*Biotechnol. Bioprocess Eng.* **2002**, 7: 234-236

# Production of Mannitol Using *Leuconostoc mesenteroides* NRRL B-1149

**Chang Yong Kim[1], Jin Ha Lee[2], Byung Hoon Kim[3], Sun Kyun Yoo[5], Eun Seong Seo[1], Kab Su Cho[4], Donal F. Day[6], and Doman Kim[1, 2]***

[1] Department of Material, Chemical and Biochemical Engineering, [2] Faculty of Applied Chemical Engineering, [3] Engineering Research Institute, [4] Department of Molecular Biotechnology, Chonnam National University, Gwangju 500-757, Korea
[5] Department of Food and Biotechnology, Joong Bu University, Geumsan-Gun, Chungnam 312-702, Korea
[6] Audubon Sugar Institute, Louisiana State University, Baton Rouge, LA 70803, USA

**Abstract** A process for the production of mannitol from fructose (5% to 25%) using *Leuconostoc mesenteroides* NRRL B-1149 was investigated. Fermentations were carried out in batch or fed-batch fermentations without aeration at 28°C, pH 5.0. When 5% fructose was used in batch culture fermentation, the yield of mannitol was 78% of that expected theoretically. When the fructose concentration was increased to 10%, the yield dropped to 59.6% of the theoretical value. However, in the fed-batch culture, using 10% fructose, the yield was 81.9% of the theoretical value. In a 15% fructose fed-batch culture, with 5% fructose being added initially and the other 10% fructose being added as a continuous supply, the final yield was 83.7% of the theoretical yield. When 20% fructose was used in the same manner, the yield was 89.5% of theoretical yield.

*Keyword*: fructose, mannitol, *Leuconostoc mesenteroides*, fed-batch fermentation

Mannitol is a common six-carbon sugar alcohol (hexitol). It is one of the most abundant carbohydrates in nature, occurring in bacteria, yeasts, fungi, algae and some plants and fruits [1]. It has various applications. It is about half as sweet as sucrose and has a cool taste. It is used in 'sugar free' chewing gum and pharmaceutical formulation for chewable tablets [1,2]. It is used as a food additive in so called 'functional foods', and in pharmaceutical preparation, because it is only partially metabolized by humans. It is also used as a powerful osmotic diuretic, which makes it useful for diabetics and for use in diets [1,2]. Mannitol is industrially produced by catalytic hydrogenation, but the removal of the by-products (like sorbitol) is relatively difficult resulting in low yields (below 50%) [3]. Mannitol is a common reserve product of many fungi and yeasts and its production by fermentation has often been tried, but with low yields and productivities (below 50%), which has hindered its commercialization [4-7]. The production of mannitol by lactic acid bacteria, and other food-grade microorganisms, offers several advantages; food-grade microorganisms and their products are directly applicable to food products, with some lactic acid bacteria being beneficial in the intestine [4,5]. *Leuconostoc mesenteroides*, a lactic acid bacterium, and its enzymes can be used to produce a range of carbohydrates and derivatives, as diverse as dextran (biopolymer), fructose, mannitol (polyol), leucrose (non-cariogenic disaccharide), glucose-1-phosphate, and many others. Recently, a new fermentation process, capable of converting fructose quantitatively to mannitol, has been developed [8]. The process uses the *Leuconostoc mesenteroides* mannitol dehydrogenase. This method yields almost equal amounts of mannitol and by product, sorbitol [9]. In this study, we reported on a process that produces only mannitol using the *Leuconostoc mesenteroides* NRRL B-1149.

Fructose and mannitol were used as carbon sources. Standards for TLC analysis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thin layer chromatography (TLC) plates were purchased from Merck (Germany). All other chemicals were of reagent grade and commercially available. The *Leuconostoc mesenteroides* NRRL B-1149 was grown on LM medium (yeast extract, 5 g/L; peptone, 5 g/L; $K_2HPO_4$, 2 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; NaCl, 0.01 g/L; $FeSO_4$, 0.01 g/L; $MnSO_4 \cdot H_2O$, 0.01 g/L; $CaCl_2 \cdot 2H_2O$, 0.015 g/L) containing 2% (w/v) sucrose [10], in an 8-L fermenter (BioTron, Korea) The pH of the medium was adjusted to between 5.0 and 7.0 throughout the fermentation, and the culture incubated at 28°C with a stirrer speed of 150 rpm without aeration. In the batch culture fermentations, either 5% or 10% fructose was used. In the fed-batch culture fermentations, after an initial 5% fructose had been consumed, additional (5-20%) fructose was supplied continuously. The cell growth was monitored by measuring the absorbance at 660 nm ($OD_{660}$). In the fed-batch culture fermentation, fructose (5%) was added

* **Corresponding author**
Tel: +82-62-530-1844, Fax: +82-62-530-1844
e-mail: dmkim@chonnam.ac.kr

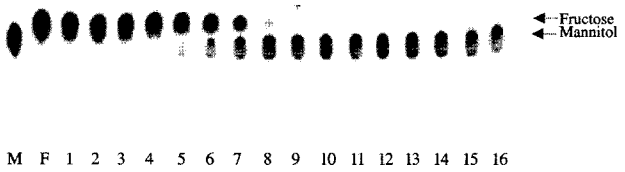


**Fig. 1.** Thin layer chromatogram of batch culture supernatant using 5% fructose. Batch fermentation was performed at 28°C for 45 h with 5% fructose and each sample was taken at the time designated (M: 1%, mannitol, F: 1%, fructose, 1ane 1: 0 h, lane 2: 3 h, lane 3: 6 h, lane 4: 9 h, lane 5: 12 h, lane 6: 15 h, lane 7: 18 h, lane 8: 21 h, lane 9: 24 h, lane 10: 27 h, lane 11: 30 h, lane 12: 33 h, lane 13: 36 h, lane 14: 39 h, lane 15: 42 h, lane 16: 45 h).

* The NIH Image Program was used to quantify the amounts of carbohydrate following TLC using standard carbohydrates [12,13]. The yield of mannitol was calculated from the conversion of fructose to mannitol.

for the initial growth of the cells, and was then added at a constant rate, with a peristaltic pump, until the desired concentration was reached.

After the cells had been removed by centrifugation (12,000 × g for 3 min), the supernatant was analyzed for mannitol using TLC. The products were separated by two ascents using a solvent mixture of acetonitrile: ethyl acetate: 1-propanol: water (85:20:20:15, v/v/v/v) at 20°C. Reducing sugar was detected by dipping the plate with $AgNO_3$-acetone for 5 min, alkaline-methanol for 2 min, and $Na_2S_2O_3$ (1.5 M), $Na_2S_2O_3$ (0.08 M), and $NaHSO_3$ (0.25 M) for 2 min, respectively [11]. The NIH Image Program was used to quantify the amounts of carbohydrate following TLC using standard carbohydrates [12,13]. The yield of mannitol was calculated from the conversion of fructose to mannitol (Fig. 1).

All cultures were grown without aeration at pH 5.0 and 28°C. The highest mannitol yield was obtained in the stationary phase ($OD_{660}$=4, 20 h after inoculation) (Fig. 1). When 5% (300 g in 6 L medium) fructose was used in a batch culture, 78% of theoretical yield (238 g mannitol) was produced. All the fructose (5%) was consumed within 22 h post-inoculation and the mannitol production rate was 2.91 g $L^{-1}h^{-1}$. However, when 10% fructose (600 g fructose in 6 L medium, w/v) was used in batch fermentation, the yield was reduced to 59.6% of the theoretical yield (Table 1). The high concentration of fructose inhibited the cell growth (data not shown) and this might be caused by a substrate inhibition. Fed-batch culture fermentations were conducted by adding 5% fructose at a fixed time: an initial 5% fructose was added as the carbon source for cell growth and for mannitol production, and then a further 5% was added within 22 h post-inoculation. This was mostly converted to mannitol (95.0% of the theoretical yield). The mannitol yield was increased to 81.8% of the theoretical yield, with a mannitol production rate of 3.61 g $L^{-1}h^{-1}$. For 15% fructose, in fed-batch fermentations, 254.5 g mannitol was produced from 300 g (5% in 6 L of medium, w/v) of fructose within 20 h post-inoculation, 80.8% of the theoretical yield. When the remains of the fructose (10%) were added, 83.7% of the theoretical yield was obtained (829.08 g mannitol produced from 1,290 g fructose in a 6-L fermentation). The mannitol production yield was increased a further 20% than with 10% fructose in the fed-batch fermentations.

**Table 1.** Production of mannitol by *Leuconostoc mesenteroides* NRRL B-1149 using various concentrations of fructose

| | Batch culture | | Fed-batch culture* | | | |
|---|---|---|---|---|---|---|
| Final Fructose concentration | 5% | 10% | 10% (5+5)** | 15% (5+10)** | 20% (5+15)** | 25% (5+20)** |
| Yield | 78.0% | 59.6% | 81.8% | 83.7% | 89.5% | 64.8% |

* Starting fructose concentration as a carbon source was 5%.

** 5% fructose was fed initially and the rest 5%, 10%, 15% or 20% fructose were added continuously.

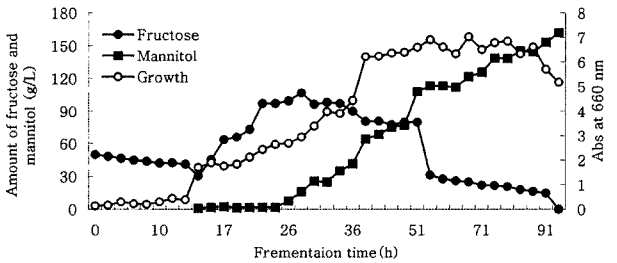


**Fig. 2.** Production of mannitol by *Leuconostoc mesenteroides* NRRL B-1149 using 20% fructose in fed-batch fermentations. 5% fructose was initially added by 15 h post-inoculation, and then 15% fructose was added continuously at a feed rate of 3.136 g $L^{-1}h^{-1}$.

For the 20% fructose fed-batch fermentations, 5% fructose was used as a substrate within 15 h post-inoculation ($OD_{660}$=4), a further 15% fructose was added at a constant rate with a pump (3.136 g $L^{-1}h^{-1}$). After 24 h, 0.07% yeast extract was added to facilitate the cell growth. Then about 21 g/L mannitol was produced from 41 g/L fructose after 15 h. 1,113 g fructose was then added, which was converted to 1,061 g of mannitol, which was 95.3% of the theoretical yield. The final mannitol yield was 89.5% of that expected theoretically (Fig. 2). Using the same procedure, 25% fructose fed-batch fermentations were studied. However, the final yield decreased significantly, to only 64.8% of the theoretical yield. This might be caused by a substrate inhibition. The optimum condition for the production of mannitol using *Leuconostoc mesenteroides* NRRL B-1149 was 20% fructose as a carbon source in fed-batch fermentation. This value is significant higher than the 83%, or less, reported by other researchers [8, 14,15].

**Acknowledgements** This work was supported by a grant from the Korea Health R&D Project (HMP-00-B-20507-00187), Ministry of Health and Welfare, Korea.

## REFERENCES


[1] Debord, B., C. Lefebvre, A. M. Guyot-Hermann, J. Hubert, R. Bouche, and J. C. Guot (1987) Study of different forms of mannitol: Comparative behaviour under compression. *Drug Develop. Industrial Pharm.* 13: 1533-1546.

[2] Soetaert, W. (1996) *Synthesis of D-Mannitol and L-Sorbitose by Microbial Hydrogenation and Dehydrogenation of Monosaccharide*. Ph. D. Thesis. University of Gent, Gent, Belgium.

[3] Makkee, M., A. P. G. Kieboom, and H. Van Bekkum (1985) Production methods of D-mannitol. *Starch* 37: 136-141.

[4] Jennings, D. H. (1984) Polyol metabolism in fungi. *Adv. Microb. Physiol.* 25: 149-193.

[5] Wisselink, H. W., R. A. Weusthuis, G. Eggink, J. Hugenholtz, and G. J. Grobben (2001) Mannitol production by lactic acid bacteria: A review. *Int. Dairy J.* 1-11.

[6] Vandamme, E. J., J. Van Loo, and A. De Laporte (1987) Dynamics and regulation of sucrose phosphorylation formation in *Leuconostoc mesenteroides* fermentations. *Biotechnol. Bioeng.* 29: 8-15.

[7] Grobben, G. J., S. W. P. G. Peters, H. W. Wisselink, R. A. Weushuis, M. H. N. Hoefnagel, J. Hugenholts, and G. Eggink (2001) Spontaneous formation of a mannitol-producing variant of *Leuconostoc mesenteroides* grown in the presence of fructose. *Appl. Environ. Microbiol.* 67: 2876-2870.

[8] Yun J. W and D. H. Kim (1998) A comparative study of mannitol production by two lactic acid bacteria. *J. Ferment. Bioeng.* 85: 203-208.

[9] Makkee. M., A. P. G. Kieboom, and H. V. Bekkum (1985) Production methods of D-mannitol. *Starch* 37: 136-141.

[10] Kim, D. and J. F. Robyt (1995) Dextransucrase constitutive mutants of *Leuconostoc mesenteroides* NRRL B-1299. *Enzyme Microb. Technol.* 17: 1050-1056.

[11] Nam S. H and J. F. Robyt (1998) Separation and detection of sugars and alditols on thin layer chromatograms. *Carbohydr. Res.* 313: 117-123.

[12] Kim, D., J. S. Baek, and J. H. Lee (1995) Characteristics of dextransucrase of *Leuconostoc mesenteroides* NRRL B-1299 mutants, and enzyme modification amylose and pullulan *Ann. RCNBMA* 4: 41-57.

[13] Remoud, M., F. Paul, and P. Monsan (1992) Characterization of $\alpha(1\rightarrow3)$ branched oligosaccharides synthesized by acceptor reaction with the extracellular glycosyltransferase from *L. mesenteroides* NRRL B-742. *J. Carbohydr. Chem.* 11: 359-378.

[14] Grobben, G. J., S. W. P. G. Peters, H. W. Wisselink, R. A. Weusthuis, M. H. N. Hoefnagel, J. Hugenholtz, and G. Eggink (2001) Spontaneous formation of a mannitol-producing variant of *Leuconostoc pseudomesenteroides* grown in the presence of fructose. *Appl. Environ. Microbiol* 66: 2867-2870.

[15] Onishi, H. and T. Suzuki (1970) Microbial production of D-mannitol and D-fructose from glycerol. *Biotechnol. Bioeng.* 12: 913-920.


[Received May 31, 2002; accepted July 19, 2002]