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Production of Glucooligosaccharides and Mannitol from *Leuconostoc mesenteroides* B-742 Fermentation and its Separation from Byproducts

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Abstract *Leuconostoc mesenteroides* B-742 fermentations with maltose as an acceptor were tested for glucooligosaccharides and mannitol co-production. *Leuconostoc* oligosaccharides were produced that were oligomers with a size range of DP 2 to 7 and were primarily DP 3, 4, 5, and 6, containing mainly α-1,4 and α-1,6 linkages. Maltose was linked to the reducing end of the isomaltosyl residues. The Ca^{2+} form of cation-exchange column could separate glucooligosaccharides from byproducts.

Key words: Glucooligosaccharides, acceptor reaction, *Leuconostoc*

Prebiotics are compounds, mainly carbohydrates, which are not degraded by human digestive enzymes, pass though the digestive tract to the colon, and selectively stimulate proliferation and/or activity of desired populations of bacteria *in situ* [13, 16]. Some examples of prebiotics are lactulose, inulin, and a range of oligosaccharides [5, 6].

The term oligosaccharide is applied to short sugar oligomers, joined by glycosidic bonds, ranging from two to ten monosaccharide units. Of the many oligosaccharides, fructooligosaccharides have attracted serious commercial interest as prebiotics. In the United States, these compounds are marketed commercially as Raftilose and Nutraflora. Another group of oligosaccharides that have potential as prebiotics are branched α-glucooligosaccharides (GOS) and isomaltooligosaccharides [1, 8, 12]. These oligosaccharides are produced by either hydrolysis of starch or transglycosylation with an enzyme called dextransucrase and an acceptor from sucrose. Enzymatic hydrolysis of starch requires multi-steps such as gelatination, liquefaction, saccharification, and purification, and leaves always some mono- and/or disaccharides in the final product [24]. In the presence of an efficient acceptor such as maltose, the reaction of a dextransucrase can be shifted towards oligosaccharide synthesis. The molecular weight and polydispersity of the enzyme products are dependent upon the specific dextransucrase, the sucrose-to-acceptor ratio, and the characteristics of the intermediate in the reaction [15, 20]. Acceptor reactions using dextransucrase from *L. mesenteroides* NRRL B-1299 produce α-glucooligosaccharides containing one or more D-glucopyranosyl branch units linked via α-1,2 glycosidic bonds, when maltose is supplied as an acceptor [7, 17, 19]. However, this process also requires a purified dextransucrase and leaves fructose that is not used from sucrose in the final product. *L. mesenteroides* NRRL B-742 (ATCC 13146) produces a highly branched dextran exhibiting a comb-like structure, containing as high as 50% α-1,3 linkages, but no α-1,4 linkages [4, 20]. The branches are single glucose molecules in length [18]. Oligosaccharides synthesized by the dextransucrase from this bacterium had α-1,6 backbones with α-1,3 and/or α-1,4-branched side chains, when maltose was used as an acceptor [18]. These dextran and oligosaccharides demonstrate resistance to endodextranase [18, 22, 23]. GOS from fermentation by this strain also showed its potential as a prebiotic [2, 3]. Yoo *et al.* [25, 26] previously showed that this strain can also produce mannitol and dextran (or GOS with the mixed culture fermentation) together when sucrose is supplied. The goals of the present research are to develop an industrial pilot-scale production and separation method for oligosaccharides and mannitol, using a *L. mesenteroides* ATCC 13146 whole cell fermentation, with maltose as an acceptor.

Leuconostoc mesenteroides B742 (ATCC 13146) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.), and maintained on MRS agar slants (Difco, Detroit, MI, U.S.A.) at 4°C and transferred monthly. Bacterial growth was measured by turbidimetry at 660 nm, calibrated against cell dry weight. Batch fermentations were conducted in a 2-l or 30-l BioFlo fermentor (New Brunswick Scientific Co.) with a working

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volume of 1.0 l or 20.0 l. The media had the following composition: sucrose (100 g/l), maltose (50 g/l), yeast extract (5 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $FeSO_4 \cdot 7H_2O$ (0.01 g/l), NaCl (0.01 g/l), $MnSO_4 \cdot 7H_2O$ (0.01 g/l), $CaCl_2$ (0.05 g/l), KH_2PO_4 (3 g/l), with pH 7.2. Fermentors were inoculated from late log phase flask cultures at 1.0–2.5% of working volume. Fermentations were conducted at pH 6.5, 28°C, and 200 rpm. After harvesting, cells were removed by centrifugation at 10,400 ×*g* for 20 min (Dupont Sorvall RC5C, Newtown, CT, U.S.A.) or by membrane filtration. Activated charcoal (5 g/l, Sigma Chem. Co., St. Louis, MO, U.S.A.) and Celite 545 (1 g/l, Fisher Scientific, Fair Lawn, NJ, U.S.A.) were added to cell-free culture broth and mixed at 50°C for 20 min. The broth was then filtered through No. 6 filter paper (Whatman International Ltd., Maidstone, England) to remove the charcoal and celite. The filtered broths were desalted using ion-exchange columns filled with an anion-exchange resin (Amberite 92RF) in the hydroxide form and a cation-exchange resin (Amberite 252RF) in the hydrogen form (Rohm and Haas, Philadelphia, PA, U.S.A.). The eluents were concentrated by vacuum evaporation (Brinkmann Instrument Inc., Westbury, NY, U.S.A.) to 65% solids. Mannitol crystallized upon cooling of the concentrates and was removed by decantation. The oligosaccharide fractions were reconcentrated by vacuum evaporation. Separation and qualitative identification of oligosaccharides was conducted using TLC. Whatman K6F silica gel plates of sizes 10×20 cm obtained from Fisher Scientific (Chicago, IL, U.S.A.). A homologous series of isomaltodextrins (DP 1-10) was kindly donated by Dr. D. Kim at Chonnam National University in Korea. Maltopentaose, maltohexaose, maltoheptaose, panose, and isomaltotriose (Sigma Chem. Co., St. Louis, MO, U.S.A.) were used as standards. The plates were developed at ambient temperature, using a mixture of solvents [acetonitrile, ethyl acetate, propanol, and water in volume (ml) proportions of 85:20:50:70]. After development was complete, the plates were dried, and the carbohydrates were visualized using a spray of an ethanol solution containing 0.3% (w/v) α-naphthol and 5% (v/v) H_2SO_4. GOS were identified by comparing their chromatographic behavior with those of the standards. Different cation resins (Na, K, Ca forms) were tested for separation of oligosaccharides from other fermentation products. Resins (Duolite CR-1320, Rohm and Haas, Philadelphia, PA, U.S.A.) in glass jacketed columns [10 mm (inner diameter)×100 mm (length), working volume 70 ml] were regenerated using 5% solutions of NaCl, KCl, or $CaCl_2$. Temperature of the water eluent was 92°C, and the circulating water temperature for the glass jacket was 80°C. Injection volume was 1 ml (15 Brix° GOS). Highly pure oligosaccharides were separated from the remaining solution after mannitol crystallization at 4°C using a cation-exchange column, illustrated in Fig. 4 (in calcium form; Duolite CR-1320, Rohm and Haas, Philadelphia, PA, U.S.A.). The detector was a differential refractometer (Waters). High-performance ion chromatography using a CarboPac MA1 column (Dionex, Sunnyvale, CA, U.S.A.) and a pulsed amperometric detector (PAD, Dionex) were used to analyze glucose, fructose, sucrose, mannitol, and maltose concentrations in solution. The samples were eluted at 0.4 ml·min^{-1} with 0.48 M NaOH solution. Oligosaccharide concentrations were calculated from peak areas on high-performance liquid chromatography on an Aminex-HPX-87K Bio-Rad column (Bio-Rad Lab. Hercules, CA, U.S.A.) run at 85°C with K_2HPO_4 as eluent, at a constant flow rate of 0.5 ml/min, using glucose as a standard. The oligosaccharides (DP 1 to DP 8) were analyzed using a DPX 250 (63 MHz ^{13}C) system. The chemical shifts were expressed in ppm relative to the methyl signal of acetone in deuterium oxide solvent which was used as an internal standard at δ=29.92 ppm. The various signals were assigned as described by Seymour *et al.* [22] and Remaud *et al.* [18].

GOS production was completed by the late log phase of about 10 h post-inoculation, and concentrations were stable. Sucrose disappeared rapidly during the log phase of growth, the depletion corresponding to the transition point to the stationary phase. Upon sucrose depletion, the accumulated fructose was epimerized to mannitol. Fructose concentrations peaked at the end of the log phase, and then decreased slowly. Mannitol production occurred through the lag phase into the stationary phase and was linked to the fructose concentration, where the rate of fructose disappearance was the inverse of the rate of mannitol formation. Oligosaccharide production was associated with cell growth. The conversion of fructose to mannitol was associated with fructose concentration. The weight percent yield of oligosaccharide and the conversion of fructose to mannitol were 82%, and 71% of theoretical values, respectively (Fig. 1). High sucrose utilization in the early stages of the fermentation fuels

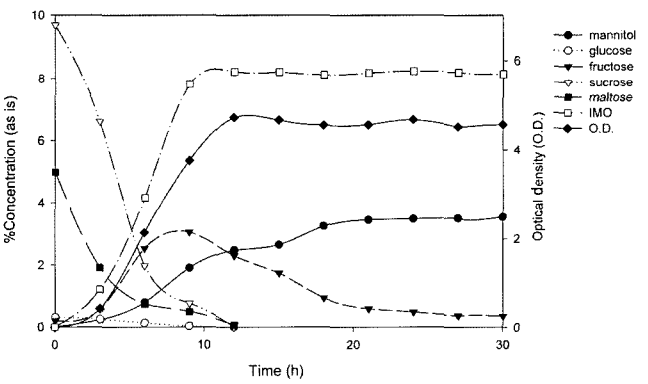

Fig. 1. Batch fermentation profile of *L. mesenteroides* as a function of time; IMO, produced oligosaccharides.
% yield of oligosaccharide=(product produced×100/[(160×mole of sucrose consumed)+(342×mole of maltose consumed)]).

oligosaccharide production and cell growth. Kim *et al.* [14] reported 86.7% of isomaltodextrin production, when the ratio of sucrose to maltose was 2.5. In their report, the amount of dextran and acceptor-products increased as the concentration of sucrose or the ratio of maltose to sucrose increased. At a ratio of sucrose to maltose of 2.5, their end products had a more complex composition of oligosaccharides and significant amount of leftover fructose. In this work, some of the fructose, liberated intracellularly by sucrose phosphorylase, was probably utilized by the cells, accounting for the observed conversion efficiency of 71%. As sucrose was depleted, the fructose was metabolized and mannitol was produced. Fructose that accumulates is a byproduct of sucrose cleavage during synthesis of oligosaccharides. Dols *et al.* [9, 10] proposed that *L. mesenteroides* possesses an efflux mechanism for fructose, such that the cell excretes fructose and then reassimilates the fructose *via* a phosphoenolpyruvate (PEP)-dependent fructose phosphotransferase mechanism [11]. This accumulation of fructose has been observed in other *Leuconostoc* species. [10, 11, 23]. The intracellular and NADH-dependent microbial enzyme, D-mannitol dehydrogenase (E.C. 1.1.1.67), catalyzes the reversible reduction of fructose to mannitol

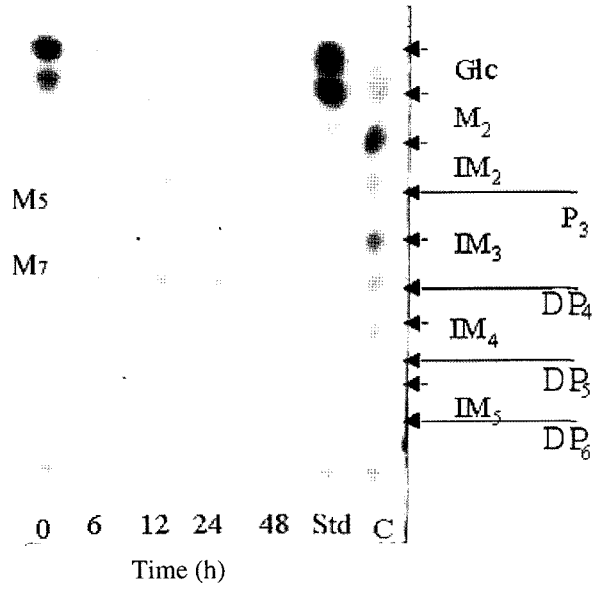

Fig. 2. TLC of glucooligosaccharide production as a function of time.

M_5, maltopentaose; M_7, maltoheptaose; Std, a mixture of glucose and maltose; C, Commercial isomaltooligosaccharides (Wako Pure Chemical Industry Ltd., Osaka, Japan); Glc, glucose; M_2, maltose; IM_2, isomaltose; P_3, panose; IM_3, isomaltotriose; DP_4, isomaltosyl-glucooligomer DP4; IM_4, isomaltotetraose; DP_5, isomaltosyl-glucooligomer DP5; IM_5, isomaltopentaose; DP_6, isomaltosyl-glucooligomer DP6. The solvent system was a mixture of acetontrile, ethyl acetate, propanol, and water [in volume (ml) proportions of 85:20:50:70]. After development was complete, the plates were dried and the carbohydrates were visualized using a spray of an ethanol solution containing 0.3% (w/v) α-naphthol and 5% (v/v) H_2SO_4. Samples were taken during fermentation at the time indicated.

[21]. Thin layer chromatography (TLC) clearly showed the course of GOS production (Fig. 2). As the fermentation proceeded, mono- and disaccharides disappeared as the higher DP polysaccharides were formed. By 24 h, all mono- and disaccharides had been converted to higher polymer oligosaccharides. This oligomer composition was compared with a commercial oligosaccharide product of known composition. The *Leuconostoc* oligosaccharides were a series of oligomers with a size range of DP 2 to 7 and were primarily DP 3, 4, 5, and 6 (Fig. 2). The degree of polymerization (DP) for most prebiotic oligosaccharides falls in the range of DP 2 to 8 [16]. The oligosaccharides, based on their Rf values on TLC, did not match with either maltodextrin or isomaltodextrins. The migration of the *Leuconostoc* oligosaccharides was faster than the equivalent isomaltodextrins (α-1,6 linkage), but slower than the equivalent maltodextrins (α-1,4 linkage). Structural analysis of these GOS by C^{13} NMR showed that they were linked by α-1.4 and α-1.6 linkages (Fig. 3). Two closely separated peaks were present at 100.44 ppm, α-(1→4). They were also observed in the spectrum of maltose, corresponding to a glucose molecule linked to a reducing residue of maltose by an α-(1→4). This implies that an α-(1→4) linkage is located at the reducing end of isomaltosyl residues containing α-1.6 linkages. The peaks corresponding to the region of 98.0–99.0 ppm showed α-(1→6) linked residues. Resonances for α-(1→3) bonds, around 100.0 and 80.6–81.2 ppm, were not present. Remaud *et al.* [18] reported that, when maltose was used as an acceptor, synthesized

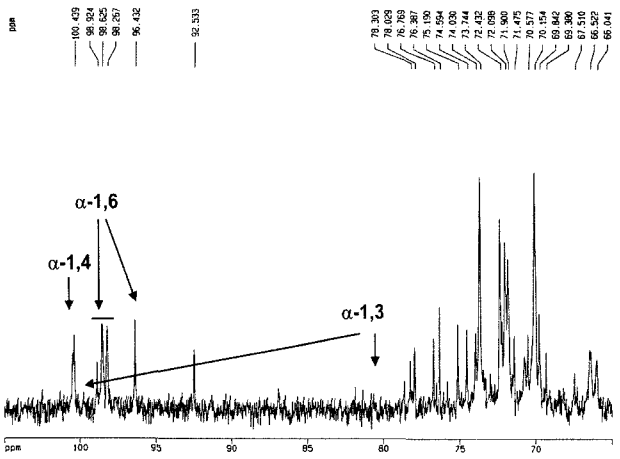

Fig. 3. ^{13}C NMR of *Leuconostoc* glucooligosaccharides.

Oligosaccharides were analyzed using a DPX 250 (63 MHz ^{13}C) system. The chemical shifts are expressed in ppm relative to the methyl signal of acetone in a deuterium oxide solvent, which was used as an internal standard at δ=29.92 ppm. The various signals were assigned as described by Seymour *et al.* [22] and Remaud *et al.* [18]; 85–105 ppm; the anomeric region (mainly 97–103 ppm, as there is only an infinitesimal proportion of reducing sugar in any of the polymers), 70–75 ppm; C-2,3,4, and 5, 60–70 ppm; bonded and non-bonded C-6 atoms, 75–85 ppm (signals of bonded C-2, C-3, C-4, C-5).

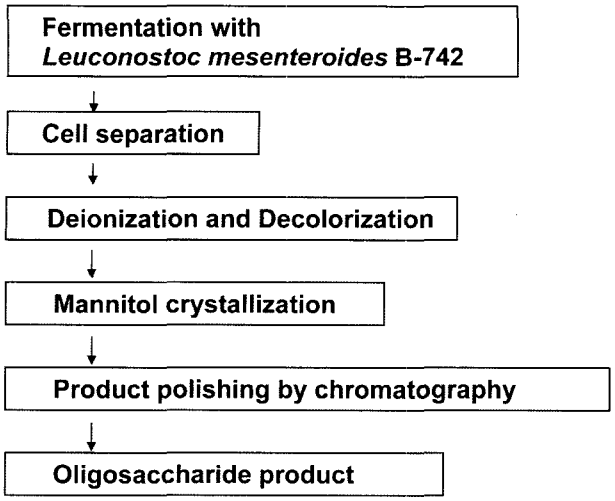

Fig. 4. Flow chart for glucooligosaccharide production.
Cell separation was done by centrifugation at 10,400 ×*g* for 20 min. Deionization and decolorization were carried out by ion exchange. Anion-exchange resin was in hydroxide form and cation-exchange resin was in hydrogen form. Mannitol crystallized out at 4°C. Ca^{2+} cation resin was used for removing residual impurities.

oligosaccharides by dextransucrase from this strain had α-1,6 backbones with α-1,3 and/or α-1,4-branched side chains. However, it seems that the GOS produced in this study contained mainly α-1,4 and α-1,6 linkages, and maltose was linked to the reducing end of the isomaltosyl residues, judged by ^{13}C NMR data. It is quite possible that the ratio of sucrose to maltose (S/M=2) used was not high enough to synthesize α-1,3 linkage. The fermentation broth after cell separation contained essentially oligosaccharides, mannitol, and some organic acids. Most of the mannitol (86.4%, data not shown) was recovered without further processing by crystallization at 4°C. Mannitol harvested showed high purity (>99.0%, calculated by HPLC peak area, Fig. 5A). An impure oligosaccharide was left in the broth after crystallization. Yoo *et al.* [25] also investigated co-production of GOS and mannitol by mixed culture fermentation. They obtained a mixture of GOS, mannitol, and dextran. However, it is necessary to separate mannitol from the oligosaccharides if they are to be used as prebiotics, since mannitol can also be a carbon source for microorganisms such as *E. coli* or *Salmonella* species. Because oligosaccharides are neutral polymers and the other components (acids, color compounds, and salts) are charged molecules, cation resins were used for separation. Neither K^+ nor H^+ cation columns clearly separated oligosaccharides from mannitol and other products, whereas Ca^{2+} cation columns produced two well-separated peaks (data not shown). Oligosaccharides eluted in the first peak from a Ca^{+2} resin, followed by a mixture of mannitol and organic acids such as acetic and lactic acid (data not shown). Smaller mannitol molecules were eluted

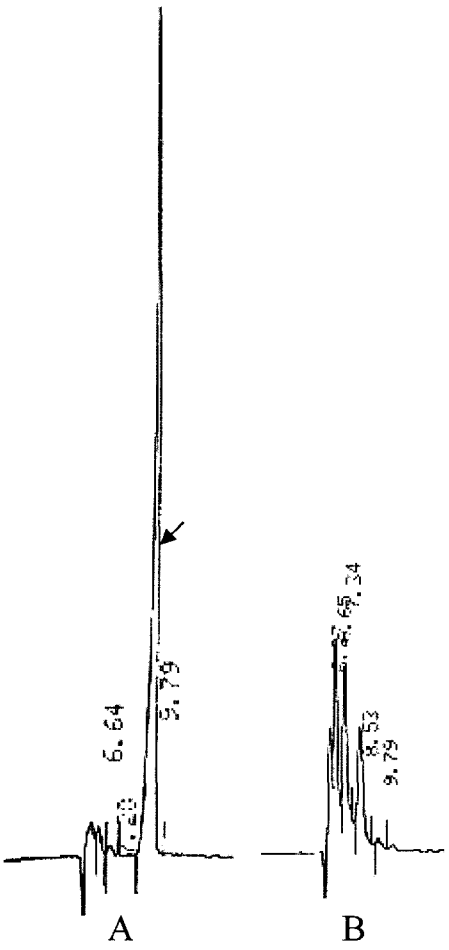

Fig. 5. HPLC chromatogram of the products.
A. Mannitol product (>99.0% purity). B. Oligosaccharide product (>98.8% purity). The arrows indicate the mannitol peak.

after oligosaccharides, in part because of partial ionization of mannitol at pH 6.5. Based on these results, a process for producing a pure oligosaccharide product was developed (Fig. 4). Using the suggested process, a purified oligosaccharides solution (>98.8% w/w) was obtained (Fig. 5B). The purified product contained only trace amounts of monosaccharides (<0.2%) and no polysaccharide larger than DP 7. There was 6.9% of DP 2, 28.4% of panose, 36.7% of DP 4, 19.1% of DP5, 7.4% of DP6, and 1.2% of DP7.

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