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Microbial production of xylitol from L-arabinose by metabolically engineered *Escherichia coli*

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An *Escherichia coli* strain, ZUC99(pATX210), which can produce xylitol from L-arabinose at a high yield, has been created by introducing a new bioconversion pathway into the cells. This pathway consists of three enzymes: L-arabinose isomerase (which converts L-arabinose to L-ribulose), D-psicose 3-epimerase (which converts L-ribulose to L-xylulose), and L-xylulose reductase (which converts L-xylulose to xylitol). The genes encoding these enzymes were cloned behind the *araBAD* promoter in tandem so that they were polycistronically transcribed from the single promoter, like an operon. Expression of the recombinant enzymes in the active form was successfully achieved in the presence of L-arabinose. A xylitol production profile of the recombinant strain was evaluated by shake-flask fermentation. ZUC99(pATX210) produced 2.6 g/l xylitol using 4.2 g/l L-arabinose with a xylitol yield of 0.62 g/g L-arabinose in 36 h. It was determined that utilization of glycerol as a co-substrate significantly improved xylitol production and yield. In the presence of 11.8 g/l glycerol, ZUC99(pATX210) produced 9.7 g/l xylitol from 10.5 g/l L-arabinose with a xylitol yield of 0.92 g/g L-arabinose in 36 h. ZUC99(pATX210) also exhibited efficient conversion in fermentor experiments with 1 l medium containing L-arabinose and glycerol. The strain produced 14.5 g/l xylitol from 15.2 g/l L-arabinose with a xylitol yield of 0.95 g/g L-arabinose in 30 h.

[**Key words:** Xylitol; L-Arabinose; *Escherichia coli*; Metabolic engineering; Cofactor regeneration]

Xylitol, a five-carbon sugar alcohol, is used commercially as a natural sweetener in food products such as chewing gum, soft drinks, and confectionery. A number of advantageous properties make xylitol an extremely useful food ingredient. It has the same order of sweetness as sucrose with fewer calories and no unpleasant aftertaste. It is anticariogenic and helps to keep teeth healthy (1). Xylitol has other unique properties that offer further applications. Its metabolism is not regulated by insulin (2) and does not involve glucose 6-phosphate dehydrogenase (3). Therefore, it is an ideal sweetener for diabetics and individuals with a deficiency in glucose 6-phosphate dehydrogenase.

Xylitol is found naturally in fruits such as strawberries, raspberries, plums, and pears (4), but its small quantities in fruits make extraction difficult and uneconomical. On an industrial scale, xylitol is currently produced by chemical reduction of D-xylose derived from plant materials, mainly hemicellulose hydrolysates from birch trees and corn stalks (5), which are extremely rich in xylose (about 50–75% of total aldose) (6, 7). Although hydrolysis of other agricultural residues such as wheat straw, flax straw, and peanut hulls also yields hemicellulose hydrolysates, these hydrolysates contain less D-xylose (about 20–30% of total aldose) and also other sugars such as L-arabinose, D-mannose, D-galactose, and D-glucose (7). In order to produce pure xylitol from these hemicellulose hydrolysates, the competing substrates and reaction byproducts must be removed through expensive separation and purification steps (5). In particular, L-arabinose is a problem in xylitol production because it can be converted to L-arabitol during the reaction, which is practically impossible to separate from xylitol in a cost-effective way.

In addition to the difficult separation and purification steps, xylitol production by chemical reduction has other drawbacks, such as requirements of high pressure (up to 50 atm) and temperature (80–140 °C) and use of an expensive catalyst (8). Therefore, it has been worthwhile to explore methods for effective production of xylitol using microorganisms. Xylitol can be produced by natural xylose-assimilating yeasts and fungi such as *Candida tropicalis*, *C. guilliermondii*, *Pachysolen tannophilus*, and *Petromyces albertensis* (9–13). These yeasts and fungi can convert D-xylose to xylitol because they contain xylose reductase (XR). Furthermore, genetically engineered *Saccharomyces cerevisiae* expressing the XR gene from *Pichia stipitis* was reported to produce xylitol (14).

Microbial production of xylitol using hemicellulose hydrolysates of agricultural residues is an attractive method for reduction of manufacturing costs. These products, however, contain a non-negligible amount of L-arabinose (about 5% of total aldose) in addition to D-xylose (7). The previous methods using xylose-assimilating yeasts and fungi appear to convert L-arabinose to L-arabitol due to broad substrate specificity of XR (15–18). Conversion of L-arabinose to xylitol (but not to L-arabitol) would allow a greater variety of raw

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]
ARAF	GCGTCTAGATAAGGAGGATCATCTATGACGATTTTTGATAA
ARAR	GGCATGCTTAGCGACGAAACCCGTAATA
DPEF	GCGAATTCTAAGGAGGATATATTATGAAACACGGCATCT
DPER	GCGGATCCTCAGCCACCAAGAAC
LXRF	GCGGATCCATAAAGGAGGATATATAATGACTGACTACATTCC
LXRR	GCTCTAGATTATTTGTCTTGCAACATGACCTTAATGTTACCATTAGATG
LYXF	GATGTGCCGCAGGGAGCTGATCTGCTGAAAGCCGTGGATG**GTGTAGGCTGGAGCTGCTTC**
LYXR	GATGAGTGGTCGAGGGCCAGTTGCAGAAGTGGTCGGCTCA**ATTCCGGGGATCCGTCGACC**

[a] The restriction sites and the ribosome binding sequences introduced into the primers are underlined and boxed, respectively. Boldface type in the LYXF and LYXR primers indicates segments that anneal to plasmid pKD13 for amplification of the FRT-kanamycin resistant cassette.

materials to be used for xylitol production and would improve xylitol yield from agricultural residues. In this study, we have developed a novel pathway for bioconversion of L-arabinose to xylitol without L-arabitol formation. The pathway was introduced into *Escherichia coli* and the recombinant *E. coli* was able to convert L-arabinose to xylitol with a good yield. To our knowledge, this is the first time evidence has been shown of efficient production of xylitol from L-arabinose.

MATERIALS AND METHODS

Strains and DNAs *Ambrosiozyma monospora* NRRL Y-1484 was obtained from the ARS Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL, USA). *E. coli* AB707, a wild type strain of K-12, was obtained from the Coli Genetic Stock Center (Yale University, New Haven, CT, USA). *E. coli* DH5α was purchased from Invitrogen (Carlsbad, CA, USA). Genomic DNA of *Rhizobium radiobacter* C58 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). *E. coli* expression vectors, pTTQ18 (19) and pBAD18-Kan (20), were obtained from Amersham Biosciences (Piscataway, NJ, USA) and ATCC, respectively. *E. coli* BW25113 (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$) (21) and plasmids pKD13, pKD46, and pCP20 were obtained from Barry L. Wanner (Purdue University, West Lafayette, IN, USA).

Plasmid construction Genes for L-arabinose isomerase (AraA), D-psicose 3-epimerase (DPE), and L-xylulose reductase (LXR) were cloned into a single plasmid in tandem. A consensus sequence of bacterial ribosome binding sites (5′-AGGAGG-3′) was inserted 8 bp upstream of the translation initiation of each gene. First, the *lxr* gene was isolated from *A. monospora* NRRL Y-1484 by reverse transcription-PCR (RT-PCR) using a primer pair, LXRF and LXRR (Table 1), designed from the published sequence (GenBank Acc. no. AJ583159) (22). *A. monospora* cells were grown overnight in YMA medium that contained 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, and 20 g/l L-arabinose. After total RNA was isolated from the cells using an RNeasy mini kit (Qiagen, Valencia, CA, USA), RT-PCR was performed using a One-Step RT-PCR kit (Qiagen). The PCR product was restricted with BamHI and XbaI and then ligated into pTTQ18 restricted with the same enzymes to yield pATX124. Second, the *dpe* gene was isolated from genomic DNA of *R. radiobacter* C58 by PCR using a primer pair, DPEF and DPER (Table 1), designed from the published sequence (GenBank Acc. no. AE008210). The PCR product was restricted with EcoRI and BamHI and then ligated into pATX124 restricted with the same enzymes to yield pATX207. Third, the *araA* gene was isolated from genomic DNA of *E. coli* AB707 by PCR using a primer pair, ARAF and ARAR (Table 1), designed from the published sequence (GenBank Acc. no. M15263) (23). The PCR product was restricted with XbaI and SphI and then ligated into pATX207 restricted with the same enzymes to yield pATX208. DNA sequences of the cloned genes were analyzed using a BigDyeTerminator v3.1 Cycle Sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Plasmid pATX208 was digested with EcoRI and SphI and then loaded onto an agarose gel to isolate a 3.3-kb DNA fragment including the *lxr*, *dpe*, and *araA* genes. This fragment was subcloned into pBAD18-Kan restricted with the same enzymes to yield pATX210.

Strain construction Disruption of the L-xylulokinase gene (*lyxK*) in the chromosome of *E. coli* BW25113 was carried out by the method of Datsenko and Wanner (21). A kanamycin-resistant gene flanked by FRT (FLP recognition target) sites at both sides was amplified from the template plasmid pKD13 using primers (LYXF and LYXR) that contained 40- and 39-nt extensions homologous to the genomic sequences of the *lyxK* flanking regions (Table 1). This FRT-kanamycin resistant cassette was electroporated into BW25113 competent cells carrying the lambda-red recombinase plasmid pKD46. The shocked cells were suspended in 1 ml Luria-Bertani (LB) medium, incubated at 37 °C for 1 h, and plated on LB agar supplemented with 50 μg/ml kanamycin. The mutant cells were then cured of the inserted kanamycin resistant gene using the helper plasmid pCP20 that expressed FLP recombinase to yield *E. coli* ZUC99 (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$ Δ*lyxK*). *E. coli* ZUC99 was transformed with pATX210 by electroporation. The transformant, designated ZUC99 (pATX210), was isolated using kanamycin as a selective marker.

SDS-PAGE analysis ZUC99(pATX210) was inoculated into LB medium with or without 0.2% (w/v) L-arabinose and incubated at 30 °C for 12 h. Cells were harvested from a 1 ml aliquot of each culture by centrifugation at 16,000 ×*g* for 10 min and then suspended in 100 μl BugBuster protein extraction reagent supplemented with 0.2% (v/v)

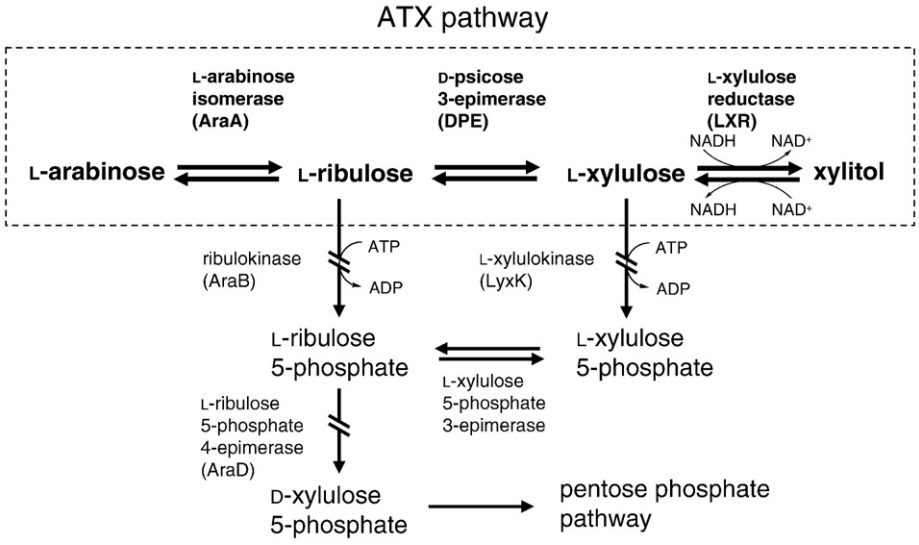

FIG. 1. The ATX pathway for bioconversion of L-arabinose into xylitol. L-Arabinose is converted to L-ribulose by AraA, L-ribulose is converted to L-xylulose by DPE, and L-xylulose is converted to xylitol by LXR. The genes encoding the ATX pathway are carried in plasmid pATX210 as shown in Fig. 2. The *araBAD* operon and the *lyxK* gene are deleted from the chromosome of *E. coli* ZUC99.