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Research paper

Molecular characterization of glycosyl hydrolases of *Trichoderma harzianum* WF5 – A potential strain isolated from decaying wood and their application in bioconversion of poplar wood to ethanol under separate hydrolysis and fermentation

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ABSTRACT

Populous deltoides wood is a suitable raw material for ethanol production, since it has high cellulose content. Efficient degradation and subsequent conversion of lignocellulosic biomass to bio-ethanol, requires physico-chemical pretreatments followed by enzymatic hydrolysis to obtain high overall yields of sugars. Microbial hydrolytic enzymes are actively utilized for biomass degradation and bio-ethanol production. Four glycosyl hydrolases (GHs) namely; three subunits of cellulase, endoglucanase (*ThWF5-endoglucanase*), exoglucanase (*ThWF5-exoglucanase*) and β-glucosidase (*ThWF5-glucosidase*), and xylanase (*ThWF5-xylanase*), from fungal strain *Trichoderma harzianum* WF5 - isolated from decayed wood, were pulled out and subsequently characterized. Analysis of full length sequences and prediction of secondary and tertiary structure showed that these enzymes shared homology with the respective enzymes of other *Trichoderma* species and have comparatively evolved structure to express catalytic activity under broad range of environmental conditions. Respective GHs were partially purified and used for the enzymatic hydrolysis of *P. deltoids* wood after pretreatments. The pretreatment of *P. deltoides* wood with sulfuric acid and hydrogen peroxide ($H_2SO_4 + H_2O_2$) at 121 °C for 60 min was determined as the best condition to high recovery of reducing sugars after enzymatic hydrolysis. The overall high ethanol yield of 15.6 g/l and fermentation efficiency of 61.1% was obtained when fermentation was performed on pretreated poplar wood slurry followed by detoxification with calcium hydroxide. High bio-ethanol production from *P. deltoides* wood and molecular characterization of glycosyl hydrolases used, provide insights that this wood offers a potential source for biofuel production while *T. harzianum* WF5 is a treasure house of robust degrading enzymes.

1. Introduction

The search for new technologies aimed at the production of renewable biofuels has been intensified in recent years. There is an increasing world-wide interest in the limitation of environmental impacts and climate change, by replacing petrochemical products with environment-friendly analogues in order to move towards a sustainable economy [1,2]. Fossil fuels are being replaced by alternative fuels from renewable sources all over the world [3].

Woody lignocellulosic biomass is an especially attractive feedstock for biofuels by enzymatically hydrolyzing complex cellulose and hemicelluloses into simple sugars and in turn to ethanol. Eucalyptus, poplar and pine are commercially important fast-growing trees having implications in construction sector, pulp and paper industries [4], and are also being used as potential biomass sources for biofuel production [2–4]. In particular, hybrid poplar (*Populus* spp.) a short rotation woody bioenergy crops, are currently being grown in temperate regions, as feedstocks for liquid transportation fuels having lots of logistic and agronomic advantages. It has been shown that hybrid poplar can be grown on

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marginal agricultural lands with low energy and chemical input, and produce biomass with high energy density at moderately high productivities [5,6]. This inculcates the interest and motivation for developing effective and economic conversion technologies for biofuel production from woody feedstocks [7] such as poplar.

As the lignocellulosic biomass consists of a network of cellulose and hemicellulose bound by lignin, the lignin sheath as well as the crystallinity of cellulose presents major challenges for efficient hydrolysis of lignocellulosic biomass. In general, pretreatment presents the most practical and economic challenges in the attempt to commercialize cellulosic bioethanol [8–10]. Due to the inherent properties of lignocellulosic biomass, a chemical, thermal, or physical pretreatment step is necessary to facilitate the biochemical production of biofuels from polysaccharides [7]. However, dilute acid solutions can effectively remove lignin and reduce cellulose crystallinity [11–13]. Determining the optimal pre-treatment conditions thus, are necessary to develop efficient fermentation and subsequent ethanol production from lignocellulosic biomass [14].

The degradation of lignocellulosic biomass into the sugar monomers is necessary for fermentation process, where the cellulose and hemicellulose chains needs to be hydrolyzed. Microorganisms produce a variety of glycosyl hydrolases that are able to degrade the complex sugars in the lignocellulosic biomass into simple monomeric forms. Glycosyl hydrolases such as the cellulase and xylanase catalyze these reactions and converts complex biomass into monomer sugars [15]. These enzymes can be used individually as well as in combination after optimization of conditions depending upon the nature of biomass and enzyme catalysis. Interestingly, due to the crystallinity of cellulose, effective hydrolysis can be achieved by the synergistic catalysis by different cellulolytic sub-enzymes used together, which results in higher activities as compared to the sum of their individual effects [16]. The cellulolytic complex involves three main groups of enzymes. The first group is constituted by the β-1,4-exoglucanases, which are represented by the cellobiohydrolases (CBH, EC 3.2.1.91) and glucanohydrolases (GH, EC 3.2.1.74). These enzymes catalyze the production of either cellobiose or glucose units from the reducing (CBH I) and nonreducing (CBH II) ends of cellulosic fibrils, and are generally inhibited by their hydrolysis products. The second group involves the β-1,4-endoglucanases (EG, EC 3.2.1.4), which randomly break internal glycosidic linkages of the amorphous region of cellulose, liberating oligosaccharides of various lengths. Finally, the third group is composed of β-1,4-glucosidases, which hydrolyze cellobiose and soluble oligosaccharides into glucose [17]. Xylanases (E.C.2.8.1.8), a group of hemicellulolytic enzymes, are required for the hydrolyisis of β-1, 4-xylans present in lignocellulosic materials [18].

Considering the above facts the present work was aimed to explore the potential of microbial hydrolytic enzymes for lignocellulosic biomass degradation and subsequent bioethanol production. Potentially robust hydrolytic enzymes; cellulase and xylanase from fungus *Trichoderma harzianum* WF5 isolated from decayed wood, were used and evaluated for the effective hydrolysis of pretreated poplar wood. Also the full length gene sequences of the respective enzymes were pulled out and characterized. The study provides insights, and their potential for utilizing the respective microbial hydrolytic enzymes for maximizing the yield of sugars from complex biomass and subsequent fermentation to ethanol under separate hydrolysis and fermentation (SHF).

2. Material and methods

2.1. Fungal strain

A potential fungal strain *T. harzianum* WF5 was isolated from decayed wood from northern parts (Himalayan Belt) of India. It was identified at genomic level using ITS 5.8S rRNA gene technique.

2.2. Cloning of full length gene sequences of hydrolytic enzymes

Primers specific to endoglucanase, exoglucanase, β-glucosidase and endo-β-1,4-xylanase were designed on the basis of multiple alignment of respective gene sequences from different strains of *T. harzianum* (Table 1). DNA was isolated from the culture of *T. harzianum* WF5. Fifty nanogram of this DNA was used in a 50 μl PCR reaction, which contained 5 μl of 10X PCR buffer with $MgCl_2$, 1 μl of 10 mM dNTPs mix, 1 μl of each of the gene specific primers (10 p mol μl^{-1}), and 2 unit of Taq polymerase. The reaction (94 °C, 30 s; Tm °C for 30 s, 72 °C for 1 min and 72 °C for 7 min) was run for 35 cycles (Table 1). The presence and yield of PCR products were determined on 1% agarose gel. The PCR products were purified using QIAquick gel extraction kit (Qiagen, USA) and sequenced (Xcelris, India) using gene specific primers from both ends. Sequences from both ends were then aligned for overlaps using BLAST algorithm of NCBI (http://www.ncbi.nlm.nih.gov/). ORF finder in NCBI was used to determine the 5′ UTR, ORF and 3′UTR in the respective gene sequences.

2.3. In-silico sequence, phylogenetic and structure analysis

The deduced amino acid sequences were analyzed by ProtParam [19] and Prosite tools [20] at ExPASy proteomics server (http://ca.expasy.org/), and CD search [21] at NCBI, to calculate composition of amino acids, molecular mass, isoelectric point and to scan the motifs/domains present in the deduced amino acid sequence. Signaling peptide was searched using SignalP [22] algorithm (http://www.cbs.dtu.dk/services/SignalP/). Subcellular localizations were predicted using PSORTb [23], TargetP 1.1 Server [24], ProtComp Version 9.0 and CELLO v.2.5: subCELlular LOcalization predictor [25]. Multiple sequence alignments were generated by ClustalW (version 2.0.9) program (http://www.ebi.ac.uk/clustalw/). The secondary structure was predicted using SOPMA (http://npsa-pbil.ibcp.fr/) [26]. Tertiary (3D) structure and function prediction was carried out using Expasy's SWISS MODEL [27], an automated protein homology-modeling server (http://swissmodel.expasy.org/interactive).

2.4. Production and partial purification of glycosyl hydrolases (cellulase and xylanase) from T. harzianum WF5

The fungus *T. harzianum* WF5 was cultured in Vogel's media for enriched production of glycosyl hydrolases which were then partially purified using Ammonium sulfate method. Briefly, to the 90 ml of Vogel's media (containing 2% cellulose for cellulase and 2% xylan for xylanase), 10% of 7 days old *T. harzianum* WF5 culture containing 1×10^7 spores/ml was added and incubated at 30 °C for 6 days. Afterwards, culture contents were centrifuged at 10,000 x*g* for 15 min at 4 °C. The supernatants collected were subjected to ammonium sulfate precipitation to obtain different saturation level for each enzyme. Both, endo- and exoglucanase were precipitated at 30–60% whereas β-glucosidase at 20–30% level of saturation of ammonium sulfate. Xylanase was precipitated at 30–70% level of saturation of ammonium sulfate. Precipitates obtained were dissolved in phosphate buffer (0.1 M, pH 6.9) and were dialyzed against same buffer overnight at 4 °C using dialysis membrane of 14 kDa cut off and kept at −80 °C until further use. Enzyme activities were determined in the culture supernatant and purified fractions according to standard units. Enzyme activities of Endoglucanase (carboxymethyl cellulase) and exoglucanase were calculated as described by Reese and Mandel [28]. One Unit (IU) of