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 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 synthesized 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* DH5 α competent cells were purchased from TaKaRa Biotechnology (Dalian, China). Luria-Bertani (LB) medium supplemented with 200 μ g/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 *lox71-spc-lox66* 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 *lox71-spc-lox66* cassettes. The plasmid pTSC containing the Cre recombinase expression gene was then introduced into the verified true positive transformants. The *lox71* and *lox66* sites can be recombined into a double-mutant *lox72* 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

Table 1. Oligonucleotides used for construction of plasmids and DNA fragments¹

Primer	Sequence (5'–3')
P1	TTACCTTCTCTCTTCTAAGTACCGTTCGTATAGCAT
P2	CAAGCAAAGCTGTTTTATCTACCGTTCGTATAATGT
P3	TACAAAGCAAAGCGGAAAATGACCATC
P4	ATGCTATACGAACGGTACTTAGAAGAGAGAAGGTAA
P5	ACATTATACGAACGGTAGATAAAACAGCTTTGCTTG
P6	CAGCTGATAGGATTCTTGCTCGCTTA
P7	<u>CATTATAGGTAAGAGAGGAATGTACACATGGAGAAAATAGTACATGAAAT</u>
P8	<u>CTGTACGTTTCCTTAAGGAATTCCTTAGTGGTGGTGGTGGTGAACCTCACTGTTTTTCACC</u>
P9	<u>ATTTCATGTACTATTTTCTCCATGTGTACATTCCTCTCTTACCTATAATG</u>
P10	<u>CACCACCACCACCACCCTAAGAATTCCTTAAGGAACGTACAG</u>

¹The bold letters indicate the introduction of histidine tags. The underlined letters indicate the reverse complementary sequences of the corresponding primers.

used for the plasmid construction. The DPEase expression gene region was replaced by the sequence of CEase from *T. saccharolyticum* JW/SL-YS485 (NCBI accession number: AFK87331.1) in 4 steps. (1) The CEase gene was amplified from plasmid pET-Thsa-CE used in our previous study (Chen et al., 2015) by PCR with the primers P7/P8; P7 and P8 contain 23 and 22 bp reverse complementary sequences at the 3'-terminus of primers P9 and P10 (underlined), respectively. A C-terminal 6 × His-tag fused with a stop codon (bold) was added to primer P8 to facilitate the subsequent purification. (2) The primers P9 and P10 were similarly designed to amplify the vector backbone (the linear fragment outside the DPEase expression gene region of pUB-P43-DPE-dal). (3) The large DNA multimers of the PCR products in the previous 2 steps in tandem were formed via prolonged overlap extension PCE without primers (Zhang and Zhang, 2011). (4) The desired plasmid, pUB-P43-TsCE-dal, was obtained by directly transforming the prolonged overlap extension PCR products (DNA multimer) into *B. subtilis* 1A751 (*dal*[−]) competent cells.

Segregational Stability

The segregational stability of antibiotic resistance gene-free plasmid was determined according to the method described by Yu et al. (2012). One milliliter of LB medium in the absence of D-alanine was inoculated with a 1A751/pUB-P43-TsCE-dal colony and incubated for 24 h. This culture was used to inoculate into 1 mL of fresh LB at 1% inoculum, incubated for 24 h, and inoculated into 1 mL of LB with D-alanine at 1% inoculum to begin the segregation stability test. The culture was subcultured in LB supplemented with D-alanine at 1% inoculum every 24 h to reach the identical cell density. The cultures at 72 h were diluted and plated on LB plates (containing 200 µg/mL of D-alanine; plate D), and replica-plated on an LB plate (plate C). The numbers of colonies on each LB plate were counted as

C_D and C_C, respectively. The segregational stability of the recombinant plasmid was expressed as the value of C_C/C_D.

Purification of the Recombinant Protein

The histidine tag was used to facilitate the purification of the target protein. The recombinant cells were cultivated in 2-L flasks, which contained 100 mL of LB medium for 36 h, and harvested by centrifugation at 6,000 × *g* for 20 min. The resuspended cells were disrupted with a Scientz-II D ultrasonic homogenizer (Scientz Biotechnology, Ningbo, China). The crude CEase obtained from the previous step was filtered and purified by Ni²⁺ affinity chromatography from GE Healthcare (Uppsala, Sweden). All purification steps were implemented according to the manufacturer's protocol (Instruction 71–5001–87 AE, http://wolfson.huji.ac.il/purification/PDF/Tag_Protein_Purification/Ni-NTA/AMERSHAM_Chelating_Instruction.pdf; GE Healthcare) at 4°C. The purified protein was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) for 8 h.

Molecular Mass Determination

The subunit molecular masses of the crude and purified enzyme solutions were examined by SDS-PAGE. A 5% (wt/vol) stacking gel and a 12% (wt/vol) separating gel were used to perform SDS-PAGE. The gel was then stained and destained with Coomassie Blue G250 (Sangon Biotech, Shanghai, China) and a mixture of acetic acid, methanol, and water (10:10:80 vol/vol/vol, respectively).

An HPLC system (Agilent Technologies, Santa Clara, CA) was used to determine the native molecular mass of the purified recombinant enzyme. The separation was performed in a TSK gel G3000 SWXL (Tosoh Bioscience LLC, Tokyo, Japan) column with 0.1 mol/L phosphate buffer (pH 6.8), which contained 0.1 mol/L

of Na₂SO₄, and 0.05% NaN₃ at a flow rate of 1 mL/min. The samples and protein standards (669, 443, 200, 150, 66, and 29 kDa) were detected at 280 nm using a UV detector.

Enzyme Assay

The activity of recombinant CEase was determined based on the whole-cell reactions. One milliliter of the aqueous reaction mixture contained 200 mM lactose, 50 mM sodium phosphate buffer (pH 7.0), and 200 μ L of resuspended recombinant cell solution. The reaction mixture was incubated at 60°C for 15 min, and HCl was subsequently added with a final concentration of 250 mM. The activities of the cells permeabilized by 20 to 70% (vol/vol) ethanol were measured based on 15 min of reactions using lactose as substrate. One unit of CEase activity was defined as the amount of enzyme that produced 1 μ mol of epilactose per minute at pH 7.0 and 60°C. Epilactose produced during the enzymatic reactions was detected using an HPLC system (Waters 2695, Milford, MA) equipped with a Shodex VG-50 4E (4.6 mm id $\times$ 250 mm, 5 μ m; particle size, Shodex, Tokyo, Japan). The mobile phase was a mixture of methanol, acetonitrile, and water (75: 20: 5, vol/vol/vol) with a flow rate of 1.0 mL/min at 40°C. The compounds with approximately equal or longer retention time than that of the trisaccharide standard were added together as galactooligosaccharides (GOS), including trisaccharides, tetrasaccharides, and pentasaccharides. The amount (<1%) of disaccharides generated by the transgalactosylation reaction was ignored.

Bioconversion of Epilactose from Lactose

After 36 h of incubation, the recombinant *B. subtilis* was harvested by centrifugation at 6,000 $\times$ g, 4°C, 20 min. It was then resuspended to a final concentration of 150 g of wet cells/L by 50% (vol/vol) ethanol solution with agitation for 20 min at 25°C, and washed twice with sodium phosphate buffer (pH 7.0) to remove the residues of raw materials in the fermentation media. The recombinant cells suspension was adjusted to 18 U/mL of the original volumetric activity. Then, 200 mL of biocatalyst was permeabilized and added to 800 mL of 375 g/L of lactose in a sodium phosphate buffer (pH 7.0) for a final lactose concentration of 300 g/L. The yields of the epilactose in this section were detected by sampling following the method of enzyme assay. When equilibrium was reached, the enzymatic reaction was stopped by immersing the vessels in a boiling water bath for 10 min. After centrifugation (8,000 $\times$ g, 4°C, 20 min), the supernatant of 375 g/L of cheese whey

powder solution (pH 7.0) was filtered and used for bioconversion in the described manner.

Purification of Epilactose

The total sugar concentration of the reaction mixture was adjusted to 100 g/L with sodium phosphate buffer (pH 7.0). Then, β -galactosidase was added to a final concentration of 25 U/mL (250 U/g of initial lactose). One unit of β -galactosidase can hydrolyze 1 μ mol o-nitrophenyl- β -D-galactopyranoside per minute at 25°C and pH 7.5. The mixture was incubated with shaking at 25°C for 12 h. After the hydrolysis reaction, fresh baker's yeast *Saccharomyces cerevisiae* (Angel Yeast Co. Ltd., Yichang, China) was used with the addition of 15 g (wet weight) of yeast per 100 g of sugar to remove the monosaccharides. The fermentation mixture was stirred at 28°C for 12 h in an aerobic flask. Finally, the mixture was centrifuged (6,000 $\times$ g, 4°C, 20 min) and the GOS and remaining monosaccharides in the supernatant were removed with a preparative cation-exchange chromatograph equipped with a preparative column (1.6 cm $\times$ 1 m) packed with Na-type cation-exchange resin DTF-01 (Jiangsu Suqing Water Treatment Engineering Group Co. Ltd., Wuxi, China). The mobile phase was deionized water with a flow rate of 1 mL/min at 60°C. The injection volume was 5 mL.

RESULTS

Selection of the *dal* Gene Deficiency Strains

The scheme to knockout the structural *dal* gene from the *B. subtilis* 1A751 chromosome was presented in detail in a previous report from our laboratory (He et al., 2016). The *dal* gene deficiency variant *B. subtilis* 1A751 (*dal*⁻) was obtained on the LB agar with D-alanine and confirmed by PCR. This deficiency variant strains cannot grow on the general culture medium without the addition of D-alanine.

Construction of Food-Grade Recombinant Strains

Plasmid pUB-P43-TsCE-dal, which derives from pUB110, can be used as an expression vector to produce recombinant proteins in *B. subtilis*. This plasmid lacks an antibiotic resistance gene (kanamycin and bleomycin) unlike pUB110. A CEase expression gene with 2 tandem promoters (*Hpa*II and P43) and an in-frame 6 $\times$ His-tag sequence was substituted for the Mob gene in pUB-P43-TsCE-dal. The flow diagram to construct the food-grade plasmid pUB-P43-TsCE-dal is illustrated in Figure 1. The PCR multimer product

Figure 1. Flow diagram for constructing the food-grade plasmid pUB-P43-TsCE-dal. The plasmid pUB-P43-DPE-dal, featuring 2 tandem promoters *HpaII* and P43, was constructed from the original vector pUB110. The D-psicose 3-epimerase expression gene was inserted into the Mob gene region and 2 antibiotic resistance genes (kanamycin and bleomycin) of pUB110 were replaced by the D-alanine racemase gene. Plasmid pUB-P43-TsCE-dal derived from pUB-P43-DPE-dal was constructed by a general, restriction enzyme-free and ligase-free method, as described in Materials and Methods. POE = prolonged overlap extension. Color version available online.

was directly transformed into *B. subtilis* 1A751 (*dal*⁻) competent cells, yielding plasmid pUB-P43-TsCE-dal. The recombinant *B. subtilis* 1A751/pUB-P43-TsCE-dal screened by LB agar without D-alanine was simultaneously obtained. The C_C/C_D ratio of plasmid pUB-P43-TsCE-dal was higher than $99.5 \pm 0.2\%$ after the culture in the presence of D-alanine for 72 h.

Fermentation of the Recombinant Strain

A single colony of 1A751/ pUB-P43-TsCE-dal was picked for the LB medium. After 12 h of cultivation at 37°C, the precultures were inoculated at a 1:50 dilution into 300 mL of LB medium in a 3-L flask, and incubated at 37°C on a rotary shaker at 200 rpm. Samples (1 mL) were taken regularly to monitor cell growth at 600 nm. It took approximately 30 h for the culture to grow to the highest optical density at 600 nm. The

maximum concentration of the wet cells reached 16.5 ± 1.8 g (3.3 ± 0.3 g for dry cells) per liter of culture medium. The enzymatic activity curve of the whole cell of 1A751/ pUB-P43-TsCE-dal, which lagged behind the cell growth curve, reached a plateau after 48 h of incubation (Figure 2). The maximum volumetric activity of the fermentation broth measured by HPLC was more than 7 U/mL. The crude enzyme was obtained by sonication and, thus, the target proteins' expression percentage after 48 h was then determined to be $37.1 \pm 4.7\%$ based on the activity of the permeabilized medium divided by specific activity.

Purification of Recombinant Protein

Purified enzyme was produced by 1-step nickel-affinity chromatography to electrophoretic homogeneity with a single band at approximately 47 kDa using

Figure 2. Enzyme activity curve and growth curve of the recombinant strain. The enzyme units per milliliter fermentation broth (○) and optical density at 600 nm (OD₆₀₀; ■) were used to describe the enzyme activity curve and growth curve. The values in the figure are the average of 3 independent measurements and are given as the mean $\pm$ SD.

SDS-PAGE (Figure 3A), which was consistent with the molecular mass obtained using a gel filtration analysis (Figure 3B). These analyses indicate that the native CEase overexpressed by 1A751/pUB-P43-TsCE-dal existed as a 47-kDa monomer and the recombinant CEase was identical in size to CEase from the *E. coli* expression system (Chen et al., 2015).

Production of Epilactose by Whole-Cell Recombinant *B. subtilis* and Ethanol Treatment

The cell sediment that harbors CEase can be vacuum freeze-dried; thus, 1 L of 48-h fermentation broth ($\sim 7,200$ U of total activity) was dried to 2.81 g of dry cells. The activity of dry cells was determined to be 3,250 U/g, which was an increase of 27% compared with the activity of wet cells. The freezing and thawing, which contribute to the permeability of cells for the substrates, may cause this improvement (Chen, 2007). To investigate the effect of permeabilization, the activity of the cells preprocessed by ethanol permeabilization was measured based on the 15 min of reaction (Figure 4). The enzyme activity steadily increased when the ethanol concentration increased from 20 (vol/vol) to 50% (vol/vol), and a decrease in activity was observed with further increase in concentration. All the reaction mixtures had nearly identical final epilactose yields ($\sim 29\%$).

The laboratory-scale production reaction by the whole-cell biocatalyst of permeabilized recombinant *B. subtilis* was conducted in a shake flask. First, 300 g/L of lactose was converted into 88.5 ± 0.6 g/L of epilactose ($29.5 \pm 0.2\%$ in yield) with an original activity of 3.6 U/mL of the whole cells (raised to 13.3 U/mL by permeabilization) at pH 7.0 and 60°C for 1.5 h (Figure 5). The in situ production of epilactose from lactose was performed with supernatant of cheese whey powder solution. The reaction mixture containing 228.2 ± 1.2 g/L of lactose (76% of total weight of cheese whey

Figure 3. Estimation of the molecular mass of cellobiose 2-epimerase from *Thermoanaerobacterium saccharolyticum* by SDS-PAGE and gel filtration. (A) SDS-PAGE analysis of Thsa-CE. Lanes: 1, molecular mass marker proteins. 2, purified enzyme. 3, crude enzyme from *Bacillus subtilis* pUB-P43-TsCE-dal. 4, *B. subtilis* 1A751 (negative control). (B) Gel filtration chromatography of Thsa-CE.

powder) was incubated in condition identical to those mentioned previously. The existence of the other components in whey did not strongly influence the activity of the whole-cell biocatalyst. As the concentration of the lactose in cheese whey powder solution was less than the previous reaction, 66.9 ± 0.7 g/L of epilactose was produced in 1 h with 13.3 U/mL of permeabilized biocatalyst (Figure 5). Both the sources of the lactose and the permeabilization process did not change the reaction equilibrium.

Purification of Epilactose from the Reaction Mixture

A food-grade strategy to remove of the residual lactose in the reaction mixtures is described in this work. Figure 6 demonstrates the flow diagram to purify epilactose. First, lactose was selectively degraded with β -galactosidase that hydrolyzed lactose into galactose and glucose. The second step is optional, where yeast fermentation was conducted to remove the generated monosaccharides. The last step is preparative cation-exchange chromatography. Most monosaccharides and GOS were successfully separated from lactose and epilactose (Table 2); 87% of the epilactose applied to the column was finally collected with purity >98%, which indicates that 24.0 g of epilactose can be obtained from 100 g of lactose.

Figure 4. Effect of sonication or permeabilization on the activity of the recombinant cells. The initial activity without treatment was taken as 100%. A represents activity of the cells disrupted by sonication; B, C, D, E, F, and G represent activities of the permeabilized cells using 20, 30, 40, 50, 60, and 70% (vol/vol) ethanol, respectively. The values are the means of 3 replications $\pm$ SD.

Figure 5. Time course of the enzymatic lactose conversion. The enzymatic reaction was performed from 300 g/L of lactose (■) and 300 g/L of cheese-whey powder solution (▲) at pH 7.0 and 60°C. The values are the means of 3 replications $\pm$ SD.

DISCUSSION

D-Alanine is involved in the growth of the cell wall, which makes it an essential component that is not found in common media. The deficiency of the *dal* gene provides a strong selectable marker, without which D-alanine cannot be converted from L-alanine. As a result, *B. subtilis* 1A751 (*dal*⁻) is a good host strain for a food-grade expression system. Antibiotic resistance genes and antibiotics were not required for this recombinant cell to maintain replicative plasmids. Therefore, the fermentation product of recombinant cells can be directly used in the bioconversion of epilactose from lactose, which eliminates the removal procedure of antibiotics and hidden dangers for food security.

The plasmid segregational stability is expressed as the percentage or probability of the host retaining the plasmid per generation, which is an essential issue in industrial application. The segregational stability of the recombinant plasmid pUB-P43-TsCE-dal is as stable as pUB-P43-DPE-dal (He et al., 2016). The plasmid pUB-P43-DPE-dal can be used as a backbone to construct other expression vectors with only 4 steps and 4 primers. The PCR amplifications were conducted using Phusion polymerase because of its high fidelity and high speed (You et al., 2012). This flexible, low-cost, and time-saving method requires no consideration of restriction sites and is independent of restriction enzymes and ligases, which can be used as a tool in other food-grade expressions for different heterologous genes.

Figure 6. Strategy to purify epilactose from the reaction mixture. A, B, C, and D show the HPLC profiles of the products of steps 1, 2, 3, and 4, respectively. Color version available online.

Table 2. Purity and yield of epilactose after each purification step¹

Step	Concentration of epilactose (g/L)	Purity (%)	Yield (%)
Epimerization	88.5 $\pm$ 0.6	29.5	29.5
Dilution	29.5 $\pm$ 0.2	29.5	29.5
Degradation of the lactose	27.6 $\pm$ 0.2	27.6	27.6
Yeast fermentation	27.5 $\pm$ 0.2	45.4	27.5
Cation exchange chromatography	24.0 $\pm$ 0.1	98.5	24.0

¹The concentration values are the means of 3 replications $\pm$ SD.

This new pUB110-based expression vector has 2 advantages. (1) The plasmid pUB110 has a high copy number, greater than 40 U/chromosome, replicating by a rolling-circle mechanism, and the plasmid pUB-P43-TsCE-dal with the rep proteins gene of pUB110 retains the expression level of the target protein. (2) A strong promoter P43 was added immediately behind the original promoter *HpaII*, which constructs a double promoter plasmid. The dual-promoter system is often used for better protein expression. For instance, the *T7-tac* promoter was constructed using different regulation mechanisms for different expression levels at different conditions (Huang et al., 2016). In this case, the dual promoter *HpaII*-P43 using the same regulation mechanism was built to further improve the expression level. Therefore, the recombinant strains 1A751/pUB-P43-TsCE-dal accumulate heterologous proteins with a distinct band on SDS-PAGE.

This laboratory-scale fermentation was conducted in the widely used LB medium. In our previous work, the recombinant strains containing the DPEase gene were cultivated using a super-rich medium (tryptone 25 g/L, yeast extract 20 g/L, K_2HPO_4 3 g/L, glucose 30 g/L) as the fermentation medium. The maximum enzyme activity appeared earlier (13 h of fermentation time) and the maximum optical density value was slightly higher than that of 1A751/pUB-P43-TsCE-dal in the current work (He et al., 2016). Thus, a super-rich medium is recommended to satisfy the nutritional requirements of the recombinant strains and significantly shorten the fermentation time. However, super-rich media might be too costly to produce epilactose for industrial production. Nevertheless, the fermentation product of 1A751/pUB-P43-TsCE-dal with the LB medium exhibited excellent catalytic ability compared with the *E. coli* expression system (Chen et al., 2015).

Further studies were performed to increase the utilization rate of the biocatalyst. The recombinant cells were permeabilized to help substrates and products hurdle the barrier that is the cell envelope. Compared with untreated cells, up to a 3.7-fold increase in epilactose production activity was obtained for treated cells. An identical concentration of epilactose can be obtained with a smaller volume of permeabilized *B. subtilis* than with the untreated biocatalyst. Surprisingly, the permeabilized cells performed better (1.68 fold) than the cells that were disrupted by sonication. This difference may be because of the protective effects of permeabilized cells or presence of other proteins. It was suggested that a better stabilization of enzyme will be performed in the presence of other cellular proteins due to in vitro macromolecular crowding effect (Myung and Zhang, 2013). By comparison, the synthesis activ-

ity of lactulose from lactose catalyzed with yeast cells containing β -D-galactosidase were increased by up to 1.5 fold after ethanol permeabilization (Panesar et al., 2007). In another study, the use of 40% (vol/vol) ethanol to permeabilize *E. coli* cells that harbored CEase from *Caldicellulosiruptor saccharolyticus* increased the production of lactulose by just 1.1 fold (Wang et al., 2015), indicating that the treatment with ethanol to enhance the permeability of the gram-negative cell envelope for lactose and its derivatives was not efficient compared with that of gram-positive bacterium *Bacillus subtilis*. This interesting phenomenon is seemingly quite different from the case of the Gram staining reaction, where the gram-positive microorganisms with a relatively thick cell wall are impermeable and resist the decolorization of 96% (vol/vol) ethanol (Popescu and Doyle, 1996). Because other processes, such as toluene permeabilization, freezing and thawing, and electroporation, are either unsuitable for the food industry or time-consuming, treatment with ethanol may be the best method for this gram-positive whole-cell biocatalyst.

The activity of the recombinant enzyme and productivity of epilactose were nearly identical to the lactose in the buffer matrix, which indicates that the whole-cell biocatalyst worked well in the presence of various components of whey powders. Such enzymes can reduce the lactose content of milk or whey and provide prebiotics, which provide benefits to the well-being and health of humans. For example, CEase from *Caldicellulosiruptor saccharolyticus* was directly used in milk to produce epilactose (Krewinkel et al., 2014, 2015) or lactulose (Rentschler et al., 2015) in previous studies.

For purification of epilactose, lactose was selectively degraded with β -galactosidase. Apart from the lactose hydrolysis, β -galactosidase also catalyzed a transgalactosylation reaction, yielding a mixture of GOS (5.3%, wt/wt), disaccharides (28.2%, wt/wt), and monosaccharides (66.5%, wt/wt). The transgalactosylation reaction was weakened by diluting the total concentration of sugar in the mixtures. More importantly, β -galactosidase preferentially hydrolyzes lactose other than epilactose, resulting in epilactose accounting for 98.2% of the disaccharides. This supplementary evidence shows that epilactose is a nondigestible food ingredient.

Although the yeast treatment has been widely used to purify GOS (Goulas et al., 2007), difructose dianhydride III, and kojibiose, the result that 41.3% of monosaccharides remained after purification was not ideal. The reason may be that the galactose and mannose in the mixture cannot be completely metabolized (Goulas et al., 2007). Nevertheless, removal of the monosaccha-

rides before the implementation of liquid chromatography is still recommended for better performance.

A similar method with 4 steps was used to purify epilactose by Saburi et al. (2010), where monosaccharides were successfully fermented into glycerol after yeast treatment. However, the selectivity of hydrolysis by *Bacillus circulans* β -galactosidase and purity of product (91.1%) were relatively low (Saburi et al., 2010). Kuschel et al. (2016) developed a non-food-grade method to produce epilactose; under the best conditions, the final epilactose purity was 99% with a total yield of 51%.

CONCLUSIONS

An efficient epilactose-producing *B. subtilis* strain was developed based on an antibiotic resistance gene-free plasmid. These recombinant cells can be directly used as a whole-cell biocatalyst in industrial production without unsafe factors and complex purification steps. In our results, 66.9 ± 0.7 g/L of epilactose was produced from 300 g/L of whey powder solution for 1 h with 13.3 U/mL of permeabilized biocatalyst. Further purification of epilactose from lactose was described in this work. We found that epilactose with purity >98% was produced from 300 g/L of lactose solution with a yield of 24.0%, which may direct a new enzymatic route for the production of epilactose.

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