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Continuous Production of Lactosucrose by Immobilized *Sterigmatomyces elviae* Mutant

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Abstract In this study, in order to develop a continuous production process of lactosucrose in a packed-bed reactor, *Sterigmatomyces elviae* ATCC 18894 was selected and mutated. The mutant strain of *S. elviae* showed 54.3% higher lactosucrose production than the wild type. Reaction conditions such as temperature, pH, substrate concentration and flow rate were also optimized. Under optimized reaction conditions (50°C, pH 6.0, 25% sucrose and 25% lactose as substrate, flow rate 1.2 ml/min), the maximum concentration of lactosucrose (192 g/l) was obtained. In a packed-bed reactor, continuous production of lactosucrose was performed using *S. elviae* mutant immobilized in calcium alginate, and about 180 g/l of lactosucrose production was achieved for 48 days.

Keywords: Continuous production, lactosucrose, packed-bed reactor, *Sterigmatomyces elviae* mutant, whole-cell immobilization

Lactosucrose (lactosylfructoside) is a newly synthesized compound that is an indigestible oligosaccharide produced from lactose and sucrose by the action of β-fructofuranosidase (E.C. 3.2.1.26) from *Arthrobacter* sp. K-1 [5, 6, 16]. Lactosucrose is noncariogenic and suitable for low-calorie food. Furthermore, it supports the growth of intestinal *Bifidobacteria* [11, 13, 18]. Lactosucrose has already been used to play a major role in the refinement of sugars in the food industry. The degree of sweetness is similar to that of sucrose, and its functional properties are well characterized compared with other oligosaccharides such as fructooligosaccharides, isomaltooligosaccharides, and soybean oligosaccharides. The enzyme source of lactosucrose synthesis can be divided into two classes: β-fructofuranosidase from *Bacillus* sp. and *Arthrobacter* sp., and galactosyltransferase (E.C. 2.4.1.167) from *Sporobolomyces* sp. and *Rahnella* sp. [7, 15]. However, lactosucrose-producing enzymes catalyze the transfer reaction, and catalyze the hydrolysis of sucrose and lactosucrose to form fructose, glucose, and lactose [6, 8]; therefore, these backward and side reactions should be reduced to enhance the production of lactosucrose. Kawase *et al.* [8] studied lactosucrose production by β-fructofuranosidase from *Arthrobacter* sp. K-1 in a simulated moving bed reactor, but the lactosucrose yield was low owing to strong product hydrolysis. It is, therefore, necessary to develop a continuous process that can prevent the reversible reaction of the enzyme. Nevertheless, continuous production of lactosucrose using either whole-cell immobilization or enzyme from *S. elviae* has never before been reported. Therefore, in this study, *S. elviae* was selected among various strains and mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) to improve lactosucrose-producing activity. Then, continuous production of lactosucrose was carried out in a packed-bed reactor by using whole-cell immobilization of the *S. elviae* mutant.

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MATERIALS AND METHODS

Microorganisms and Culture Conditions

Sterigmatomyces elviae ATCC 18894, *Arthrobacter mysorens* ATCC 33408, *Klebsiella pneumoniae* ATCC 25306, and *Rahnella aquatilis* ATCC 55046 were provided from CJ Corporation, Korea. They were subcultured on a sucrose and yeast extract agar plate. The culture media consisted of 1% sucrose, 1% yeast extract, 1% peptone, 0.5% ammonium sulfate, 0.3% K_2HPO_4, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.0 before autoclaving. The main culture was carried out at 30°C and 150 rpm for 90 h in a rotary shaking incubator.

Selection and Mutation of Microorganism

Lactosucrose-producing activities of four microorganisms were compared. The whole cells of four microorganisms (10 g) were reacted with the substrate consisting of 30% sucrose and 30% lactose for 15 h at 50°C and pH 6.0. Among the four microorganisms, *S. elviae* ATCC 18894 showed the highest activity and was then mutagenized with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) as described by Fiedurek *et al.* [4]. The survival ratio (%) after NTG treatment was 6.8%, and mutation was performed until productivity was increased.

Whole-Cell Immobilization

S. elviae mutant cells (10%, w/v) were washed with distilled water and immobilized on calcium alginate beads by mixing them with 2% sodium alginate solution and then extruding the mixture dropwise into 0.2 M $CaCl_2$ [12]. The average bead diameter was about 2 mm, and they were kept in $CaCl_2$ solution at 4°C for 24 h before use.

Batch and Continuous Production of Lactosucrose

To determine optimal reaction conditions for lactosucrose production, 10 g of immobilized cells was added into 100 ml of sucrose- and lactose-containing solution (30% and 30%, respectively, w/v), and the production of lactosucrose was allowed for 15 h at 50°C with shaking at 100 rpm.

The continuous production of lactosucrose was carried out in a packed-bed reactor (Fig. 1). A jacketed glass column (ID 45 mm×200 mm) was used as a packed-bed reactor, where the immobilized whole cells (220 g) were charged. Samples were taken every 24 h, and temperature was controlled at 50°C by circulating water in the water jacket.

The products were analyzed by HPLC (YOUNG-LIN Instrument Co. Ltd., Korea) using a ZOBAX NH_2 column (150×6.0 mm, Agilent, Inc., U.S.A.) and a refractive index detector (YOUNG-LIN Instrument Co. Ltd., RI750F, Korea). The column temperature was maintained at 50°C. As a mobile phase, 70% (v/v) acetonitrile and 30% (v/v) water were used at a flow rate of 1.0 ml/min.

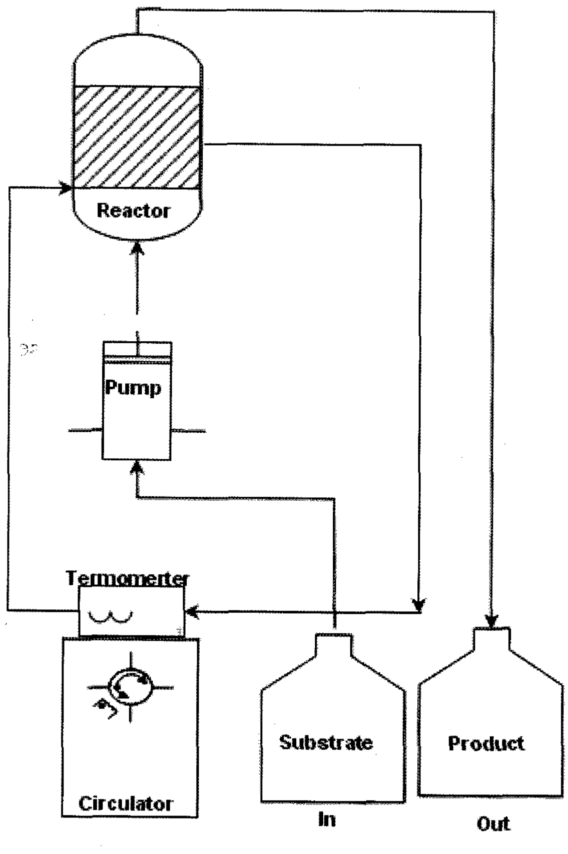

Fig. 1. Schematic diagram of the experimental setup for lactosucrose production.

RESULTS AND DISCUSSION

Selection and Mutation of *S. elviae* ATCC 18894

Table 1 shows the lactosucrose production by various microorganisms in batch reactions. Among them, *S. elviae* ATCC 18894 showed the highest production of lactosucrose, which was about 91 g/l after 15 h. Lactosucrose productions by *A. mysorens* ATCC 33408 and *K. pneumoniae* ATCC 25306 were low. Lactosucrose by *R. aquatilis* ATCC 55046 was 79 g/l at 15 h, but lactosucrose was rapidly hydrolyzed to glucose, fructose, and lactose at 30 h. In pact Park *et al.* [15] suggested that *Bacillus substilis* most plentifully produced the lactosucrose. However, because of the small cell size of bacteria such as *R. aquatilis* and *B. substilis*, the maintenance time (about 7 days) was very short and cell mass (about 1.2 g/l) was very low (data not shown). Therefore, they were not suitable for use in the industrial immobilization process. Consequently, *S. elviae* ATCC 18894 was selected, but its activity was very low. To increase lactosucrose production, the mutation of *S. elviae* was carried out with NTG by selection sequence in a batch reaction. Table 2 shows the lactosucrose production by selection sequence in the batch reaction. According to

Table 1. Comparison of lactosucrose production by various microorganisms in batch reactions.

Microorganism	Lactosucrose production for 15 h (g/l)	Lactosucrose production for 30 h (g/l)
A. mysorens	15±0.35	32±2.38
K. pneumoniae	16±1.92	19±1.16
R. aquatilis	79±2.07	21±4.11
S. elviae	91±1.55	110±3.68

Ten g of each microorganism was reacted with substrate solution, containing 30% sucrose and 30% lactose, for 15 h at 50°C and pH 6.0.

Table 2. Comparison of lactosucrose production by selection order on NTG mutation.

Selection order	Lactosucrose production (g/l)
1	97.33±2.91
2	96.87±2.01
3	96.65±4.43
4	94.55±0.97
5	94.91±0.56
6	91.11±3.66
7	90.69±1.48
8	91.38±1.15
9	91.99±0.74
10	90.94±2.36

Table 2, colonies of fast growth rate had a higher production of lactosucrose than those of late growth rate. Therefore, in other NTG mutation experiments, the mutated colonies were selected based on the fast growth rate (6 colonies). NTG mutation was carried out until the production of lactosucrose was increased. In the 5th mutation, lactosucrose production and cell mass were increased by about 54% and 8%, respectively, compared with those of the wild type (Table 3). Accordingly, the *S. elviae* mutant that showed the highest lactosucrose production (141 g/l) was screened and used for lactosucrose production.

Effect of Reaction Conditions on Lactosucrose Production

To determine the optimal reaction conditions for continuous lactosucrose production using whole-cell immobilization of the *S. elviae* mutant, the effects of pH, temperature, and substrate concentration on lactosucrose production were performed with batch reactions.

In the study on the effect of pH on lactosucrose production, the lactosucrose production of *S. elviae* was measured at various pHs ranging from 3.0 to 10.0 [10]. The reaction solution was incubated for 30 h at 50°C. As shown in Fig. 2, the optimum pH was found to be 6.0. Specifically, at pH below 4.0, lactosucrose production was significantly inhibited. At pH 6.0, lactosucrose production was about 188 g/l (Fig. 2). The optimum temperature for lactosucrose production was also determined (Fig. 3).

Table 3. NTG treatment to mutate *Sterigmatomyces elviae.*

Frequency of mutation	Residual living cells (%)	Lactosucrose production (g/l)
1	6.33	97.33±2.91
2	6.55	103.83±2.83
3	7.55	99.65±4.44
4	5.61	96.55±6.62
5	6.76	140.92±1.99
7	6.12	141.33±2.61
8	4.12	140.13±3.23

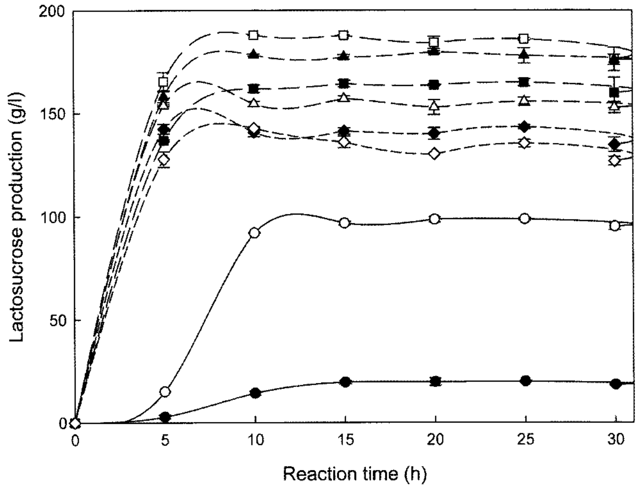

Fig. 2. Effect of pH on lactosucrose production.
Immobilized cells (10 g) were reacted with substrate solution, containing 30% sucrose and 30% lactose, for 30 h at 50°C and various pHs: 3 (●), 4 (○), 5 (■), 6 (□), 7 (▲), 8 (△), 9 (◆), 10 (◇).

When the reaction temperatures were 40°C, 45°C, and 50°C, maximum productions of lactosucrose were 133 g/l, 144 g/l, and 189 g/l, respectively. At 55°C, lactosucrose production was almost similar to that of 50°C. At 60°C, the lactosucrose-producing enzyme was thought to be inactivated. Thus, 50°C was found to be an optimum reaction temperature for lactosucrose production.

To investigate the effect of substrate concentration on lactosucrose production, solution containing various concentrations of sucrose (10%–35%) and lactose (10%–35%) were used. Some studies on the production of lactosucrose

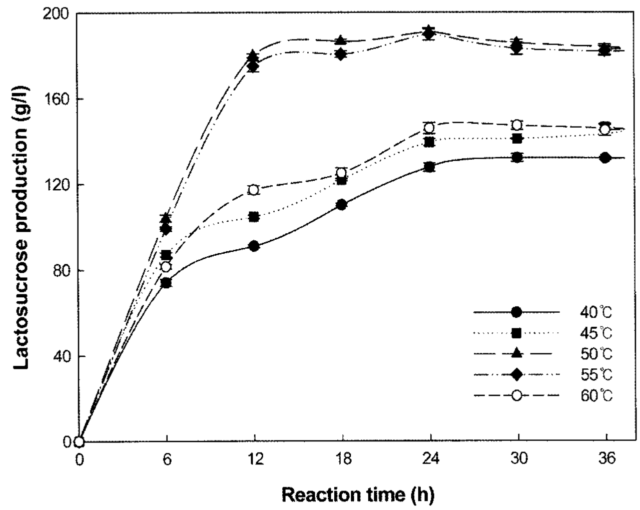

Fig. 3. Effect of temperature on lactosucrose production.
Immobilized cells (10 g) were reacted with substrate solution, containing 30% sucrose and 30% lactose, for 35 h at pH 6.0 and various temperatures: 40°C (●), 45°C (■), 50°C (▲), 55°C (◆), 60°C (○).

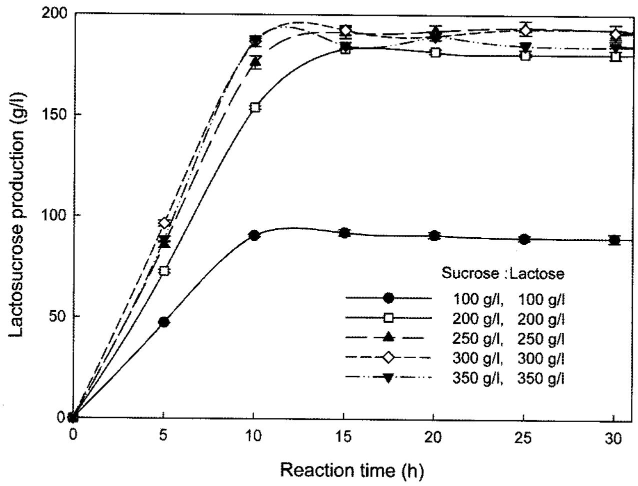

Fig. 4. Effect of substrate concentration on lactosucrose production.

Immobilized cells (10 g) were reacted with substrate solution, containing various concentrations of substrate: 10% sucrose and 10% lactose (■), 20% sucrose and 20% lactose (○), 25% sucrose and 25% lactose (▲), 30% sucrose and 30% lactose (□), 35% sucrose and 35% lactose (●), for 20 h at 50°C and pH 6.0.

reported that lactosucrose was produced most plentifully in the presence of equal amounts of sucrose and lactose [1, 2]. Therefore, equal amounts of sucrose and lactose were applied in this reaction. Among various substrate concentrations, the maximum lactosucrose production (192 g/l) was obtained at 15 h when 25% sucrose and 25% lactose were reacted (Fig. 4). With 30% sucrose and 30% lactose, a similar result was obtained at 15 h. However, lactosucrose production was found to decrease after 15 h, because of hydrolysis. Thus, 25% sucrose and 25% lactose were used for continuous production of lactosucrose.

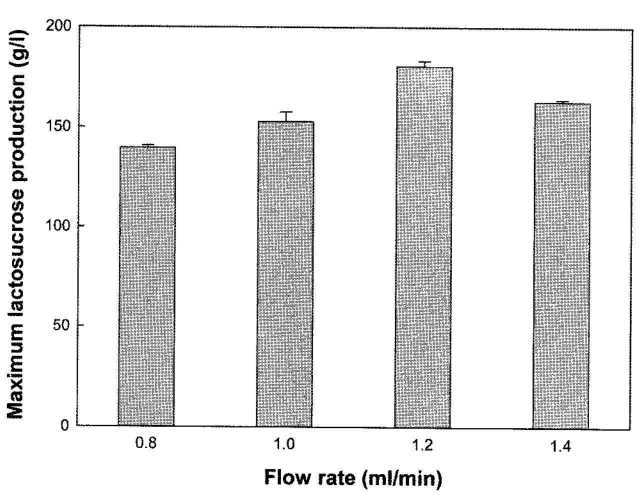

Fig. 5. Effect of flow rate on lactosucrose production by cell-immobilized beads in packed-bed reactor.

Continuous Production of Lactosucrose in a Packed-Bed Reactor Using Whole-Cell Immobilization of *S. elviae* mutant

Lactosucrose was produced continuously in a packed-bed reactor at optimized reaction conditions. Fig. 5 shows the effect of the feed flow rate on the lactosucrose production. The lactosucrose production increased as the feed flow rate increased to 1.2 ml/min, and then maximum lactosucrose production (182.63 g/l) was obtained at the feed flow rate of 1.2 ml/min. However, at 0.8 and 1.0 ml/min of the feed flow rates, lactosucrose production decreased to 138.92 g/l and 156.44 g/l, respectively. This result implies that the feed flow rate can influence the lactosucrose production, by accelerating the degradation of sucrose and inhibiting the fructosyltransfer reactions. In fact, the glucose produced inhibits the fructosyltransfer reaction. Therefore, to increase the fructosyltransfer reaction rate, Yun *et al.* [20] used glucose isomerase and glucose oxidase in the reaction mixture to eliminate glucose. Ultimately, the reaction rates increased when glucose was eliminated, and higher production yields were obtained.

The packed-bed reactor was also employed for the long-term operation. Fig. 6 shows the continuous production of lactosucrose in a packed-bed reactor at optimal reaction conditions and the feed flow rate of 1.2 ml/min. Lactosucrose production was maintained at about 180 g/l for longer than 48 days. Each sample was tested in plate by incubation to detect cell leakage. Cells were not leaked from immobilized bead until 48 days. However, the beads were swollen and cell leakage occurred after 48 days. Consequently, lactosucrose production was decreased to 170 g/l. This operation time is significantly long, compared with other oligosaccharide productions in a packed-bed reactor [3, 9, 14, 17, 19]. This

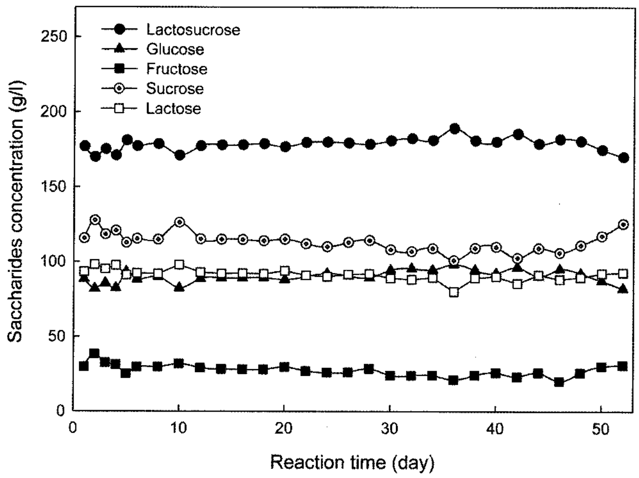

Fig. 6. Continuous production of lactosucrose by immobilized *S. elviae* mutant in a packed-bed reactor.

Production was carried out at 50°C, pH 6.0, and 1.2 ml/min of feed flow rate. Substrate solution contains 25% sucrose and 25% lactose: fructose (■), glucose (▲), sucrose (◎), lactose (□), lactosucrose (●).

immobilization process was, therefore, highly effective for the continuous production of lactosucrose from sucrose and lactose. In summary, based on the results of long-term operation and high level of lactosucrose production in a packed bed-reactor, it is suggested that a continuous process by immobilized *S. elviae* mutant has potential for application to industrial lactosucrose production.

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