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Improvement of lactulose synthesis through optimization of reaction conditions with immobilized β-galactosidase

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Abstract–*Kluyveromyces lactis* β-galactosidase was immobilized on silica gels using a covalent bonding method. To improve lactulose synthesis using immobilized β-galactosidase, the optimal reaction conditions, such as lactose and fructose concentrations, pH and ionic strength of the buffer, loading amount of the enzyme and temperature, were determined. Lactulose synthesis using the immobilized β-galactosidase was markedly improved after optimization of the reaction conditions. When the lactulose synthesis was carried out at 47 °C using 40% (w/v) lactose, 20% (w/v) fructose and immobilized β-galactosidase of 12 U/ml in 50 mM sodium phosphate buffer at pH 7.5, the lactulose concentration and specific productivity were 15.80 g/*l* and 1.32 mg/U·h, respectively. In addition, when the immobilized β-galactosidase was reused for lactulose synthesis, its catalytic activity retained 60.5% after 10 reuses.

Key words: β-Galactosidase, Lactulose, Optimization, Transgalactosylation, Immobilization

INTRODUCTION

Galactooligosaccharides (GOSs) have recently been shown to be functional components in the food and pharmaceutical fields. One compound of industrial interest among galactooligosaccharides is lactulose (non-digestible GOS, 4-*O*-β-D-galactopyranosyl-D-fructose), due to its many uses [1]. Lactulose has been used in a wide variety of foods as a bifidus factor or as a functional ingredient for intestinal regulation [2]. In the pharmaceutical field, lactulose is used mainly for the treatment of constipation, hepatic encephalopathy, complication of liver disease and maintenance of blood glucose and insulin level [3].

Methods for lactulose synthesis can be divided into chemical and biological. In the chemical methods, lactulose is synthesized by alkaline isomerization of lactose [4]. However, lactulose synthesis by chemical methods has many problems, including product purification, lactulose degradation, side reactions and waste management [1]. To solve these, enzymatic synthesis of lactulose was recently studied. β-Galactosidase is a very important enzyme in the dairy industry and catalyzes the conversion of lactose into glucose and galactose [5,6]. It has been broadly used for the preparation of lactose-hydrolyzed products for lactose-intolerant or lactase-deficient people [7,8]. β-Galactosidases can be used to synthesize lactulose from lactose by a transgalactosylation reaction, using fructose as a galactosyl acceptor [1,4,9-12].

The major industrial β-galactosidases are derived from *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus niger* and *Aspergillus oryzae*. Of these, *K. lactis* β-galactosidase is one of the most important and widely used enzymes for the food and pharmaceutical industries [13]. The lactulose synthesis by *K. lactis* β-galactosidase was first reported by using whole cells of *K. lactis* [10]. Subsequently, Hua et al. [9] reported the lactulose synthesis in organic-aqueous media using β-galactosidase immobilized into magnetic chitosan microspheres. However, magnetic chitosan microspheres are an expensive material for commercial application. By contrast, using silica gels as the solid support for enzyme immobilization would be more cost-effective.

For these reasons, we applied *K. lactis* β-galactosidase as the biocatalyst and silica gels as the solid support for enzyme immobilization. Also, we have recently developed a pretreatment method that prevents the loss of β-galactosidase activity during enzyme immobilization through covalent bonds [14]. Therefore, in this study, β-galactosidase was immobilized on silica gels using the previously reported immobilization method. The transgalactosylation activity of β-galactosidase is highly dependent on the reaction conditions [4]. Accordingly, we focused on the optimization of the reaction conditions for lactulose synthesis, such as concentrations of lactose and fructose, pH and ionic strength of buffer, loading amount of enzyme and temperature. In addition, we examined the reusability of the immobilized β-galactosidase through repeated batch reactions.

MATERIALS AND METHODS

1. Materials

β-Galactosidase (EC 3.2.1.23) from *Kluyveromyces lactis*, lactulose, fructose, (3-aminopropyl)triethoxysilane (3-APTES), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 3-(*N*-morpholino)propanesulfonic acid (MOPS) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) were purchased from Sigma (St. Louis, MO, USA). Lactose monohydrate was purchased from Junsei Chemical Co. (Tokyo, Japan). Glutaraldehyde solution was purchased from the Fluka Co. (Switzerland). All other chemicals used were of reagent grade.

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2. Preparation of Activated Silica Gel

One gram of silica gels was silanized with 20 ml of an acetone solution containing 0.75 M 3-APTES at 50 °C for 2 h, washed with distilled water, and dried at 60 °C for 2 h. The dried silica gels were added to 20 ml of a 0.21 M glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 8.0), after it was incubated at 20 °C for 2 h [15]. The activated silica gels were washed with distilled water and dried at 60 °C for 2 h.

3. β-Galactosidase Pretreatment before Immobilization

Lactose (30 mM) was added to 20 ml of 10% (v/v) β-galactosidase solution in 0.1 M sodium phosphate buffer (pH 7.2) [14]. The mixture was then incubated at 37 °C and 150 rpm for 1 h. This β-galactosidase solution was then used for immobilization as described below.

4. β-Galactosidase Immobilization

The activated silica gels were added to the pretreated β-galactosidase solution and reacted at 20 °C for 12 h with constant stirring [14]. The immobilized β-galactosidase was washed with 0.1 M sodium phosphate buffer and then recovered by filtration.

5. Determination of β-Galactosidase Activity

The activity of immobilized β-galactosidase was assayed using ONPG as the substrate [16]. The reaction mixture contained 10 ml of 20 mM potassium phosphate buffer at pH 7.0, 5 mM ONPG and immobilized β-galactosidase, and the enzyme reaction was conducted at 37 °C and 150 rpm for 10 min. Two milliliters of 1 M Na_2CO_3 was then added to the reaction mixture to stop the reaction. The liberated 2-nitrophenol was measured at 410 nm and its concentration was calculated using standard 2-nitrophenol solutions. One unit of enzyme activity (EU) was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of ONPG per minute under the reaction conditions. The activity recovery was calculated as the ratio of the activity of immobilized β-galactosidase to the activity of free β-galactosidase and expressed as a percentage.

6. Lactulose Synthesis

For lactulose synthesis by immobilized β-galactosidase, the following reaction conditions were used: lactose (20-60%, w/v), fructose (5-40%, w/v), immobilized β-galactosidase (3-15 U/ml), various buffers, pH (6.0-8.0), ionic strength (20-200 mM) and temperature (32-57 °C). To reuse immobilized β-galactosidase, after a single batch reaction, immobilized β-galactosidase was filtered and washed with sodium phosphate buffer, and then reused for the next batch reaction. Reactions were carried out in a shaking water bath at 150 rpm, and experiments were conducted in duplicate.

7. Determination of Immobilized Protein

The amount of immobilized protein was determined from the difference between total protein and the amount remaining in the solution after immobilization, and the immobilization yield was expressed as a percentage. The protein concentration was determined using a Bio-Rad protein assay reagent according to the manufacturer's protocols (Bio-Rad Laboratories, Hercules, USA), and the absorbance was measured at 750 nm [17].

8. Determination of Sugars

Lactose, fructose, glucose, galactose and lactulose were analyzed by high performance liquid chromatography (HPLC) with an RI detector using a high performance carbohydrate column (4.6 mm× 250 mm, Waters Co., Milford, MA, USA) at a flow rate of 1.4 ml/min at 35 °C [10,18]. The mobile phase consisted of a mixture of acetonitrile : deionized water (8 :2 , v/v).

9. Estimation of Immobilized β-Galactosidase Performance

To estimate the performance of immobilized β-galactosidase under each reaction condition, conversion rate of lactose (defined as $([S_s]_0-[S_s])/[S_s]_0\times100$, %), lactulose yield (defined as $[P]/[S_s]_0\times100$, %) and specific productivity (defined as $[P]/([E]\times t)$, mg/U·h) were calculated. Notations, $[S_s]_0$, $[S_s]$, [P], [E] and t represent the initial concentration of lactose and fructose, final concentration of lactose and fructose, lactulose concentration, enzyme concentration and reaction time, respectively.

RESULTS AND DISCUSSION

1. Immobilization of β-Galactosidase

In our previous study, a pretreatment method was developed to prevent the activity loss during β-galactosidase immobilization by covalent bonding [14]. The results suggested that the effect of enzyme pretreatment prior to immobilization was due to a steric effect at the enzyme active site by the substrate and that enzymatic regions far from the active site would react with the solid surface. Accordingly, in this study, β-galactosidase pretreated with lactose was immobilized covalently on activated silica gels, and the immobilized β-galactosidase was used for lactulose synthesis. The immobilization yield and activity recovery were 31.4% and 47.8%, respectively, and the activity of immobilized β-galactosidase was 754 U/g matrix.

2. Effect of Lactose and Fructose Concentration

Lactose is a substrate that acts as a galactose donor in the produc-

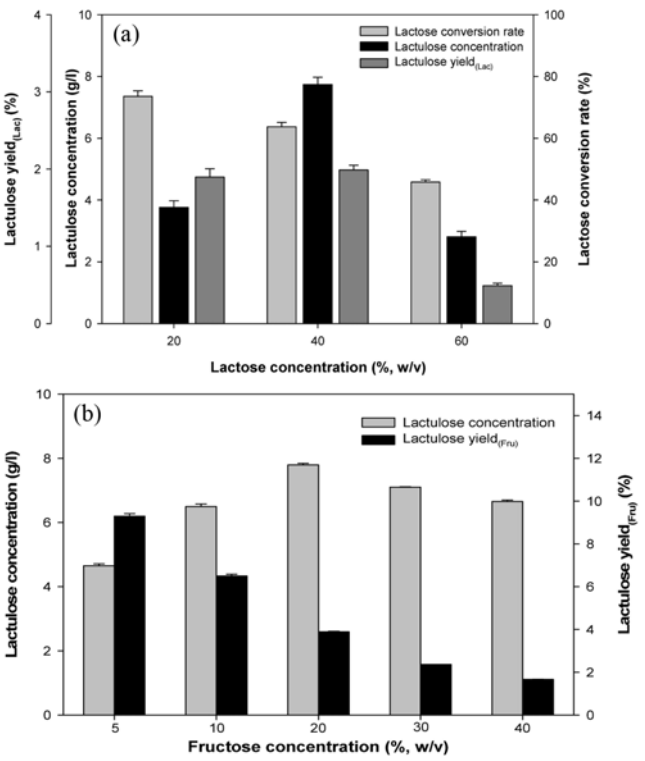

Fig. 1. Effect of lactose (a) and fructose concentration (b) on lactulose synthesis. (a); 20% (w/v) fructose, 10 U/ml of immobilized β-galactosidase, 20 mM potassium phosphate buffer, pH 7.0, 37 °C. (b); 40% (w/v) lactose, 10 U/ml of immobilized β-galactosidase, 20 mM potassium phosphate buffer, pH 7.0, 37 °C.

tion of lactulose. To determine the optimum concentration of lactose for maximum lactulose synthesis and yield, the effect of lactose concentration on lactulose synthesis was evaluated using lactose concentrations of 20, 40 and 60% (w/v) and a fixed fructose concentration of 20% (w/v). Fig. 1(a) shows the lactulose concentration, $yield_{(Lac)}$ and lactose conversion rate at different lactose concentrations. A higher conversion rate of lactose was observed at lower substrate concentrations. Lactulose synthesis by *K. lactis* β-galactosidase was strongly dependent on the lactose concentration. At lactose concentrations of 20 and 40%, the lactulose concentration and $yield_{(Lac)}$ increased at the higher lactose concentration, but at higher concentration (60%), those significantly decreased. Compared to lactose concentration of 20% (1.9%), the lactulose $yield_{(Lac)}$ was increased by 5.3% at 40% (2.0%), indicating that the lactose concentration of 40% was more effective in improving lactulose synthesis. Therefore, the highest lactulose concentration (7.74 g/*l*) and $yield_{(Lac)}$ were found at a lactose concentration of 40%.

On the other hand, fructose is the substrate that accepts galactosyl moiety through the transgalactosylation reaction of β-galactosidase from lactose hydrolysate. Thus, the effect of fructose concentration on lactulose synthesis was also investigated using different fructose concentrations (5-40%, w/v) and a fixed lactose concentration of 40% (w/v). As shown in Fig. 1(b), the highest lactulose $yield_{(Fru)}$ was at 5% fructose (9.3%) and it progressively decreased with an increase in fructose concentration. In contrast, the lactulose concentration increased to a fructose concentration of 20%, above which it started to decrease. Several researchers have reported a decrease in the lactulose synthesis when the total initial concentration of lactose and fructose was increased [4,9,11]. In this study, total sugar concentrations above 60% (w/v) were found to decrease the lactulose synthesis. This may have been due to inhibition of β-galactosidase activity, as well as mass transfer limitations caused by an increase in the viscosity of the reaction mixture [4,9]. Maximum lactulose concentration (7.79 g/*l*) was obtained at 20% fructose. The optimal concentrations of lactose and fructose on lactulose synthesis were determined to be 40% lactose and 20% fructose, respectively, indicating that the optimum ratio of lactose to fructose was 2 : 1 on a mass basis and 1 : 1 on a molar basis. Therefore, the ratio of galactose donor to acceptor influences the transgalactosylation reaction during lactulose synthesis.

3. Effect of the Loading Amount of Immobilized β-Galactosidase

To improve lactulose synthesis at the optimum lactose and fructose concentrations, the effect of loading amount of immobilized β-galactosidase was evaluated. Fig. 2 shows the lactulose concentration and specific productivity at various loading amounts of immobilized β-galactosidase (3-15 U/ml). Overall, the lactulose concentration and specific productivity increased as the amount of immobilized β-galactosidase increased, but the specific productivity did not increase any further when the loading amount was greater than 12 U/ml. An increase in the loading amount of immobilized β-galactosidase resulted in maximum lactulose synthesis in a shorter reaction time. At loading amounts below 9 U/ml, the maximum lactulose synthesis was achieved after 3 h, whereas maximum lactulose synthesis at a loading amount of 12 and 15 U/ml was reached at 2 h. Therefore, higher specific productivity was obtained when 12 and 15 U/ml of immobilized β-galactosidase were used. These

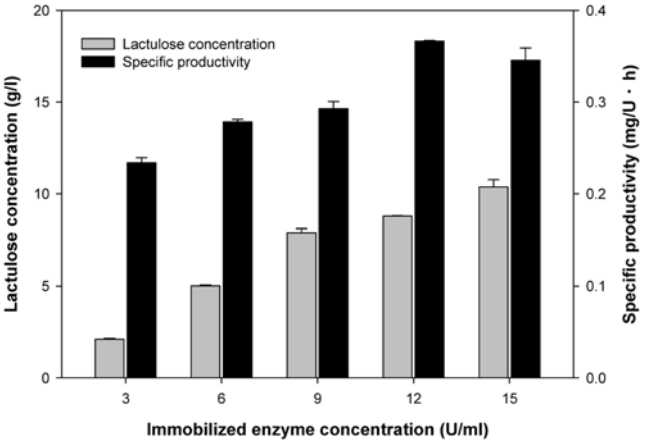

Fig. 2. Effect of the loading amount of immobilized β-galactosidase on lactulose synthesis. Reactions were conducted at 37 °C using 40% (w/v) lactose and 20% (w/v) fructose in 20 mM potassium phosphate buffer at pH 7.0.

results indicated that the high reaction rate caused by the increased loading of immobilized β-galactosidase was closely related to improved lactulose synthesis. Although the maximum lactulose concentration (10.37 g/*l*) was obtained with 15 U/ml of immobilized β-galactosidase, the specific productivity was not higher when compared to 12 U/ml (0.37 mg/U·h) of immobilized β-galactosidase. Thus, 12 U/ml of immobilized β-galactosidase was determined to be the most effective loading amount for lactulose synthesis.

4. Effect of the Various Buffers, pH and Ionic Strength

Enzymatic reactions are usually carried out in aqueous buffer systems. However, the effect of buffers on lactulose synthesis by β-galactosidase has not yet been examined. Accordingly, the effect of various buffers on lactulose synthesis by immobilized β-galactosidase was evaluated. Each buffer had an ionic strength of 20 mM at pH 7.0. Lactose and fructose were dissolved in the various buffers and then reacted with immobilized β-galactosidase. Fig. 3(a) shows the effect of buffers on lactulose synthesis. Among the various buffers, the sodium phosphate buffer was determined to be the most effective buffer for lactulose synthesis, followed by potassium phosphate buffer. The lactulose concentration and specific productivity in sodium phosphate buffer were 10.43 g/*l* and 0.87 mg/U·h, respectively. The specific productivity was significantly higher because of the shorter reaction time. Lactose was hydrolyzed more rapidly in potassium phosphate buffer (lactose conversion rate of 72.5%) than sodium phosphate buffer (lactose conversion rate of 59.8%) (data not shown), but the concentration of synthesized lactulose and specific productivity in potassium phosphate buffer were significantly lower than those in sodium phosphate buffer. Buffers can cause subtle changes in protein domains and the shift of enzyme catalytic activities. The above result may have occurred because the catalytic activity of immobilized β-galactosidase may have shifted to hydrolysis over transgalactosylation in the potassium phosphate buffer [9].

The pH range for catalytic activity of β-galactosidase from *K. lactis* is between pH 5.5 and 8.5. Accordingly, the effect of pH on lactulose synthesis was examined at pH values ranging from 6.0 to 8.0, and the results are presented in Fig. 3(b). The lactulose concentration and specific productivity increased as the pH increased

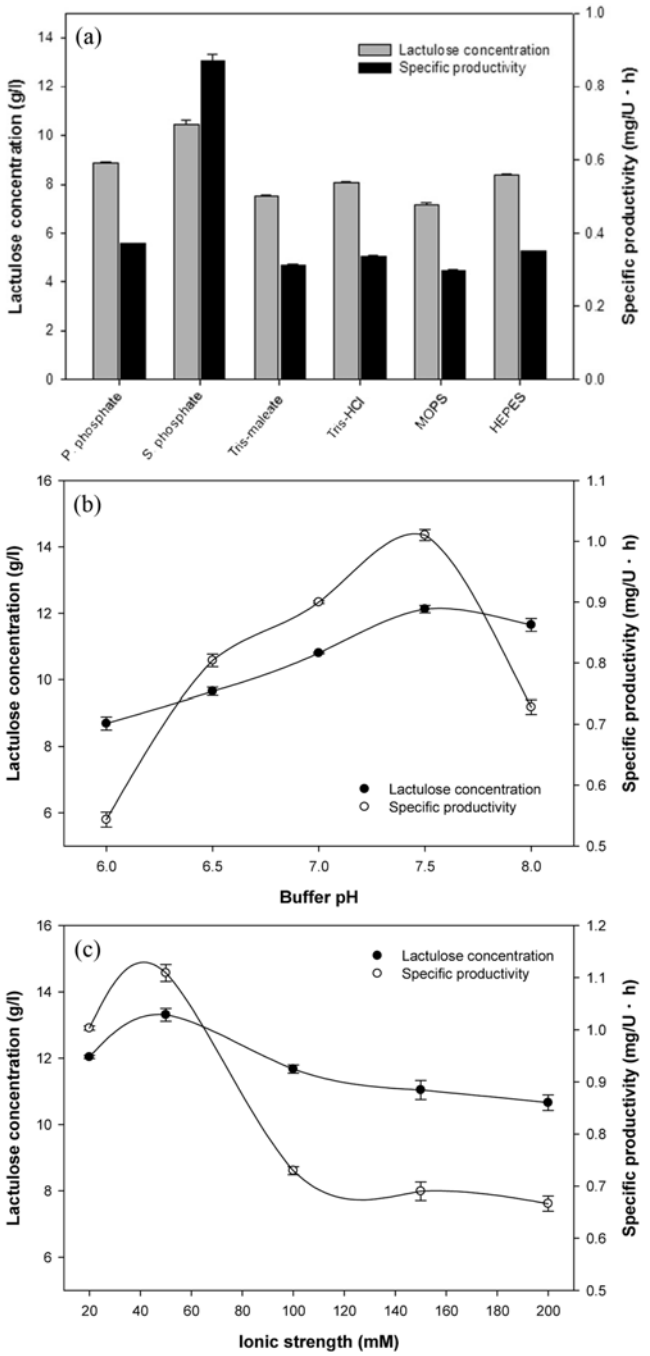

Fig. 3. Effect of the various buffers (a), pH (b) and ionic strength (c) on lactulose synthesis. (a) 40% (w/v) lactose, 20% (w/v) fructose, 12 U/ml of immobilized β-galactosidase, 20 mM ionic strength at pH 7.0, 37 °C. (b) 40% (w/v) lactose, 20% (w/v) fructose, 12 U/ml of immobilized β-galactosidase, 20 mM sodium phosphate buffer, 37 °C. (c); 40% (w/v) lactose, 20% (w/v) fructose, 12 U/ml of immobilized β-galactosidase, sodium phosphate buffer at pH 7.5, 37 °C.

to pH 7.5 and the specific productivity sharply decreased at pH 8.0. At pH 7.5, the lactulose concentration and specific productivity were 12.12 g/*l* and 1.01 mg/U·h, respectively. The optimum pH for lactulose synthesis was 7.5, which was 0.5 units higher than the optimum pH 7.0 reported by Lee et al. [10] and 0.5 units lower than the optimum pH 8.0 reported by Hua et al. [9]. These results may be due to the immobilization of β-galactosidase. Moreover, the optimum pH value for lactulose synthesis was consistent with the previously reported optimum pH value for ONPG hydrolysis [14].

The effect of ionic strength (20-200 mM) of the sodium phosphate buffer (pH 7.5) on lactulose synthesis was also investigated. As shown in Fig. 3(c), lactulose synthesis was the highest at a molarity of 50 mM, where the lactulose concentration and specific productivity were 13.31 g/*l* and 1.11 mg/U·h, respectively. The lactulose concentration increased with an increase in the ionic strength up to 50 mM, above which it continuously decreased up to 200 mM. The rate (specific productivity) of the transgalactosylation reaction of *K. lactis* β-galactosidase was found to be dependent on the molarity of the buffer. The specific productivity decreased dramatically when the ionic strength was greater than 50 mM. Lee et al. [19] reported that the decrease in enzyme activity at high ionic strengths occurred because the protein became dehydrated, due to the hydrating effect of the salt molecules. Taken together, these results indicate that the increase in lactulose synthesis by β-galactosidase was closely related to the buffer, pH and ionic strength.

5. Effect of Temperature

The effect of temperature on lactulose synthesis was evaluated from 32 to 57 °C. Fig. 4 shows the effect of reaction temperature on lactulose synthesis. The lactulose concentration and specific productivity increased as the temperature increased and maximum values were observed at 47 °C, above which the values sharply decreased. This may have been due to enzyme inactivation at elevated temperature. The lactulose concentration and specific productivity at 47 °C were 15.80 g/*l* and 1.32 mg/U·h after 60 min of reaction, respectively. The optimum temperature was observed at 47 °C. However, Lee et al. [10] found that the optimum temperature was 60 °C. The reason for this difference was because they used permeabilized *K. lactis* cells as the enzyme source. In this study, the lactulose productivity by immobilized β-galactosidase was 15.8 g/*l*·h, which was higher than the values reported by Kim et al. [1], Lee et al. [10], Mayer et al. [11] (8.3, 6.8 and 8.2 g/*l*·h, respectively).

Lactulose synthesis by β-galactosidase is the combined result of

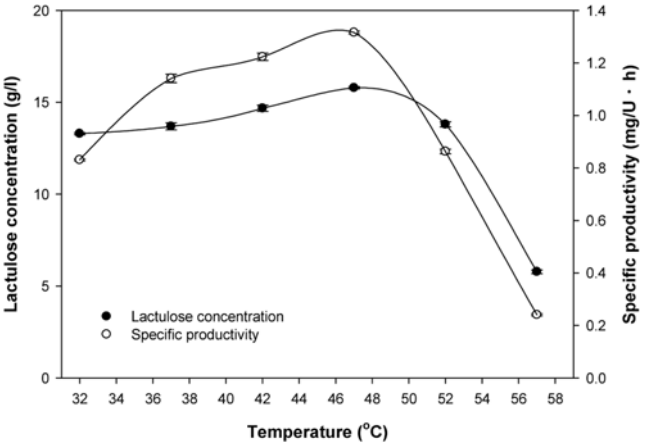

Fig. 4. Effect of temperature on lactulose synthesis. Reactions were conducted using 40% (w/v) lactose, 20% (w/v) fructose and 12 U/ml of immobilized β-galactosidase in 50 mM sodium phosphate buffer at pH 7.5.

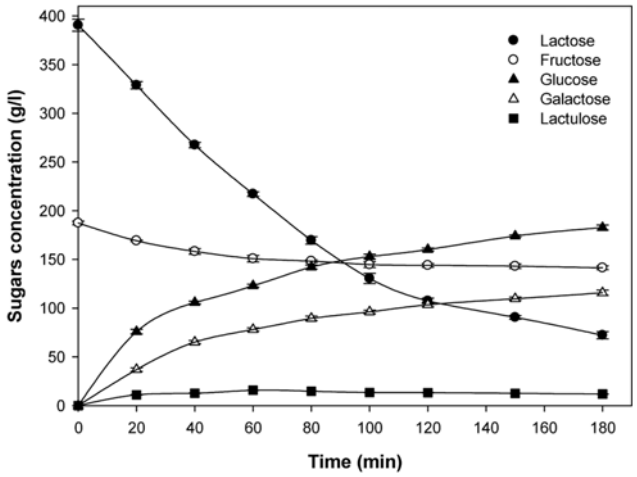

Fig. 5. Time course of the synthesis of lactulose from lactose and fructose. Reactions were conducted at 47 °C using 40% (w/v) lactose, 20% (w/v) fructose and 12 U/ml of immobilized β-galactosidase in 50 mM sodium phosphate buffer at pH 7.5.

hydrolysis and transgalactosylation activity. This balance is altered by pH and temperature changes and by the presence/absence of sodium ions located at the active site [13]. Accordingly, the obtained results indicated that the activity of β-galactosidase was shifted to transgalactosylation through optimization of the reaction conditions and therefore improved the lactulose synthesis.

Fig. 5 shows the lactose, fructose, glucose, galactose and lactulose content during the time course of the reaction under the following optimum conditions: 40% (w/v) lactose, 20% (w/v) fructose, immobilized β-galactosidase of 12 U/ml, 50 mM sodium phosphate buffer at pH 7.5 and 47 °C. As the reaction time increased, the concentrations of lactose and fructose gradually decreased due to the hydrolysis and transgalactosylation reaction of β-galactosidase, whereas glucose and galactose gradually increased. Glucose was present at higher concentrations than galactose, indicating that galactose was used in the synthesis of other galactooligosaccharides [1]. The maximum lactulose concentration (15.80 g/*l*) was obtained after 60 min of the reaction, after which it gradually decreased to 11.91 g/*l*. This occurred because β-galactosidase also hydrolyzes lactulose [9].

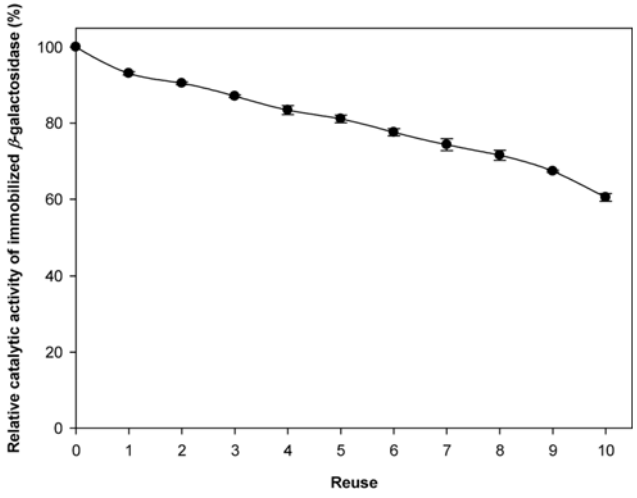

Fig. 6. Reusability of immobilized β-galactosidase on lactulose synthesis. Reactions were conducted at 47 °C for 1 h using 40% (w/v) lactose, 20% (w/v) fructose and 12 U/ml of immobilized β-galactosidase in 50 mM sodium phosphate buffer at pH 7.5.

6. Reusability of Immobilized β-Galactosidase

The reusability of immobilized enzymes is of key importance for industrial applications. To assess reusability, the immobilized β-galactosidase was used for consecutive batches of lactulose synthesis. The relative catalytic activity of immobilized β-galactosidase on lactulose synthesis is shown in Fig. 6, and the lactulose concentration (15.84 g/*l*) of the first batch was taken as 100%. The relative catalytic activity of immobilized β-galactosidase decreased as the number of reuses was increased. After 10 reuses, the synthesized lactulose concentration was 9.58 g/*l*, and the immobilized β-galactosidase retained 60.5% of the relative catalytic activity.

CONCLUSION

The objective of this study was to improve lactulose synthesis by covalently immobilized β-galactosidase. β-Galactosidase was pretreated with lactose prior to immobilization, and then immobilized on silica gels. Optimization of the reaction conditions for lactulose synthesis greatly improved the transgalactosylation activity of immobilized β-galactosidase. The optimal concentrations of lactose and fructose, pH and ionic strength of buffer, loading amount of enzyme and temperature for lactulose synthesis were 40% (w/v) lactose, 20% (w/v) fructose, 50 mM sodium phosphate buffer at pH 7.5, immobilized β-galactosidase of 12 U/ml and 47 °C, respectively, and the lactulose concentration and specific productivity under these conditions were approximately 2.0 and 5.1 times higher than before optimization, respectively. Moreover, the immobilized β-galactosidase retained 60.5% of its original catalytic activity after 10 reuses. Subsequent studies will have to evaluate the use of alternatives to lactose for cost-effective production of lactulose.

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