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Production of xylitol from D-xylose and glucose with recombinant *Corynebacterium glutamicum*

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ABSTRACT

The product spectrum of a soil bacterium *Corynebacterium glutamicum* was extended to include a functional sugar xylitol. The recombinant *C. glutamicum*, engineered to express the xylose reductase gene *XYL1* of *Pichia stipitis*, produced xylose reductase with a specific activity of ca. 0.6 U/mg protein. Due to the absence of xylose isomerase and xylitol dehydrogenase genes, loose catabolite repression, high NADPH regeneration capacity, and tolerance against sugar-induced osmotic stress, the recombinant biocatalyst was able to efficiently produce xylitol from D-xylose using glucose as source of reducing equivalents. A fed-batch culture-based biotransformation allowed xylitol to accumulate to a concentration of 34.4 g/L (226 mM) in the medium with the specific productivity and product yield of xylose of 0.092 g/g dry cells/h and over 97%, respectively. The molar yield of xylitol to energy source during the biotransformation reached approximately 1.6 mol of xylose/mol of glucose.

1. Introduction

Xylitol is widely used as a functional sweetener in the food and confectionary industry. It is low in calories, has a cooling taste, and high quality sweetness. Moreover, xylitol has been shown to be independent of insulin and anticariogenic [1–3]. In addition, xylitol can serve as a building block for organic synthesis and was listed as one of the top twelve value-added materials produced from biomass [4].

Xylitol is commercially produced by chemical hydrogenation of D-xylose derived from hemicellulose–xylan hydrolysates of biomass materials. Conversion yields of xylitol from xylose remained below 60% [5]. In an attempt to increase product yields, biocatalytic processes based on whole-cell or cell-free enzyme biocatalysts have been investigated [6–11]. Some of the bioprocesses enabled high product yields with high volumetric productivities. For instance, a *Candida tropicalis*-based bioprocess was used to produce xylitol from D-xylose at a 90% yield with volumetric productivities higher than 4.8 g/L/h [9]. The recombinant biocatalysts based on *Lactococcus lactis* [12], *Escherichia coli* [13], and *Saccharomyces cerevisiae* [14,15] produced xylitol from D-xylose with a high yield often over 95%. However, large amounts of organic acids and/or ethanol tended to be formed as a result of fermentative metabolism of the energy sources (e.g., glucose). These metabolites may negatively influence the metabolic activity of microbial biocatalysts and reduce the amount of reduction equivalents produced per glucose molecule. Furthermore, the bacterial biocatalysts could be susceptible to sugar/sugar alcohol-induced osmotic stress during biotransformation.

Corynebacterium glutamicum is an ideal organism for industrial production of fine chemicals due to the ease of gene and pathway manipulations as well as rapid growth and ease of high-cell density cultivation in inexpensive growth media. In addition, the microorganism is GRAS (generally recognized as safe) and structurally stable owing to its lipid-rich outer layer mainly consisting of mycolic acid. The outer layer is supposed to be involved in tolerance to lysis by lytic enzymes (e.g., lysozyme) and to osmotic stress [16,17].

Since *C. glutamicum* is a promising biocatalyst for biotransformations and it lacks the xylose isomerase gene [18,19], we developed the microorganism as a biocatalyst for the production of xylitol from D-xylose. After engineering *C. glutamicum* ATCC13032 to express the xylose reductase gene (*XYL1*) of *Pichia stipitis*, its biocatalytic performance was investigated by conducting biotransformations in fed-batch culture mode.

2. Materials and methods

2.1. Bacterial strains, cloning vector, and culture media

E. coli MC1061 was grown in Luria-Bertani (LB) medium at 37 °C. *C. glutamicum* ATCC13032 was grown in brain–heart infusion (BHI) at 30 °C. When required, kanamycin was added to the medium at a final concentration of 15 μg/ml. The *E. coli*/*C. glutamicum* shuttle vector pEKEx2 [20] was obtained from Forschungszentrum Juelich GmbH (Juelich, Germany).

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2.2. Plasmid construction for expression of XYL1 in C. glutamicum

The *XYL1* gene of *P. stipitis* was ligated into the expression vector pEKEx2. A ribosomal binding site (rbs) was encoded upstream of the *XYL1* gene. The *XYL1* gene was amplified from the *E. coli* template vector pY2-XR [10] with the GeneAmp PCR System 2400 (Applied Biosystems, USA). The PCR forward primer was 5′-TGC*CTGCAG*AAAGGAGATATAGATGTGTCAGATGCCTTCTATTAAGTTGAACTCTGGT-3′ (XR-PstI(*CTGCAG*)-rbs-F) and reverse primer was 5′-ATA*GAGCTC*TTA-GACGAAGATAGGAATCTTGCCC A-3′ (XR-SacI(*GAGCTC*)-R). The oligonucleotides were obtained from Bioneer (Daejeon, Korea). The amplification generated the restriction sites PstI and SacI, and the construct was cloned into the *C. glutamicum* expression vector pEKEx2, resulting in vector pEKEx2-*XYL1*. The restriction enzymes were obtained from Bionics (Korea).

2.3. Enzyme assays and protein electrophoresis

Cells were harvested by centrifugation (17,000 × *g*, 10 min, 4 °C) and washed in 100 mM potassium phosphate buffer, pH 6.5. Cell disruption was carried out by sonication, and crude extracts were centrifuged at 17,000 × *g* for 10 min at 4 °C. The supernatants were used as cell-free extracts. The activities of xylose reductase were determined according to a method described in our previous report [11]. The reaction was initiated by adding xylose to a concentration of 50 mM in the potassium phosphate buffer (pH 6.0) containing NADPH. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the conversion of 1 μmol NADPH per min at 30 °C. Protein concentrations were determined by the method of Bradford [21], using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide) analysis was carried out according to the method described by Sambrook and Russell [22].

2.4. Biotransformation

The recombinant *C. glutamicum* pEKEx2-*XYL1*, which was cultivated in BHI medium containing 15 μg/ml of kanamycin, was used to inoculate the main cultures. The main cultures were grown in BHI medium containing 10 g/L glucose and 15 μg/ml kanamycin at 30 °C. The pH was maintained at 6.0 by feeding the culture broth with a 25% ammonium hydroxide solution. The agitation speed and aeration rate were set to 600 rpm and 2.0 vvm, respectively. The glucose feed was started when the carbon source in the culture medium was completely consumed and its rate was controlled to prevent glucose from accumulating in the culture broth. Biotransformation was initiated by adding xylose and isopropylthiogalactoside (IPTG) at a concentration of 50 g/L and 0.7 mM, respectively, in the medium.

2.5. Determination of metabolite concentration

Concentrations of glucose, xylose, xylitol, and organic acids (i.e., acetic acid, lactic acid) were measured by high performance liquid chromatography (HPLC) (Waters, USA) equipped with an HPX-87H column (Bio-rad Aminex, USA). The column was eluted with 5 mM H_2SO_4 at a constant rate of 0.6 ml/min at 45 °C. The Waters 410 RI refractive index detector (Waters, USA) was used.

3. Results

3.1. Expression of XYL1 in C. glutamicum

With the goal of constructing a recombinant *C. glutamicum* that is able to produce xylitol from D-xylose, the *XYL1* gene of *P. stipitis* was subcloned under the control of the strong inducible promoter P_{tac} which is present in an *E. coli*/*C. glutamicum* shuttle vector pEKEx2 [20]. The PCR product of *XYL1* was cloned into the plasmid pEKEx2, yielding pEKEx2-*XYL1*. To ensure translational initiation, the ribosome-binding site, AAGGAGATATAGAT, was included at the proper distance upstream from the start codon of the *XYL1* gene.

C. glutamicum ATCC13032 was transformed with pEKEx2-*XYL1* by electroporation. Transformants were selected on the BHI agar medium containing kanamycin and subjected to a xylose reductase activity assay. When the recombinant cell cultures were induced with 0.1 mM IPTG in the BHI medium at 30 °C, the xylose reductase activity of cell extracts was below 0.1 U/mg protein. To increase the enzyme activity, induction conditions for the expression of *XYL1* (e.g., inducer concentration, culture temperature) were optimized. When induced with 0.7 mM IPTG at 30 °C, the xylose reductase activity increased up to ca. 0.6 U/mg protein.

The cell extracts from the samples induced with 0.7 mM IPTG and without IPTG were further analyzed by SDS-PAGE. A clearly visible band at the appropriate xylose reductase molecular weight (M.W.: 35.9 kDa) was present in the samples obtained from the induced cultures, but not in the sample obtained from the culture without the inducer added (data not shown).

Table 1
Effect of the source of reducing equivalents on biotransformation of xylose into xylitol.

Energy source[a]	Final cell concentration (g CDW/L)	Final xylitol concentration (g/L)
Glucose	5.8	2.2
Fructose	4.1	2.1
Lactic acid	1.0	1.7
Acetic acid	1.8	<0.1
Citric acid	0.6	<0.1

The errors were less than 10%.

[a] Glucose and fructose were added to 10 g/L but lactic acid, acetic acid, and citric acid were added to 5 g/L at the CGXII medium containing 10 g/L xylose. The biotransformations were conducted in a baffled flask (200 rpm and 30 °C).

3.2. Biotransformation of xylose into xylitol

One of the most critical factors in the biotransformation of xylose into xylitol using whole-cell biocatalysts is the regeneration of nicotinamide cofactors (i.e., NADPH) and the transport of xylose into the cytoplasm, where the catalytic enzymes are present. The first step in the development of the xylitol production process was to choose an energy source, which would serve as a source of reducing equivalents without inhibiting the transport of xylose into the cytoplasmic space. Sugars (i.e., glucose, fructose) and organic acids (i.e., lactic acid, acetic acid, citric acid) were examined as energy sources during biotransformation.

When the biotransformation was carried out in the CGXII medium [23] containing xylose and glucose at a concentration of 10 g/L, the bioconversion of xylose into xylitol was fully activated just after depletion of glucose in the medium. Xylitol accumulated in the medium to a concentration of 2.2 g/L (Fig. 1A). This result indicated that xylose transport into the cells was subject to loose catabolite repression, as shown in an earlier study [19]. A similar biotransformation profile was observed when fructose was used as the energy source (data not shown). However, use of lactic acid as the source of reducing equivalents did not lead to catabolite repression, which allowed the production of xylitol before entering the stationary growth phase. Xylitol concentration reached 1.7 g/L even under poor growth (Fig. 1B). On the other hand, only low levels of xylitol were produced when acetic acid and citric acid were used as the source of reducing equivalents (Table 1).

Biotransformation of xylose into xylitol was carried out during fed-batch cultivation under glucose-limited conditions. One hour after initiation of fed-batch cultivation, the biotransformation was started by adding IPTG and xylose to a concentration of 0.7 mM and ca. 50 g/L, respectively, in the culture medium. The glucose feed rate was controlled so that glucose did not accumulate in the medium (Fig. 2).

The linear cell growth was followed by exponential growth and cell growth ceased at $t = 16$ h. The xylose reductase activity of the cells remained over 0.4 U/mg protein during biotransformation. Xylitol was produced under glucose-limited conditions. Otherwise, the biotransformation was markedly inhibited (see the bioconversion profile at $t = 8$–14 h). Xylitol accumulated in the reaction medium to a concentration of 30.1 g/L ($t = 39$ h). The product yield based on the amount of xylose consumed was over 97%, whereas the molar yield of xylitol to glucose consumed was approximately 1.4 mol/mol. Only low levels of byproduct formation, such as acetic acid and lactic acid, were observed in the culture medium during the biotransformation.