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Evaluation of glycosyl hydrolases from thermophilic fungi for their potential in bioconversion of alkali and biologically treated *Parthenium hysterophorus* weed and rice straw into ethanol

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HIGHLIGHTS

- *Malbranchea cinnamomea* a rich source of cellulases/hemicellulases.
- In depth analysis of hydrolysis products from biologically and alkali treated carrot grass.
- Secretome of *M. cinnamomea* contains xylose isomerase for conversion of xylose to xylulose.
- First report on bioconversion of carrot grass into ethanol.

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ABSTRACT

The aim of this work was to evaluate glycosyl hydrolases produced by diverse thermophilic fungal strains for saccharification of alkali and biologically (*Trametes hirusita*/*Myrothecium roridum*) treated *Parthenium hysterophorus* and rice straw. The compositional analysis of hydrolysates by HPLC showed distinct profiles of hexose, pentose and oligomeric sugars. *Malbranchea cinnamomea* was most efficient source of glycosyl hydrolases producing 283.8, 35.9, 129.6, 27,193, 4.66, 7.26 (units/gds) of endoglucanase, cellobiohydrolase, β-glucosidase, xylanase, α-αrabinofuranosidase and β xylosidase, respectively. The saccharification of alkali and biologically treated carrot grass by culture extract of *M. cinnamomea* was further enhanced by supplementation of β-glucosidase produced by *Aspergillus* sp. mutant "O". The resultant hydrolysates containing glucose/xylose were fermented efficiently to ethanol by *Saccharomyces cerevisiae* owing to presence of xylose isomerase (0.8 units/gds) activity in culture extract of *M. cinnamomea* resulting in production of 16.5 and 15.0 g/l of ethanol from alkali treated rice straw and carrot grass, respectively.

1. Introduction

Parthenium hysterophorus (hereafter referred to as carrot grass), an obnoxious weed that is known to cause allergies and rice straw an agro-residue containing high amount of silica is often burnt on fields because of intensive cropping practices reflects the environmental and health hazards which require immediate attention for developing means for their utilization. Being lignocellulosics these can be utilized for bioconversion into ethanol and other fine chemicals using bio-refinery concept employing multistep bioconversion platform comprising of pre-treatment, enzyme production, saccharification and fermentation (Clark et al., 2012). The pre-treatment is primarily carried out to disintegrate and delignify lignocellulosics by an array of energy intensive methods (physical, chemical, thermochemical) that accounts for ~20% of the total cost involved in bioconversion of lignocellulosics into ethanol (Foust et al., 2009). Therefore, researchers are evaluating the use of benign and energy saving biological pre-treatment methods employing ligninolytic fungi (Saritha et al., 2013; Tiwari et al., 2013). The other important factor in developing commercially viable bioconversion technology is the availability of catalytically efficient mixture of glycosyl hydrolases (cellulase/hemicellulases) capable of releasing fermentable sugars from a variety of treated lignocellulosics that differ in their inherent structural complexity as well as changes occurring during different pre-treatment processes

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(Mohanram et al., 2013). Therefore, bioprospecting for novel microbial strains capable of producing a variety of glycosyl hydrolases (endoglucanases, cellobiohydrolases, β-glucosidases, xylanases, β-xylosidases, α-arbinofuranosidase) is an area of intense research (Berrin et al., 2012; Brink et al., 2013).

This study evaluates the potential of glycosyl hydrolases produced by ten different thermophilic fungal strains, isolated from composting material, for saccharification of differently pretreated (alkali/biologically) carrot grass and rice straw through an in-depth analysis of the released sugars using HPLC. The paper further identifies a thermophilic fungal strain *Malbranchea cinnamomea* as a rich source of cellulases and xylanase in addition to producer of xylose isomerase for subsequent efficient fermentation of xylose and glucose present in hydrolysates into ethanol by *Saccharomyces cerevisiae*. This study reports the bioconversion of carrot grass into ethanol for the first time.

2. Methods

2.1. Isolation of fungal cultures

Ten thermophilic fungal strains namely, CM-2T, CM-3T, CM-4T, CM-5T, CM-6T, CM-7T, CM-8T, CM-10T, CM-11T and DS5 were isolated from composting soil/agricultural residues collected from Amritsar (India). These cultures were grown at 45 °C on yeast potato soluble starch agar (YpSs, pH 7.0) of following composition (%; w/v) starch 1.5, yeast extract 0.4, KH_2PO_4 0.23, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.05, citric acid 0.057 and agar 2.0 (Cooney and Emerson, 1964) and maintained on same medium at 4 °C. The strains were identified on the basis of morphological features as well as sequencing of PCR amplified rDNA (ITSI-5.8S-ITSII) region (Sharma et al., 2008).

2.2. Isolation of DNA and PCR amplification of ITS region

DNA was extracted from 48 h old fungal mycelium grown on glucose broth (Sharma et al., 2008) The resultant DNA was amplified for internal transcribed spacers (ITSI-5.8S-ITSII) rDNA coding region using universal primers, ITS1 (forward 5′TCCGTAGGTGA ACCTGCGG3′) and ITS4 (reverse 5′ TCCTCCGCTTATTGATATGC 3′) (White et al., 1990). The PCR amplification was carried out using 25 μl PCR master mix (Genei, Bangalore, India), 2.5 μl of DMSO, 1 pmol l^{-1} of each primer and 100 ng of template DNA and the reaction was carried out using protocol described previously (Sharma et al., 2008). The amplified product in the range of 550–600 bp was sequenced using single primer analysis (SPA) services (Genei, Bangalore, India). The generated ITS sequences from different fungal isolates were aligned with each other and DNA sequences retrieved from NCBI databases using multiple sequence alignment software (CLUSTAL X) for generating phylogenetic tree using neighbor joining (NJ) plot. The ITS sequence data was submitted to NCBI gene bank and the accession numbers were obtained (Fig. 1).

2.3. Solid substrate culturing for enzyme production

Solid-state fermentation was carried out in Erlenmeyer flasks (250 ml) containing ground rice straw (5 g) as a carbon source and 15 ml basal medium of following composition (%; w/v) KH_2PO_4, 0.4, CH_3COONH_4, 0.45, and $(NH_4)_2SO_4$, 1.3, pH adjusted to 7.0 (Badhan et al., 2007). The flasks were inoculated with 24 h old mycelial suspension (2 ml) grown on glucose pre-culture medium (Kaur et al., 2013) and incubated in a water saturated atmosphere at 45 °C for 7 days in an incubator. Thereafter, the enzymes were harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks kept at 45 °C for 1 h under mild shaking. The resultant slurry was filtered through a muslin cloth and centrifuged at 8800×*g* for 20 min. The extracts were used for enzyme assay, SDS–PAGE, zymograms development, as well as saccharification of differently treated substrates.

2.4. Enzymatic assay

Endoglucanase and xylanase activities were determined using 1% CM-cellulose prepared in sodium citrate buffer (50 mM, pH 6.0) and 1% Birch wood xylan (Sigma chemicals) prepared in sodium acetate buffer (50 mM, pH 5.0). The reaction mixture containing equal amounts of suitably diluted enzyme and substrate was incubated at 50 °C for 10 and 5 min, respectively. Total cellulase activity (FPase) was measured by incubating 50 mg Whatman No. 1 filter paper strip (1 × 6 cm) dipped in 0.5 ml of sodium citrate buffer (50 mM, pH 6.0) and 0.5 ml of suitably diluted enzyme at 50 °C for 1 h. The reactions were stopped by adding 3 ml dinitrosalicylic acid (DNS) followed by boiling and the developed color was read at 540 nm using Novaspec II spectrophotometer (Pharmacia). The amount of reducing sugars released was quantified using glucose/xylose standards (Kaur et al., 2014). β-Glucosidase, cellobiohydrolase (CBH I), α-arabinofuranosidase, α-galactosidase and β-xylosidase were assayed using p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenyl-β-D-lactopyranoside (pNPL), 4-nitrophenol-α-L-arabinofuranoside (pNPAf), p-nitrophenyl-α-D-galactopyranoside (pNPGal) and 4-nitrophenyl-β-D-xylopyranoside (pNPX), as respective substrates, employing micro-titre plate based method (Kaur et al., 2013). Appropriately diluted enzyme (25 μl) was mixed with 50 μl of sodium acetate buffer (50 mM, pH 5.0) and the reaction was initiated by adding 25 μl of pNPG (10 mM)/pNPL/pNPAf/pNPGal/pNPX (3 mM) and incubated at 50 °C for 30 min, the reaction was terminated by adding 100 μl of NaOH glycine buffer (0.4 M, pH 10.8) and the developed yellow color was read at 405 nm using an ELISA reader (Bio Rad 680 XR multi plate reader). The enzyme activities against aryl substrates were quantified using para-nitrophenol (pNP) as standard. The enzyme activities were expressed as the amount of enzyme required to release 1 μmol of reducing sugar/pNP per min under given assay conditions. Xylose isomerase activity was determined using carbazole cysteine–HCl method (Banerjee et al., 1994). The enzyme activities were expressed as units/g dry weight substrate (gds).

2.5. SDS–PAGE and activity staining

The enzyme extracts obtained from different cultures grown under SSF were resolved by SDS–PAGE (Laemmli, 1970). The protein samples (70 μg) prepared in sample buffer was boiled for 5 min for denaturation. The resolved proteins in the gel were visualized using silver staining. The endoglucanase zymogram was developed by resolving the secretomes from different fungal strains on 10% SDS–PAGE gel containing 0.1% carboxymethyl cellulose. Following electrophoresis, the gels were washed once for 20 min in 20% (v/v) isopropanol prepared in phosphate buffer saline (PBS), pH 6.8, followed by three washes (20 min each) in PBS. The gel was incubated at 55 °C in PBS for 1 h and then stained with Congo red (0.1%) for 30 min. Bands corresponding to endoglucanase appeared as clear zone against a dark background after destaining the gel with 1 M NaCl followed by treatment with 10% (v/v) acetic acid solution (Kenneth et al., 2006). The β-glucosidase activity in gel was detected by developing zymogram using 10 mM 4-methylumbelliferyl-β-D-glucoside (Sigma) as substrate prepared in sodium acetate buffer (50 mM, pH 5.0). Upon completion of electrophoresis, the gel was incubated in renaturation buffer containing 20 mM piperazine-N,N-bis(2-ethanesulphonic acid), 2.5% Triton X-100, 2 mM dithiothreitol (DTT), and 2.5 mM $CaCl_2$ for