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Bioprocessing of enhanced cellulase production from a mutant of *Trichoderma asperellum* RCK2011 and its application in hydrolysis of cellulose

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HIGHLIGHTS

- The hyper cellulase mutant of *Trichoderma asperellum* RCK2011 was developed.
- The mutant produced higher cellulase with lower catabolite repression.
- Mutant strain cellulase showed 1.6-fold increase in sugar release compared to wild.

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ABSTRACT

A mutant strain of *Trichoderma asperellum* RCK2011 was developed through UV-irradiation for enhanced cellulase production and lower catabolite repression. The production of FPase, CMCase and β-glucosidase was optimized under solid state fermentation; up to 20 mM of glucose did not inhibit cellulase production. The mutant strain *T. asperellum* SR1-7 produced FPase (2.2 IU/gds), CMCase (13.2 IU/gds), and β-glucosidase (9.2 IU/gds) under optimized conditions, which is, 1.4, 1.3, 1.5-fold higher than the wild type. The wild as well as mutant strain produced the cellulases at pH range, 4.0–10.0. Saccharification of pretreated corn cob, wheat straw, and sugarcane bagasse by cellulase from mutant strain SR1-7 resulted in release of reducing sugar at the rate of 530.0 mg/g, 290.0 mg/g, and 335.0 mg/g of substrate, respectively; this is 1.6-fold higher than the wild type strain.

1. Introduction

The constant increase in energy consumption, depletion of fossil fuels and increased environmental concerns have globally shifted the focus to generate energy from non-renewable carbon sources [1] such as agricultural and forest byproducts. Regardless of the source, majority of these materials are lignocellulosic in nature. Lignocellulose complex consists of three main polymers; cellulose, a linear polymer of anhydro-glucopyranose molecules, connected by β-1,4-glycosidic bonds; hemicellulose, a heteropolymer of pentoses and hexoses; and lignin, an amorphous polymer of phenyl propanoid units [2]. On an average, plant material contains 40–50% of cellulose, making this polysaccharide as a huge organic carbon reservoir on earth. The renewable nature of cellulose projects it as a restorable source of energy. The cellulose locked in plant cell wall, with hemicellulose and lignin, is not easily exploitable unless it is hydrolyzed to monomeric sugars. The key step in the utilization of cellulose is its enzymatic hydrolysis into monomeric sugars and subsequent conversion into valuable chemicals and energy [3].

Unlike most other enzymes, cellulase, is a complex of three enzymes that work synergistically to attack native cellulose. These are; endoglucanases (EC 3.2.1.4) which act randomly on soluble and insoluble cellulose chains; exoglucanases (cellobiohydrolases EC 3.2.1.91) that act to liberate cellobiose from the reducing and non-reducing ends of cellulose chains and β-glucosidases (EC 3.2.1.21) which liberate glucose from cellobiose [4].

A large number of microorganisms such as bacteria, actinomycetes and fungi [2,5] are known to degrade cellulose. 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 significant amount of exoglucanases and endoglucanases but low levels of β-glucosidase, which results in accumulation of cellobiose; this inhibits FPase and CMCase and results in overall low rate of substrate

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hydrolysis [6]. Other important species of *Penicillium* and *Aspergillus* produce cellulolytic enzyme complex with higher β-glucosidase activity than *Trichoderma reesei*, but the total cellulase (FPase) activity is relatively low [7]. In bioconversion strategies, the cellulase required for biomass conversion may account for as much as 40% of the total process cost [8]. Therefore, large-scale, low cost production of complete cellulase enzyme complex is very important.

Beside the enzymatic saccharification of lignocellulosic biomass, cellulase enzyme has been potentially utilized in enzymatic deinking of office waste paper, paper pulping, detergent composition, as a fabric softener, in textile industry for removing excess dye from denim fabric and for biopolishing of cellulosic fabric [9].The industrial significance of cellulases can further be improved by investigating the functional efficiency of these enzymes under extreme conditions of pH, as most of the applications require alkaline conditions [10]. Although *Fusarium* sp. [9] and *Chaetomium* sp. [11] are reported to produce cellulase under alkaline conditions, but most of the microbial cellulases reported so far are produced under acidic conditions. Therefore, it is of paramount importance to look for new and hyper cellulase producing microorganisms.

In this study, a strain of *Trichoderma asperellum* RCK2011, a newly isolated ascomycetous fungus, was found to secrete alkaline cellulase. An attempt was made to develop a UV mutant for enhanced production of cellulase with reduced sensitivity to catabolite repression. The cellulase production from *T. asperellum* RCK2011 and its mutant strain SR1-7 has been investigated on lignocellulosic substrates under solid state fermentation (SSF).

2. Materials and methods

2.1. Raw material

Wheat straw (WS), rice straw (RS), wheat bran (WB), corn cob (CC), corn stover (CS), cotton stacks (CSS), and sugarcane bagasse (SB) were obtained locally. They were dried and, except for WB, chopped into small pieces and ground to smaller particles in a hammer mill (Metrex Scientific Instrumentation Pvt. Ltd., New Delhi, India); they were separated through a 20 mesh sieve.

2.2. Microorganism and culture conditions

Fungal isolate RCK2011 was procured from the culture collection of Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, New Delhi, India. The wild type and the mutants were grown and maintained on malt extract agar (MEA) composed of ($g\,l^{-1}$): 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 [12,13]. The fungal cultures were maintained by periodical sub-culturing on MEA at 30 °C and stored at 4 °C.

The medium used for selection of mutants was composed of ($g\,l^{-1}$): 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; carboxy methyl cellulose, 5.0 and agar, 20.0.

2.3. Identification of the fungus

2.3.1. Isolation of genomic DNA

Fungal isolate RCK2011 was grown in malt extract broth (MEB) composed of ($g\,l^{-1}$): 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; (pH 5.5) [12,13] at 30 °C under static cultivation conditions for 3 d. Mycelial mat was harvested by filtering the content of the flasks through Whatman No. 1 filter paper. It was thoroughly washed with Milli Q water and ground under liquid nitrogen; genomic DNA was isolated following the method of Kuhad et al. [14].

2.3.2. Phylogenetic study of the fungus

Internal transcribed spacer (ITS) region of fungal isolate RCK2011 was amplified by polymerase chain reaction (PCR) using pITS (primer internal transcribed spacer)-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and pITS-4 (5′-TCCTCC GCTTATTGATATGC-3′) primer pair. Following thermal cycling parameters were used: 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 it was sequenced at the DNA sequencing facility at the University of Delhi South Campus, New Delhi, India. The sequence was compared with ITS sequences available in the database, GenBank.

2.4. Time course of cellulase production under SSF

This was studied in 250 ml Erlenmeyer flasks, each containing 5.0 g dry WB moistened with mineral salt solution containing ($g\,l^{-1}$): $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; $MgSO_4$, 0.5, pH 5.5; and moisture was maintained at substrate to moisture ratio of 1:3.5 (w/v). Flasks were sterilized by autoclaving at 121 °C (15 psi), they were inoculated with 1 ml spore suspension (10^6–10^7spores/ml) from 4 d old fungal culture and incubated at 30 °C. The fungal fermented WB was aseptically removed from the flasks at desired interval, suspended in 50 ml of 100 mM citrate phosphate buffer (pH 5.5) and shaken gently for 45 min. The extracted contents were filtered, squeezed through muslin cloth, and centrifuged at 10,000 rpm at 4 °C for 10 min. The crude enzyme solution thus obtained was assayed for cellulase activities [15].

2.5. Optimization of cellulase production under solid state fermentation (SSF)

Various process variables effecting cellulase production from *T. asperellum* RCK2011 and mutant SR1-7 were monitored under SSF. This included incubation period (up to 11 d), pH (2.0–12.0), incubation temperature (15–45 °C), substrate to moisture ratio (1:2–1:5 w/v), inoculum size (10–50%, v/w) and carbon sources (WB, CC, CS, SB, WS, RS, CSS).

2.6. Mutant selection

Wild strain RCK2011 was grown on MEA plates for 4 d at 30 °C. Plates were UV-irradiated (at 260 nm, 20 cm distance) for different time periods; sub-culturing was carried out for selection of improved enzymatic activity employing a plate assay [16] of Wood.

2.7. Study of catabolite repression

For catabolite repression study, the wild and mutant strains were grown on the fermentation medium supplemented with 5.0 to 25.0 mM glucose.

2.8. Pre-treatment and enzymatic saccharification of agricultural by-products

Grounded WS, CC and SB were pre-treated with 2% (w/v) NaOH solution at 121 °C for 1 h; the residue was washed to neutral pH and dried at 60 °C till constant weight.

Enzymatic saccharification of untreated and pre-treated materials WS, CC and SB was carried out at 2% (w/v) consistency in 50 mM citrate phosphate buffer (pH 4.8) containing 0.005% (w/v) sodium azide. For this crude enzyme solution was used (equivalent to 5 IU/g substrate FPase activity) and incubated at