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Production of Lactosucrose from Sucrose and Lactose by a Levansucrase from *Zymomonas mobilis*

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Lactosucrose (4^G-β-D-galactosylsucrose) is an oligosaccharide consisting of galactose, glucose, and fructose. In this study, we prepared lactosucrose from lactose and sucrose using a levansucrase derived from *Zymomonas mobilis*. Optimum conditions for lactosucrose formation were 23°C, pH 7.0, 18.0% (w/v) lactose monohydrate, and 18% (w/v) sucrose as substrates, and 1 unit of enzyme/ml of reaction mixture. Under these conditions, the lactosucrose conversion efficiency was 28.5%. The product was purified and confirmed to be *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→2)-β-D-fructofuranoside, or lactosucrose. A mixed-enzyme system containing a levansucrase and a glucose oxidase was applied in order to increase the efficiency of lactose and sucrose conversion to lactosucrose, which rose to 43.2% as a result.

Keywords: Glucose oxidase, lactosucrose, levansucrase, *Zymomonas mobilis*

A number of health benefits have been claimed for prebiotics, and many products containing them are available worldwide [11, 18]. Gibson and Roberfroid [7] defined a prebiotic as a food ingredient that is nondigestible and nonabsorbable in the upper gastrointestinal tract and that supports the growth and/or activity of one or more particular bacteria in the colon, thereby improving the health of the host.

Lactosucrose (4^G-β-D-lactosylfructoside, galactosylsucrose) is a trisaccharide consisting of glucose, galactose, and fructose. Lactosucrose can be obtained *via* a transfructosylation reaction catalyzed by either a levansucrase (E.C. 2.4.1.10) or a β-fructofuranosidase (E.C. 3.2.1.26), with lactose and sucrose serving as substrates [2, 6, 15]. Lactosucrose is indigestible in the human digestive tract and is therefore low in calories and suitable for use in low-calorie foods. It is a putative growth stimulator of intestinal bifidobacteria [1] and therefore could be classified as a prebiotic. Until now, most trials in process development for the production of lactosucrose have concentrated on using whole-cell systems [14–16]. However, microbial levansucrases are usually produced extracellularly or in the outer membrane-bound form [17]. With the exception of a few classes of proteins, such as toxins [3] and hemolysins [8], *E. coli* normally does not secrete foreign proteins extracellularly; therefore, Yun *et al.* [22] have suggested that whole-cell immobilization of recombinant *E. coli* containing an endoinulinase gene is suitable for the production of inulo-oligosaccharides from inulin. Previously, we isolated the levansucrase gene from *Z. mobilis* and successfully expressed it in *E. coli* [9, 19]. In the present work, the optimal reaction conditions for batchwise production of lactosucrose using *E. coli* lysate are described.

Materials and Methods

Materials

Fructose, glucose, and sucrose were purchased from Sigma (St. Louis, MO, U.S.A.) and α-lactose monohydrate was obtained from Samchun Chemical Inc. (Seoul, Korea). Gluzyme, a mixture of glucose oxidase and catalase, was obtained from Novo Nordisk (Bagsvaerd, Denmark).

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Preparation of Levansucrase

Plasmid pELCHis24 carrying the levansucrase gene *levU* of *Z. mobilis*, which is expressed when induced by the T7 promoter in *E. coli* BL21 (DE3, F^- ompT r-Bm-B) [20], was used for levansucrase production [19]. *E. coli* harboring pELCHis24 was grown aerobically at 37°C for 12 h. Cells were harvested and then disrupted by ultrasonication. Cell debris was removed by centrifugation at 10,000 ×*g* for 10 min at 4°C. The supernatant was used as the source of levansucrase without further purification.

Measurement of Enzyme Activity

Levansucrase activity was determined by either sucrose hydrolysis or lactosucrose formation activity. One unit (U) of levansucrase activity was defined as the amount of enzyme releasing one mmole of glucose per minute at pH 5.0 and 37°C. One unit of glucose oxidase (GOD) activity was defined as the amount of enzyme consuming one mmole of oxygen per minute at pH 5.1 and 35°C. To calculate the conversion efficiency (%), the concentration of lactosucrose was divided by the sum of the initial concentrations of lactose monohydrate and sucrose, and then expressed as a percentage value.

Analysis of Products

Glucose, fructose, sucrose, lactose, and lactosucrose were quantitatively determined by HPLC (Agilent Technologies Inc., Santa Clara, CA, U.S.A.) with a refractive index and a Kromasil 100-10NH2 column (Eka Chemicals AB, Bohus, Sweden) at 50°C. The mobile phase consisted of 75% acetonitrile:25% water and was used at a flow rate of 1.0 ml/min. The transfructosylation reaction was performed and the product was fractionated by HPLC. The purified sugar was concentrated by rotary evaporation and subjected to NMR measurements. ^{1}H NMR and ^{13}C NMR spectra were obtained in pyridine-*d5* on a Varian 500 MHz NMR system (Varian, Palo Alto, CA, U.S.A.) equipped with a cold probe, with standard pulse sequences operating at 500 and 125 MHz.

Optimization of Reaction Conditions for Lactosucrose Production

The effect of temperature on lactosucrose production was determined by varying the temperature from 5 to 50°C. The enzymatic reaction was carried out in a 2-ml mixture containing 18.0% (w/v) sucrose and 18.0% (w/v) lactose monohydrate in 50 mM sodium acetate buffer (pH 6.0) and 8 µl (2 U) of enzyme solution for 30 min and 6 h. The reaction was terminated by heating the tubes at 95°C for 5 min and the filtrate was analyzed by HPLC.

To determine the optimal pH for lactosucrose production, the reaction was carried out at varying pH (3.0 to 7.0) in a 2-ml reaction mixture containing 18.0% (w/v) sucrose and 18.0% (w/v) lactose monohydrate at 23°C under the conditions described above.

To determine the optimal substrate concentration, lactose monohydrate and sucrose were used at the same concentration (0.9%, 1.8%, 9.0%, 13.5%, and 18.0% [w/v]) [14]. The enzymatic reaction was carried out at pH 7.0 and 23°C under the conditions described above.

To determine the optimal ratio of lactose monohydrate to sucrose, the enzymatic reaction was performed with varying concentrations of sucrose (4.5%, 9.0%, 18.0%, and 27.0% [w/v]) whereas the concentration of lactose monohydrate was maintained at 18.0% (w/v). Enzymatic reactions were carried out at pH 7.0 and 23°C as described above.

The effect of enzyme concentration on lactosucrose production was determined by varying the concentration from 0.02 to 4 U. Reactions containing 18.0% (w/v) sucrose and 18.0% (w/v) lactose monohydrate were carried out at pH 7.0 and 23°C as described above.

For the time course, the enzymatic reaction was carried out in a 20-ml mixture containing 18.0% (w/v) sucrose and 18.0% (w/v) lactose monohydrate in 50 mM sodium acetate buffer (pH 7.0) and 80 µl (20 U) of enzyme solution at 23°C.

Mixed Enzymatic Reactor System

A Marubishi (Tokyo, Japan) MJ-N14L jar fermenter (7.5 l) was employed as a mixed enzymatic reactor to produce lactosucrose. The reaction was carried out in a 2-l mixture containing 18% sucrose and 18% lactose monohydrate in 10 mM sodium acetate buffer (pH 6.0), 10 ml (2,500 U) of levansucrase, and 1.2 g (12,000 GOD U) of gluzyme. The reaction temperature was maintained at 30°C and the pH was kept at 6.0 through the automatic addition of a $CaCO_3$ slurry. Oxygen was continuously supplied at a velocity of 10 l/min and the agitation speed was 500 rpm.

The reproducibility of analyses was verified as follows: five injections were carried out using various concentrations of fructose, glucose, sucrose, lactose, and lactosucrose; there was a linear relationship between the peak area detected corresponding to each carbohydrate and the amount of carbohydrate applied, with a less than 3% variation in detected amounts between injections. For analysis of carbohydrate composition following the mixed enzyme reaction, independent experiments were performed. Results reported for each type of experiment were consistent between replicates within any one experiment but some quantitative variation in carbohydrate composition occurred between separate runs. The trends seen for the relative proportions of carbohydrates were identical in every analysis; therefore, representative figures are shown in the results and discussion rather than an average of percentage composition among experiments, unless otherwise stated.

Results and Discussion

Effect of Temperature on Lactosucrose Formation Activity

The optimal temperature for lactosucrose formation was 23°C (Fig. 1), which is low compared with those of other bacterial levansucrases. For example, this temperature is 55°C in the case of levansucrases from *Bacillus subtilis*, *Geobacillus stearothermophilus,* and *Paenibacillus polymyxa* [16] and is 40°C for levansucrases from *Pseudomonas syringae* and *Rahnella aquatilis* [16]. For further experiments, a temperature of 23°C was selected for lactosucrose production because levansucrase from *Z. mobilis* is less thermostable than other levansucrases [9, 10].

Effect of pH on Lactosucrose Formation Activity

Differences in pH gave rise to marked differences in lactosucrose production. The optimum pH for enzyme activity was found to be 7.0 (Fig. 2). In contrast, the optimum pH for lactosucrose production by levansucrases from *B. subtilis*, *G. stearothermophilus, P. polymyxa, P. syringae*, *R. aquatilis*, *Sterigmatomyces elviae*, and *Microbacterium laevaniformans* is 6.0 [13, 14, 16]. A pH of 7.0 was selected

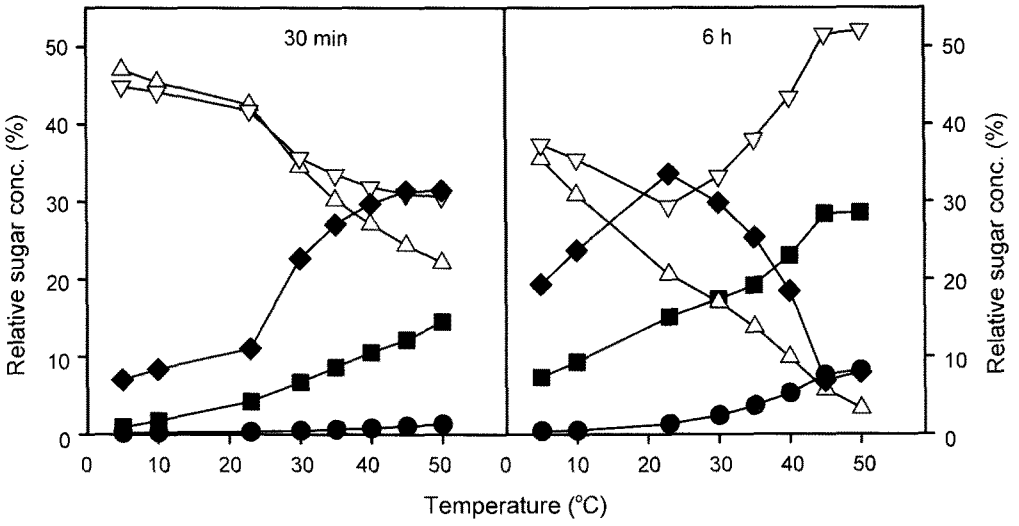

Fig. 1. Effect of temperature on lactosucrose production.
The enzymatic reaction was carried out with 18% (w/v) sucrose and 18% (w/v) lactose monohydrate in 50 mM sodium acetate buffer (pH 6.0) and 2 U enzyme for 30 min and 6 h. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

for further experiments since levansucrase is highly unstable at alkaline pH [10].

Effect of Substrate Concentration on Lactosucrose Formation Activity

To investigate the effect of substrate concentration, enzyme reactions were performed using equal concentrations of lactose monohydrate and sucrose. Maximum lactosucrose was produced at the highest concentration (18.0% w/v) (Fig. 3). This concentration was chosen for further experiments rather than designing experiments to cover the full range of possible substrate concentrations since the solubility of lactose monohydrate was fairly low at 18.0% (w/v) at room temperature. It is interesting to note that in the presence of a high concentration of sucrose as the sole substrate in the reaction mixture, synthesis of low-molecular-weight fructooligosaccharide is the dominant reaction catalyzed by the levansucrase rather than synthesis of high-molecular-weight levan [5].

Effect of the Ratio of Sucrose to Lactose Monohydrate on Lactosucrose Formation Activity

In order to improve the efficiency of lactosucrose production, the concentration of sucrose was varied (4.5%, 9.0%, 18.0%,

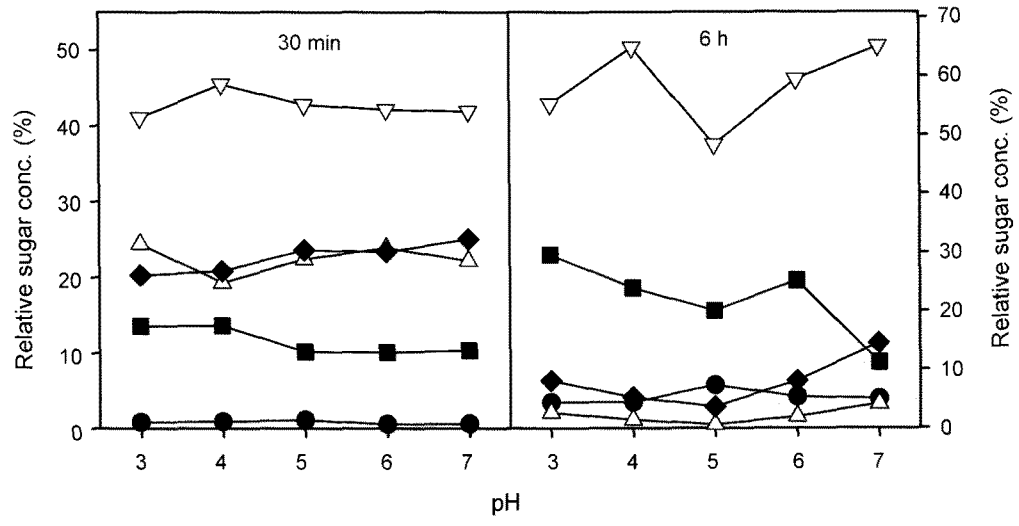

Fig. 2. Effect of pH on lactosucrose production.
The enzymatic reaction was carried out at varying pH (3.0–7.0) with 18% (w/v) sucrose and 18% (w/v) lactose monohydrate in 50 mM sodium acetate buffer and 2 U enzyme at 23°C for 30 min and 6 h. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

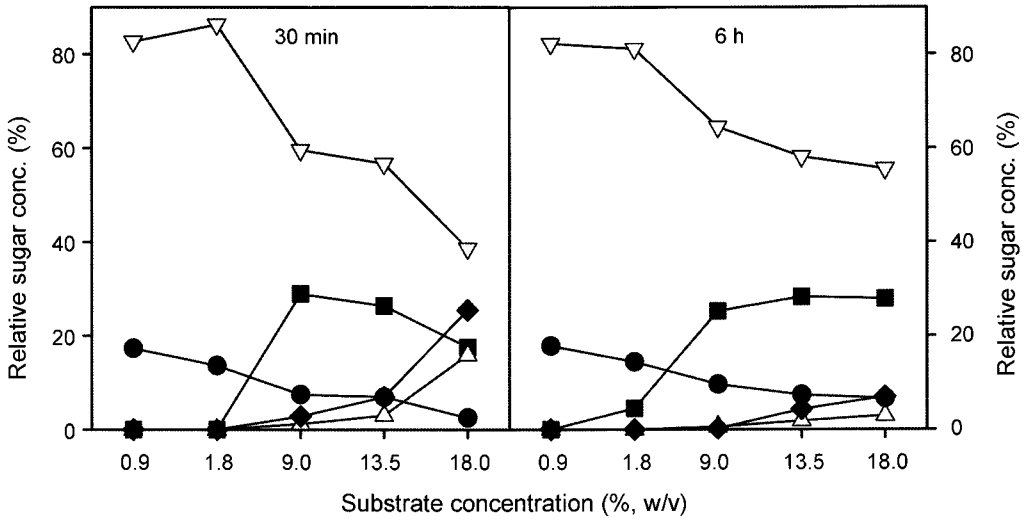

Fig. 3. Effect of substrate concentration on lactosucrose production.
The enzymatic reaction was performed with various substrate concentrations in 50 mM sodium acetate buffer (pH 7.0) and 2 U enzyme at 23°C for 30 min and 6 h. Equal concentrations of the two substrates were used: 0.9%, 1.8%, 9.0%, 13.5%, 18% (w/v) each of lactose monohydrate and sucrose. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

and 27.0% [w/v]) while the concentration of lactose monohydrate was kept at 18.0% (w/v). At the lower ratios of sucrose to lactose monohydrate, a lower conversion efficiency was observed (Fig. 4). The conversion efficiency increased as the ratio of sucrose to lactose monohydrate increased. The conversion efficiency was 26.4% when the ratio of sucrose to lactose monohydrate was 1:1.

Effect of Enzyme Concentration on Lactosucrose Formation Activity

As the enzyme concentration was increased up to 2 units (5.4 units enzyme per gram of lactose monohydrate), the production of lactosucrose was increased. However, a further increase in enzyme concentration up to 4 units resulted in a slight decrease in the efficiency of conversion to lactosucrose from the two substrates (Fig. 5). This result is probably due to the lactosucrose hydrolysis activity of the levansucrase.

Time Course of Lactosucrose Production

Levansucrase transfers the fructosyl moiety of sucrose to lactose and then lactosucrose is formed [12]. However, levansucrase catalyzes not only the fructose transfer reaction but also the hydrolysis of lactosucrose to fructose and lactose [6, 12]. Therefore, the hydrolysis reaction should

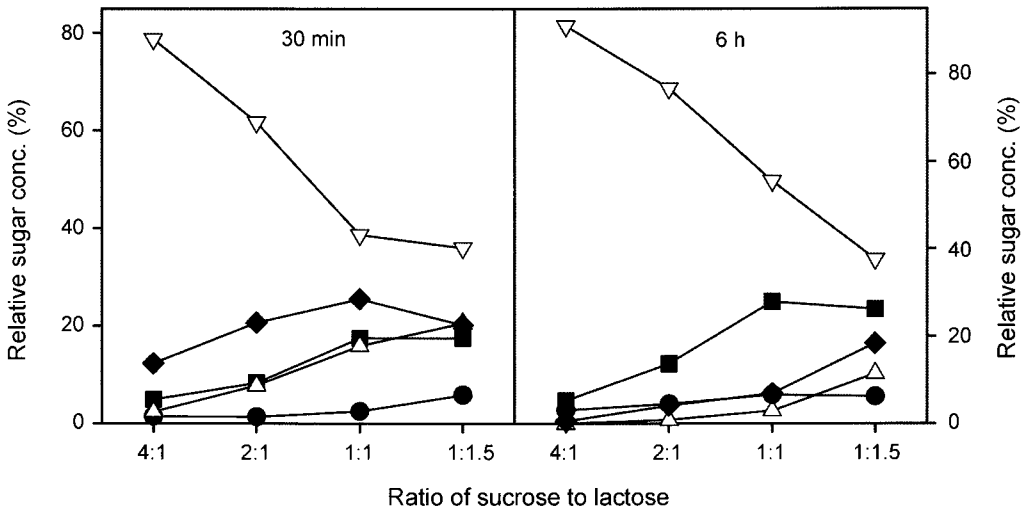

Fig. 4. Effect of the concentration ratio of the two substrates on lactosucrose production.
The enzymatic reaction was performed with a varying ratio of the two substrates in 50 mM sodium acetate buffer (pH 7.0) and 2 U enzyme at 23°C for 30 min and 6 h. Substrate ratios: 18.0%:4.5%, 18.0%:9.0%, 18.0%:18.0%, 18.0%:27.0% (w/v) lactose monohydrate:sucrose. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

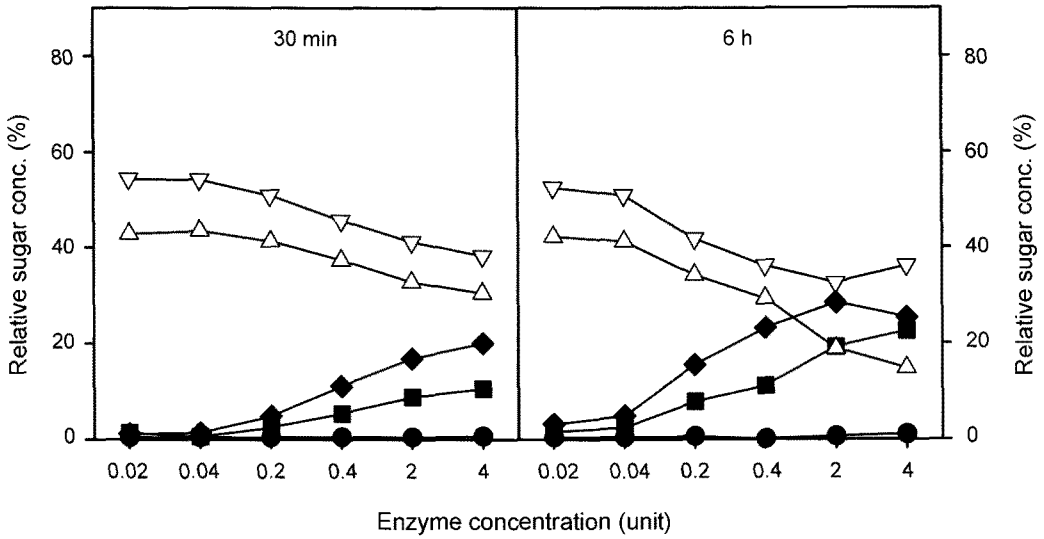

Fig. 5. Effect of enzyme concentration on lactosucrose production.
The enzymatic reaction was performed with 18% (w/v) sucrose and 18% (w/v) lactose monohydrate in 50 mM sodium acetate buffer (pH 7.0) with varying concentrations of enzyme. Enzyme concentrations: 0.02 units, 0.04 units, 0.2 units, 0.4 units, 2 units, 4 units. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

be minimized by monitoring the enzymatic reaction time. Fig. 6 shows the time course of enzyme activity over a period of 24 h. The production of lactosucrose increased with increasing cultivation time and consequently reached a conversion efficiency of 28.5% after 2 h but began to decrease thereafter. HPLC analysis revealed that a prolonged enzymatic reaction of more than 2 h increased the production of glucose and lactose, whereas the amount of sucrose and lactosucrose were reduced, a result that has been described by others [6, 12]. Glucose can be produced from sucrose by the sucrase activity of levansucrase, whereas lactose can be released from lactosucrose by the lactosucrose hydrolysis activity of the enzyme.

Measurements of NMR Specta

The structure of the sugar was elucidated through analysis of 1D and 2D NMR data primarily from ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, and HSQC experiments. The ^{13}C NMR spectrum showed 18 carbons including three anomeric sugar carbons (105.80, 92.81, 105.80 ppm) and four methylene oxylated carbons (62.70, 61.39, 61.76, 64.39 ppm), indicating the presence

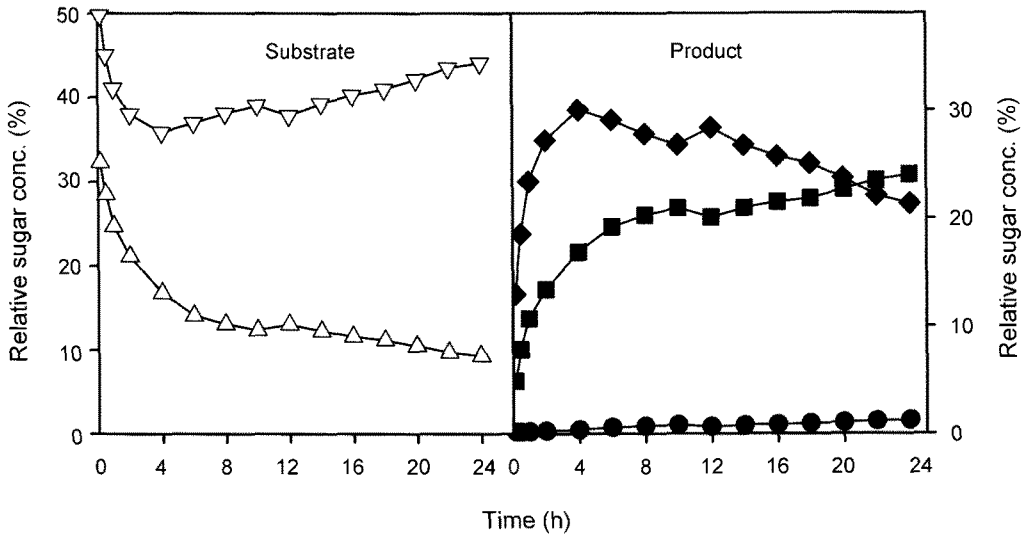

Fig. 6. Time course of lactosucrose production.
The enzymatic reaction was performed in a 20-ml reaction mixture containing 18% (w/v) sucrose and 18% (w/v) lactose monohydrate in 50 mM sodium acetate buffer (pH 7.0) and 20 U enzyme at 23°C. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

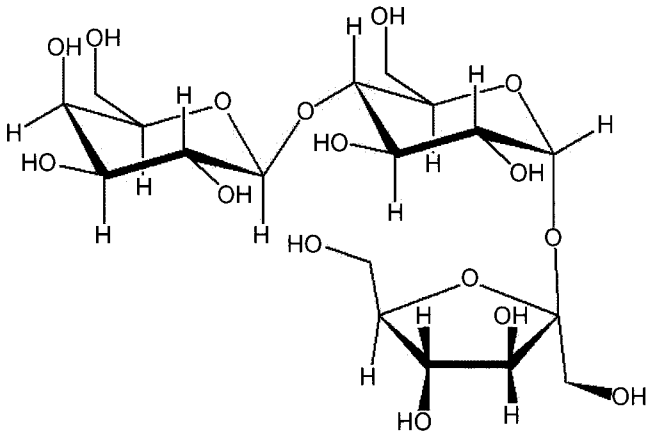

Fig. 7. Structure of lactosucrose.

of three hexose sugars in the structure. The ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC spectra confirmed that the 3-sugar moiety consisted of galacopyranose, glucopyranose, and fructopyranose. The ^{1}H-^{13}C HMBC spectrum showed a correlation of glucose H-1' (6.10 ppm) to fructose C-2'' (105.80 ppm), indicating a glucose (1→2) fructose linkage. The HMBC correlation of galactose H-1 (5.07 ppm) to glucose C-4' (82.02 ppm) indicated a galactose (1→4) glucose linkage. The results of these experiments suggest that the sugar product was *O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside, or lactosucrose (Fig. 7). The full assignment of ^{1}H and ^{13}C NMR data are shown in Table 1.

Table 1. Chemical shifts in ^{1}H and ^{13}C NMR spectra of lactosucrose produced from lactose and sucrose by *Z. mobilis* levansucrase.

Group	Carbon atoms	^{13}C NMR [a]	^{1}H NMR [a]
Galactose	1	105.80	5.07
	2	72.21	4.46
	3	72.68	4.12
	4	69.73	4.09
	5	76.99	4.11
	6	62.70	4.29/4.26
Glucose	1'	92.81	6.10
	2'	74.92	4.13
	3'	72.38	4.71
	4'	82.02	4.28
	5'	72.88	4.66
	6'	61.39	4.48/4.44
Fructose	1''	61.76	4.43/4.36
	2''	105.80	-
	3''	79.57	5.02
	4''	75.40	4.97
	5''	84.50	4.51
	6''	64.39	4.34/4.29

[a]NMR data acquired in Pyr-*d5*.

Production of Lactosucrose by a Mixed Enzyme System

It has been suggested that a conversion approaching 100% of the theoretical yield would be difficult to obtain with levansucrase in a batchwise process owing to the high level of inhibition by released glucose, which competes with the glucose moiety of sucrose for the enzyme [17]. Yun *et al.* [21] reported an enzymatic method to enhance fructooligosaccharide conversion efficiency by removing glucose using glucose oxidase. Using a similar strategy, we applied a mixed enzyme system containing levansucrase and glucose oxidase. As shown in Fig. 8, an increase in lactosucrose conversion efficiency was achieved by converting glucose to gluconic acid with glucose oxidase. The maximal conversion efficiency was 43.2%. The efficiencies of lactose and sucrose conversion to lactosucrose

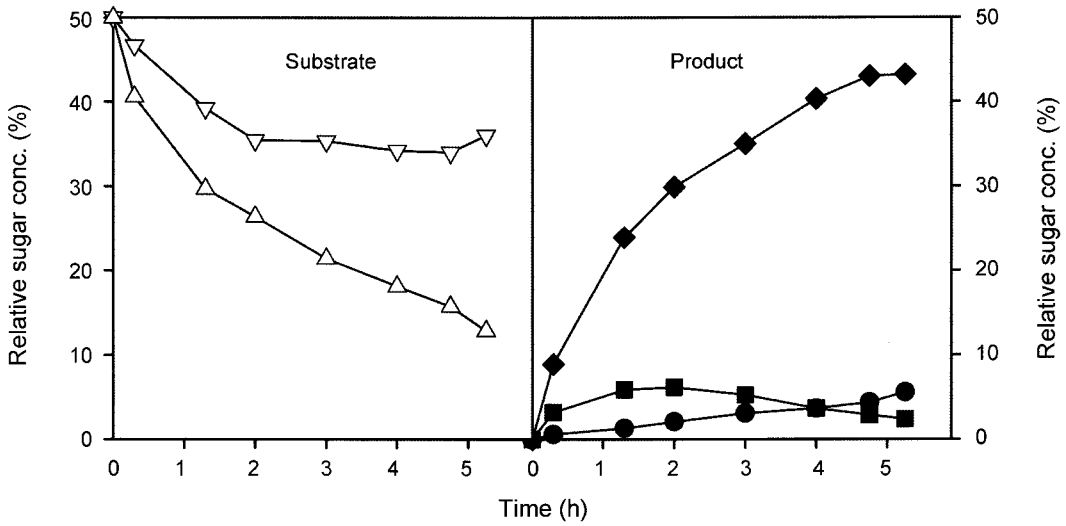

Fig. 8. Time course of lactosucrose production in a batch system with *Z. mobilis* levansucrase and glucose oxidase.
The reaction was performed in a 7.5-l fermenter with a working volume of 2 l with 2,500 units of levansucrase and 12,000 units of gluzyme. Reaction conditions: 500 rpm agitation, 10 l/min air flow, 30°C, pH 6.0. Symbols: (△), sucrose; (▽), lactose; (◆), lactosucrose; (■), glucose; (●), fructose.

Table 2. Lactosucrose yields obtained using enzymes from various microorganisms.

Enzyme source	Substrate (g/l) (lactose+sucrose)	Conversion efficiency (%)	Reference
Arthrobacter mysorens	300+300	5.3	[14]
Klebsiella pneumoniae	300+300	3.2	[14]
Rahnella aquatilis	300+300	13.2	[14]
Sterigmatomyces elviae	250+250	38.4	[14]
Bacillus subtilis	225+225	40.7	[16]
Paenibacillus polymyxa	225+225	37.8	[4]
Zymomonas mobilis	180+180	28.5	This work
Z. mobilis (+glucose oxidase)	180+180	43.2	This work

reported by others and from this work are summarized in Table 2. Lee *et al.* [14] achieved a 38.4% conversion efficiency in a batch reaction with an *S. elviae* strain harboring levansucrase activity. In the same paper, the authors reported a conversion efficiency of 36.0% for 48 days using immobilized *S. elviae* cells for continuous lactosucrose production in a packed-bed reactor. In another report, an efficiency of 40.7% was observed using *B. subtilis* levansucrase to convert 225 g lactose/l and 225 g sucrose/l [16]. The conversion efficiency obtained in this work using a mixed-enzyme system was higher than those obtained using a single enzyme in isolation or in a whole-cell system. Because we have not optimized the enzymatic conditions for the mixed-enzyme system, further experimentation is warranted. From the present data, it is evident that *Z. mobilis* levansucrase was successfully applied to the production of lactosucrose from lactose and sucrose substrates. However, the results of this work suggest that eliminating glucose during the enzymatic reaction is a necessity, for which the use of glucose oxidase may be applicable.

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