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Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation

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ABSTRACT

Culture conditions for enhanced cellulase production from a newly isolated brown rot fungus, *Fomitopsis* sp. RCK2010 were optimized under solid state fermentation. An initial pH of 5.5 and moisture ratio of 1:3.5 (solid:liquid) were found to be optimal for maximum enzyme production. Of the different carbon sources tested wheat bran gave the maximum production of CMCase (71.526 IU/g), FPase (3.268 IU/g), and β-glucosidase (50.696 IU/g). Among the nitrogen sources, urea caused maximum production of CMCase (81.832 IU/g), where as casein and soyabean meal gave the highest FPase (4.682 IU/g) and β-glucosidase (69.083 IU/g) production, respectively. Among amino acids tested glutamic acid gave the highest production for CMCase (84.127 IU/g); however 4-hydroxy-L-proline stimulated maximum FPase production (6.762 IU/g). Saccharification of pretreated rice straw and wheat straw by crude enzyme extract from *Fomitopsis* sp. RCK2010 resulted in release of 157.160 and 214.044 mg/g of reducing sugar, respectively.
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1. Introduction

The growing concern for depleting fossil fuel requires a transition from non-renewable carbon sources to renewable bioresources such as lignocellulose. Regardless of the source, lignocellulosic materials consist of three main polymers; cellulose, a homopolymer of glucose; hemicellulose, a heteropolymer of pentoses and hexoses; and lignin, an amorphous polymer of phenyl propanoid units (Kuhad et al., 1997). Among these cellulose fits in the role perfectly and thus is also referred as the "biological currency" (Himmel et al., 1999). Every year plants produce about 180 billion tons of cellulose, making this polysaccharide a huge organic carbon reservoir on earth. The cellulose synthesis rate is estimated to be equivalent to 70 kg per person per day (Lutzen et al., 1983). Therefore, the importance of cellulose as a renewable source of energy has become a subject of both, intense research and of commercial interest. The key step in the utilization of cellulose is its hydrolysis into monomeric sugars and their eventual conversion into valuable chemicals and energy (Olofsson et al., 2010).

The enzyme, which governs the hydrolysis of cellulose, is known as "cellulase". Unlike most of the enzymes cellulase is a complex of enzymes that work synergistically to attack native cellulose. Cellulase is a family of at least three groups of enzymes: firstly endoglucanases (EC 3.2.1.4) which act randomly on soluble and insoluble cellulose chains; secondly exoglucanases (cellobiohydrolases EC 3.2.1.91) that act to liberate cellobiose from the reducing and non-reducing ends of cellulose chains and finally, β-glucosidases (EC 3.2.1.21) which liberate glucose from cellobiose. The cellulases give us an opportunity to reap the tremendous benefits of biomass utilization in an eco-friendly manner (Himmel et al., 1999). Besides this, cellulases have many other potential applications as well, for example, formulation of washing powder, animal feed production (Han and He, 2010), textile industry, pulp and paper industry, starch processing, grain alcohol fermentation, malting and brewing, extraction of fruits and vegetable juices (Bhat, 2000). Therefore, like cellulose, a wide range of applications have made cellulase one of the most desirable enzyme systems. An efficient cellulose hydrolysis requires a high enzyme loading and except recombinants, the level of cellulase production from majority of the microorganisms has been generally low. The requirement of high amount of the enzyme makes the hydrolysis process economically less favorable. In many bioconversion strategies, the cellulase required for biomass conversion may account for as much as 40% of the total process cost (Ahamed and Vermette, 2008). Therefore large –scale 2008). Therefore, large-scale low cost production of cellulase is very important for the overall process economics for bioconversion of lignocellulosics into value added chemicals such as ethanol as biofuel.

A large number of microorganisms such as bacteria, actinomycetes and fungi (Kuhad et al., 1997; Kalogeris et al., 2003) are known to degrade cellulose. Among fungi, soft rot and white rot have been extensively studied while brown rots have not been studied much. Cellulolytic enzymes from soft rot and white rot fungi have been studied in model organisms such as *Trichoderma viride* and *Phanerochaete chrysosporium*, respectively. *T. viride* produces fairly good amount of exoglucanases and endoglucanases

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but low level of β-glucosidase, which is insufficient for effective conversion of cellulose to glucose. However, the brown rot fungi differ substantially from soft-rot and white rot fungi with respect to the cellulolytic enzymes produced and the pattern of cellulose degradation (Kuhad et al., 1997). These fungi are generally reported to lack the exoglucanases that can hydrolyze crystalline cellulose (Kuhad et al., 1997), yet they cause the most destructive type of wood decay and are important contributors to biomass recycling. Recently, brown rot fungi such as *Fomitopsis* has been reported to hydrolyze microcrystalline cellulose and therefore has been studied in some laboratories (Yoon et al., 2008). Yoon and Kim (2005) have reported the degradation of crystalline cellulose by *Fomitopsis palustris*. However, to the best of our knowledge the cellulase production from *Fomitopsis* sp. has not been optimized.

Beside the fungus type, the cellulase production is also greatly influenced by media components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature and moisture (Lynd et al., 2002). In order to obtain maximum enzyme production, development of a suitable medium and culture conditions is obligatory. Solid-state fermentation (SSF) conditions have shown to be potential for enzyme production by the filamentous fungi (Holker et al., 2004). The commercial production of enzymes is carried out through SSF for its obvious advantages over liquid cultivation (Viniegra-Gonzalez et al., 2003; Holker et al., 2004). The substrate used in SSF for cellulase production is deterimental in economizing the enzyme production process. Therefore, various cellulosics substrates such as sugarcane bagasse, corn stover, wheat straw, and wheat bran have been tested by several workers for production of cellulases.

In this paper, we report the optimization of various physiological and nutritional parameters for cellulase production from newly isolated brown rot fungus *Fomitopsis* sp. RCK2010 using cost effective substrates under solid state fermentation cultivation conditions. Moreover an attempt has been made to study the application of cellulase(s) in hydrolysis of lignocellulosic substrates such as wheat straw and rice straw.

2. Methods

2.1. Raw materials and their pretreatment

Lignocellulosic substrates like wheat straw (WS), rice straw (RS), wheat bran (WB), corn cob (CC), corn stover (CS), *Prosopis juliflora* (PJ), and *Lantana camera* (LC) were obtained locally. They were first dried and chopped into small pieces by a chopper, then ground into smaller particles in a hammer mill (Metrex Scientific Instrumentation Pvt. Ltd., New Delhi, India) and finally separated by 20 mesh sieve.

The pretreatment of both the substrates (rice straw and wheat straw) was carried out separately with 0.5% (w/v) H_2SO_4 and 2.5% NaOH at 121 °C for 15 min. The pretreated residues were washed extensively to neutral pH and dried at 60 °C till constant weight.

2.2. Microorganism and culture conditions

The fungal isolate RCK2010 procured from the culture collection of Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, New Delhi, India was grown and maintained on malt extract agar (MEA) composed of (gl^{-1}): 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 and Kuhad, 2002; Vasdev et al., 2005). The fungal cultures were maintained by periodical subculturing on MEA at 30 °C and stored at 4 °C.

2.3. Identification of the fungus

2.3.1. Isolation of genomic DNA

Fungal isolate RCK2010 was grown in malt extract broth (MEB) as described elsewhere (Dhawan and Kuhad, 2002; Vasdev et al., 2005) at 30 °C under static cultivation conditions for 168 h. The cultures were harvested by filtering through Whatman No. 1 filter paper. The fungal mycelium was thoroughly washed with Milli Q water and ground in liquid nitrogen. Genomic DNA of fungal mycelium was isolated using method modified by Kuhad and coworkers as reported earlier (Kuhad et al., 2004).

2.4. Phylogenetic studies of the fungus

ITS sequence of fungal isolate RCK2010 was amplified by PCR using pITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and pITS-4 (5′-TCCTCC GCTTATTGATATGC-3′) primer pair. Following cycling parameters: an initial denaturation at 94 °C (4 min), 35 cycles of primer annealing at 58 °C (40 s), elongation at 72 °C (1 min) and denaturation at 94 °C (1 min). A final elongation step was allowed at 72 °C for 8 min. The PCR product was eluted using gel extraction kit (Quigen Sciences, Maryland, USA) and was sequenced at The Centre for Genomic Application (TCGA) Okhla, New Delhi, India. Sequence obtained was compared with ITS sequences available in GenBank, using Clustal W and a dendrogram was constructed to establish the taxonomic rank of the fungus (DNA STAR, Madison, WI, USA).

2.5. Inoculum preparation

Each Erlenmeyer flask (250 ml) containing 50 ml of MEB was inoculated with four mycelial discs (0.8 cm dia each) 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 primary inoculum for further experiments.

2.6. Cellulase production under solid state fermentation

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks, each having 5.0 g of dry wheat bran moistened with mineral salt solution (gl^{-1}:$(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; $MgSO_4$, 0.5 and pH 5.5) to attain the final substrate-to-moisture ratio of 1:3.5. The flasks were sterilized by autoclaving at 121 °C (15 psi), and thereafter cooled to room temperature and inoculated with desired volume of inoculums to obtain 0.25 ± 0.013 g of fungal dry mass (0.5% w/w). The contents of the flasks were mixed well with sterilized glass rod to distribute the inoculum through out the substrate and incubated at 30 °C. The fungal fermented wheat bran (mycobran) was aseptically removed from flasks after an appropriate interval, suspended in 50 ml citrate buffer (100 mM, pH 5.5) and shaken gently for 45 min. The extrudates were squeezed through muslin cloth for maximizing the enzyme extraction and centrifuged at 10,000 rpm at 4 °C for 10 min. The enzyme solution thus obtained was assayed for various cellulase activities to study their time course production.

2.7. Optimization of cellulase production

The cellulase production by the fungus was optimized following one factor at a time (OFAT) approach. The effect of various factors such as initial pH (3.0–10.0), incubation temperature (25–40 °C), substrate to moisture ratio (1:1–1:4), metal ions (2 mM), and different carbon and nitrogen sources, as described in the text, was tested. In addition effect of amino acids (0.2% w/w), vitamins

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