



Enhanced conversion of sucrose to isomaltulose by a mutant of *Erwinia rhapontici*

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Abstract

Mutagenesis of *Erwinia rhapontici* was performed to enhance the production of isomaltulose from sucrose. A mutant strain, BN 68089, was obtained through a screening process involving automated and miniaturized cultivation in Bioscreen C. This high-throughput, miniaturized screening system was optimized to identify the mutant strain, which had a conversion yield (90%) and productivity ($194 \text{ g l}^{-1} \text{ h}^{-1}$). The BN 68089 mutant cells were immobilized in sodium alginate and when operated in a packed bed reactor gave a yield of 89% and a productivity of $144 \text{ g l}^{-1} \text{ h}^{-1}$ at 30°C , the optimal temperature. Immobilized BN 68089 cells exhibited 8% and 15% higher yield and productivity, respectively, than those of the wild-type strain.

Introduction

Isomaltulose (6-*O*- α -D-glucopyranosyl-D-fructofuranose) is a structural isomer of sucrose and is naturally present in honey in very small quantities (Low & Sporns 1988). This sugar has physical properties similar to those of sucrose but is only about half as sweet (Maki *et al.* 1983). Since it is non-cariogenic and releases monosaccharides into the bloodstream more slowly than sucrose, it has a potential value as a food ingredient, either unchanged or in a hydrogenated form (Cheetham *et al.* 1982). Efficient systems for producing isomaltulose could be of substantial value to industry.

Several microorganisms, including *Protaminobacter rubrum* (Weidenhagen & Lorenz 1957), *Serratia plymuthica* (Fujii *et al.* 1983), *Klebsiella planticola* (Park *et al.* 1992, Huang *et al.* 1998), and *Erwinia rhapontici* (Cheetham 1984), can convert sucrose into isomaltulose. *E. rhapontici* is of par-

ticular interest because of its ability to produce isomaltulose in high yield. Isomaltulose is produced from sucrose by a reaction involving a single enzyme, α -glucosyltransferase. The enzyme is located in the periplasmic space and its kinetic properties have been studied (Cheetham 1984). The relevant genes from *E. rhapontici* have been cloned and characterized (Bornke *et al.* 2001).

An increased conversion yield and productivity can be achieved through optimization of the fermentation process (Cheetham *et al.* 1985). However, the conversion yield of sucrose to isomaltulose in *E. rhapontici* did not exceed 80% (Yun *et al.* 1992).

Strain improvement plays a central role in the commercial development of microbial fermentation processes. Genetic engineering has begun to make a significant contribution to this activity but random screening (mutagenesis and selection) is still a cost-effective procedure (Rowlands 1984), although it is

a tedious and time-consuming process. Therefore, an efficient screening strategy is necessary to achieve the goal within a limited time period. In this study, we demonstrate the use of a high-throughput screening system to identify a mutant strain with improved conversion yield and productivity. In preparation for possible industrial application, cells of the mutant strain were immobilized, and a packed bed reactor (PBR) was operated to confirm the stability of the conversion yield of the strain.

Materials and methods

Strain, media, and culture conditions

Erwinia rhapontici ATCC 29283 was used as a wild-type strain. The agar plating medium used in the study contained 5% (w/v) sucrose, 1% (w/v) yeast extract, 0.5% (w/v) $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 1.5% (w/v) agar. The same medium without agar was used as the flask and jar fermenter culture medium. The medium was autoclaved at 121 °C for 15 min. The pH was neither adjusted nor regulated during cultivation. A single colony from a plate culture was inoculated into a 500 ml Erlenmeyer flask containing 50 ml medium and incubated at 30 °C and 230 rpm for 12 h. The 5 l jar fermenter (3 l working vol.) was inoculated with a 5% (v/v) seed flask culture and incubated at 30 °C for 12 h with stirring at 500 rpm and aeration at 1 vvm.

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis and screening of mutants

One ml of the seed culture was centrifuged at 10 000 g for 5 min and washed with 0.1 M citrate buffer (pH 5.5) twice, and NTG solution was added to the cell suspension to give 100 mg l⁻¹. After incubation at 30 °C for 30 min with shaking, the cells were centrifuged, and the pellets were washed with 0.05 M phosphate buffer (pH 7) twice. The suspension was diluted into the same buffer and spread on an agar plate. Plates were incubated at 30 °C for 12 h. Colonies that appeared on the agar plates were inoculated into each well of Bioscreen C. After Bioscreen C culture (see below for the detail), mutants with higher conversion activity than the wild were selected using 3,5-dinitrosalicylic acid (DNS) method (Henriksson 1997) and further screened using TLC followed by densitometric analysis of the isomaltulose spot. Finally, α -glucosyltransferase activity assay was

performed using the cells from flask or jar fermenter culture to give promising mutants.

Bioscreen C culture

The culture conditions in Bioscreen C (LabSystems, Helsinki, Finland) were as follows: temperature, 30 °C; shaking intensity, medium; shaking interval, 30 min; shaking time, 20 s; preheating time, 10 min; total incubation time, 10 h; volume of culture broth, 350 μ l. After each mutant cell was inoculated into the medium, the growth was automatically checked at 600 nm for 10 h. For the *in situ* micro-fed batch culture, 100 μ l sucrose (to give 80 g l⁻¹) was fed into each well and incubated for a further 8 h.

α -Glucosyltransferase activity assay

α -Glucosyltransferase activity was analyzed by measuring isomaltulose formation using sucrose as the substrate. The assay mixture (1 ml) consisted of 40% (w/v) sucrose and 75 g l⁻¹ cell suspension (wet cell w/v) in 50 mM Tris/HCl (pH 7). The mixture was incubated at 30 °C for 90 min in a shaking water bath to allow the enzymatic reaction to take place. The tube was then incubated at 100 °C for 10 min to stop the reaction and cooled to room temperature with tap water. The reaction mixture was centrifuged and filtered for HPLC injection. One unit of α -glucosyltransferase activity was defined as the amount of enzyme producing 1 μ mol of isomaltulose per min at 30 °C, pH 7.

Immobilization and the operation of PBR

Cells grown in a 5 l jar fermenter were collected by centrifugation at 1000 g for 20 min at 4 °C and washed with 0.9% (w/v) NaCl twice. The 40% (wet cell w/v) cell suspension was fully mixed with the same volume of 5% (w/v) sodium alginate. The mixture was immobilized by extruding dropwise in 0.15 M CaCl_2 to form immobilized beads. After hardening in the CaCl_2 solution at 4 °C overnight and being treated in 55% (w/v) sucrose solution at 30 °C for 2 d, the beads were packed into the PBR columns (300 ml, 18 \times 5 cm diam.) at 30 °C and supplied with 55% (w/v) sucrose solution (pH 7) to determine the conversion yield and productivity. Sucrose solution was continuously fed into the reactor using a peristaltic pump at a flow rate adjusted to 1% (w/v) sucrose remained in the effluent solution of the column. The PBR temperature was constantly maintained by circulating water

into the column jacket with water circulator (Jeiotech, Korea).

Analytical method

Isomaltulose was determined using the following methods: i) dinitrosalicylate (DNS) method, ii) TLC and iii) HPLC. In order to easily detect a reducing sugar, isomaltulose, the DNS method was performed on the 100 well plates of Bioscreen C. Mutant strains showing higher absorbance than that of wild-type strain were analyzed again with TLC (Silica gel 60, Merck, Germany). The solvent system was 65% 2-propanol/ethyl acetate (1:1, v/v). After being developed and dried, the plate was briefly dipped into 2% (v/v) H_2SO_4 solution (in ethanol), and then heated at 110°C for 5 min. The spot size and density were quantified by Scion Image software (Scion Corp., USA). The samples from flask or jar cultures were analyzed with HPLC coupled to a refractive index detector (Waters 410, USA) and a High Performance Carbohydrate column (4.6×250 mm, Waters, Milford, MA). The mobile phase was acetonitrile/water (8:2 v/v) and the flow rate was 1.5 ml min^{-1} .

Results and discussion

Two stage micro-fed batch culture

We developed a micro-volume liquid culture and *in situ* enzyme reaction system using Bioscreen C to effectively isolate *E. rhapsontici* mutants showing an improved conversion yield of isomaltulose.

Cell growth in Bioscreen C (1st stage)

To determine the appropriate concentration of sucrose solution for micro-scale Bioscreen C culture, the *E. rhapsontici* wild-type strain was cultured in $350 \mu\text{l}$ of media with various sucrose concentrations. Sucrose, when at 20 and 30 g l^{-1} , was consumed in 14 h, and at 5 and 10 g l^{-1} was consumed in 10 and 12 h, respectively (Figure 1A). Cell growth in the medium containing $10 \text{ g sucrose l}^{-1}$ was faster than that at the other concentrations; the lag time was short, and the final cell mass was similar to that obtained with media containing 20 or $30 \text{ g sucrose l}^{-1}$. However, growth in the $5 \text{ g sucrose l}^{-1}$ medium was slow, and the final cell mass was low (Figure 1B). Considering the maximum growth rate (μ_{max}) and the final cell mass, $10 \text{ g sucrose l}^{-1}$ was chosen as the optimal sucrose concentration for Bioscreen C culture.

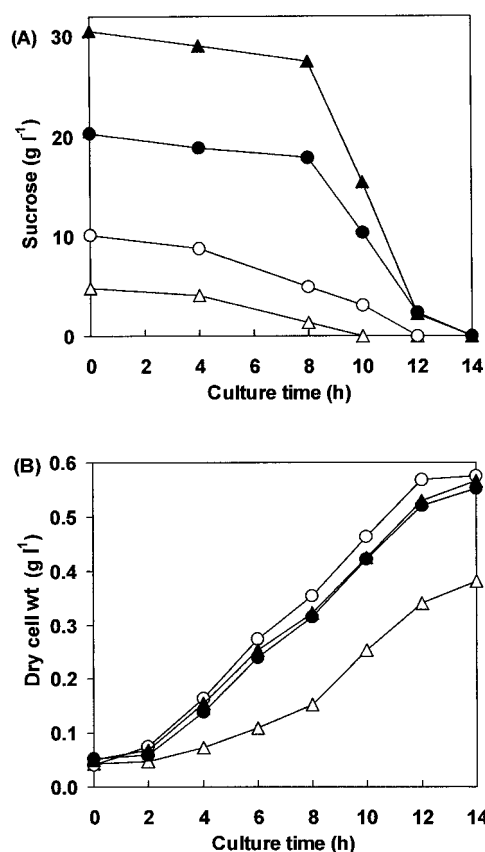


Fig. 1. Culture profiles of *E. rhapsontici* in Bioscreen C with varying sucrose concentration of 5 g l^{-1} (Δ), 10 g l^{-1} (○), 20 g l^{-1} (●) and 30 g l^{-1} (▲). (A) Sucrose consumption; (B) cell growth.

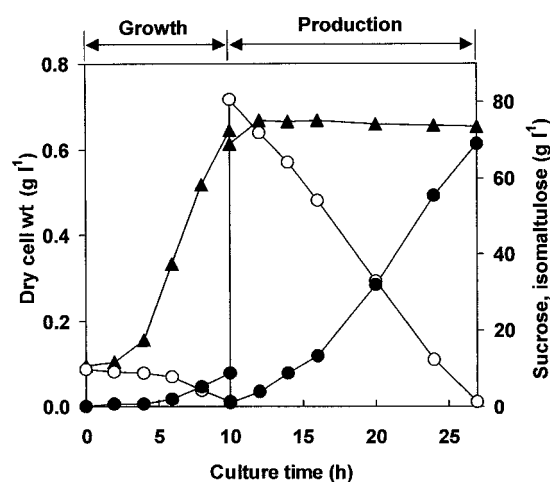


Fig. 2. Typical profiles of the two-stage micro-fed batch culture. One hundred microliter of 80 g l^{-1} (final concentration) sucrose solution was fed at the end of the growth stage, and the conversion of sucrose to isomaltulose proceeded during the production stage. Cell mass (▲), sucrose (○) and isomaltulose (●).

Table 1. Conversion yield, productivity, and α -glucosyltransferase activity of *E. rhapsodic* wild and mutant strains. α -Glucosyltransferase activity assay was performed with free cells. One unit of α -glucosyltransferase activity was defined as the amount of enzyme producing 1 μ mol of isomaltulose per min at 30 °C and pH 7. Mean values from three separate reactions \pm SD are presented.

Strain	α -Glucosyltransferase (U g ⁻¹ cell)	Yield (%)	Productivity (g l ⁻¹ h ⁻¹)
Wild	253 \pm 7.4	82 \pm 1.4	124 \pm 2.1
BN 35070	237 \pm 5.8	85 \pm 1.5	116 \pm 3.5
BN 47150	334 \pm 6.3	81 \pm 0.9	163 \pm 3.9
BN 68089	397 \pm 8.6	90 \pm 1.2	194 \pm 2.8

Sucrose conversion into isomaltulose (2nd stage)

After the cell growth stage, sucrose was directly converted into isomaltulose enzymatically in Bioscreen C and the time profiles of sugar concentration and cell growth were investigated. After the sucrose solution (final concentration 80 g l⁻¹) was fed to each well containing 350 μ l cell broth in 100-well plates, the conversion profile was obtained (Figure 2). It took about 18 h for the sucrose to be consumed and converted into isomaltulose. The cell mass did not increase as the enzymatic reaction proceeded. Eight to 10 h were required after sucrose feeding for completion of this second stage. The isomaltulose concentration was measured *in situ* using the DNS method. From these experiments, the two-stage (growth and production) micro-fed batch enzyme reaction was properly optimized and miniaturized.

Isolation of an enhanced isomaltulose producer, BN 68089

Random screening with a large number of mutants is a very laborious and tedious work. Automation and miniaturization of routine microbiological procedures, which is now widespread, improves screening efficiency and the possibility of finding desirable mutants (Rowlands 1984). In our screening system, two 100-well plates in Bioscreen C replaced two hundred test tubes, which also provided for automatic analysis of cell mass and product. Automated, miniaturized systems have a low resolution due to the small scale. However, with our screening system, we overcame this problem by using a multi-level screen that gradually enriched the proportion of potential candidates in the population. From the starting mutants in the Bioscreen C culture, the top 10% of isolates were re-

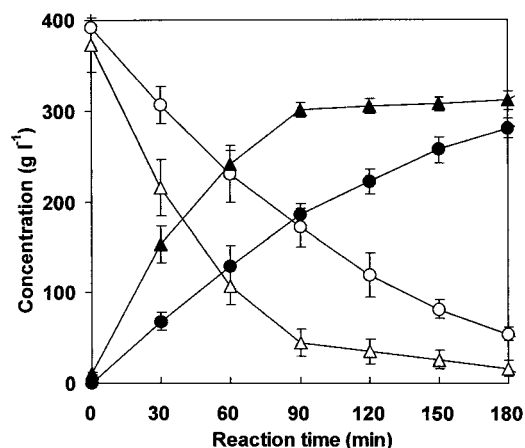


Fig. 3. Comparison of sucrose conversion profiles between *E. rhapsodic* wild strain (sucrose, \circ ; isomaltulose, \bullet) and mutant BN 68089 (sucrose, \triangle ; isomaltulose, \blacktriangle) free cells. Mean values from three separate reactions \pm SD are presented with error bar.

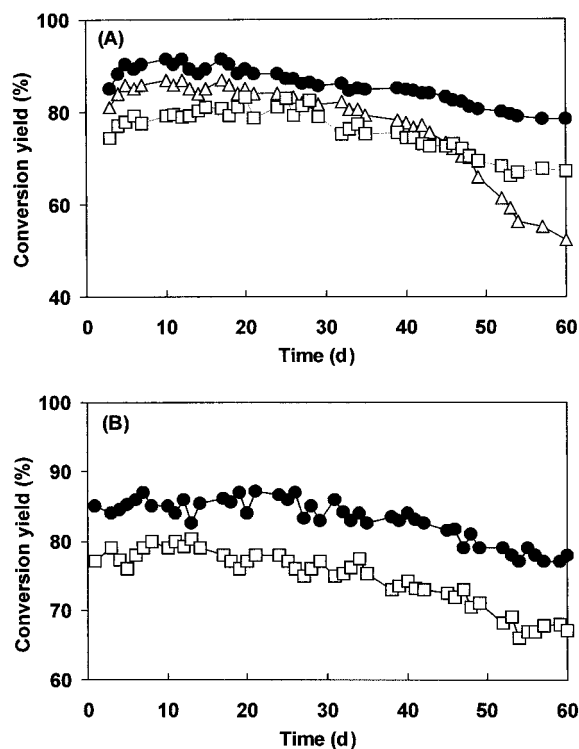


Fig. 4. Packed Bed Reactor (PBR) operation with the immobilized cells of *E. rhapsodic* wild and mutant BN 68089. (A) Conversion yields of immobilized BN 68089 at varying temperatures of 27 (\square), 30 (\bullet) and 33 °C (\triangle); (B) conversion yields of immobilized wild (\square) and mutant BN 68089 (\bullet) cells at 30 °C for 60 d.

cycled for secondary screening using TLC analysis. Then, approx. 20% of the isolates from the TLC analysis were incubated in shake flasks, and the conversion yields of the mutant cells were compared with that of the wild-type strain. Finally, promising candidates from the flask experiment were cultured in the large-scale fermentor. The use of this multi-level screening system increased the probability of isolating improved mutants. Using this system, we isolated three potentially promising mutant strains. Based on conversion yields and productivity, as shown in Table 1, we chose strain BN 68089 as the best producer of isomaltulose. Conversion profiles of the wild-type strain and the BN 68089 mutant are compared in Figure 3.

Isomaltulose production in PBR containing immobilized cells

If cells or enzymes are immobilized, their bioconversion characteristics and properties, including optimal temperature and pH, may be altered (Cheetham *et al.* 1985). To investigate the optimal temperature of the immobilized mutant cells, three PBRs were constructed and operated at different temperatures, 27, 30, and 33 °C, for 60 d (Figure 4A). The conversion yields at the initial stage, 79, 89, and 85% were decreased to 67, 79, and 57%, respectively, after 60 d. Thus 30 °C was optimal for activity and stability of PBR operation with immobilized BN 68089 cells. The optimal pH of immobilized BN 68089 was the same as that of the free cells (pH 7, data not shown). Based on these experiments, isomaltulose was produced continuously by immobilized wild-type and mutant cells in PBR. Sucrose substrate solution (550 g l⁻¹) was fed continuously to a bioreactor. The pH and temperature were maintained at 7 and 30 °C, respectively. The initial conversion yields of the immobilized wild-type and mutant cells were 79% and 87%, respectively. Productivity of the immobilized mutant cells was 144 g l⁻¹ h⁻¹, while that of the wild-type strain was 125 g l⁻¹ h⁻¹. Although the conversion yield and flow rate decreased with time, the conversion yield and productivity of BN 68089 remained about 8% and 15% higher than those of the wild-type strain for 60 d (Figure 4B).

In conclusion, we used a high-throughput screening system to isolate a mutant (BN 68089) of *E. rhapsontici* with enhanced ability to produce isomaltulose from sucrose. Immobilized and free cells of the mutant

strain, BN 68089, showed high stability and a highly enhanced conversion yield and productivity. This is the first report on the use of a high-throughput screening system on a micro-scale to obtain an isomaltulose-producing mutant strain with the potential for use in industrial production. These results and this screening system may contribute to better industrial production of isomaltulose, offer a better chance to efficiently obtain a high-producing strain, and increase the competitiveness of isomaltulose production.

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