J. Microbiol. Biotechnol. (2001), **11**(4), 700–703

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Highly Branched Glucooligosaccharide and Mannitol Production by Mixed Culture Fermentation of *Leuconostoc mesenteroides* and *Lipomyces starkeyi*

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Received: January 18, 2001
Accepted: June 1, 2001

Abstract The influence of process conditions on highly branched glucooligosaccharides production by mixed culture of *Leuconostoc mesenteroides* ATCC 13146 and *Lipomyces starkeyi* ATCC 74054 was studied. We divided the batch culture fermentations into two groups according to inoculation method. One-point inoculation was performed by coinoculation of *L. mesenteroides* and *L. starkeyi* at the ratio of 10 to 1, and two-point inoculation by *L. mesenteroides* inoculation first and *L. starkeyi* inoculation after *L. mesenteroides* grew to the end of the log phase of growth. Two-point inoculation improved the yield of oligosaccharide by 1.5 to 2.0 fold more than one-point inoculation. In this process, the highest yield of oligosaccharides (48% of theoretical yield) and productivity (0.85 g/l/h) were obtained with starch as an initial substrate for *L. starkeyi* growth. The estimated composition of the end product consisted of 31.5% oligosaccharides, 17.6% dextran, and 46.5% mannitol.

Key words: Glucooligosaccharide, *Leuconostoc mesenteroides*, *Lipomyces starkeyi*, mixed culture fermentation, functional foods

The production of functional foods providing health benefit is one of the fastest growing fields in the food industry [6]. Of the most popular functional food, non- or partially digestible glucooligosaccharides (GOS) encourage the growth of *Bifidobacteria* and limit the growth of competing pathogenic organisms [16, 23]. Branched GOS are more beneficial because they may be more resistive to be utilized by harmful microorganisms in the intestine [2]. Extracellular dextransucrase (EC 2.4.1.5) of *L. mesenteroides* ATCC 13146 or its constitutive mutant *L. mesenteroides* B-742CB synthesizes two different structures of dextran [12-14]. Their structures exhibit the highest percent of branches possible [8]. One is a water soluble dextran containing 50% of an α-(1 → 6) glucose backbone and 50% of α-(1 → 3) glucose branched linkages [21]. The other dextran is composed of 83% of an α-(1 → 6) glucose backbone and 17% of α-(1 → 3) glucose branched linkages [22]. This enzyme or other dextransucrase synthesizes GOS by successive transfers of glucosyl units from sucrose to the nonreducing end of acceptors such as maltose, isomaltose, and oligosaccharides [1, 11-14, 20]. When *L. mesenteroides* ATCC 10830 (a strain producing a linear type of dextran) and *L. starkeyi* ATCC 74054 (a derepressed and constitutive mutant producing dextranase) grow together in a simple batch fermentation, they produce a linear type of clinical size dextran and GOS [1, 7, 9, 15]. This process may be valid since metabolites of both strains generate an acceptor reaction that limits the polymerization of dextran [9]. Therefore, we hypothesized the mixed culture of *L. starkeyi* ATCC 74054 and *L. mesenteroides* ATCC 13146 synthesize highly branched GOS of small size [1, 7, 15]. In this work, we developed a simple process for the production of highly branched GOS from "a mixed culture" fermentation.

Bacterial culture was maintained on a mineral medium agar slant supplemented by 0.5% (w/v) yeast extract and 3% (w/v) sucrose. The mineral medium consisted of 3.0 g KH_2PO_4, 0.01 g $FeSO_4 \cdot H_2O$, 0.01 g $MnSO_4 \cdot 4H_2O$, 0.2 g $MgSO_4.7H_2O$, 0.01 g NaCl, and 0.05 g $CaCl_2$ per 1-l deionized water. The medium pH was adjusted to 6.0 prior to sterilization. Working culture of *L. mesenteroides* was prepared by incubating bacterium in a test tube containing 10 ml of mineral medium supplemented with 2% (w/v) sucrose and 0.5% (w/v) yeast extract and subcultured every two days. *L. starkeyi* was maintained on mineral medium agar slants fortified by 0.5% (w/v) $(NH_4)_2SO_4$ and 2% (w/v) soluble starch. The medium pH was adjusted to 4.5 prior to sterilization. Working culture was prepared by incubating yeast in a 500-ml Erlenmeyer flask containing mineral

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medium supplemented with 0.5% (w/v) $(NH_4)_2SO_4$ and 1% (w/v) soluble starch. The flask was incubated in a shaker at 30°C and 200 rpm. The culture was subcultured weekly.

For growth determination, the total amount of biomass was estimated by turbidity determined at 660 nm, and the turbidity was calibrated to cell dry weight by a method developed by Otts and Day [17]. For the quantification of dextran, cells after harvesting were removed by centrifugation at 10,400 ×g for 20 min (Dupont Sorvall RC5C: Newtown, CT, U.S.A.). The supernatant solution was adjusted to pH 7.0. The dextran was precipitated by slow addition of ethanol (95%) to the supernatant with stirring (final ethanol volume is two-thirds of the total volume). The solution was allowed to stand for 2 h and then centrifuged at 3,000 ×g for 20 min. The precipitated dextrans were assayed by the phenol-sulfuric acid method [5]. For quantification of oligosaccharides, oligosaccharides in the cell-free supernatant adjusted to pH 7.0 were separated using an ion-exchange column (Aminex HPX-42A: Bio-Rad, CA, U.S.A.) of HPLC (Millipore Co., MA, U.S.A.). Sample (50 µl) filtered through 0.22 µm membrane was injected. The column temperature was 85°C. The flow rate of an eluent (deionized water) was 0.4 ml/min. The detector was a refractometer (410 Differential Refractometer: Millipore, MA, U.S.A.). The size of oligosaccharides produced was estimated by comparing the retention time of peaks of sample with standard isomaltooligosaccharides mixtures (BioEurope, Toulouse, U.S.A.). Isomaltose, pannose, and isomaltopentose were used as internal standards (Sigma, MO, U.S.A.). The eluted total amount of oligosaccharides were collected and assayed by the phenol-sulfuric assay. The amount of sucrose, fructose, and mannitol were determined by HPLC using an ion exchange/size exclusion column (Sugarpak: Millipore, MA, U.S.A.) under the following conditions: 90°C column temperature, 0.5 ml/min eluent (deionized water) flow rate, and 50 µl injection volume. Sorbitol, 5% (w/v), as an internal standard was added to the cell-free supernatant before injection.

Batch fermentations were conducted in a bioreactor (2-l Bio Flo II Fermentor: New Brunswick Scientific, NJ, U.S.A.) with a working volume of 1.2 l. Culture temperature and pH were adjusted to 28°C and 5.2, respectively, and controlled during the fermentation. The agitation and aeration were 200 rpm and 0.1 vvm, respectively. Four batch fermentaions were established based on different inoculation time and sucrose supply. In batch A, 1% (v/v) *L. mesenteroides* and 10% (v/v) *L. starkeyi* from the working culture were inoculated at the start of fermentation (culture medium: mineral medium containing 0.5% yeast extract and 15% sucrose). In batch B, the procedure followed batch A except that 30% (w/v) sucrose was continuously added at a rate of 11.2 g/l/ h until the total sucrose concentration reached 15%. In batch C, *L. starkeyi* grew first at mineral medium containing 0.5% (w/v) $(NH_4)_2SO_4$ and 2% (w/v) sucrose until the growth of *L. starkeyi* reached mid-log phase ($absorbance_{660}$=5.0), and then 1% (v/v) *L. mesenteroides* was inoculated, followed by sucrose addition as per batch B. Procedure of batch D followed the way of batch C except that *L. starkeyi* grew first with mineral medium containing 0.5% (w/v) $(NH_4)_2SO_4$ and 2% (w/v) soluble starch. All the fermentations continued until sucrose was utilized completely.

The dextransucrase of *L. mesenteroides* ATCC 13146 effects an acceptor reaction in the presence of maltose. This reaction leads to the synthesis of the same GOS series as that obtained with the *L. mesenteroides* ATCC 10830 dextransucrase [20]. In contrast, increasing the ratio of sucrose to maltose synthesizes α-(1 → 3) glucose branched GOS [19]. We performed the production of highly branched GOS by balancing the interaction of mixed cultures without adding extra acceptor, because the size determination relates closely with the result of both dextranase of *L. starkeyi* and acceptor reaction in the mixed culture fermentation

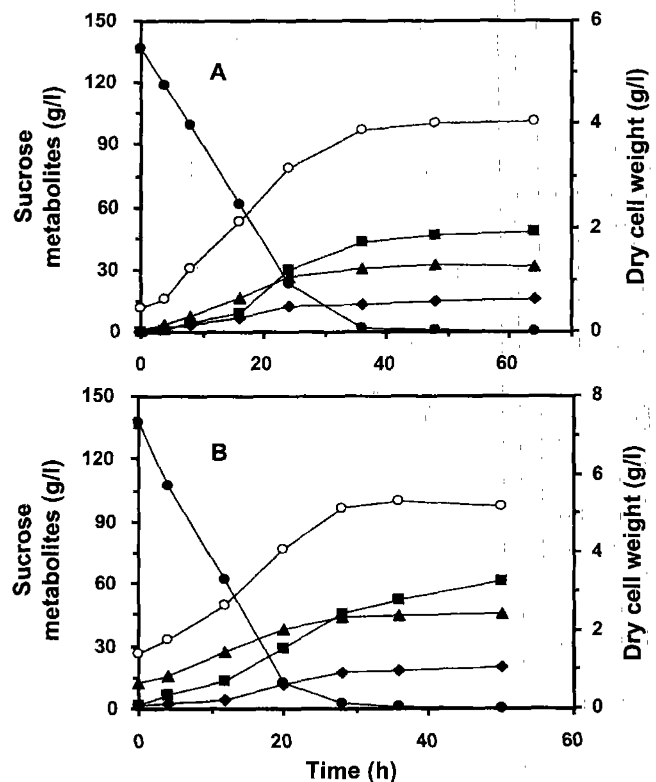

Fig. 1. Time course of glucooligosaccharides production by mixed culture of *L. mesenteroides* ATCC 13146 and *L. starkeyi* ATCC 74054 (one-point inoculation).

Batch A; 1% (v/v) *L. mesenteroides* and 10% (v/v) *L. starkeyi* were inoculated at the start of fermentation (Fig. 1A). Batch B: procedure followed batch A except that 30% (w/v) sucrose was continuously added at a rate of 11.2 g/l/h (Fig. 1B). All the amount of sugars and metabolites on the figures were obtained from the end of sucrose addition. ●: sucrose; ○: biomass; ■: mannitol; ◆: oligosaccharide; ▲: dextran.

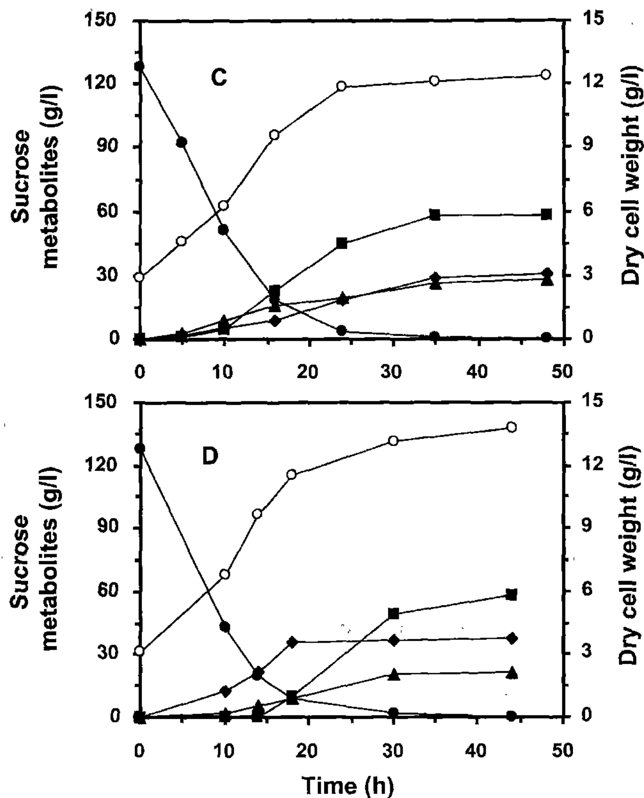

Fig. 2. Time course of glucooligosaccharide production by mixed culture of *L. mesenteroides* ATCC 13146 and *L. starkeyi* ATCC 74054 (two-point inoculation).

Batch C: *L. starkeyi* grew first, then 1% (v/v) *L. mesenteroides* was inoculated and followed by the sucrose addition (Fig. C). Batch D: followed batch C except that the yeast grew first at 2% (w/v) soluble starch (Fig. D). All the amount of sugars and metabolites on figures were obtained from the end of sucrose addition. ●: sucrose; ○: biomass; ■: mannitol; ◆: oligosaccharide; ▲: dextran.

[7, 9, 15]. The kinetic relationship between the growth and the GOS production in batch fermentation is illustrated in Fig. 1 for one-point inoculation and Fig. 2 for two-point inoculation. The yield and productivity of GOS varied depending on the method of inoculation and sucrose supply used. In batch A, the GOS production was related to the dextran formation and closely associated with biomass increase (Fig. 1A). At the highest yield of oligosaccharides (27%), the converted amount of sucrose to oligosaccharides was half of that of sucrose to dextran. Because the amount of oligosaccharides synthesized is dependent on how much acceptors can receive glucose from sucrose in mixed culture, sucrose was continuously supplied at the rate of 11.2 g/l/h in batch B to reduce the rate of dextran formation and bacterial growth at the beginning of fermentation. It was found that total biomass was increased by about 25%, although there was no notable difference in the oligosaccharides production pattern between the two batches. Productivity of oligosaccharides in batch B was 18% higher than that of batch A (Table 1). The conversion of sucrose to dextran and to mannitol was not significantly different between the two batches. It indicates that the metabolic and energetics properties of *L. mesenteroides* were not altered in the two batches [3, 4]. We set up two-point inoculation strategies to allow *L. starkeyi* to grow first to maximize the dextranase production prior to the inoculation of *L. mesenteroides* and the sucrose supply (Figs. 2A and 2B). *L. starkeyi* grew to late log phase when it presumably maximized dextranase production (data not shown), and then sucrose was added at rate of 11.2 g/l/h together with *L. mesenteroides*. This process was equivalent to coinoculation of *L. starkeyi* to *L. mesenteroides* with the ratio of 66 to 1 based on the number of cells. The sucrose was completely consumed after 36 h from inoculation without a noticeable lag phase, and the biomass was increased exponentially to 12.9 g/l at the end of the fermentation (Fig. 2A). This process ensured a 39% oligosaccharides yield: its value was about 56% higher than that of batch A or of batch B and made the sucrose supplied to be equivalently shared by dextran (36%) and oligosaccharide (39%) synthesis. In batch D, we used starch as a starting carbon source for *L. starkeyi* because dextranase production is greater with starch than with sucrose, and the other process condition was the same as

Table 1. Fermentaion parameters related to production of glucooligosaccharides (GOS), dextran, and mannitol by mixed cultures fermentation of *L. mesenteroides* ATCC 13146 and *L. starkeyi* ATCC 74054.

Fermentations[a]	Biomass (g/l)	Yield[b]			Productivity		
		GOS	Dextran	Mannitol	GOS	Dextran	Mannitol
Batch A	4.1	27	54	86	0.32	0.65	1.01
Batch B	5.2	25	58	80	0.39	0.90	1.21
Batch C	12.4	39	36	76	0.66	0.60	1.25
Batch D	13.8	48	27	74	0.85	0.48	1.26

[a]Batch A: 1% bacteria and 10% (v/v) yeast were inoculated at the start of the fermentation. Batch B: procedure followed batch A except that 30% (w/v) sucrose was continuously added at a rate of 11.2 g/l/h until total sucrose concentration reached 15%. Batch C: yeast grew first until its growth reached the mid-log phase, then 1% (v/v) bacterial culture was inoculated, followed by the sucrose addition like batch B. Batch D: followed batch C except that the yeast grew first at 2% (w/v) soluble starch.

[b]Oligosaccharide yield: oligosaccharide (g/l)/[0.47 sucrose (g/l)]×100; mannitol yield: mannitol (g/l)/[0.53 sucrose (g/l)]×100; and dextran yield: dextran (g/l)/[0.47 sucrose (g/l)]×100.

that of batch C [10, 15]. This process improved the yield of GOS by 82%, 92%, or 26% higher than the yield in batch A, B, or C, respectively. In summary, the two-point inoculation process (Batches C and D) improved the yields of GOS production by 1.5 to 2.0 fold compared to that of one-point inoculation. The highest yield (48%) of GOS production was determined when starch was used as an initial substrate for *L. starkeyi*. The productivity (0.85 g/l/h) was about 1.23-fold higher than batch C when sucrose was used alone. The estimated composition of the end product in batch D consisted of 31.5% GOS, 17.6% dextran, and 46.5% mannitol. This GOS may be a commercially desirable product as either a functional food or food ingredients.

Acknowledgment

This work was supported by the Korea Research Foundation Grant. (KRF-Y00-290).

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