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A New Way of Producing Isomalto-Oligosaccharide Syrup by Using the Transglycosylation Reaction of Neopullulanase

TAKASHI KURIKI,[1]* MICHIYO YANASE,[1] HIROKI TAKATA,[1] YOSHIAKI TAKESADA,[2] TADAYUKI IMANAKA,[3] AND SHIGETAKA OKADA[1]

Biochemical Research Laboratories, Ezaki Glico Co., Ltd., Nishiyodogawa-ku, Osaka 555,[1] Nagase Biochemicals Co., Ltd., Nagatano-cho, Fukuchiyama, Kyoto 620,[2] and Department of Biotechnology, Faculty of Engineering, Osaka University, Yamadaoka, Suita, Osaka 565,[3] Japan

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A new way of producing isomalto-oligosaccharide syrup from starch was developed. Isomalto-oligosaccharides contain one or more α-(1→6)-glucosidic linkages with or without α-(1→4)-glucosidic linkages. The isomalto-oligosaccharide syrups are receiving increased attention as food additives because it is thought that they help prevent dental caries and improve human intestinal microflora, acting as a growth factor for bifidobacteria. The new system for production of isomalto-oligosaccharide syrup is based on the strong α-(1→6)-transglycosylation reaction of neopullulanase. *Bacillus subtilis* saccharifying α-amylase was simultaneously used with neopullulanase to improve the yield of isomalto-oligosaccharides. The yield of isomalto-oligosaccharides was increased to more than 60%, compared with a yield of 45.0% obtained by the conventional system. To reduce the costs, the use of immobilized neopullulanase was investigated. Almost the same yield of isomalto-oligosaccharides was obtained by using immobilized neopullulanase.

Oligosaccharides enzymatically derived from sucrose, starch, or other saccharides have useful properties as food additives. Maltooligosyl-sucrose (coupling sugar) (24) and 6-*O*-α-D-glucopyranosyl-D-fructofuranose (palatinose) (23) can prevent dental caries, while fructo-oligosaccharide (neosugar) acts as a growth factor for bifidobacteria in the human intestine (7).

Isomalto-oligosaccharides are glucosyl saccharides with α-(1→6)-glucosidic linkages, such as isomaltose, panose (6^2-*O*-α-glucosyl-maltose), and isomaltotriose. They occur naturally in various fermented foods and sugars in honey (13). Isomalto-oligosaccharide syrups have been developed to prevent dental caries (26) or improve the intestinal microflora because they act as a growth factor for bifidobacteria (13). The sole method for isomalto-oligosaccharide production from starch is via two enzymatic steps (30). The first step is the hydrolysis of starch by α-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41), and β-amylase (EC 3.2.1.2) to yield accumulated maltose. The second step is the transglucosylation of the glucose moiety of maltose by α-D-glucosidase (EC 3.2.1.20) to produce α-(1→6)-linked oligosaccharides (isomalto-oligosaccharides). However, the maximum concentration of isomalto-oligosaccharides accumulated in the final reaction mixture is around 40%, and about 40% of the glucose remains in the syrup (30).

We have been studying a new type of pullulan-hydrolyzing enzyme, neopullulanase from *Bacillus stearothermophilus*. This enzyme hydrolyzes mainly the α-(1→4)-glucosidic linkages of pullulan to produce panose (16). Further study revealed that neopullulanase hydrolyzed not only α-(1→4)-glucosidic linkages but also α-(1→6)-glucosidic linkages of several branched oligosaccharides (10). The neopullulanase gene (*nplT*) has been cloned (16) and sequenced (15). We also presented evidence that one active center of neopullulanase participates in dual activity toward α-(1→4)- and α-(1→6)-glucosidic linkages (19). Furthermore, we obtained mutant enzymes which have different specificities toward α-(1→4)- and α-(1→6)-glucosidic linkages by replacing the amino acid residues which were probably involved in substrate recognition (19, 20). On the basis of this background, we recently discovered that neopullulanase catalyzed not only the hydrolyses of α-(1→4)- and α-(1→6)-glucosidic linkages but also transglycosylations to form α-(1→4)- and α-(1→6)-glucosidic linkages [these are referred α-(1→4)- and α-(1→6)-transglycosylations, respectively, in this paper] when the enzyme acted on a high concentration (10%, wt/vol) of maltotriose (31).

We have previously described a continuous production system for panose, one of the isomalto-oligosaccharides, from pullulan by immobilized neopullulanase (21). However, the cost for pullulan as a starting material is much higher than the cost for starch, and the use of the panose which is obtained from this system is consequently limited in food industries.

Within the context of the background described above, in this paper we describe a new way of producing isomalto-oligosaccharides syrup from starch by using the transglycosylation catalyzed by neopullulanase. We investigated the conditions which maximized the isomalto-oligosaccharide concentration in the syrup. The immobilization of neopullulanase and its use in the system are also described.

MATERIALS AND METHODS

Enzyme. Neopullulanase was prepared from *Bacillus subtilis* NA-1 (*arg-15 hsmM hsrM* Amy^- Npr^-) carrying recombinant plasmid pPP10 (Tc^r $nplT^+$ [structural gene of neopullulanase from *B. stearothermophilus* TRS40]) (16). The 36-h broth from L medium (9) at 37°C (neopullulanase activity, 2.7 U/ml of broth) was used for enzyme purification. Partially purified neopullulanase (specific activity, 33.5 U/mg of protein) was obtained by ammonium sulfate treatment (the fraction was collected at final saturations between 35 and

* Corresponding author.

60%), as previously described (21). This preparation produced almost a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and was used for all experiments described in this paper. The bacterial saccharifying type of α-amylase (EC 3.2.1.1) was prepared from *B. subtilis* as described previously (4). Taka-amylase (α-amylase from *Aspergillus oryzae*) was purchased from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. The bacterial liquefying type of α-amylase from *B. subtilis* was obtained from Nagase Biochemicals Co., Ltd., Kyoto, Japan. Glucoamylase (exo-1,4-α-D-glucosidase) (EC 3.2.1.3) was obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

Substrate and reagents. Maltotriose, maltotetraose, isomaltose, panose, and pullulan were purchased from Hayashibara Co., Ltd., Okayama, Japan. 6^3-*O*-α-Glucosyl-maltotriose was obtained from Sigma Chemical Co., St. Louis, Mo. Soluble starch (Pinedex no. 1) was obtained from Matsutani Chemical Industry Co., Ltd., Hyogo, Japan. Isopanose (6-*O*-α-maltosyl-glucose) was prepared from the hydrolysate of pullulan with isopullulanase as described by Sakano et al. (29). 6^2-*O*-α-Maltosyl-maltose was prepared as described elsewhere (10). Unless otherwise specified, the sources of all other reagents have been described previously (17, 18).

Assay of enzyme activity. Neopullulanase activity was assayed by measuring the amount of reducing sugars obtained from pullulan as a substrate at 50°C as described previously (10). α-Amylase activity was assayed by measuring the amount of reducing sugars obtained from starch as a substrate. The reaction mixture contained 1% soluble starch in 0.1 M sodium acetate buffer (pH 6.0) and the enzyme. The reaction was stopped after an appropriate incubation period at 50°C by the addition of 3,5-dinitrosalicylic acid reagent (10); 1 U of neopullulanase or α-amylase activity was defined as the amount of enzyme which released 1 μmol of reducing sugar as glucose per minute under the assay conditions described above.

Analysis of reaction products. The sugar composition of reaction products was quantitatively analyzed by high-performance liquid chromatography by using a LiChrosorb NH_2 column (Merck AG, Darmstadt, Germany) as previously described (31). For the analysis and isolation of reaction products, paper chromatography was also carried out in the ascending mode as described previously (10). To elucidate the structure of the oligosaccharides, proton nuclear magnetic resonance (NMR) spectra and methylation analysis (5) of the purified samples were also employed as described previously (31). Glucoamylase treatment of a hydrogenated sample (31) was used to ascertain the structures of branched oligosaccharides with a degree of polymerization (DP) of 3.

Protein assay. Protein concentration was measured with a bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.), using bovine serum albumin as the standard.

Immobilization of neopullulanase on porous chitosan beads. Chitopearl BCW series beads (Fuji Spinning Co., Ltd., Tokyo, Japan) were porous chitosan beads that were cross-linked with an aliphatic or aromatic compound and used as a carrier for enzyme immobilization. The carrier beads (diameter, 0.3 mm) were washed with deionized water and equilibrated with 20 mM sodium phosphate buffer (pH 7.0). A 1-g portion of the carrier beads and 6 ml of a solution containing neopullulanase (5 to 180 U) in the same buffer were mixed in a 50-ml Erlenmeyer flask. The mixture was shaken with a reciprocal shaker (120 strokes per min; stroke width, 3 cm) for 30 to 150 min at room temperature to immobilize the enzyme on the carrier. After centrifugation (1,000 × *g*, 10 min), the neopullulanase activity and protein concentration of the supernatant were assayed. The apparent activity of the immobilized enzyme was defined as follows (33): apparent activity of the immobilized enzyme (in units per gram) = (enzyme activity applied) − (enzyme activity in the supernatant). Immobilized enzyme prepared by the method described above was washed with 20 mM sodium phosphate buffer (pH 7.0) until no protein was detected in the washing buffer solution. The activity of the immobilized enzyme was assayed as follows. A 10-mg portion (wet base) of immobilized enzyme was suspended in 0.5 ml of 20 mM sodium phosphate buffer (pH 6.0) in a 50-ml Erlenmeyer flask, and the preparation was incubated with 4.5 ml of a 1.11% pullulan solution in 20 mM sodium phosphate buffer (pH 6.0) at 50°C for 10 min with reciprocal shaking (120 strokes per min; stroke width, 3 cm). No displacement of the enzyme from the carrier was observed at pH 6.0. After incubation, 200 μl of the supernatant of the mixture was withdrawn, and the amount of reducing sugars was measured by adding 3,5-dinitrosalicylic acid reagent as described above. This activity was defined as the exhibited activity.

RESULTS

Products from maltotriose obtained by transglycosylation of neopullulanase. Since maltotriose is the simplest substrate of the hydrolysis reaction of neopullulanase (10), we analyzed the products obtained from maltotriose by the transglycosylation catalyzed by neopullulanase. The reaction mixture (50 μl) contained 10% (wt/vol) maltotriose in 10 mM phosphate buffer (pH 6.0) and 0.025 U of neopullulanase (5 U/g of substrate). In addition to the formation of glucose and maltose as hydrolysis products, branched oligosaccharides with DP of 2, 3, and 4 (oligosaccharides B2, B3, and B4, respectively) were simultaneously detected on the paper chromatogram (Fig. 1). The retention times during high-performance liquid chromatography of oligosaccharides B2, B3, and B4 were identical to those of isomaltose, isopanose (6-*O*-α-maltosyl-glucose)–panose (6^2-*O*-α-glucosyl-maltose), and 6^2-*O*-α-maltosyl-maltose, respectively (Fig. 2). The difference in the retention times of isopanose and panose was indistinguishable by this system. These branched oligosaccharides were purified by preparative paper chromatography, and their structures were analyzed by proton NMR and methylation analysis (31). The proton NMR spectra of oligosaccharides B2 and B4 coincided with those of isomaltose and 6^2-*O*-α-maltosyl-maltose, respectively. The results of a methylation analysis of oligosaccharide B4 supported this result. 2,3,4,6-Tetra-*O*-methylglucitol acetate, 2,3,4-tri-*O*-methylglucitol acetate, and 2,3,6-tri-*O*-methylglucitol acetate were detected at a molar ratio of 1:1:2 after methylation of oligosaccharide B4. These results clearly indicated that the branched oligosaccharides with DP of 2 and 4 were isomaltose and 6^2-*O*-α-maltosyl-maltose, respectively (Fig. 1). The proton NMR spectrum of oligosaccharide B3 was like that of a mixture of isopanose and panose. 2,3,4,6-Tetra-*O*-methylglucitol acetate, 2,3,4-tri-*O*-methylglucitol acetate, and 2,3,6-tri-*O*-methylglucitol acetate were detected at a molar ratio of 1:1:1 after methylation of oligosaccharide B3. Therefore, oligosaccharide B3 includes isopanose and/or panose and no 6^1-*O*-α-glucosyl-maltose. In this context, we employed glucoamylase treatment of hydrogenated products to confirm the structure of oligosaccharide B3 (Fig. 3), as described previously (31). We identified the structures of the branched oligosaccharides with a DP of 3 as a mixture of isopanose and panose, because both isomaltitol

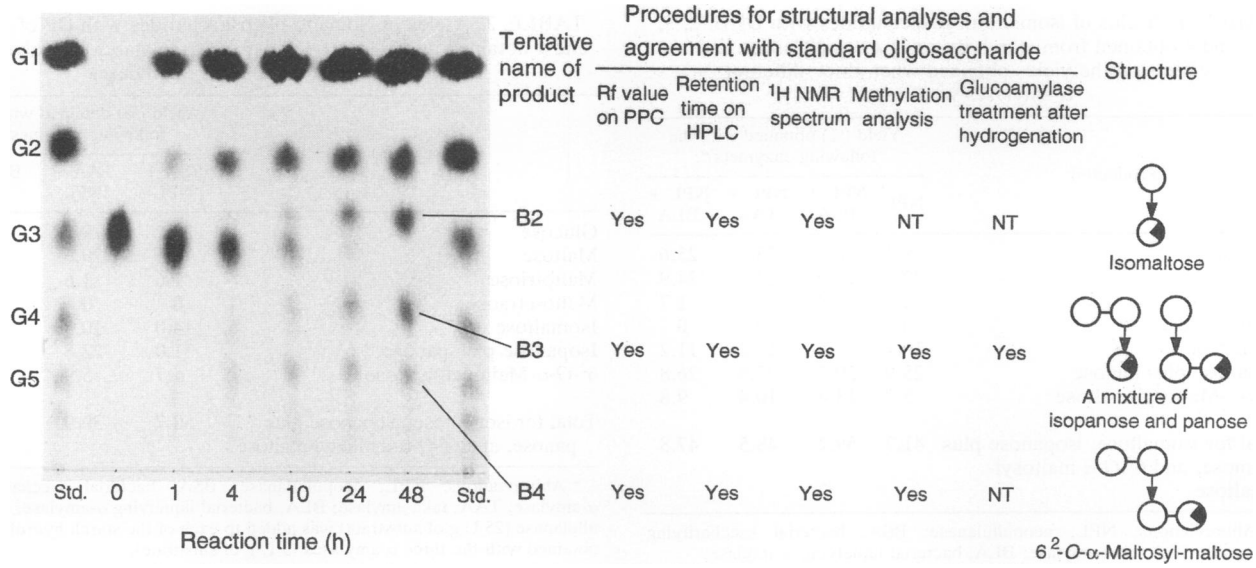

FIG. 1. Chromatographic characterization of the products from maltotriose by transglycosylation of neopullulanase: paper chromatographic analysis. Abbreviations: G1, G2, G3, G4, and G5, glucose, maltose, maltotriose, maltotetraose, and maltopentaose, respectively; B2, B3, and B4, branched oligosaccharides with DP of 2, 3, and 4, respectively; Std., standard mixture of oligosaccharides; PPC, HPLC, and ^{1}H NMR, paper chromatography, high-performance liquid chromatography, and proton NMR, respectively; NT, not tested. Symbols: ○, glucose; ◔, glucose with reducing end; ——, α-(1→4) linkage; ↓, α-(1→6) linkage.

and maltitol were detected by high-performance liquid chromatography after glucoamylase digestion of hydrogenated branched oligosaccharides with a DP of 3 (Fig. 3). Since almost equal amounts of isomaltitol and maltitol were detected by the analysis (data not shown), we concluded that nearly equal amounts of isopanose and panose existed in the mixture.

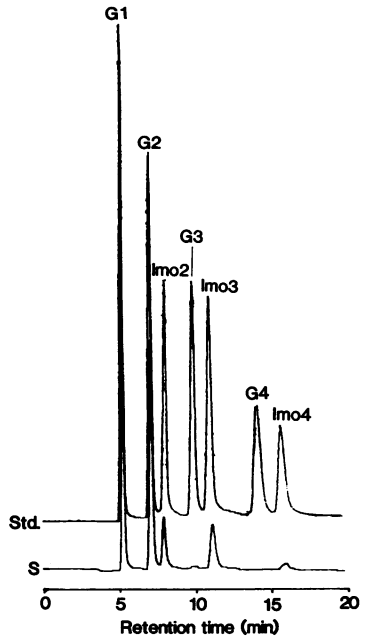

FIG. 2. Analysis of products obtained from maltotriose by transglycosylation with neopullulanase: high-performance liquid chromatography. The elution profiles for the standard oligosaccharide mixture (Std.) (each oligosaccharide was present at a concentration of 0.5%) and the sample (S) (total concentration, 1.0%) are shown. A reaction mixture incubated for 24 h (Fig. 1) was used as the sample. Abbreviations: Imo2, Imo3, and Imo4, isomaltose, isopanose, 6^2-*O*-α-maltosyl-maltose, respectively. For other abbreviations see the legend to Fig. 1.

Production of a new type of isomalto-oligosaccharide syrup by neopullulanase from starch. A neopullulanase solution (25 U/g of substrate) was added to a 30% soluble starch solution in 20 mM sodium phosphate buffer (pH 6.0), and the mixture was incubated at 50°C for 32 h. The reaction products were analyzed by high-performance liquid chromatography (Table 1). More than 40% of the branched oligosaccharides with DP of 2, 3, and 4 accumulated in the products (Table 1). The structures of branched oligosaccharides were confirmed by proton NMR, methylation, and glucoamylase treatment analyses, as described above. The branched oligosaccharides with DP of 2, 3, and 4 were isomaltose, a mixture of isopanose and panose, and 6^2-*O*-α-maltosyl-maltose, respectively.

Although neopullulanase could hydrolyze pullulan and

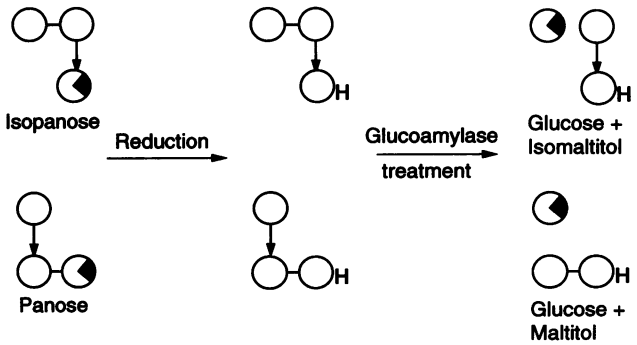

FIG. 3. Identification of branched oligosaccharides with a DP of 3 by glucoamylase treatment after hydrogenation. Symbols: ○H, sorbitol; ○, glucose; ◔, glucose with reducing end; ——, α-(1→4) linkage; ↓, α-(1→6) linkage.

TABLE 1. Yields of isomalto-oligosaccharides with DP of 2, 3, and 4 obtained from starch by using neopullulanase and effects on the yields obtained when three different α-amylases were used

Product(s)	Yield (%) obtained with the following enzyme(s)[a]:			
	NPL	NPL + BSA	NPL + TAA	NPL + BLA
Glucose	29.4	24.3	25.2	25.6
Maltose	27.2	15.0	24.3	24.9
Maltotriose	1.6	1.4	1.9	1.7
Maltotetraose	0	0	0	0
Isomaltose	10.1	15.5	12.2	11.2
Isopanose plus panose	25.9	30.3	25.9	26.8
6^2-*O*-α-Maltosyl-maltose	5.7	13.4	10.4	9.8
Total for isomaltose, isopanose plus panose, and 6^2-*O*-α-maltosyl-maltose	41.7	59.2	48.5	47.8

[a] Abbreviations: NPL, neopullulanase; BSA, bacterial saccharifying α-amylase; TAA, taka-amylase; BLA, bacterial liquefying α-amylase.

TABLE 2. Yields of isomalto-oligosaccharides with DP of 2, 3, and 4 obtained by using neopullulanase from starch hydrolysates produced by three different α-amylases

Product(s)	Yield (%) obtained with the following enzymes[a]:		
	BSA + NPL	TAA + NPL	BLA + NPL
Glucose	30.2	29.7	26.4
Maltose	17.5	29.6	27.1
Maltotriose	1.6	1.6	1.4
Maltotetraose	0	0	0
Isomaltose	14.0	10.3	11.0
Isopanose plus panose	31.0	22.8	24.3
6^2-*O*-α-Maltosyl-maltose	5.7	5.9	9.7
Total for isomaltose, isopanose plus panose, and 6^2-*O*-α-maltosyl-maltose	50.7	39.0	45.0

[a] Abbreviations: NPL, neopullulanase; BSA, bacterial saccharifying α-amylase; TAA, taka-amylase; BLA, bacterial liquefying α-amylase. Neopullulanase (25 U/g of substrate) was added to each of the starch hydrolysates obtained with the three α-amylases (5 U/g of substrate).

various oligosaccharides, it hydrolyzed starch less efficiently (16). Therefore, we tested the dosage effect of the addition of three kinds of α-amylases which had different patterns of action on starch for improvement of the yield of isomalto-oligosaccharides from starch (Table 1). The bacterial saccharifying α-amylase hydrolyzes starch to produce glucose, maltose, and maltotriose, whereas the bacterial liquefying α-amylase hydrolyzes starch to produce glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose (32). Taka-amylase hydrolyzes starch to produce maltose and glucose (25). Each α-amylase (5 U/g of substrate) was used in conjunction with neopullulanase to act simultaneously on starch. All three α-amylases increased the yield of isomalto-oligosaccharides with DP of 2, 3, and 4 (Table 1); bacterial saccharifying α-amylase from *B. subtilis* notably improved the yield of these compounds (the yield increased from 41.7 to 59.2%) (Table 1).

However, the yield of isomalto-oligosaccharides with DP of 2, 3, and 4 was not so improved when the starch hydrolysates of these three α-amylases were used as the substrates for the transglycosylation of neopullulanase (Table 2). In this context, we analyzed the sugar compositions of the starch hydrolysates obtained with these three α-amylases and confirmed that they contained only glucose and α-(1→4)-linked oligosaccharides, such as maltose, maltotriose, and maltotetraose, along with α-limit dextrin (data not shown).

It is important to reduce the cost of production of isomalto-oligosaccharide syrups from the viewpoint of industrial applications. Enzyme cost is an important factor. The reaction temperature is also important for accelerating the enzyme reaction. Generally, industrial starch enzymes should be used at temperatures above 55°C (8, 21, 28). We investigated these parameters and found that 2 U of neopullulanase per g of substrate and 5 U of bacterial saccharifying α-amylase from *B. subtilis* per g of substrate were enough to obtain the same yield of isomalto-oligosaccharides with DP of 2, 3, and 4 as the yield described above (about 60%) when the reaction was run for 92 h. The reaction temperature could be raised to 58°C (data not shown).

Pilot scale production of a new type of oligosaccharide syrup from starch. On the basis of the laboratory-scale results, we produced a new type of oligosaccharide syrup on a pilot scale. Neopullulanase (2 U/g of substrate) and bacterial saccharifying α-amylase (5 U/g of substrate) from *B. subtilis* were added to 10 liters of a 30% soluble starch solution, and the mixture was incubated at 58°C for 92 h. Figure 4 shows the time course of sugar composition of the syrup as analyzed by high-performance liquid chromatography. The syrup contained isomaltose, a mixture of isopanose and panose, and 6^2-*O*-α-maltosyl-maltose as branched oligosaccharides with DP of 2, 3, and 4, respectively. Isomalto-oligosaccharides with DP of 5 and more were also obtained (Fig. 4 and Table 3). We analyzed the structure of the isomalto-oligosaccharides with DP of 5 and more and confirmed that they had one or more α-(1→6)-glucosidic linkages. The precise structures will be described elsewhere.

The total yield of isomalto-oligosaccharides was 60.6%, a

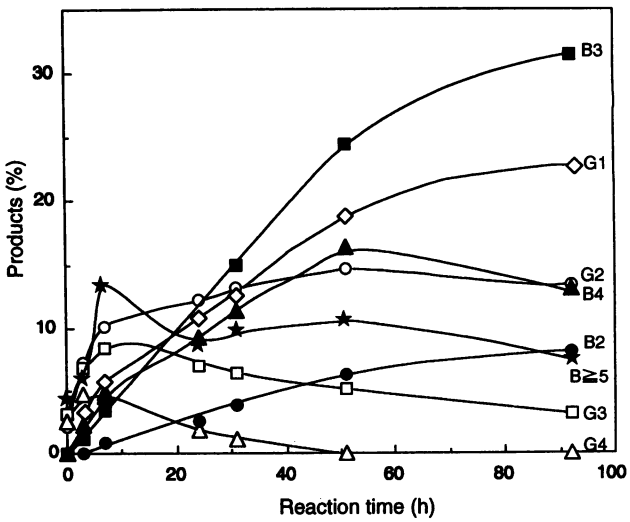

FIG. 4. Time course of sugar composition of products obtained from starch on a pilot scale. Symbols: ◇, glucose (G1); ○, maltose (G2); □, maltotriose (G3); △, maltotetraose (G4); ●, isomaltose (B2); ■, isopanose and panose (B3); ▲, 6^2-*O*-α-maltosyl-maltose (B4); ★, isomalto-oligosaccharides with DP of 5 and more (B ≥ 5).

TABLE 3. Comparison of the yields of isomalto-oligosaccharides and total branched oligosaccharides obtained with the new system and the conventional system

Product(s)	Yield (%) obtained with:	
	New system	Conventional system[a]
Glucose	22.8	40.5
Maltose	13.4	6.7
Maltotriose	3.2	0.8
Maltotetraose	0	0
Isomaltose	8.1	16.9
Isopanose plus panose	31.4	12.5
Isomaltotriose	0	3.4
6^2-*O*-α-Maltosyl-maltose	13.1	0
Isomalto-oligosaccharides with DP of ≧5	8.0	
Other branched oligosaccharides with DP of 2[b]	0	4.7
Other branched oligosaccharides with DP of 3[b]	0	2.3
Branched oligosaccharides with DP of 4[c]		8.9
Branched oligosaccharides with DP of 5[c]		3.3
Total for isomalto-oligosaccharides[d]	60.6	32.8–45.0
Total for branched oligosaccharides[e]	60.6	52.0

[a] Data are from reference 30.
[b] Oligosaccharides branched with an α-(1→2)- or α-(1→3)-glucosidic linkage.
[c] Oligosaccharides branched with an α-(1→6)-, α-(1→2)-, or α-(1→3)-glucosidic linkage.
[d] Total for oligosaccharides branched with an α-(1→6)-glucosidic linkage(s).
[e] Total for oligosaccharides branched with an α-(1→6)-, α-(1→2)-, or α-(1→3)-glucosidic linkage(s).

value much higher than the total yield obtained by the conventional system (maximum yield, 45.0%) (Table 3). A small amount of the other branched oligosaccharides which have α-(1→2)- or α-(1→3)-glucosidic linkages, such as kojibiose (2-*O*-α-glucosyl-glucose) or nigerose (3-*O*-α-glucosyl-glucose), are also produced by α-(1→2)- or α-(1→3)-transglycosylation catalyzed by α-D-glucosidase in the conventional system (30). The functions (such as prevention of dental caries or improvement of intestinal microflora) of the α-(1→2)- or α-(1→3)-linked oligosaccharides are not clear yet. However, the yield produced by the new system (60.6%) was significantly higher than the yield produced by the conventional system (52.0%) even when the two systems were compared on the basis of the amount of total branched oligosaccharides produced (Table 3). The glucose in oligosaccharide syrups often prevents their use in food industries. The concentration of glucose obtained by our system (22.8%) was much lower than the concentration obtained by the conventional system (40.5%) (Table 3).

Production of isomalto-oligosaccharide syrup by the immobilized neopullulanase. To reduce enzyme costs, enzyme immobilization has been widely used industrially (1, 11, 12, 27). We therefore evaluated immobilized neopullulanase for the production of the isomalto-oligosaccharide syrup. Porous chitosan beads have been used recently for the immobilization of exomaltotetraohydrolase to produce maltotetraose (12). Since porous chitosan beads have large pore sizes and a large surface area, they are suitable for the immobilization of the enzymes acting on large polymeric substrates, such as starch (12). Therefore, we chose porous chitosan beads as a carrier for the immobilization of neopullulanase.

We tested four kinds of cross-linked porous chitosan beads, BCW-2503, BCW-2603, BCW-3003, and BCW-3503, which had different functional groups. BCW-2603 beads exhibited the highest activity of neopullulanase after immobilization (data not shown). BCW-2603 beads have tertiary amine groups for ionic bonding with proteins. Figure 5 shows the relationship between the amount of neopullulanase applied and the apparent and exhibited activities of the enzyme immobilized on the chitosan beads. The exhibited activity of the immobilized enzyme reached a maximum at a concentration of around 80 U/g of carrier. The difference between the apparent and exhibited activities of the immobilized enzyme was remarkably large when more than 80 U/g of carrier was used (Fig. 5). The relationship between the ratio of immobilization and the shaking time during immobilization was also examined. The apparent and exhibited activities reached a constant value after more than 60 min of treatment (data not shown). On the basis of the results described above, we set up standard conditions for the immobilization of neopullulanase on porous chitosan beads as follows: (i) about 70 U of neopullulanase was applied per 1 g of carrier; and (ii) the shaking time during immobilization treatment was 90 min at room temperature. We obtained 50 U of exhibited activity per g of carrier of the immobilized neopullulanase under these standard conditions.

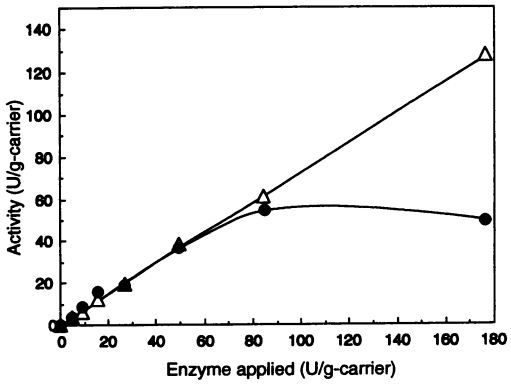

FIG. 5. Relationship between amount of enzyme applied and apparent (△) and exhibited (●) activities of immobilized neopullulanase.

The characteristics of the immobilized neopullulanase were compared with those of native enzyme (16). The optimum pH and optimum temperature for reaction and the effects of temperature on the stability of the immobilized neopullulanase were similar to those of the native enzyme. However, the pH range for stability of immobilized neopullulanase (pH 5.5 to 8.0) was narrower than that of native enzyme (pH 5.5 to 9.0) (data not shown).

The immobilized neopullulanase (2 U/g of substrate) was added to a 30% soluble starch solution simultaneously with bacterial saccharifying α-amylase (5 U/g of substrate) from *B. subtilis*, and the mixture was incubated at 58°C for 92 h. This mixture yielded an isomalto-oligosaccharide syrup which contained more than 60% of the total amount of isomalto-oligosaccharides (Table 4).

DISCUSSION

It is widely known that bifidobacteria can be classified as beneficial intestinal bacteria for humans in terms of both health and nutrition (22). Isomalto-oligosaccharides have been shown to act as a growth factor for bifidobacteria by in

TABLE 4. Yields of total isomalto-oligosaccharides from starch obtained by using immobilized neopullulanase

Product	Yield (%)
Glucose	20.4
Maltose	14.4
Maltotriose	4.1
Maltotetraose	0
Isomaltose	8.2
Isopanose plus panose	25.3
6^2-*O*-α-Maltosyl-maltose	14.6
Isomalto-oligosaccharides with DP of ≧5	12.8
Total for isomalto-oligosaccharides	61.0

vitro and in vivo experiments (13). Our approach to producing isomalto-oligosaccharide syrup is superior to the conventional methods for the following reasons. First, the yield of isomalto-oligosaccharides from starch obtained by our method (more than 60%) is significantly higher than the yields of previously described methods (maximum yield, 45%). Second, our method produces less glucose (about 20%) than the other methods (about 40%). Since glucose can be utilized by all intestinal bacteria, not only beneficial bacteria like bifidobacteria but also unfavorable bacteria like clostridia (2, 3), the syrup made by our method may be more effective for improving the intestinal microflora than the syrup made by the other methods. Furthermore, the syrup containing less glucose may be better from the point of view of prevention of dental caries because glucose is fermentable by *Streptococcus mutans*, which synthesizes water-insoluble glucan and produces acids that lead to localized decalcification of the enamel surfaces of teeth (6, 14). Third, our system is simpler than the conventional one; our method uses only two enzymes, neopullulanase and α-amylase, whereas the conventional method uses four enzymes, α-amylase, pullulanase, β-amylase, and α-D-glucosidase (30).

We are presently studying the continuous production system of the isomalto-oligosaccharide syrup by using column-immobilized neopullulanase. Further work to examine precisely the effect of the new isomalto-oligosaccharide syrup on the improvement of the human intestinal microflora by in vitro and in vivo tests is also in progress.

REFERENCES

1. **Bachler, M. J., G. W. Strandberg, and K. L. Smiley.** 1970. Starch conversion by immobilized glucoamylase. Biotechnol. Bioeng. **12**:85–92.
2. **Benno, Y., K. Endo, N. Shiragami, K. Sayama, and T. Mitsuoka.** 1987. Effect of raffinose intake on human fecal microflora. Bifidobacteria Microflora **6**:59–63.
3. **Dombou, M., H. Yamamoto, H. Nakajima, K. Tomita, and T. Komaki.** 1991. Utilization of 4-β-galactosyl-lactose by the intestinal bacteria. Denpun Kagaku **38**:365–367.
4. **Fukumoto, J., and S. Okada.** 1963. Studies on bacterial amylase. Amylase types of *Bacillus subtilis* species. J. Ferment. Technol. **41**:427–434.
5. **Hakomori, S.** 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55**:205–208.
6. **Hamada, S., and M. Torii.** 1980. Interaction of glucosyltransferase from *Streptococcus mutans* with various glucans. J. Gen. Microbiol. **116**:51–59.
7. **Hidaka, H.** 1988. Fructooligosaccharides, p. 228–230. *In* The Amylase Research Society of Japan (ed.), Handbook of amylase and related enzymes. Pergamon Press, Oxford.
8. **Hyun, H. H., and J. G. Zeikus.** 1985. General biochemical characterization of thermostable pullulanase and glucoamylase from *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. **49**:1168–1173.
9. **Imanaka, T., M. Fujii, I. Aramori, and S. Aiba.** 1982. Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmid between *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol. **149**:824–830.
10. **Imanaka, T., and T. Kuriki.** 1989. Pattern of action of *Bacillus stearothermophilus* neopullulanase on pullulan. J. Bacteriol. **171**:369–374.
11. **Kimura, T., M. Ogata, M. Yoshida, and T. Nakakuki.** 1988. Continuous production of maltotetoraose using immobilized *Pseudomonas stutzeri* amylase. Biotechnol. Bioeng. **32**:669–676.
12. **Kimura, T., M. Yoshida, K. Oishi, M. Ogata, and T. Nakakuki.** 1989. Immobilization of exo-maltotetraohydrolase and pullulanase. Agric. Biol. Chem. **53**:1843–1848.
13. **Komoto, T., F. Fukui, H. Takaku, Y. Machida, M. Arai, and T. Mitsuoka.** 1988. Effect of isomalto-oligosaccharides on human fecal flora. Bifidobacteria Microflora **7**:61–69.
14. **Krasse, B., and J. Carlsson.** 1970. Various types of streptococci and experimental caries in hamsters. Arch. Oral Biol. **15**:25–32.
15. **Kuriki, T., and T. Imanaka.** 1989. Nucleotide sequence of the neopullulanase gene from *Bacillus stearothermophilus*. J. Gen. Microbiol. **135**:1521–1528.
16. **Kuriki, T., S. Okada, and T. Imanaka.** 1988. New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. J. Bacteriol. **170**:1554–1559.
17. **Kuriki, T., J.-H. Park, and T. Imanaka.** 1990. Characteristics of thermostable pullulanase from *Bacillus stearothermophilus* and the nucleotide sequence of the gene. J. Ferment. Bioeng. **69**:204–210.
18. **Kuriki, T., J.-H. Park, S. Okada, and T. Imanaka.** 1988. Purification and characterization of thermostable pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. Appl. Environ. Microbiol. **54**:2881–2883.
19. **Kuriki, T., H. Takata, S. Okada, and T. Imanaka.** 1991. Analysis of the active center of *Bacillus stearothermophilus* neopullulanase. J. Bacteriol. **173**:6147–6152.
20. **Kuriki, T., H. Takata, S. Okada, and T. Imanaka.** 1992. Alteration of the specificity of neopullulanase by protein engineering. Denpun Kagaku **39**:75–84.
21. **Kuriki, T., M. Tsuda, and T. Imanaka.** 1992. Continuous production of panose by immobilized neopullulanase. J. Ferment. Bioeng. **73**:198–202.
22. **Mitsuoka, T.** 1982. Recent trends in research on intestinal flora. Bifidobacteria Microflora **1**:3–24.
23. **Nakajima, Y.** 1988. Palatinose, p. 230–232. *In* The Amylase Research Society of Japan (ed.), Handbook of amylase and related enzymes. Pergamon Press, Oxford.
24. **Okada, S., and S. Kitahata.** 1975. Preparation and some properties of sucrose-bound syrup. Nippon Syokuhin Kogyo Gakkaishi **22**:420–424.
25. **Omich, K., and T. Ikenaka.** 1988. Taka-amylase A (*Aspergillus oryzae* α-amylase), p. 32–37. *In* The Amylase Research Society of Japan (ed.), Handbook of amylase and related enzymes. Pergamon Press, Oxford.
26. **Ooshima, T., T. Fujiwara, T. Takei, A. Izumitani, S. Sobue, and S. Hamada.** 1988. The caries inhibitory effects of GOS-sugar in vitro and rat experiments. Microbiol. Immunol. **32**:1093–1105.
27. **Park, Y. K., and D. C. Lima.** 1973. Continuous conversion of starch to glucose by an amyloglucosidase-resin complex. J. Food Sci. **38**:358–359.
28. **Saha, B. C., and J. G. Zeikus.** 1989. Novel highly thermostable pullulanase from thermophiles. Trends Biotechnol. **7**:234–239.
29. **Sakano, Y., M. Kogure, T. Kobayashi, M. Tamura, and M. Suekane.** 1978. Enzymatic preparation of panose and isopanose from pullulan. Carbohydr. Res. **61**:175–179.
30. **Takaku, H.** 1988. Anomalously linked oligosaccharides mixture ("Alo mixture"), p. 215–217. *In* The Amylase Research Society

of Japan (ed.), Handbook of amylase and related enzymes. Pergamon Press, Oxford.

31. **Takata, H., T. Kuriki, S. Okada, Y. Takesada, M. Iizuka, N. Minamiura, and T. Imanaka.** 1992. Action of neopullulanase: neopullulanase catalyzes both hydrolysis and transglycosylation at α-(1→4)- and α-(1→6)-glucosidic linkages. J. Biol. Chem. **267:**18447–18452.
32. **Yamamoto, T.** 1988. Bacterial α-amylase (liquefying and saccharifying types) of *Bacillus subtilis* and related bacteria, p. 40–45. *In* The Amylase Research Society of Japan (ed.), Handbook of amylase and related enzymes. Pergamon Press, Oxford.
33. **Yoshida, M., T. Kimura, M. Ogata, and T. Nakakuki.** 1988. Immobilization of the exo-maltotetraohydrolase and some properties of the enzyme. J. Jpn. Soc. Starch Sci. **35:**245–252.