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Construction of an enzymatic route using a food-grade recombinant *Bacillus subtilis* for the production and purification of epilactose from lactose

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ABSTRACT

Lactose is a main by-product in the cheese industry. Many attempts have been made to convert the lactose to high value-added products, including epilactose. Epilactose is a valuable prebiotic and can be epimerized from lactose with cellobiose 2-epimerase (CEase). The objective of the present work was to construct a food-grade recombinant *Bacillus subtilis* that produces CEase from *Thermoanaerobacterium saccharolyticum*. The CEase was expressed in *B. subtilis* without antibiotic resistance genes. After fermentation, the maximum volumetric activity of the fermented broth was more than 7 U/mL. The activity of the recombinant *B. subtilis* was increased by up to 3.7 fold after ethanol permeabilization. Then, 66.9 ± 0.7 g/L of epilactose was produced from 300 g/L of whey powder solution in 1 h with 13.3 U/mL of permeabilized biocatalyst. In addition, an enzymatic route including degradation of the lactose, yeast fermentation, and cation exchange chromatography was described to further purify the produced epilactose from lactose. Finally, epilactose with a purity >98% was produced from 300 g/L of lactose with a yield of 24.0%. In conclusion, neither antibiotics nor pathogenic bacteria were used throughout the epilactose production and purification procedure.
Key words: lactose, epilactose, cellobiose 2-epimerase, food-grade

INTRODUCTION

Cheese whey is usually considered a liquid by-product in the cheese industry (Mu et al., 2013). As the main carbohydrate in cheese whey, lactose is produced at a high and constant rate by the dairy industry every year. According to the data from University of Wisconsin, the average annual production of condensed whey in the United States is over 50 million kg in the last 3 yr, and for dry whey the number is 420 million kg with the price of lower than $0.2/kg (University of Wisconsin, 2017). However, the presence of lactose at a concentration of 4.8 to 5.3% makes whey an environmental problem because of its high biochemical oxygen demand (Procentese et al., 2015). Although lactose removal is accompanied by its production process, the low price of the product reduces the value of this process. This fact would necessitate better uses of lactose or methods to enhance the added value of the product (Jin et al., 2016).

Biotechnologists have found enzymatic modifications to produce many valuable lactose derivatives, such as galactooligosaccharide produced with β-galactosidase (Rodriguez-Colinas et al., 2014), lactosucrose synthetized with levansucrase (Li et al., 2015), and poly (3-hydroxybutyrate) fermented through poly(hydroxyalkanoate) (**PHA**)-producing bacteria (Berwig et al., 2016). Epilactose (4-O-β-D-galactopyranosyl-D-mannose) is an epimer of lactose. This disaccharide is resistant to rat intestinal enzymes and beneficially changes the intestinal flora (Ito et al., 2008), which gives it better prebiotic properties than lactose (Watanabe et al., 2008). In addition, epilactose can considerably promote the calcium absorption in rat small intestines (Nishimukai et al., 2008; Suzuki et al., 2010) and possibly lower the risk of arteriosclerosis (Nishimukai et al., 2008).

Epilactose is difficult to chemically synthesized (Mu et al., 2013), but can be epimerized enzymatically from lactose without a co-substrate by cellobiose 2-epimerase (EC 5.1.3.11; **CEase**), which shows promising potential for industrial application. Cellobiose 2-epimerase is the only enzyme that exhibits epimerization activity

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toward disaccharides (Van Overtveldt et al., 2015). The CEase from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 is relatively thermostable and shows high epimerization activity toward lactose (Chen et al., 2015). This enzyme exhibits little isomerization activity other than CEase from *Caldicellulosiruptor saccharolyticus* (Park et al., 2011) and *Dictyoglomus turgidum* (Kim et al., 2012). All reported CEase were overexpressed in the *Escherichia coli* expression system, which features endotoxins on the cell membrane (Zobel et al., 2015) and often requires antibiotic resistance genes on the plasmid. Unlike *E. coli*, *Bacillus subtilis* is nonpathogenic and is classified as a generally recognized as safe (GRAS) strain by the US Food and Drug Administration. The D-alanine racemase gene on the chromosome of *B. subtilis* can be knocked out as an auxotrophic selective marker to avoid the usage of antibiotic. Moreover, *Bacillus subtilis* has been used in large-scale food fermentation for years and is more prolific than *E. coli*, which makes it a better and safer choice as a host microorganism in the food industry.

In contrast with enzyme immobilization, biocatalysts in the form of whole cells can avoid the loss of repeated uses and time-consuming purification steps. Besides, it is more convenient to concentrate the enzymes in cells than their free forms. After centrifugation, the whole cells can be freeze-dried for long-term storage; however, the reaction rates in whole cells are often limited by the permeability barrier of the cell envelope. Permeabilization was widely used to prepare whole-cell biocatalysts, such as yeast cells (Lee et al., 2004; Panesar et al., 2007) and gram-negative cells (Wang et al., 2015). To reduce the permeability of the recombinant *Bacillus subtilis*, ethanol permeabilization of cells was conducted to increase the use rate of the whole-cell biocatalyst. In the current study, we constructed a food-grade expression system using an antibiotic resistance gene-free plasmid and evaluated the productivity of epilactose from lactose with this recombinant organism.

MATERIALS AND METHODS

Chemicals

Epilactose and gel filtration chromatography standards were purchased from Sigma-Aldrich (St Louis, MO). Cheese whey powder was purchased from Apple Foods Tech (Shanghai, China). Ethanol to permeabilize the cells was obtained from Feizhidao Food Ingredients Factory (Henan, China), and β-galactosidase (EC 3.2.1.23) from *Bifidobacterium bifidum* purchased from Sangon Biotech (Shanghai, China); both were food-grade. The electrophoresis reagents were from Bio-Rad (Hercules, CA). Phusion HF DNA Polymerase was from New England Biolabs (Beijing, China). The primers for PCR amplification and all other chemicals in this study were obtained from Sangon Biotech (Shanghai, China).

Microorganisms and Shake Flask Fermentation

The *B. subtilis* 1A751 host strain, pET-Thsa-CE, p7S6, pTSC, and pUB-P43-DPE-dal plasmid (Chen et al., 2015; He et al., 2016) in this work were obtained from our laboratory. *Escherichia coli* DH5α competent cells were purchased from TaKaRa Biotechnology (Dalian, China). Luria-Bertani (**LB**) medium supplemented with 200 μg/mL of D-alanine was used to culture *B. subtilis* 1A751 without the *dal* (D-alanine racemase) gene.

Strategy for Cre/lox System Knockout of dal Gene

The entire *dal* gene (GenBank no. CAB12271.1), along with expression regulatory signals, were knocked out using the Cre/*lox* system (Yan et al., 2008). A fusion of the linear DNA fragments was assembled using a PCR-based method (Shevchuk et al., 2004) with the following steps. The *lox*71-*spc*-*lox*66 cassette was amplified from the p7S6 vector by PCR using primer I and II. Two flanking homology regions (~900 bp each) of the *dal* gene were separately amplified with 2 pair of primers P3/P4 and P5/P6 (Table 1). Because primers P1 and P2 contain the corresponding reverse complementary sequences of adjacent fragments P4 and P5, respectively, these gel-purified DNA fragments can be simultaneously fused into one. The final product was used to realize the in-frame deletion of the *dal* gene of the *B. subtilis* host 1A751 chromosome. Luria-Bertani agar supplemented with D-alanine and spectinomycin was used to select the transformants, whose *dal* chromosomal genes were replaced by *lox*71-*spc*-*lox*66 cassettes. The plasmid pTSC containing the Cre recombinase expression gene was then introduced into the verified true positive transformants. The *lox*71 and *lox*66 sites can be recombined into a double-mutant *lox*72 site with Cre recombinase. The temperature-sensitive plasmid pTSC was then eliminated by heating up to 51°C

Construction of Antibiotic Resistance Gene-Free Plasmid

An antibiotic resistance gene-free plasmid pUB-P43-DPE-dal to produce D-psicose 3-epimerase (**DPEase**) fused with endogenous promoter P43 was previously constructed in our laboratory (He et al., 2016). The plasmid in our study was recombined based on pUB-P43-DPE-dal. The method developed by You et al. (2012) without restriction enzymes and ligases was