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A novel pathway construction in *Candida tropicalis* for direct xylitol conversion from corncob xylan

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HIGHLIGHTS

- A new β-xylanase gene was discovered from *Aspergillus terreus*.
- The co-expression plasmid of xylanase and xylosidase was constructed.
- A novel xylan transforming pathway was integrated in *Candida tropicalis* BIT-Xol-1.
- Xylanase and xylosidase were secretively expressed in the engineered *C. tropicalis*.
- It simultaneously saccharify and transform xylan to xylitol efficiently.

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ABSTRACT

In this study, an integrated xylitol production pathway, directly using xylan as the substrate, was constructed in *Candida tropicalis* BIT-Xol-1 which could efficiently convert xylose into xylitol. In order to consolidate this bioprocessing, a β-1,4-xylanase gene (*atn*) and a β-xylosidase gene (*atl*) were cloned from *Aspergillus terreus*, and were constructed onto episomal plasmid pAUR123. Additionally, combination of the individual *atn* and *atl* expression cassette was also cloned onto pAUR123. After transforming, the positive *C. tropicalis* transformants co-expressing xylanase and xylosidase produced larger hydrolysis zones than those expressing xylanase alone, when incubated on xylan-congo red plates. The engineered *C. tropicalis*/pAUR-*atn*-*atl*-3 (*C. tropicalis* PNL3) secrete heterologous xylanase and xylosidase simultaneously, with the activities of 48.17 and 11.56 U/mL, respectively. The xylitol yields by *C. tropicalis* PNL3 utilizing xylan and corncob were 77.1% and 66.9%, respectively. The integrated pathway of xylitol production was feasible and efficient in utilization of xylan-rich renewable biomass via combining saccharification and transformation of xylan in engineered *C. tropicalis*.

1. Introduction

Xylitol, a natural occurring five carbon sugar alcohol, is one of the most expensive polyol sweeteners and has specific health claims in the world market. As an alternative sugar, owning the properties of low energy and inhibition against the metabolism of dental plaque formation, xylitol was widely used in oral hygiene and pharmaceutical products to reduce tooth decay and ear infection (Mäkinen, 2000). Additionally, xylitol also works as a sucrose substitute for diabetics since it does not require insulin for its metabolic regulation (Emodi, 1978).

The traditional production of xylitol involves direct chemical hydrogenation of D-xylose derived from hemicelluloses xylan hydrolysates of biomass materials over a Raney–Nickel catalyst, which includes high pressure and temperature as well as expensive separation and purification steps. Some research efforts have focused on xylitol production using *Escherichia coli* by xylose reduction during growth on glucose (Cirino et al., 2006; Khankal et al., 2008) or xylose (Cirino et al., 2006; Cirino and Akinterinwa, 2009). Additionally, the biotransformation of D-arabitol into xylitol was also investigated with focus on the conversion of D-xylulose into xylitol (Zhou et al., 2012). Alternately, xylitol production from D-xylose through bioconversion has been proposed as an alternative process utilizing microorganisms such as yeasts, bacteria and filamentous fungi (Chang and Knight, 1960; Antti et al., 2005; Converti and Dominguez, 2001). Among these, yeasts are generally considered to be more efficient producers of xylitol than bacteria or filamentous fungi. Many studies have investigated biological methods of xylitol production by three different types of yeasts (Jin et al., 2005). First, there are wild type xylose utilizing yeasts, such as *C. guilliermondii* (Meyrial et al., 1991), *C. boindii* (Vandeska et al., 1995), *C. tropicalis* (Kim et al., 1999), *C. parapsilosis* and *Debaryomyces hansenii* (Prakash et al., 2011). In addition, recombinant

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Saccharomyces cerevisiae (Hallborn et al., 1991; Meinander et al., 1999) expressing XYL1(xylose reductase gene) from *Pichia stipitis* is known to convert xylose into xylitol. Moreover, mutant strains of some native xylose metabolizing yeasts such as *P. stipitis* (Kim et al., 2001) and *C. tropicalis* (Ko et al., 2006; Yoon et al., 2010) were screened and could produce xylitol from xylose more efficiently. Obviously, the substrate for all these yeast strains to produce xylitol is xylose, which is obtained by acid hydrolysis of xylan present in the hemicellulose. Environmental pollution and equipment corrosion caused by industrial hemicellulose hydrolysate preparation using sulfuric acid is seriously significant (Boussarsar et al., 2009). Moreover, several by-products derived from sugars and lignin during the pretreatment of hemicellulosic raw materials negatively affect the fermentation efficiency (Zhang et al., 2012). The development of a new environment friendly xylitol producing bioprocessing with moderate reaction conditions and low pollution was practically imperative.

Corncobs is one of the most abundant agricultural wastes containing about 30% xylan-type hemicelluloses (Santos et al., 2005), which were recognized as satisfactory sources of xylitol synthesis (Yuan et al., 2004). In the degradation process of xylan, endo-β-1,4-xylanase (β-1,4-D-xylan xylanohydrolase, E.C.3.2.1. 8) and β-xylosidase (β-1,4-D-xylan xylohydrolase, EC 3.2.1.37) play very important roles that the former one randomly cleaves the β-1,4-glycosidic linkages of xylan into short xylo-oligosaccharides and the latter one hydrolyze xylo-oligosaccharides to D-xylose (Beg et al., 2001). Many bacterial and fungal species are able to utilize xylans as a carbon source, among which *A. terreus* is an efficient producers of cellulolytic and xylanolytic enzymes (Hrmova et al., 1989; Ghanem et al., 2000). Also, an *A. terreus* Li-20 strain previously obtained can produce high xylanase and xylosidase activitiy, during the properties investigation of β-glucoronidase (Liu et al., 2012).

In the previous work, a new strain named *Candida tropicalis* BIT-Xol-1 which could efficiently convert xylose to xylitol was obtained. In this paper, in order to achieve direct xylitol production from corncob hemicellulose, a new xylan fermentation pathway was constructed in the xylitol producing *C. tropicalis* BIT-Xol-1 by heterologously co-expressing xylanase and xylosidase from *A. terreus*. It leaves out the use of toxic catalyst, the expensive separation and purification steps of xylose, and has the benefit of energy saving and environmental protection. This is the first report of direct xylitol production from xylan in an engineered *C. tropicalis* strain without addition of xylanolytic enzymes.

2. Methods

2.1. Strains and culture conditions

Xylanolytic enzymes producing strain *A. terreus* Li-20 and xylitol producing strain *C. tropicalis* BIT-Xol-1 were both originally isolated and stored in laboratory.

Aspergillus terreus Li-20 was used as the resource of total RNA to clone the xylanase and xylosidase encoding genes. The fungus was precultured in 30 mL liquid Czapek medium for 24 h at 30 °C with 170 rpm shaking, and then the mycelia were inoculated into the induction medium containing 0.3% birchwood xylan (Sigma, US). *E. coli* DH5α and *C. tropicalis* BIT-Xol-1 were used as the cloning host and the expression host strain, respectively. The plasmids pMD19-T (TaKaRa, Japan) and pAUR123 DNA (provided by Dr. Liu, Central China Normal University) were used as cloning and expression vector, respectively. Luria–Bertani (LB) medium was used to grow *E. coli* cultures in broth and agar plates at 37 °C. Ampicillin (100 μg/mL) was added to the medium to maintain the plasmids. *C. tropicalis* cultures were maintained on YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) containing 0.5 μg/mL of Aureobasidin A (AbA) for plasmid maintenance. YPD-xylan-AbA and YPD-corncob-AbA mediums (YPD medium with the addition of 5 g/L xylan and 15 g/L corncob powder respectively and 0.5 μg/mL AbA both) were used as fermentation mediums for xylitol production. The corncob powder made by steam explosion, containing about 30% xylan-type hemicelluloses, was provided by Prof. Yuan, Beijing University of Chemical Technology.

2.2. Total RNA extraction and gene clone

Total RNA was extracted from *A. terreus* Li-20 grown in xylan induction media for 2 d using TRIzol method (Donald et al., 2010). The quality and integrity of RNA was determined by gel electrophoresis in 1% agarose containing 3.5% formaldehyde as described by Sambrook (Sambrook et al., 1989). For cloning of full-length xylanase and xylosidase coding genes without intron, the total RNA was reverse transcribed with M-MLV reverse transcriptase using M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan) following instructions provided by the manufacturers.

According to the published nucleotide sequences in GenBank, primers were designed (Table 1). The mature xylanase gene (*atn*) and xylosidase gene (*atl*) were amplified by PCR using the AtNF/AtNR and AtLF/AtLR as the primers from the *A. terreus* Li-20 cDNA under the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min with Ex Tag (TaKaRa, Japan). The PCR products were purified using a DNA gel purification kit (BioTeKe, Beijing, China) and cloned into the pMD19-T plasmid. The resulting plasmids were then transformed into *E. coli* DH5α using the calcium chloride method (Cohen et al., 1972). Transformants were screened on LB-amp plates supplemented with 20 μg/mL X-Gal by convenient blue-white selection. Plasmids (pMD19-T-*atn* and pMD19-T-*atl*) were extracted and the *atn* and *atl* genes were sequenced (Sangon, Shanghai, China).

The nucleotide sequences were entered into DNAMAN (Lynnon Biosoft, Version 5.2.2) to predict the molecular weight and deduce the amino acid sequences. The signal peptides were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). Homology searches were carried out by comparing the nucleotide and the deduced amino acid sequences against GenBank database using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST).

2.3. Construction of expression vectors and transformation

For the construction of xylanase expression vector, the *atn* gene double-digested from the pMD19-T-*atn* plasmid with *Kpn*I and *Sal*I (TaKaRa, Japan) was cloned into the multiple cloning sites of the plasmid pAUR123 DNA, which had also been previously digested with the same enzymes. The resulting plasmid was designated pAUR-*atn* (Fig. 1A). Likewise, the 1.7-kp *Kpn*I-*Xba*I DNA fragment carrying the *atl* gene was obtained by double-digestion from the

Table 1
Oligonucleotides used in this study.

Primer names	Sequence (5′ → 3′)	Restriction sites
AtNF	GG***GGTACC***ATGGTTCGTCTTACTGTTCTTGCA	*Kpn*I
AtNR	ACGC***GTCGAC***TTATAAGGCGGAGA TAATTGCG	*Sal*I
AtLF	GG***GGTACC***ATGACGAACGACGACCACG	*Kpn*I
AtLR	GC***TCTAGA***TCACTTCAAGTGGATATCTCCC	*Xba*I
Fpnt	C***CACTAGGTG***GGATCCTCTAGCTCCCTAACATGTA	*Dra*III
Rpnt	C***CACTAGGTG***ATCCGTGTGGAAGAACGATTACAAC	*Dra*III