw under various conditions (untreated, pretreated and saccharified), it was observed that both CMCase and xylanase activities were maximum in saccharified substrates. Figure 4 revealed that CMCase specific activity was maximum in saccharified bagasse ( $5.1 \pm 0.8$  U/mg), saccharified wheat straw ( $4.7 \pm 0.4$  U/mg) and saccharified rice straw ( $5.7 \pm 0.6$  U/mg) as compared to untreated and pretreated substrates. Xylanase specific activity was also found maximum on saccharified substrates (Figure 5). Saccharified bagasse ( $64.2 \pm 2.8$  U/mg), saccharified wheat straw ( $68 \pm 1.7$  U/mg) and saccharified rice straw ( $64.3 \pm 2.1$  U/mg) yielded high xylanase production.

These findings indicated that *Trichoderma viride* has the ability to utilize the excess amount of glucose present in the medium.



**Figure 4.** CMCase specific activity of *Trichoderma viride* grown on different substrates for 7days of fermentation period at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

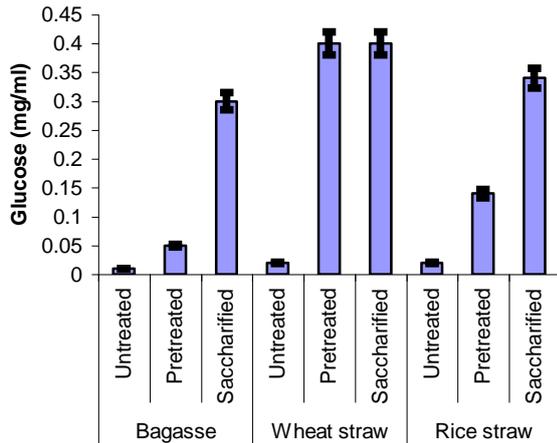


**Figure 5.** Xylanase specific activity of *Trichoderma viride* grown on different substrates for 7days of fermentation period at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

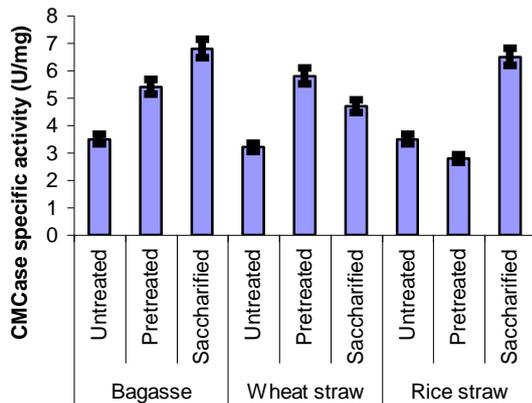
Figure 6 presented the glucose production from substrates in submerged fermentation. In bagasse and rice straw maximum glucose production was observed in saccharified substrates which indicated that the enzyme released by the fungus effectively hydrolyzed the substrates. In case of wheat straw both pretreated and saccharified substrate yielded almost equal quantity of glucose i.e. 0.4 mg/ml.

Co-cultures were also cultivated on various substrates such as bagasse, wheat straw and rice straw under different conditions (untreated, pretreated and saccharified) in submerged fermentation. When bagasse was used it was observed that highest CMCase activity was found in saccharified bagasse ( $6.8 \pm 0.5$  U/mg) as compared

to untreated ( $3.5 \pm 0.3$  U/mg) and pretreated bagasse ( $5.4 \pm 0.3$  U/mg) respectively. These results indicated that enzyme production was increased upto 100% in saccharified bagasse as shown in Figure 7. In case of wheat straw enzyme production was increased in pretreated substrate ( $5.8 \pm 0.8$  U/mg) and reduced in saccharified ( $4.7 \pm 0.4$  U/mg) and untreated substrate ( $3.2 \pm 0.27$  U/mg). In case of rice straw highest levels of CMCase production was observed in saccharified ( $6.5 \pm 0.7$  U/mg) but decreased in pretreated ( $2.8 \pm 0.16$  U/mg) as compared to untreated ( $3.5 \pm 0.24$  U/mg) rice straw.



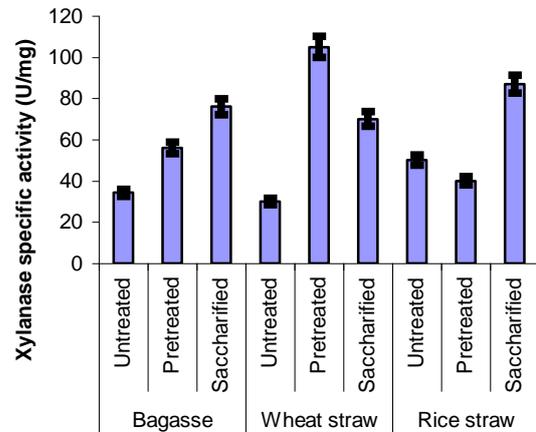
**Figure 6.** Glucose production from *Trichoderma viride* grown on different substrates for 7 days of fermentation period at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.



**Figure 7.** CMCase specific activity from consortium of *Aspergillus niger* and *Trichoderma viride* grown on different substrates at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

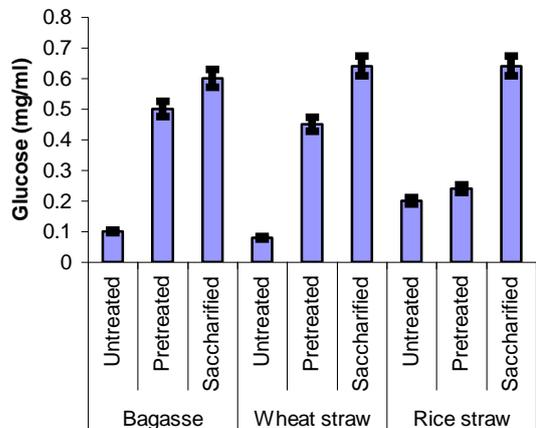
Figure 8 explains the production of xylanase from co-cultures on various substrates under different conditions in submerged fermentation. Results indicated that production of xylanase follow the same pattern as in CMCase production. In these experiment highest titers of xylanase production was  $105 \pm 2.8$  U/mg observed with pretreated wheat

straw. Similar findings were also reported by Haq et al [16] using co-culture of *A.niger* and *T.viride* for CMCase and xylanase production.



**Figure 8.** Xylanase specific activity from consortium of *Aspergillus niger* and *Trichoderma viride* grown on different substrates at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

They also reported that co-cultures produce high levels of enzyme production as compared to mono cultures. Glucose production was also found better due to the synergistic action of these two fungi (Figure 9). For efficient sacchrification of substrate a complete cellulose system is very essential. *Trichoderma* species have deficient in  $\beta$ -glucosidase which is cover come by co-culturing with *Aspergillus* sp [17]. Both species acts synergistically for each other for better growth, enzyme production and saccharification. Mixed cultures of lignocellulolytic or cellulolytic microorganisms increases the protein content and efficient in degrading lignocellulosic substrates by producing high activities of enzyme as compared to pure cultures [18,19]. Pure cultures or mixed cultures of cellulolytic origin are used for improved conversions of cellulosic biomass to a variety of chemicals and fuels [20,21].



**Figure 9.** Glucose production from consortium of *Aspergillus niger* and *Trichoderma viride* grown on different

substrates at 30°C with agitation of 120rpm. The error bars represent the standard deviation of the means based on three replicates.

#### 4 Conclusions

Results of this study indicated that pretreatment has a potential influence on enzyme production and this process is very helpful particularly in bioethanol production from plant biomasses. Co-culturing of fungus proved to be beneficial for increased enzyme production in submerge fermentation.

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