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Fungal pretreatment improves amenability of lignocellulosic material for its saccharification to sugars

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ABSTRACT

The sugarcane bagasse was biologically pretreated with three white-rot fungi; *Pleurotus florida*, *Coriolopsis caperata* RCK 2011 and *Ganoderma* sp. rckk-02, individually under solid-state fermentation. *P. florida*, *C. caperata* RCK 2011 and *Ganoderma* sp. rckk-02 degraded lignin up to 7.91, 5.48 and 5.58%, respectively. The lignocellulolytic enzymes produced by these fungi were also monitored during solid state fermentation of sugarcane bagasse. The fungal fermented sugarcane bagasse when hydrolyzed with crude cellulases from brown-rot fungus, *Fomitopsis* sp. RCK2010, released comparatively 1.5–2.4 fold higher sugars than in case of untreated sugarcane bagasse. The study demonstrated that white-rot fungal pretreatment improved the amenability of plant material for enzymatic hydrolysis.

1. Introduction

The annual production of lignocellulosic materials has been estimated about 10^{10} metric tons worldwide (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010). Lignocellulosic biomass chiefly consists of cellulose, a homopolymer of glucose; hemicellulose, a heteropolymer of pentoses and hexoses; and lignin, a polymer of phenyl propanoid units (Kuhad, Singh, & Eriksson, 1997). The polysaccharides (cellulose and hemicellulose) on average accounts for 55–75% on dry weight basis in plant cell wall and can be deconstructed into simple sugars, which further can be fermented to alcohols such as ethanol and butanol (Swana, Yang, Behnam, & Thompson, 2011), organic acids, acetone and glycerol (Celińska and Grajek, 2009; Tran, Cheirsilp, Hodgson, & Umsakul, 2010). Among these, ethanol from lignocellulosics, is one of the valuable energy sources, which could meet the growing energy demand (Lynd et al., 2008).

The lignocellulosic residues which don't compete with food demand, provide a low cost feedstock for production of fuels and commodity chemicals and thereby can offer economic, environmental and strategic advantages (Sukumaran et al., 2010). Sugarcane bagasse (SB) is one of the abundantly available low-cost plant residue, which could be used for production of biofuels (Rocha et al., 2011; Soares, Travassos, Baudel, Benachour, & Abreu, 2011). Nearly, 101.3 million metric tons of SB is annually produced in India (Sukumaran et al., 2010).

The most critical step in conversion of plant material into ethanol is to bring about fractionation of plant material in individual components (Dyk & Pletschke, 2012). The partial or complete removal of lignin is essential because its cross linking with hemicellulose makes it highly recalcitrant and obstructs any chemical or enzymatic pretreatment (Dyk & Pletschke, 2012). Thus to enhance the overall chemical or enzymatic hydrolysis, the crystalline structure of holocellulose needs to be disintegrated and the lignin to be broken down to increase the accessibility of cellulases to the substrates. Therefore, an efficient pretreatment method is necessary to produce cellulose largely free of hemicellulose and lignin. Variety of chemical and physiochemical pretreatment methods; steam explosion, steam treatment with diluted sulfuric acid or alkali, organosolv extraction and ammonia fiber expansion have been largely used to improve the enzymatic hydrolysis of plant biomass (Alvira et al., 2010). However, most of these pretreatments are energy intensive, require costly equipments and often generate toxic compounds, which make the process environment unfriendly and commercially uncompetitive (Alvira et al., 2010).

Recently researchers have started working on biological pretreatment as an alternative method either to partially replace or bring down the severity of chemicals generally used in different pretreatment methods (Gupta, Mehta, Khasa, & Kuhad, 2011; Menon & Rao, 2012). Biological pretreatment is advantageous in improving enzymatic saccharification of plant biomass, because of low energy input, cost, reduction in chemical requirement and mild processing conditions (Saritha, Arora, & Nain, 2012). The

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potential of biological pretreatment has been explained by the ability of certain microorganisms to disrupt the plant cell wall by partial breakdown of the lignin-carbohydrate complex (Sanchez, 2009; Pinto et al., 2012). Among microorganisms, the white rot fungi are the most promising lignin degraders and thus have potential for biological breakdown of plant materials (Dias et al., 2010). Different white-rot fungi vary greatly in their capabilities of degrading lignin and carbohydrates in plant cell wall. Recently, *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, *Crinipellis* sp. RCK-1, *Pleurotus ostreatus* and *Trametes versicolor* have been tested for their lignin degradation abilities, when grown under solid-state fermentation (SSF) (Gupta, Mehta, Khasa, & Kuhad, 2011; Kuhar, Nair, & Kuhad, 2008; Shrivastava et al., 2011).

The present study was aimed at evaluating the abilities of three white rot fungi; *Pleurotus florida*, *Coriolopsis caperata* RCK2011 and *Ganoderma* sp. rckk-02 to ferment SB for transforming it into a more amenable material for enzymatic hydrolysis. Additionally an attempt has been made to test the efficacy of cellulases from *Fomitopsis* sp. RCK2010, a brown-rot fungus, for saccharifying the fungal treated SB.

2. Materials and methods

2.1. Raw material

Lignocellulosic substrates, wheat bran (WB) and sugarcane bagasse (SB), were obtained locally. SB was processed through a combination of chipping and milling to attain a particle size of 1–2 mm using a laboratory knife mill (Metrex Scientific Instrumentation Pvt. Ltd., New Delhi, India). The grounded material was used throughout the study.

2.2. Microorganisms and culture conditions

Pleurotus florida, *Coriolopsis caperata* RCK2011, *Ganoderma* sp. rckk-02 and *Fomitopsis* sp. RCK2010 were obtained from the culture collection of Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, New Delhi, India. The fungal cultures were grown on malt extract agar (MEA) composed of (g/L): malt extract, 20.0; $Ca(NO_3)_2{\cdot}4H_2O$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 0.5; and agar, 20.0 (pH 5.5) at 30 °C (Dhawan & Kuhad, 2002; Vasdev, Dhawan, Kapoor, & Kuhad, 2005). The fungal cultures were maintained periodically on MEA at 30 °C and stored at 4 °C.

2.3. Inoculum preparation

Fungal inoculum was prepared by growing each fungus separately in 250 ml Erlenmeyer flasks containing 50 ml of sterile malt extract broth (MEB) having (g/L): malt extract, 20.0; $Ca(NO_3)_2{\cdot}4H_2O$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 0.5 (pH 5.5). Each flask was inoculated with four mycelial discs (0.8 cm diameter each) of the respective fungal culture and incubated at 30 °C under static cultivation conditions for 7 days. The mycelial mat thus obtained was homogenized with pestle and mortar under sterile conditions and used as inoculum for biological pretreatment experiments.

2.4. Solid state fermentation of sugarcane bagasse

The fungal pretreatment was carried out in 500 ml Erlenmeyer flasks, each with 20.0 g of the air dried sugarcane bagasse moistened with mineral salt solution (MSS) containing (g/L): KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $Ca(NO_3)_2$, 0.5 and pH 5.5 to obtain a substrate to moisture ratio of 1:4 and autoclaved at 15 psi for 15 min. Each flask was inoculated with 0.25 g ± 0.01 fungal biomass (dry weight) of the respective fungus (*P. florida*, *C. caperata* RCK 2011, *Ganoderma* sp. rckk-02). The contents of the flasks were mixed well under aseptic conditions for homogenous distribution of inoculum through out the substrate and incubated at 30 °C for 25 days. The samples (myco-substrate) from flasks were harvested periodically at regular time intervals of 5 days and used for compositional analysis and enzyme assays. The non-fungal inoculated flasks containing sterilized substrate served as control.

For enzyme assays, the myco-substrate harvested aseptically from flasks after various intervals, was suspended in 50 ml of citrate phosphate buffer (pH 5.0) and vortexed for 45 min at room temperature. The extrudates were squeezed through muslin cloth and centrifuged at 10,733 × *g* at 4 °C for 10 min (Deswal, Khasa, & Kuhad, 2011; Shrivastava et al., 2011). The supernatant thus obtained was used to assay ligninolytic (laccase, MnP, LiP) and cellulolytic (CMCase and FPase) enzymes.

2.5. Cellulase production from Fomitopsis sp. RCK2010 under SSF

Cellulase production under SSF by *Fomitopsis* sp. RCK2010 was carried out in 250 ml Erlenmeyer flasks as described else where (Deswal et al., 2011).

2.6. Enzymatic saccharification of fungal treated substrate

The untreated SB served as negative control, while SB treated with H_2SO_4 (0.5%, w/v) and NaOH (0.5%, w/v) separately were kept as positive controls. Enzymatic saccharification of fungal pretreated and controls were carried out at 2% (w/v) consistency in citrate phosphate buffer (pH 4.8) containing 0.005% (w/v) sodium azide. Crude enzyme solution equivalent to cellulase activity (FPase) of 20 IU/g substrate was added to each flask and incubated at 50 °C and 150 rpm for 24 h. Samples were withdrawn at regular intervals, centrifuged at 8000 rpm for 10 min and the supernatants were analyzed for release of reducing sugars.

2.7. Analytical methods

The chemical composition (holocellulose, klason lignin) of the control and fungal treated SB was determined following standard TAPPI (1992) protocols.

The saccharification rate was calculated as

$$= \frac{\text{Amount of sugar released}}{\text{Total carbohydrate in pretreated substrate} \times \text{time of incubation}}$$

CMCase and FPase activities were determined following the IUPAC method (Ghose, 1987). The laccase activity was determined as described previously (Dhawan & Kuhad, 2002). The manganese peroxidase (MnP) and lignin peroxidase (LiP) activities were estimated as reported by Martínez, Ruiz-Dueñas, Guillen, and Martínez (1996) and Heinfling, Martinez, Bergbauer, and Szewzyk (1998), respectively. The reducing sugars were quantified by DNSA method (Miller, 1959).

All the experiments were carried out in triplicates and data presented is the mean value ± standard deviation.

3. Results and discussion

3.1. Diversity of lignocellulolytic enzymes during fungal pretreatment

The oxidative (laccase, LiP and MnP) and cellulolytic enzymes (CMCase and FPase) produced by the three fungi tested for biological pretreatment of lignocellulosic substrates were studied. It was observed that irrespective of the fungus used, the maximum laccase production was observed on 15th day and decreased thereafter