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High efficiency co-production of ferulic acid and xylooligosaccharides from wheat bran by recombinant xylanase and feruloyl esterase

Hongli Wu [a], Hailong Li [b], Yong Xue [a], Gan Luo [a], Lihui Gan [a], Jian Liu [a,*], Liuhao Mao [a], Minnan Long [a,*]

[a] College of Energy, Xiamen University, Xiamen 361005, PR China
[b] Guangdong Key Laboratory of New and Renewable Energy Research and Development, CAS Key Laboratory of Renewable Energy, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, PR China

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ABSTRACT

The enzymatic hydrolysis of lignocellulosic biomass has been studied for years, but this hydrolysis has been minimally used in the industry due to its high costs and low conversion yields. In the work reported here, ferulic acid (FA) and xylooligosaccharides (XOS) were generated from wheat bran at high yields based on the synergistic action of two xylan-degrading enzymes, xylanase (AnXyn11A) and feruloyl esterase (AnFaeA), which were cloned from *Aspergillus niger* BE-2 and heterologously expressed at high levels in *Pichia pastoris*. AnXyn11A exhibited a maximal activity of 240 U mL^{-1} at pH 5.0 and 60 °C and less thermostability above 50 °C. AnFaeA showed a maximal activity of 21 U mL^{-1} at pH 5.0 and 45 °C and high thermostability below 55 °C. The ratio of FA released from destarched wheat bran (DSWB) under the synergistic action of AnXyn11A and AnFaeA increased to 70% in comparison with that of the individual enzyme acting alone (only 16.8% of FA was released). Moreover, at the optimum level of enzyme addition, the XOS yield was double that under the single enzyme action.

1. Introduction

Recently, intense interest has been focused on environmentally friendly methods to utilize lignocellulosic biomass to lower the dependency on fossil fuel feedstocks [1]. Agricultural waste materials such as wheat bran, rice straw, corncob and bagasse, which have been adopted for biofuels and high value-added compounds, are considered to be a potential substitute for fossil resources. The enzymatic hydrolysis of lignocellulosic biomass has been a hot research topic recently. The major obstacle to the efficient and economically feasible bioconversion of lignocellulose is its recalcitrance to enzymes due to its complexity of composition and structure [2]. To obtain any of these biomass constituents, the synergistic action of several types of enzymes is required [3]. Ferulic acid (FA) and xylooligosaccharides (XOS) liberated from lignocellulose are two promising functional foods [4,5]. FA is the most abundant hydroxycinnamic acid and is distributed widely throughout the plant kingdom. Most graminaceous plants such as wheat, bamboo, barley, maize, and tropical grasses contain up to 3% (w/w) FA, which is esterified to the C-5 position of α-L-arabinofuranosyl. As a renewable resource for biocatalysis and chemical conversion, FA can be used as an antioxidant, antimicrobial agent, photo-active compound in sunscreens and food preservative or enzymatically converted to vanillin as an essential flavor in the food and perfume industry [6–8,9]. XOS generated from lignocellulose has great potential as an agent to maintain a balanced intestinal flora for health [10,11]. Fig. 1 shows a simplified structure of wheat bran arabinoxylan, giving a visual guide to the pattern of most substituents. The main xylan backbone is composed of β-1,4-D-xylpyranose residues. Arabinofuranosyl substituents are attached to D-xylose residues via an α-1,3 and/or α-1,2 linkage. The feruloyl residues are attached via ester bonds at the C-5 position of the arabinofuranosyl substituents.

Endo-β-1,4-xylanases (EC 3.2.1.8, EXs) are critical enzymes in the degradation of xylan that are able to cleave the xylan main chain and release various XOS species [3]. According to the amino acid sequence similarity and hydrophobic cluster analysis, we can identify characterized *endo*-β-1,4-xylanases in families 5, 8, 10, 11, 43 and 62 [12] among all the glucoside hydrolase (GH) families. GH10 and 11 EXs are widely used, and the majority of researches has been focused on them. There are great differences between these two families. Compared with GH10 EXs, GH11 EXs are more

* Corresponding authors.
E-mail addresses: jianliu@xmu.edu.cn (J. Liu), longmn@xmu.edu.cn (M. Long).

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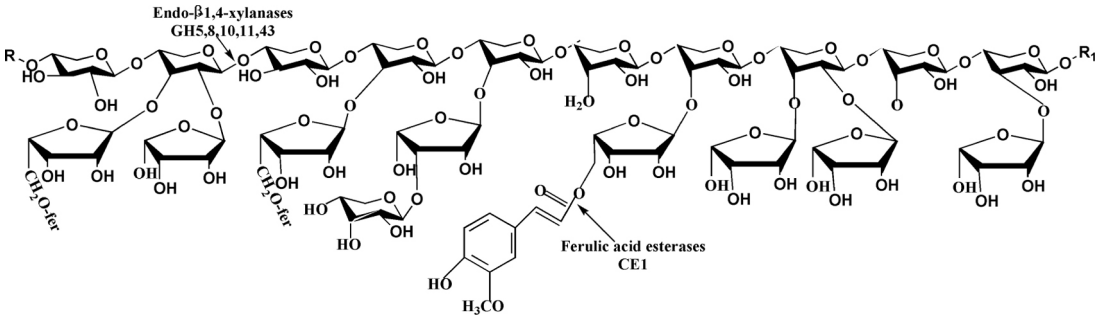

Fig. 1. Sketch of arabinoxylan structure of wheat bran. fer, feruloyl residue. Xylans consist of a backbone of β-1,4-D-xylpyranose residues. Arabinofuranosyl substituents are attached in D-xylose residues via α-1,3 and/or α-1,2 linkages. Feruloyl residues are attached via ester bonds at the C-5 position of the arabinofuranosyl substituents.

liable to be influenced by side chains [13]. The molecular weight of GH11 xylanases are lower, providing great advantages for penetrating into insoluble substrates [14]. Ferulic acid or feruloyl esterases (EC 3.1.1.73, FAEs) are core enzymes for cleaving ester linkages between hydroxycinnamic acids and carbohydrates in the process of the biodegradation of plant cell walls [15,16]. The evidence accumulated in recent years suggests that FAEs produced by microorganisms differ in structure, biochemical properties and substrate specificity. Thus, four types of FAEs (A, B, C, and D) were identified based on those characteristics [17].

So far, multiple efforts have been devoted to the construction of engineering microorganisms. Various EXs and FAEs have been isolated and characterized from fungi and bacteria, but few were able to be applied on the industrial level due to their low levels of expression and weak catalytic activities. The heterologous expression of novel hemicellulases still remains a considerable concern. Herein, we studied the heterologous expression of *Anxyn11A* and *AnFaeA* genes in the methylotrophic yeast *Pichia pastoris*, and the biochemical properties were also investigated. To further explore the hydrolytic ability on complex substrates and obtain high-value-added products efficiently, a synergistic action between AnXyn11A and AnFaeA on destarched wheat bran (DSWB) for the co-production of FA and XOS was also investigated.

2. Materials and methods

2.1. Strains, vectors and culture media

A. niger strain BE-2 (GenBank accession No. JQ867187) was maintained as the source of the *Anxyn11*A and *Anfae*A genes. *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) was used for the heterologous expression of *Anxyn11A* and *AnfaeA* cDNA. *Escherichia coli* DH5α, pPMD19-T (Invitrogen) pPIC9 (Invitrogen) and pPIC9 K (Invitrogen) vectors were used for the construction of the recombinant expression vector. *E. coli* DH5α was cultured at 37 °C in Luria–Bertani medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl, pH 7.2). *P. pastoris* was preserved in yeast extract peptone dextrose medium (YPD). The transformants were selected on minimal dextrose plates (MD) and cultured on buffered minimal glycerol medium (BMGY) (225 rpm, 28 °C) until the culture reached a density of OD_{600} = 2.0–6.0. The cells were resuspended and cultured in 100 mL of buffered minimal methanol medium (BMMY), which was prepared according to the manual of the Multi-Copy Pichia expression kit (Invitrogen).

2.2. Cloning of Anxyn11A and AnfaeA genes

Based on the BLAST algorithms, we designed specific primers for amplifying *Anxyn11A* (AnXyn11A-F, AnXyn11A-R) and *AnfaeA* (AnFaeA-F, AnFaeA-R) with the cDNA as a template (Table 1). The PCR product was ligated to the pPMD19-T Easy vector and transformed into *E. coli* DH5α. Three positive clones were sequenced, and the correct genes were uploaded to NCBI by sequence alignment. After sequencing, the results were identified using Blast Server at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The ProtParam program (http://web.expasy.org/protparam) was used to analyze the bioinformatics of AnXyn11A and AnFaeA. The putative N-linked glycosylation sites were analyzed by the NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The phylogenetic tree was constructed using Mega 5.0 software. The tertiary structure of AnXyn11A was predicted by the online Swiss-Model server (https://www.swissmodel.expasy.org/) based on the crystal structure of a GH11 xylanase from *Penicillium funiculosum* (PDB code 1TE1) [18]. The three-dimensional (3-D) structure of AnFaeA was determined on the same online server based on the crystal structure of a type A feruloyl esterase from *A. niger* (PDB code 1UWC) [19].

Table 1
Gene-specific primers for AnXyn11A and AnFaeA from A. niger BE-2. Restriction endonuclease sites used for cloning are underlined.

Gene	Primer sequence (Underlined restriction site) 5′-3′
AnXyn11A-F	ATGGTCGCCTACTCGTCTCT
AnXyn11A-R	TTAGCAGCTCTCCTCGGTG
AnXyn11A-F'	GAATCCTAGGCTCCCCAATGGCAAGGCCC
AnXyn11A-R'	ATAAGAATGCGGCCGCTTAGCAGCTCTCCTCGGTGCTGTC
AnFaeA-F	ATGAAGCAATTCTCTGCAAAATACG
AnFaeA-R	TTACCAAGTACAAGCTCCGCTCG
AnFaeA-F'	CCGGAATTCGCGGCCTCCACGCAAGG
AnFaeA-R'	GAATCCTAGGTTACCAAGTACAAGCTCCGCTCG

The DNA fragment encoding the mature AnXyn11A protein was amplified with two primers (AnXyn11A-F′ and AnXyn11A-R′) (Table 1**)** that contain restriction endonuclease sites *Bln*I(*Avr*II) and *Not*Ifor linking the target gene and plasmid. The gene *Anxyn11A* was inserted into the expression vector pPIC9 (Invitrogen) digested by *Bln*Iand *Not*I to generate pPIC9-*Anxyn11A*. Likewise, the DNA fragment encoding the mature AnFaeA protein was amplified with two primers (AnFaeA-F′, AnFaeA-R′) (Table 1) incorporated in *Eco*Riand *Bln*I(*Avr*II) restriction sites, respectively and inserted into the expression vector pPIC9K (Invitrogen) digested by *Eco*Riand *Bln*I to generate pPIC9K-*AnfaeA*.

2.3. Expression of AnXyn11A and AnFaeA in P. pastoris

The plasmids pPIC9-*Anxyn11A* and pPIC9K-*AnfaeA* were separately linearized by *Sal*Iand *Stu*I and then transformed into *P. pastoris* GS115 by electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The phenotypes of the transformants were identified by colony PCR using 5′AOX1 (5′-GACTGGTTCCAATTGACAAGC-3′) and 3′AOX1 (5′-GCAAATGGCATTCTGACATCC-3′) primers. The