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Malbranchea cinnamomea: A thermophilic fungal source of catalytically efficient lignocellulolytic glycosyl hydrolases and metal dependent enzymes

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HIGHLIGHTS

- Secretome analysis of *M. Cinnamomea* using LC/MS/MS orbitrap.
- Proteins in the secretome fractionated using Q Sepharose column.
- Fraction analyzed for hydrolysis of ATCG in presence of metal ions.
- Metal dependent proteins identified using PMF.
- Combination of fractions and Cellic CTec2 led to enhanced hydrolysis of ATCG.

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ABSTRACT

This study reports thermophilic fungus *Malbranchea cinnamomea* as an important source of lignocellulolytic enzymes. The secretome analysis using LC–MS/MS orbitrap showed that fungus produced a spectrum of glycosyl hydrolases (cellulase/hemicellulase), polysaccharide lyases (PL) and carbohydrate esterases (CE) in addition to cellobiose dehydrogenase (CDH) indicating the presence of functional classical and oxidative cellulolytic mechanisms. The protein fractions in the secretome resolved by ion exchange chromatography were analyzed for ability to hydrolyze alkali treated carrot grass (ATCG) in the presence of Mn^{2+}/Cu^{2+}. This strategy in tandem with peptide mass fingerprinting led to identification of metal dependent protein hydrolases with no apparent hydrolytic activity, however, showed 5.7 folds higher saccharification in presence of Mn^{2+}. Furthermore, adding different protein fractions to commercial cellulase (Novozymes: Cellic CTec2) resulted in enhanced hydrolysis of ATCG ranging between 1.57 and 3.43 folds indicating the enzymes from *M. cinnamomea* as catalytically efficient.

1. Introduction

High cost of cellulases, accounting for 20–30% of the total cost during bioconversion of lignocellulosics, is considered as one of major constraint in developing commercially viable cellulose to ethanol technology (Klein-Marcuschamer et al., 2012). Therefore search for catalytically efficient cellulases through bioprospecting of novel bacterial and fungal strains, metagenomics, protein engineering of existing cellulases, random mutation (ep PCR) and rational screening, system biology tools (genomics, transcriptomics and proteomics) are being pursued extensively (Busk and Lange, 2013; Gong et al., 2012; Liu et al., 2013). Concerted efforts employing these techniques have resulted in improved strains capable of producing high levels of cellulases thus bringing down the cost of enzymes appreciably in recent past (Payne et al., 2015). Elucidation of structural complexity of the lignocellulosics through SEM as well as fluorescent labelled probes have aided in discovering novel enzymes and understanding their mode of action on natural substrates. The concerted enzymatic action of cellulases (cellobiohydrolases) on cellulose fibers has been conclusively demonstrated through atomic force microscopy in recent past (Igarashi et al., 2011). Recent studies suggest important role of lytic polysaccharide mono-oxygenase (LPMO) and cellobiose

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dehydrogenase (CDH) as oxidative catalysts in enhancing the efficiency of cellulolytic degradation has added new dimensions to existing cellulose degradation paradigm (Horn et al., 2012). This study attempts to unveil the cellulolytic/hemicelluloytic capability of a thermophilic fungus *Malbranchea cinnamomea* by fractionating the glycosyl hydrolases and other proteins in secretome using ion exchange chromatography and analysis of their ability to hydrolyze alkali treated *Parthenium hysterophorus* (carrot grass) a wide spread obnoxious weed, in this part of the world. The study through a series of experiments involving commercial cellulases identifies catalytically efficient proteins in *M. cinnamomea* secretome that can enhance the hydrolysis of alkali treated of carrot grass (ATCG). Furthermore, the role of divalent metal ion (Mn^{2+}/Cu^{2+}) dependent proteins in mediating the catalysis of selected enzyme fractions for achieving improved hydrolysis is also being reported.

2. Methods

2.1. Culture

M. cinnamomea (CM-10T) isolated from composting soil samples was identified on the basis of morphological, microscopic as well as amplified sequence analysis of ITS1-5.8S-ITS2 region (KJ563258). The culture was grown on YG agar (Cooney and Emerson, 1964) at 45 °C and maintained at 4 °C.

2.2. Production of cellulases and hemicellulases

For production of cellulases and hemicellulases, *M. cinnamomea* was grown using solid state fermentation (SSF) using sorghum straw as carbon source. The production was carried out in Erlenmeyer flasks that contained ground 5 g sorghum straw as carbon source and basal medium (17.0 ml) of following composition (%; w/v) KH_2PO_4; 0.4: CH_3COONH_4; 0.45: and $(NH_4)_2SO_4$; 1.3 (Badhan et al., 2007). Prior to sterilization, the initial pH and the moisture content of the medium were adjusted to 7.0% and 75%, respectively. The flasks were inoculated with 2.0 ml of actively growing mycelium of 24 h old culture grown on glucose preculture medium (% w/v): glucose; 1.5, yeast extract; 0.4, K_2HPO_4; 0.2; $MgSO_4{\cdot}7H_2O$; 0.1, pH 7.0) and incubated at 40 °C. The enzyme from different flasks was harvested at 24 h intervals by adding 50.0 ml of sodium citrate buffer (50 mM, pH 6.0) and mixing for 1 h at 45 °C under mild shaking conditions (60 rpm). The resultant slurry was filtered through a muslin cloth and centrifuged at 8800×*g* for 20 min. The extracts were used for assay of endoglucanase, cellobiohydrolase, β-glucosidase, FPase, xylanase, α-arabinofuranosidase, β-xylosidase, acetyl xylan esterase and feruloyl esterase. The enzyme activities are given as units per gram dry weight substrate (units/gds). The experiment was carried out in triplicates.

2.3. Secretome analysis

The enzyme obtained from 7 day old culture flask was subjected to ultra filtration and desalting using 10 kDa membranes (PES, Permeonics, India). The resultant protein sample (3 mg/ml) was reconstituted in 0.1% AALS I (Progenta™, Protea Biosciences, Inc., WV) detergent prepared in ammonium bicarbonate buffer (200 mM). Protein (15 μg) was run on 12% SDS–PAGE and whole gel lane was cut into 3 pieces. Each gel fraction was destained, followed by reduction and alkylation using 5 mM dithiothreitol (DTT) (60 °C for 45 min) and 20 mM iodoacetamide, respectively. Peptides were extracted by three incubation rounds of increasing amounts of acetonitrile and 1% formic Acid. Peptide extracts were dried in a speedvac and resuspended in 60 μl of 5% acetonitrile: 0.1% Formic Acid. Peptide extract (5 μl) was injected onto a 17 cm × 75 μm C18 reversed-phase column connected in line with LTQ-Velos-Orbitrap. A 30 min gradient was used to separate peptides from each fraction (Kolbusz et al., 2014). Acquired data was processed with Proteome Discoverer 1.3 using Sequest for peptide identification of proteins with at least two unique peptides (Singh et al., 2014). Local false discovery rates of 1% at the peptide and protein level were used as identification confidence cutoffs. Detected proteins were grouped as per the glycoside hydrolase (GH) families as described in dbCAN server.

2.4. Fractionation of secretome proteins by liquid chromatography

Five ml of concentrated and desalted protein sample (22.8 mg/ml) equilibrated with sodium acetate buffer (50 mM; pH 5.0) was loaded onto anion exchange pre-packed Q Sepharose columns (2 × 5 ml attached in series) (GE Healthcare) pre-equilibrated with sodium acetate buffer (50 mM; pH 5.0) and eluted at flow rate of 1 ml/min. The protein fractions (5 ml each) were collected under isocratic conditions (run for 5 column volumes) using equilibration buffer followed by linear gradient of sodium acetate buffer with 1 M NaCl using AKTA Prime liquid chromatography system (Amersham Biosciences). The protein content in fractions was quantified using dye binding method (Bradford, 1976). Alternate fractions were assayed for xylanase, α-arabinofuranosidase, β-xylopyranosidase, cellobiohydrolase, cellobiosidase and β-glucosidase activities.

2.5. Enzyme assay

Briefly, xylanase was assayed in 1.0 ml reaction mixture containing 10 μl of fraction, 500 μl of birch wood xylan (1.0%; w/v prepared in 50 mM sodium citrate buffer pH 6.0) and 490 μl buffer. The reaction was carried out at 50 °C for 5 min and terminated by adding 2.0 ml DNS followed by boiling (Sharma et al., 2010). The amount of reducing sugar (xylose equivalent) released was quantified spectrophotometrically at 540 nm. The β-glucosidase, CBH I, CBH II, β-xylopyranosidase and α-L-arabinofuranosidase, activities in the fractions was assayed using micro titre plate based method (96 well Grenier-F-bottom ELISA plates). The reaction mixture (100 μl) containing equal amounts of fraction and either of β-D-glucopyranoside (10 mM), β-D-lactopyranoside (3 mM), β-cellobioside (3 mM), β-xylopyranoside (3 mM), α-L-arabinofuranoside (3 mM), as respective substrate (Gao et al., 2010) prepared in 50 mM sodium acetate buffer (pH 5.0) were incubated at 50 °C for 30 min. The reaction was terminated by adding 100 μl of 0.4 M NaOH–glycine buffer (pH 10.8). The amount of released *para* nitro-phenol (*p*NP) was determined by measuring the intensity of developed color (Gao et al., 2010) at 405 nm using ELISA plate reader (Bio-Rad; model 680 XR).

2.6. Effect of metal ions (Mn^{2+}/Cu^{2+}) on the hydrolysis of alkali treated carrot grass

The effect of metal ions Mn^{2+}/Cu^{2+} on hydrolysis of alkali treated carrot grass (ATCG) by resolved proteins in the fractions was studied. The reaction mixture containing 200 μl of $MnSO_4$/$CuCl_2$ (2 mM prepared in 50 mM sodium citrate buffer pH 6.0) and 200 μl of Q-Sepharose eluted fractions in 5 ml vial containing 0.04 g ATCG (at 10% substrate loading rate) was incubated at 50 °C for 24 h and 200 rpm. Released reducing sugars were estimated using DNS employing microtitre plate based method (Xiao et al., 2005). In this protocol 25 μl of enzymatic hydrolysate aliquot was mixed with 75 μl of the buffer and 100 μl of the DNS reagent in PCR tubes (500 μl) and kept at 100 °C for 10 min in PCR master