PRETREATMENT OF SUGARCANE BAGASSE FOR THE BIOSYNTHESIS OF CELLULASES BY *TRICHODERMA VIRIDE*

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ABSTRACT: Present study describes the pretreatment of sugarcane bagasse with different physical and chemical agents for the production of cellulolyic enzymes by *Trichoderma viride* using submerged fermentation. Bagasse was pretreated with steam (121ºC and 200ºC) and different chemicals like NaOH, H_2SO_4, H_2O_2 and $NaHClO_3$ to get the maximum yield of cellulases. Among all treatments, steam pretreatment at 200ºC gave the maximum cellulases yield (CMCase 1.57 U/ml/min, FPase 0.921 U/ml/min) as compared to control (CMCase 0.084 U/ml/min, FPase 0.0138 U/ml/min), untreated bagasse. There was a considerable enhancement in the enzyme production in terms of several folds higher than control. The amount of sugarcane bagasse in the fermentation medium was also optimized and 1% level of bagasse was found to be optimum.

Key words: Cellulases biosynthesis, bagasse pretreatment, *Trichoderma Viride*

INTRODUCTION

Pakistan being an agricultural country produces a large amount of cellulosic biomass such as agricultural (corn stover, sugarcane bagasse) and forestry (pulp mill sludge) residues, municipal solid waste (particularly waste paper) and dedicated crops (switchgrass and hybrid poplar). This provides a low-cost feedstock for biological or fermentative production of a wide range of fuels and chemicals that offer substantial economic, environmental and strategic advantages (Shao *et al*., 2009). One of the important uses of cellulosic biomass is their use as substrate for cellulases biosynthesis by microorganisms through fermentation. Different agricultural byproducts such as sugarcane bagasse, wheat bran, rice husk, oil seed cakes and corn stover have been used for this purpose (Prior and Day, 2008).

Physical and chemical pre-treatment of this cellulosic biomass is a pre-requisit and results in an enhanced production of cellulases. During chemical pretreatment, NaOH, H_2SO_4, H_2O_2 or $NaHClO_3$ are used that act as scouring, sequestering and bleaching agent to enhance the crystallinity of cellulosic biomass. It also breaks lignin and carbohydrate bonds for optimal growth of microorganism (Chahal 1983; Ladisch 1989; Haq *et al*., 2003; Martin *et al*., 2007; Rabelo *et al*., 2008,). Pretreatment by physical means include the use of water at a high temperature for the hydrolysis of cellulosic biomass. Hydrolysis has been advocated as a pretreatment for herbaceous and lignocellulosic materials prior to enzymatic saccharification (Hassan, 1999).

The advantages of steam pretreatment arise from several factors including complete hydrolysis of hemicellulose, lignin depolymerization, swelling of cell walls, increase in functional specific gravity and water holding capacity (Castro *et al*., 2003). These are associated with the improved utilization of cell wall polysaccharides by cell free enzymes (Brownell and Saddler, 1987; Castro and Machado, 1990). By applying the steam explosion process to sugar cane bagasse, Kling *et al*. (1987) demonstrated that about 60% of the hemicellulose fraction was hydrolyzed thus increasing the susceptibility of cellulose to enzymatic hydrolysis.

Different chemicals like dilute acids, alkalis, peroxides and organosolvents affect lignocellulosic materials by increasing pore size and solublizing lignin and hemicellulosic by increasing surface area (Khosravi-Darani and Zoghi, 2008, Rabelo *et al*., 2008, Martin *et al.,* 2008). The primary effect of alkali is postulated to promote the swelling and sponification of intermolecular ester bonds and facilitates the penetration of enzymes into cellulose fine structure (Khosravi-Darani and Zoghi, 2008, Cheng *et al,* 2008).

The production of cellulases is an expensive bioprocess; however the incorporation of sugarcane bagasse into the media for the production of lignocellulose enzymes helps to decrease the production costs of enzymatic complexes. The production and applications of cellulases have central importance in bioprocessing industries because it causes 30% more hydrolysis than acids (Rabelo *et al*., 2008, Prior and Day, 2008). The use of cellulases in animal feed, fruit processing, textile wet processing, preparation of dehydrated vegetables, food products, essential oils, flavours, starch processing, botanical extract, pulp and paper production, jams, juices, production of plant protoplast for genetic manipulation, wine production and pharmaceutical and biomass conversion have greatly increased the prospects of enzymatic hydrolysis over chemical processes (Mach, and Zeilinger, 2003, Mekala *et al*., 2008).

The objective of the present study was to convert sugarcane bagasse by using physical and chemical means, to an easily attackable substrate for the production of cellulytic enzymes by *Trichoderma viride* through submerged fermentation.

MATERIALS AND METHODS

Pretreatment of sugarcane bagasse: Sugarcane bagasse was collected from local market and washed with tap water for three times. After washing it was dried at 45ºC overnight in an oven and grinded in a ballmill to obtain particle size of approximately 1 mm. Then the grinded bagasse was subjected to pretreatment with different agents as described below.

Steam: steam pretreatment was carried out by autoclaving the bagasse at 121°C (15 lb/inch^2) and 200°C (25 lb/inch^2) for 15 min in water.

Sodium hydroxide (NaOH): Bagasse pretreatment was done with NaOH (0.5 - 3.0 N) for 15 min with continuous stirring. After treatment with NaOH, it was washed with tap water.

Sulfuric acid (H_2SO_4): Bagasse was treated with H_2SO_4 (0.25 - 3.0 N) for 15 min and washed with surplus water until it was neutralized.

Hydrogen per-oxide (H_2O_2): Pretreatment of bagasse was done with different concentrations of H_2O_2 ranging from 1 - 5% (w/v) for 15 min with constant stirring followed by neutralization with excessive water washing.

Sodium hypochlorite ($NaHClO_3$): Bagasse pretreatment was also carried out with sodium hypochlorite (0.5 - 5.0 %, w/v) for 15 min with continuous stirring and neutralization with distilled water washing.

Microorganism: *Trichoderma viride* GCBT-11 was obtained from the available stock culture of Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan. The strain was transferred to fresh PDA slants (3.9 %, w/v) and was allowed to grow in an incubator at 30 C for 48 hrs. After sufficient growth and sporulation, the slants were placed in cool lab at 40 C for storage.

Inoculum preparation: The spore suspension was used as inoculum in the present studies. It was prepared from a 5 days old slant by adding 10ml of sterilized 0.005 % Monoxal O.T (Diacetyl ester of sodium sulphosuccinic acid) to it. The spores were scratched with the help of a sterilized wire loop to make a homogeneous suspension of spores. Spore count was measured after Sharma (1989) with the help of Haemocytometer.

Fermentation technique: Twenty five milliliters of the fermentation medium consisting of (%, w/v); $(NH_4)_2SO_4$, 0.14: KH_2PO_4, 0.20; Urea, 0.03; $MgSO_4.7H_20$, 0.03; $ZnSO_4.7H_2O$, 0.00014; $FeSO_4.7H_2O$, 0.0005; $MnSO_4$ 0.00016; $CoCl_2$,0.0002; $CaCl_2$, 0.0002; Tween-80, 2.0 ml; Polypeptone, 0.10; and sugarcane bagasse, 1 (pH 6.0) was transferred to the individual 250 ml cotton wool plugged conical flasks and autoclaved at 15 lb/inch^2 for 15 min. The flasks were inoculated with 1 ml of this inoculum containing 1.2×10^6 ml^{-1} of spores after cooling at room temperature and incubated at 30°C at 200 rpm in an orbital shaker incubator. After 72 hrs, the fermented broth was centrifuged at 6000 rpm for 10 min and the supernatant was assayed for enzyme activity.

Enzyme assay: The cellulases were assayed for CMCase and FPase using carboxymethyl cellulose and filter paper as substrates. The released reducing sugar was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity is defined as "the amount of enzyme required to liberate one μmol of reducing sugars per minute under the assay conditions".

CM-Cellulases activity (CMCase): CMCase activity was determined after Wood and Bhat (1988). One milliliter of appropriately diluted enzyme was incubated with 1.0 ml of 0.05 M citrate buffer (pH 5.0) and 1.0 ml of 1.0 % carboxymethyl cellulose for 30 minutes at 50°C followed by determination of reducing sugar.

Filter Paper-cellulases activity (FPase): A 50 mg rolled strip of Whattman filter paper No. 1, (measuring 1x6 cm) was suspended in a mixture containing 1.0 ml of diluted enzyme extract and 1.0 ml of 0.05 M sodium citrate buffer (pH 4.8). This mixture was incubated for 1 hrs at 50ºC followed by the estimation of reducing sugar (Mandela and Sternberg, 1976).

Statistical analysis: Treatment effects were compared after Snedecor and Cochran (1980). Significance difference among the replicates has been presented as Duncan's multiple range in the form of probability (<p>) value.

RESULTS AND DISCUSSION

Effect of bagasse pretreatments on the production of cellulases: Bagasse pretreatment with different methods greatly affects both of its structure and cellulases production by microbes (Ramos *et al.*, 2004; Meza *et al.*, 2006). The inside methodology involves in the removal of hemicellulose through a combination of dissolution and autohydrolysis at high temperatures (Bigelow and Wyman, 2002). In the present study various physical (grinding, washing and steaming) and chemical agents (NaOH, H_2SO_4, H_2O_2 and $NaHClO_3$) were investigated for the pretreatment of sugarcane bagasse for cellulases production by *Trichoderma viride* GCBT-11 (Table 1). Grinding and water washing showed a slight increase in

cellulases production as compared to control. The production of cellulases was found to be maximum with steam pretreated bagasse at 200°C (25 lb/inch^2).

Among the different chemicals such as NaOH (0.5-3.0 N), H_2SO_4 (0.25-3.0 N), H_2O_2 (1.0-5.0 %) and $NaHClO_3$ (0.5-5.0 %), Sulfuric acid (0.5-3N) pretreatment at 35°C for 15 min resulted into gradual increase in cellulases biosynthesis. The maximum production (CMCase 1.175 U/ml/min, FP-ase 0.164 U/ml/min) is achieved by H_2SO_4 (1.5N) pretreated bagasse. Further increase in concentration however, decreased the cellulases production. Martin *et al.*, (2007) used dilute sulfuric acid pretreatment of agricultural and agro-industrial residues for ethanol production. The prehydrolysis of sugarcane bagasse was performed at 122 °C for 20, 40 or 60 min using 2% H_2SO_4 at a solid-to-liquid ratio of 1:10.

Sodium-hydroxide (1.5N) treated bagasse also resulted in the increased cellulases biosynthesis as shown by CMCase 1.123 U/ml/min and FP-ase 0.550 U/ml/min (Tab 1). Ladisch, *et al.,* in 1989 proved that alkali brings about the swelling of native cellulose fiber and cause modifications of cellulose. NaOH (1.25 M) at temperatures between 25-85°C solubilized lignin (60.6%), hemicellulose (71.6%) and also increased the crysatllinity index. However, Koullas *et al*. (1992) showed that enzymatic conversion of cellulose to glucose varied with NaOH concentration.

Rabelo, *et al.* (2008) compared the lime and alkaline H_2O_2 pretreatments of sugarcane bagasse for ethanol production using the bagasse as it comes from an alcohol/sugar factory and bagasse in the size range of 0.248 to 1.397 mm (12-60 mesh). His results showed that when hexoses and pentoses are of interest, lime should be the pretreatment agent chosen, as high TRS yields are obtained for nonscreened bagasse using 0.40 g lime/g dry biomass at 70°C for 36 h. When the product of interest is glucose, the best results were obtained with lime pretreatment of screened bagasse. However, the results for alkaline peroxide and lime pretreatments of nonscreened bagasse are not very different.

The effects of H_2O_2 conc. (1-5%) and $NaHClO_3$ (0.5-5%) during pretreatment of bagasse on the cellulases biosynthesis were also investigated. Bagasse pretreated with H_2O_2 (3%) resulted into maximum cellulases production i.e., CMCase 1.476 U/ml/min and FPase 0.343 U/ml/min, respectively (Table 1). However, Thompson *et al. (*1991) done biomass pretreatment by (w/v) H_2O_2 (2.5%) at 25°C and pH (11.50) for residence times (0.5, 5 and 19 h) that resulted enhanced subsequent enzymatic hydrolysis. The entire process resulted into moderate removal of lignin and hemicellulose. $NaHClO_3$ (1%) pretreated bagasse increased cellulases production (CMCase 1.4761 U/ml/min and FPase 0.452 U/ml/min) in shake flasks.

Effect of different concentrations of bagasse: The effect of different concentrations of sugarcane bagasse (0.5-2.5 %, w/v) on the biosynthesis of cellulases by *Trichoderma viride* was also investigated (Figure 1). Cellulases production was found maximum when sugarcane bagasse was added to the fermentation medium at a concentration of 1.0 %. Further increase in the concentration of sugarcane bagasse significantly decreased the cellulases production. Bigelow and Wyman (2002) examined different concentrations of bagasse (0.5-3.0%) for cellulases production. Cellulases biosynthesis (CMCase 1.605 U/ml/min and FPase 0.862 U/ml/min) was notable at the 1% conc. and decreases considerably with its increase. It might be due to the fact that there is gradual decrease in the agitation as well as aeration of fermentation medium, which is essential for microbial growth. Improper microbial growth resulted into decreased cellulases biosynthesis.

Table 1: Pretreatment of bagasse for cellulases biosynthesis by *Trichoderma viride* GCBT-11 using shake flask fermentation

Treatments		Enzyme activity (U/ml/min)	
		CMCase	FPase
STEAM (C°)	121	$0.197^{z} \pm 0.001$	$0.3113^{n} \pm 0.0032$
	200	$1.57^{a} \pm 0.001$	$0.921^{a} \pm 0.001$
NaOH (N)	0.5	$0.406^{y} \pm 0.001$	$0.468^{e} \pm 0.001$
	1.0	$0.9763^{p} \pm 0.0015$	$0.265^{q} \pm 0.001$
	1.5	$1.123^{j} \pm 0.001$	$0.550^{c} \pm 0.0095$
	2.0	$0.806^{u} \pm 0.001$	$0.1913^{u} \pm 0.0015$
	2.5	$0.85^{s} \pm 0.001$	$0.372^{g} \pm 0.001$
	3.0	$0.602^{w} \pm 0.001$	$0.138^{x} \pm 0.001$
H_2SO_4 (N)	0.25	$1.235^{h} \pm 0.001$	$0.197^{t} \pm 0.001$
	0.5	$1.348^{g} \pm 0.001$	$0.257^{r} \pm 0.001$
	1.0	$1.057^{m} \pm 0.001$	$0.414^{i} \pm 0.001$
	1.5	$1.175^{i} \pm 0.001$	$0.164^{v} \pm 0.001$
	2.0	$0.951^{q} \pm 0.001$	$0.4913^{d} \pm 0.002$
	2.5	$0.81^{t} \pm 0.001$	$0.426^{h} \pm 0.001$
	3.0	$0.488^{x} \pm 0.001$	$0.357^{x} \pm 0.001$
H_2O_2 (%)	1.0	$0.762^{v} \pm 0.001$	$0.415^{i} \pm 0.001$
	2.0	$1.095^{k} \pm 0.001$	$0.675^{b} \pm 0.001$
	3.0	$1.476^{c} \pm 0.0433$	$0.343^{l} \pm 0.0015$
	4.0	$1.063^{l} \pm 0.001$	$0.334^{m} \pm 0.001$
	5.0	$1.018^{h} \pm 0.001$	$0.433^{g} \pm 0.001$
$NaHClO_3$ (%)	0.5	$0.995^{o} \pm 0.001$	$0.222^{s} \pm 0.001$
	1.0	$1.476^{b} \pm 0.001$	$0.452^{f} \pm 0.001$
	2.0	$1.432^{d} \pm 0.001$	$0.4313^{g} \pm 0.001$
	3.0	$1.419^{e} \pm 0.001$	$0.414^{i} \pm 0.0005$
	4.0	$1.401^{f} \pm 0.001$	$0.3035^{o} \pm 0.005$
	5.0	$0.855^{r} \pm 0.001$	$0.285^{p} \pm 0.001$
Control		$0.084^{C} \pm 0.001$	$0.0138^{z} \pm 0.0076$
LSD		0.00212	0.00347

Incubation period, 72 h; Temperature, 30ºC; Initial pH, 5.5
Each value is a mean of three parallel replicates, ± denotes standard deviation, Numbers followed by different letters differ significantly at $p \leq 0.05$.

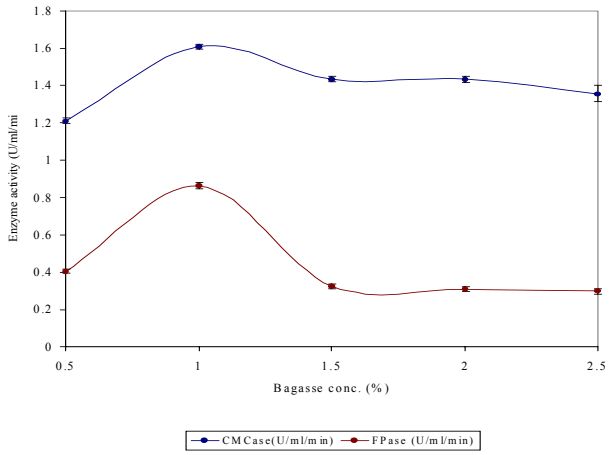

Figure 1: Effect of different concentrations of sugarcane bagasse on cellulases biosynthesis by *Trichoderma viride* GCBT-11 using shake flask fermentation

Incubation period, 72 h; Temperature, 30°C; Initial pH, 5.5. Y error bars indicate standard deviation among the three parallel replicates.

Conclusion: The present work has established the potential of *Trichoderma viride* for cellulases biosynthesis, using pretreated sugarcane bagasse in shake flask formation.

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