J Ind Microbiol Biotechnol (2015) 42:279–285
DOI 10.1007/s10295-014-1568-3

FERMENTATION, CELL CULTURE AND BIOENGINEERING

SIMB
Society for Industrial Microbiology and Biotechnology

Lime application for the efficient production of nutraceutical glucooligosaccharides from *Leuconostoc mesenteroides* NRRL B-742 (ATCC13146)

Young Hwan Moon · Lee Madsen · Chang-Ho Chung · Doman Kim · Donal F. Day

Received: 29 September 2014 / Accepted: 12 December 2014 / Published online: 23 December 2014

Abstract We have previously demonstrated the production of glucooligosaccharides via a fermentation of sucrose with *Leuconostoc mesenteroides* NRRL B-742 using sodium hydroxide (NaOH) to control the pH. Because NaOH is expensive, we sought to minimize the cost of our process by substituting hydrated lime and saccharate of lime (lime sucrate) in its place. The yield of glucooligosaccharides using either 5 % lime (41.4 ± 0.5 g/100 g) or 5 % lime sucrate (40.0 ± 1.4 g/100 g) were both similar to the NaOH control (42.4 ± 1.5 g/100 g). Based on this, it appears that the cost associated with pH control in our process can be reduced by a factor of approximately 2.4 using lime instead of NaOH. Because our chromatographic stage is based on a Ca^{2+}-form resin to separate glucooligosaccharides, the use of lime not only negates the need for costly de-salting via ion-exchange (elimination of two ion-exchange sections) prior to separation, but also greatly reduces the resin regeneration cost.

Y. H. Moon (✉) · L. Madsen · D. F. Day
Audubon Sugar Institute, Louisiana State University Agricultural Center, 3,845 Highway 75, Saint Gabriel, LA 70776, USA
e-mail: ymoon@agcenter.lsu.edu

C.-H. Chung
Department of Culinary Science and Food Service Management, Sejong University, 98 Gunja-dong, Gwangjin-gu, Seoul 143-747, South Korea

D. Kim
Graduate School of International Agricultural Technology and Institutes of Green Bio Science and Technology, Seoul National University, Pyeongchang-gun, Gangwon-do 232-916, South Korea

Keywords *Leuconostoc mesenteroides* NRRL B-742 · Glucooligosaccharides · Calcium hydroxide · Lime · Saccharate · Sucrate

Introduction

A nutraceutical is defined as any substance that is either food or a constituent thereof and that purportedly provides medical or health benefits which can include prevention and/or treatment of disease [18]. The term typically includes the following representative classes: probiotics, prebiotics, dietary fiber, omega-3 fatty acids, and antioxidants [11]. Due to increasing numbers of health-conscious consumers in Asia, the US, and Europe, the nutraceutical market has demonstrated significant growth over the past three decades [7, 15].

Of greatest interest to us are the pre- and probiotic classes. Broadly defined, probiotics are made up of living cultures of bacteria, such as yogurt, that promote the growth of healthy gut flora [6]. Health benefits are ostensibly a result of enhanced nutrition in the form of bacterial metabolic products such as butyrate [1] and the exclusion of harmful bacteria such as *Salmonella* sp. Prebiotics, however, are materials, either physical (e.g. dietary fiber) or chemical (e.g. butyrate) which can promote the growth of selected beneficial flora [3] and/or exert some beneficial effect directly to intestinal epithelial cells (thus improving uptake of nutritive calories, vitamins, etc.) [5, 19]. Additionally, they are generally compatible with most food formulations [8, 10]. By definition, glucooligosaccharides are prebiotic agents, and many forms are commercially available.

Glucooligosaccharides (GOS) are a class of prebiotics which include isomalto oligosaccharides (IMO). Strictly,

IMOs are glucosyl saccharides containing only α-(1 → 6) linkages. This definition has been expanded in past years to include glucooligosaccharides comprised of α-(1 → 6) chains with α-(1 → 4) [20], α-(1 → 3) (nigerooligosaccharides) and\or α-(1 → 2) (kojioligosaccharides) linked branches. These glucosidic linkages are found in commercial IMO syrups [7].

Commercial IMOs are generally produced from starch hydrolysates (maltose and maltodextrins) through the action of the α-transglucosidase (EC 2.4.1.24) from *Aspergillus* sp. [17], branched IMOs can be produced from sucrose with an acceptor reaction catalyzed by dextransucrase [12, 13]. Chung and Day have produced glucooligosaccharides via the action of in situ dextransucrase upon sucrose in the presence of a maltose acceptor. The IMO was an extracellular product of the fermentation of sucrose by *Leuconostoc mesenteroides* NRRL B-742. Chung and Day [4] demonstrated that these glucooligosaccharides (branched IMOs) are readily utilized by *Bifidobacterium bifidum* and *Lactobacillus johnsonii*, but not by *Escherichia coli* or *Salmonella typhimurium* in a pure-culture studies.

As a heterofermentative lactic acid bacteria, *L. mesenteroides* NRRL B-742 produces lactic and acetic acids which can rapidly accumulate to levels that will inhibit cell-proliferation and yield. So the fermentation process of Chung and Day requires pH control for optimum production of IMO (US Patent 7,291,607). In order to minimize production cost, the use of hydrated lime [$Ca(OH)_2$] was investigated as a substitute for the strong base (NaOH). Despite its poor solubility, lime is the least expensive commodity alkali available so it is used in as many industrial process areas as possible.

In this study, hydrated lime was applied as a pH control agent in lieu of sodium hydroxide (NaOH) and the yields of glucooligosaccharide were compared in fermentations to see whether lime affects the production of glucooligosaccharide during the fermentations of *L. mesenteroides* NRRL B-742. Furthermore, to overcome the solubility barrier, lime saccharate (or "sucrate", which is lime dissolved in sucrose solution rather than the salt of glucaric acid) was likewise tested and then the purification of glucooligosaccharide (GOS) produced by lime sucrate method was also tested using Ca^{2+} cation-exchange chromatography without desalting process.

Materials and methods

Bacterial strain and culture medium

L. mesenteroides NRRL B-742 was purchased from the American Type Culture Collection (ATCC 13146, Manassas, VA). After re-isolation the strain was stored at −60 °C in 20 % glycerol. This culture was grown in a medium composed of 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 (pH 6.5) at 28 °C. For 2 L fermentations, yeast extract (10 g), $MgSO_4{\cdot}7H_2O$ (400 mg), $FeSO_4{\cdot}7H_2O$ (20 mg), NaCl (20 mg), $MnSO_4{\cdot}7H_2O$ (20 mg), $CaCl_2$ (100 mg), and KH_2PO_4 (6 g) were dissolved in distilled water (1,250 mL) and adjusted to pH 6.5 using 6 M NaOH. The mixture was autoclaved for 20 min at 120 °C. Solutions of maltose (100 g/250 mL) and sucrose (200 g/500 mL) were autoclaved, as before, prior to transfer to the fermentor.

pH Control-materials and preparations

The pH control capacity of lime was compared with sodium hydroxide (NaOH). Sodium hydroxide pellets were purchased from Fisher Scientific (Hanover Park, IL) and hydrated lime powder was purchased from Batesville Marble Hydrated Lime (Arkansas Lime Company, Batesville, AR). NaOH (5 % w/v, 1.25 M, 1.25 M eq. [OH^-]) and lime (5 % w/v, 0.68 M, 1.35 M eq. [OH^-]) solutions were prepared by dissolving 50 g of each in 1 L of distilled water. To prepare a 5 % lime sucrate solution, lime powder (50 g in 1 L bottle) and sucrose (250 g in 805 mL distilled water) were autoclaved separately. After autoclaving, sucrose solution was transferred into the 50 g of lime to give a final solution concentration of 5 % lime in 25 % sucrose, named 5 % lime sucrate. Maltose solution (1 L of 12.5 %) was prepared in 5 % lime sucrate.

Fermentation and pH control

Batch fermentations were conducted using 2 L of BioFlo II fermentors (New Brunswick Scientific, New Brunswick, NJ). The fermentors were inoculated from late log-phase flask seed cultures at 1.0 % (20 mL) of working volume. Fermentations were conducted at 28 °C with stirring at 200 rpm.

The pH of the cultures decreased from 6.5 (optimal for cell growth) and automatic control began when pH reached 5.5 (optimal for dextransucrase activity), which took approximately 5.5 h. The pH was maintained at 5.5 until completion (~30 h) using either 5 % NaOH (w/v), 5 % lime (w/v), or 5 % lime sucrate (together with 12.5 % w/v maltose solution). The feed rate of 5 % lime sucrate and 12.5 % maltose was identical to the feed rate required to maintain the sucrose to maltose ratio at 2:1. For the lime sucrate method, the 5 % lime sucrate and 12.5 % maltose were fed for the initial 18 h to control the pH and then replaced with 5 % lime solution until the end (30 h) to avoid the residual fructose in the final fermentation broths. Samples (2 mL) were collected every 3 h for quantification of carbohydrate and organic acid components by HPLC.

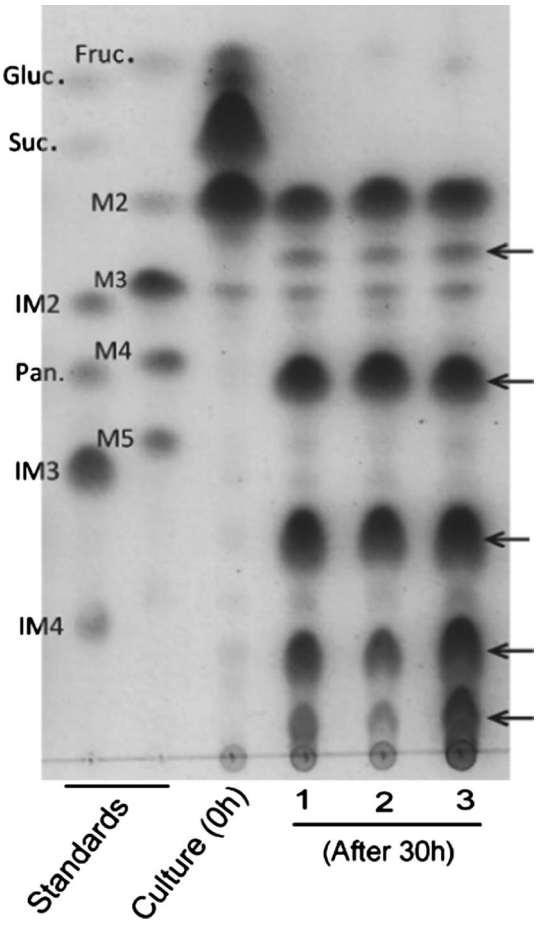

Fig. 1 TLC of glucooligosaccharides of *L. mesenteroides* NRRL B-742 fermentations with three pH control methods. *Gluc*. glucose, *Fruc*. fructose, *Suc*. sucrose, *IM2* isomaltose, *IM3* isomaltotriose, *IM4* isomaltotetraose, *M2* maltose, *M3* maltotriose, *M4* maltotetraose, *M5* maltopentaose. *Lane 1* 5 % NaOH fermentation batch, *Lane 2* 5 % lime fermentation batch, *Lane 3* 5 % lime sucrate fermentation batch

Purification of glucooligosaccharides, mannitol, and lactic acid

After harvesting, cells were removed by centrifugation at 10,400*g* for 20 min. Activated charcoal (5 g/L, Sigma, St. Louis, MO; 100–400 mesh) and Celite 545 (1 g/L, Fisher Scientific, Hanover Park, IL) were added to cell-free culture broth and mixed at 50 °C for 20 min. The broth was filtered through No. 3 filter paper (Whatman, Maidstone, England) to remove the carbon. The filtered broths were concentrated using a Yamato rotary evaporator RE71 (Yamato, Santa Clara, CA) at 85 °C to 57/100 g (°brix).

Cation-exchange chromatography (6.0 × 70 cm column) with 2 L pre-swelled Dowex Monosphere 99 320 resin (sulfonated styrene-DVB, 300–330 μm, gel, 1.5 eq/L $[H^+]$, Ca^{2+} form; Dow, Midland, MI) was used to purify the glucooligosaccharides. The sample-loading volume was 60 mL (3 % bed-volume) and the mobile-phase flow rate (18 MΩ H_2O) was 10 mL/min (0.5 % bed-volume/min) at 50 °C. Elution was monitored in real-time by periodically measuring the refractometer brix (Atago Pallet). During elution with 810 mL of water, the void volume (no brix) and 15 mL fractions were collected and analyzed for carbohydrates and acids by HPLC.

Based on the results of HPLC analysis (or brix), glucooligosaccharide (GOS) fractions containing either GOS:Acetic Acid or Mannitol:lactic acid were combined. After concentration to 30 °brix, the mannitol was separated from the lactic acid via ethanolic precipitation (70 % ethanol). The precipitated solid fraction (mannitol) was washed again with 100 % ethanol and air-dried at 55 °C. The GOS fraction and lactic acid fractions were freeze-dried. The acetic acid is volatile and the bulk of it was removed during lyophilization.

Analytical methods

Glucooligosaccharide production during fermentation was monitored using thin layer chromatography (TLC). Whatman Partisil K6F silica gel plates (10 × 20 cm) were obtained from Fisher Scientific (Hanover Park, IL). Standard materials, panose, maltooligosaccharides (DP2-5) and isomaltooligosaccharides (DP2-4) were purchased from Sigma (St. Louis, MO). Fermentation cultures (after 30 h) were spotted (0.6 μL) on TLC plates that were dried and developed five times (nitromethane:1-propanol:water = 2:5:1.5, v/v/v) at room temperature. After drying, the carbohydrates were visualized using a spray-reagent of 0.3 % *n*-(1-naphthyl)-ethylenediamine dihydrochloride (w/v) and 5 % sulfuric acid (v/v) in methanol. Final visualization occurred after heating for 10 min at 105 °C.

High-performance liquid chromatography (Agilent 1200 HPLC with a differential refractive index detector at 45 °C, BioRad Aminex HPX-87 K at 85 °C eluted with 0.01 M K_2SO_4 at 0.8 mL/min) was used for quantitative analysis of carbohydrates. A three-point curve made of maltose, panose, mannitol, glucose, and fructose was used to standardize the instrument. For the analysis of lactic acid and acetic acids, an Agilent 1100 HPLC was used with an Aminex HPX-87H column at (65 °C eluted with 1.0 mL/min of 0.005 N H_2SO_4 with detection via absorbance of the carbonyl $n \rightarrow \pi^*$ transition at 210 nm).

Table 1 GOS production by *L. mesenteroides* NRRL B-742 by pH control method (wt% of carbohydrate feed)

Product	NaOH	Lime	Lime sucrate
GOS (DP ≥ 3)	42.4 ± 1.5	41.4 ± 0.5	40.0 ± 1.4
Mannitol	32.5 ± 1.5	32.4 ± 0.8	31.2 ± 1.3
Maltose	12.9 ± 1.6	12.5 ± 0.3	13.0 ± 0.8

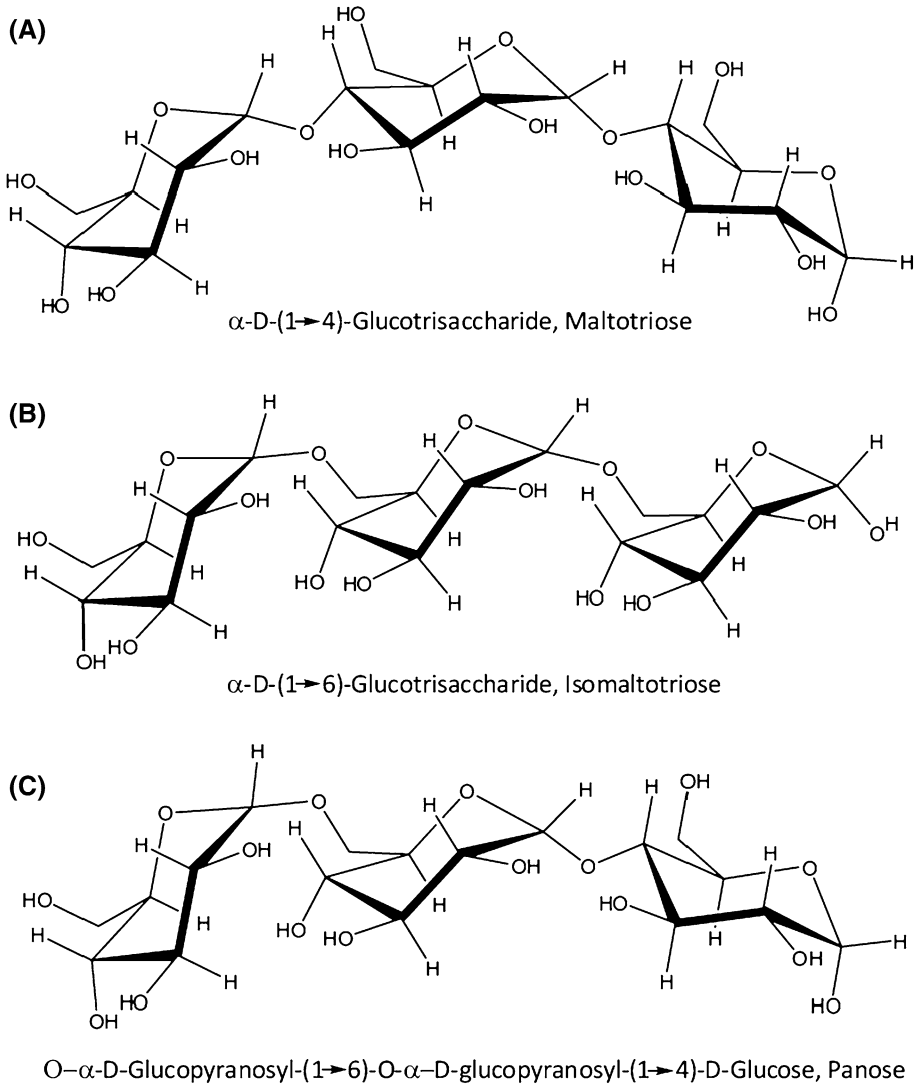

Fig. 2 Oligosaccharides by glycosidic linkage. **a** Maltooligosaccharide (MO), **b** isomaltooligosaccharide (IMO), and **c** our GOS product corresponding to the panose-type

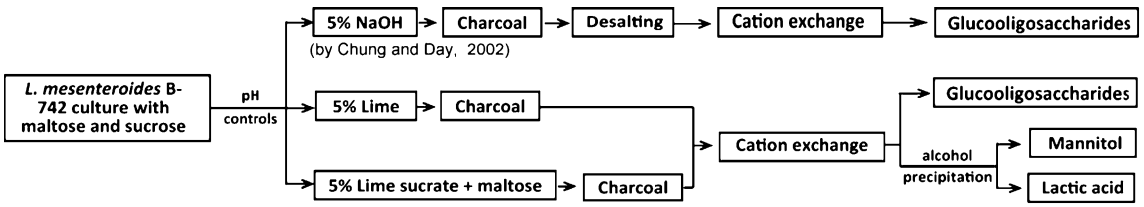

Fig. 3 Flow chart of purification of glucooligosaccharides, mannitol, and lactic acid through lime application

Results

Comparing GOS production with lime, lime-sucrate or sodium hydroxide

Using lime rather than NaOH for pH control, GOS was produced from *Leuconostoc mesenteroides* NRRL B-742 according to the method of Chung and Day [4]. Progress was monitored via TLC. The GOS products (indicated by arrows in Fig. 1) were primarily DP3 (degree of polymerization, panose) through DP6 with R_f values corresponding to neither maltooligosaccharides (M2–M5) nor isomaltooligosaccharides (IM2–IM4), which strongly suggests that they are branched isomalto oligosaccharides (Fig. 2).

Once the fermentations were complete (TLC), the yields of GOS (DP $\geq$ 3), mannitol, and maltose produced using pH control with either 5 % NaOH, 5 % lime (Fig. 3), or

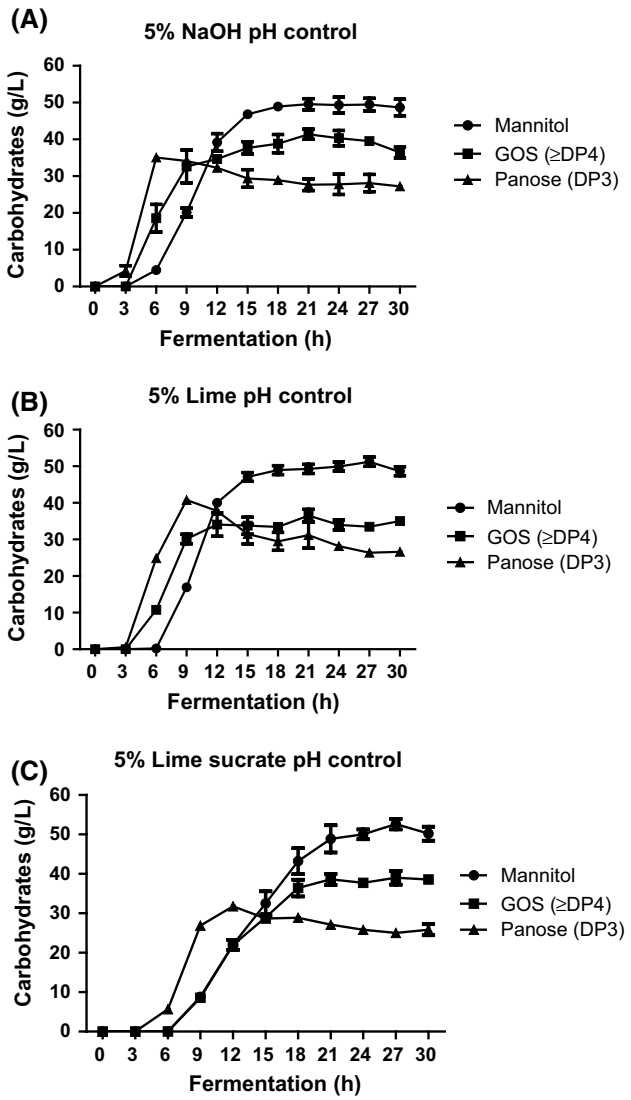

Fig. 4 The production patterns of glucooligosaccharide (GOS) and mannitol by *L. mesenteroides* NRRL B-742 from sucrose and maltose as a function of time

5 % lime sucrate with 12.5 % maltose were compared in terms of total GOS determined by HPLC (Table 1).

Using lime, the yields (% of GOS (DP ≥ 3) per total carbohydrate amount input were similar (41.4 ± 0.5 %) with the NaOH (42.4 ± 1.5 %) control (Table 1). In all fermentations, the production of GOS (DP ≥ 3) and mannitol were complete approximately 15–21 h post-inoculation (Fig. 4). With 5 % lime sucrate, the final product (GOS, mannitol, and maltose) production (193.85 g) was greater than with NaOH (Table 2) because of additional feeding of sucrose (as lime sucrate form) and maltose. The yield [40.0 ± 1.4 %, GOS (DP ≥ 3] per total carbohydrate amount input) was slightly lower than the 42.4 ± 1.5 % observed using 5 % NaOH (Table 1).

A 2-L fermentation required 8.10 g of NaOH, 13.15 g of lime, and 10.75 g of lime saccharate to maintain the optimum pH. The costs of either NaOH or lime relative to the respective product yield were calculated and are given in Table 2.

GOS produced by the lime sucrate method was separated well from mannitol and lactic acid using cation-exchange chromatography (Dowex Monosphere 99-320 resin, Ca^{2+} form) although the loading sample was not passed through two de-ashing processes as Chung and Day [4] did (Fig. 5).

Discussion

GOS are produced commercially using enzymes from *Leuconostoc* strains [2]. Enzymatic synthesis has the advantage of higher productivity when compared to either extraction from plant sources or enzymatic hydrolysis of polysaccharides. However, none of these procedures is considered to be economically feasible because enzyme isolation is costly. Conventional fermentation is considered to be a more practical approach for industrial manufacture of GOSs because dextransucrase alone yields a

Table 2 The prices of NaOH and lime by pH control method

Fermentations	NaOH[a] (g/L)	Lime[a] (g/L)	Product[b] (g/L)	NaOH/lime[c] (10 kL ferment)	GOSs[c] (10 kg product)
NaOH	8.10 g		131.75 g	$ 44.55	$ 0.33
Lime		13.15 g	128.95 g	$ 18.15	$ 0.14
Lime sucrate		10.75 g	193.85 g	$ 14.84	$ 0.07

[a] Used amounts of NaOH and lime for 1 L fermentations

[b] The final products are GOS (DP ≥ 3), mannitol, and maltose

[c] Prices of NaOH ($ 550 per metric ton) and lime ($ 138 per metric ton) was based on the year of 2013

The NaOH price in US was assessed by global chemical market intelligence service ICIS pricing on May, 2013 (http://www.icis.com/Articles/2013/05/02/9664807/three-us-producers-announce-price-initiatives-for-caustic.html)

Lime price in US was obtained from M. Michael Miller (Lime Specialist, US Geological Survey) on Dec. 3, 2013

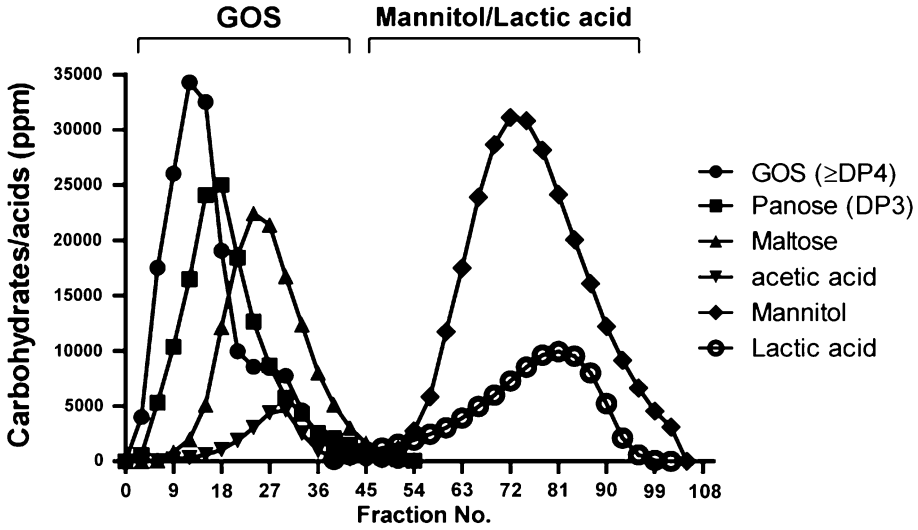

Fig. 5 Cation (Ca^{+}) exchange column (6.0 × 70 cm) chromatogram of *L. mesenteroides* NRRL B-742 fermentation using 5 % lime sucrate method

product mixture containing D-fructose that is difficult to separate. Live cultures metabolically convert the D-fructose to D-mannitol which can be economically separated by ethanolic precipitation.

As shown in Fig. 2, the GOS we produced is a family of branched isomaltooligosaccharides. It contains DP2–DP8 which are considered to be desirable prebiotics [19]. *Leuconostoc mesenteroides* NRRL B-742 (ATCC 13146) produces two exocellular α–D-glucans, a fraction L, which is comprised of an α-(1 → 6) backbone with α-(1 → 4) branch-points and a fraction S, which consists of an α-(1 → 6) backbone with α-(1 → 3) branch-points [16]. IMO synthesized by dextransucrase from ATCC 13146 had α-(1 → 6) backbones with α-(1 → 3) and/or α-(1 → 4)-branched side chains when maltose was used as an acceptor [14]. The smallest product from this fermentation was confirmed to be panose (*O*-α-D-(1 → 6)-glucopyranosyl-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucose). It appears that dextransucrase from strain 742 reliably prefers to synthesize GOS with α-(1 → 6) linkages when maltose is used as the acceptor. The larger GOS oligomers (DP4-8) may have continuous α-(1 → 6) linkages to maltose, that is, maltosyl isomaltooligosaccharides (MIMOs).

Sodium hydroxide (NaOH) is routinely used as a pH control reagent in fermentation industries. The lactic acid bacteria, including *Leuconostoc* spp. need a significant amount of NaOH to maintain the optimum pH for their active growth. Without pH control, the pH of *Leuconostoc mesenteroides* NRRL B-742 fermentation batches rapidly drop to pH 3.5 within 10 h (not data shown). Experimentally, a 1-L fermentation required 8.10 g of NaOH to maintain pH 5.5. On a bulk basis, the cost of NaOH would be $ 44.55 for a 10,000-L fermentation while the cost of equivalent lime would be $ 18.15. At industrial scale, lime ($ 0.14 per 10 kg product) can be a low-cost alternative to NaOH. The lime sucrate method ($ 0.07 per 1 kg product) is more attractive as the solubility of lime is increased up to 5 % in 22.5 % sucrose solution [9] and feeding in maltose at the same time increased the observed yield of product over time.

Chung and Day showed that cation (Ca^{2+} form) exchange resin was capable of separating GOS from mannitol and organic acids. Because Na^{+} will replace the Ca^{2+} form of the resin used for separation (necessitating costly regeneration), Chung and Day [4] had to pass the broth through two de-ashing processes (anion OH^{-} form and cation H^{+} form) prior to the separatory chromatography. Using a Ca^{2+}-based alkali negated this requirement as shown in Fig. 5.

This work furthers the art of fermentative production of GOS using *L. mesenteroides* NRRL B-742. Our work demonstrated that costs associated with alkali can be reduced by a factor of ~2.4 using $Ca(OH)_2$ rather than NaOH. Replacing $2Na^{+}$ with Ca^{2+} negates the need for de-ashing which eliminates two potentially expensive chromatographic steps. The use of lime sucrate solves the issue of solubility, and the sucrose-base is used as makeup for the fermentation. Furthermore, the optimization studies are needed because lime may affect the bacterial growth and sucrose consumption rate together with dextransucrase activities which is a crucial factor for the GOS production.

Acknowledgments The authors would like to thank Ms Chardcie Verret, Ms Shyue Lu, and Dr. Derek Dorman from the Audubon Sugar Institute at Louisisana State University Agricultural Center for their analytical HPLC support. Doman Kim was partially supported by the National Research Foundation of Korea (NRF) grant, funded by the Korea government (MEST) (No. NRF-2012R1A2A2A01045995).

References

1. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 17:1519–1528
2. Chludzinski AM, Germaine GR, Schachtele CF (1974) Purification and properties of dextransucrase from *Streptococcus mutans*. J Bacteriol 118:1–7
3. Chung CH, Day DF (2004) Efficacy of *Leuconostoc mesenteroides* (ATCC 13146) isomaltooligosaccharides as a poultry prebiotic. Poult Sci 83:1302–1306
4. Chung CH, Day DF (2002) Glucooligosaccharides from *Leuconostoc mesenteroides* B-742 (ATCC 13146): a potential prebiotic. J Ind Microbiol Biotechnol 29:196–199
5. Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1412
6. Gilliland SE, Morelli L, Reid G (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. World Health Organization, Geneva, pp 1–34
7. Goffin D, Delzenne N, Blecker C, Hanon E, Deroanne C, Paquot M (2011) Will isomalto-oligosaccharides, a well-established functional food in Asia, break through the European and American market? The status of knowledge on these prebiotics. Crit Rev Food Sci Nutr 51:394–409
8. Macfarlane S, Macfarlane GT, Cummings JH (2006) Review article: prebiotics in the gastrointestinal tract. Aliment Pharmacol Ther 24:701–714
9. Madsen LR (2009) Iron mediated precipitation of phenol: protein aggregates from sugar cane juice. Louisiana State University, Baton Rouge
10. Manning TS, Gibson GR (2004) Microbial-gut interactions in health and disease.Prebiotics. Best Pract Res Clin Gastroenterol 18:287–298
11. Pandey M, Verma RK, Saraf SA (2010) Nutraceuticals: new era of medicine and health. Asian J Pharm Clin Res 3:11–15
12. Paul F, Lopez-Munguia A, Remaud M, Pelenc V, Monsan P (1992) Method for the production of α-(1,2) oligodextrans using *Leuconostoc mesenteroides* B-1299. US Patent 5,141,858
13. Remaud-Simeon M, Lopez-Munguia A, Pelenc V, Paul F, Monsan P (1994) Production and use of glucosyltransferases from *Leuconostoc mesenteroides* NRRL B-1299 for the synthesis of oligosaccharides containing alpha-(1 → 2) linkages. Appl Biochem Biotechnol 44:101–117
14. Remaud M, Paul F, Monsan P (1992) Characterization of α-1,3 branched oligosaccharides synthesized by acceptor reaction with the extracellular glucosyltransferases from *L. mesenteroides* NRRL B-742. J Carbohyd Chem 11:359–378
15. Roberfroid MB (2002) Global view on functional foods: European perspectives. Br J Nutr 88(Suppl 2):S133–S138
16. Robyt JF (1986) Dextran, vol 4. Wiley, New York
17. Roper H, Koch H (1988) New carbohydrate-derivatives for biotechnical and chemical processes. Starch 40:453–459
18. Stephen DFL (1995) The Nutraceutical revolution, its impact on food industry R&D. Trends Food Sci Tech 6:59–61
19. Van Loo J, Cummings J, Delzenne N, Englyst H, Franck A, Hopkins M, Kok N, Macfarlane G, Newton D, Quigley M, Roberfroid M, van Vliet T, van den Heuvel E (1999) Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). Br J Nutr 81:121–132
20. Yun J, Lee M, Song S (1994) Continuous production of isomalto-oligosaccharides from maltose syrup by immobilized cells of permeabilized *Aureobasidium Pullulans*. Biotechnol Lett 16:1145–1150