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Mannitol Production by *Leuconostoc citreum* KACC 91348P Isolated from *Kimchi*

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***Leuconostoc* genus, which comprise heterofermentative lactic acid bacteria, reduces fructose to mannitol by recycling intracellular NADH. To evaluate the mannitol productivities of different *Leuconostoc* species, 5 stock cultures and 4 newly isolated strains were cultivated in MRS and simplified media containing glucose and fructose (1:2 ratio). Among them, *L. citreum* KACC 91348P, which was isolated from *kimchi*, showed superior result in cell growth rate, mannitol production rate, and yield in both media. The optimal condition for mannitol production of this strain was pH 6.5 and 30°C. When *L. citreum* KACC was cultured in simplified medium in a 2 l batch fermenter under optimal conditions, the maximum volumetric productivity was 14.83 $g \cdot l^{-1}h^{-1}$ and overall yield was 86.6%. This strain is a novel and efficient mannitol producer originated from foods to be used for fermentation of fructose-containing foods.**

Keywords: D-Mannitol, *Leuconostoc* species, volumetric mannitol productivity, yield mol%

D-Mannitol is a naturally occurring 6-carbon sugar alcohol that is widely applied in chemical, pharmaceutical, and medical industries because of its advantageous properties. It is as half sweet as sucrose with the reduced caloric value of 1.6 kcal/g [6, 20]. Currently, mannitol is produced commercially by catalytic hydrogenation of fructose, sucrose (invert sugar), or glucose-fructose syrups (*e.g.*, high-fructose corn syrup [HFCS]) [15]. The reaction results in a 25:75 mixture of mannitol and sorbitol, thus making mannitol production quite inefficient [5, 19].

Among lactic acid bacteria (LAB), only heterofermentative species are known to convert fructose into mannitol, and species belonging to the genera *Leuconostoc, Oenococcus,* and *Lactobacillus* have been reported to produce mannitol effectively [2, 8, 14]. In those species, fructose can act as an external electron acceptor in a reaction involving mannitol dehydrogenase (MDH), which catalyzes the reduction of fructose to mannitol and *vice versa* [5, 6]. *Leuconostoc* spp. are natural inhabitants of milk, grapes, and many vegetables, and they are frequently used as starter cultures in fermented milks and vegetables [3, 7].

This study was carried out to select a *Leuconostoc* sp. starter strain for the use in mannitol production by a bioconversion process in various media. For this, 9 strains of *Leuconostoc* spp., which are isolates from fermented vegetables and stock cultures frequently used in the previous studies, were compared for their mannitol productivities by using a co-metabolism strategy of glucose and fructose (ratio 1:2). Here, glucose was metabolized as a carbon source for cell growth, and fructose was mainly reduced to mannitol [13]. Subsequently, the effects of temperature and pH on mannitol productivity were analyzed on selected strains, and the strain showing the highest productivity was cultivated in a 2 l batch bioreactor for mannitol production.

Nine wild-type *Leuconostoc* species were tested; *Leuconostoc mesenteroides* ATCC9135 [1, 16], *L. mesenteroides* ATCC8293 [11], *L. mesenteroides* NRRL B-512F [10], *L. mesenteroides* KCTC3719 [9], *L. mesenteroides* NRRL B-742C [4], *L. mesenteroides* D1, *L. mesenteroides* MU3, *L. citreum* KACC 91348P, and *L. pseudomesenteroides* AJ. Among these strains, the last 4 strains were isolated in our laboratory from fermented vegetables. For cultivation of those stocks, 2 different media were used: MRS broth (10.0 g/l protease peptone, 10.0 g/l beef extract, 5.0 g/l yeast extract, 20.0 g/l glucose, 1.0 g/l polysorbate 80, 2.0 g/l ammonium citrate, 5.0 g/l sodium acetate, 0.1 g/l $MgSO_4$, 0.05 g/l $MnSO_4$, and 2.0 g/l K_2HPO_4) supplemented with 40.0 g/l fructose, and a simplified medium containing

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10.0 g/l tryptone (Difco), 5.0 g/l yeast extract (Difco), 2.0 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.01 g/l $MnSO_4$, 0.02 g/l $CaCl_2$, 0.02 g/l NaCl, 30.0 g/l fructose, and 15.0 g/l glucose. During cultivation in both media (100 ml) in shaking flasks, a sample of the cell suspension was withdrawn every 2 h and cell growth was monitored by measuring the optical density at 600 nm against clear broth. For sugar analysis, samples were taken at the middle of the stationary phase by centrifugation of culture broth followed by boiling. The clear broth was filtered, and glucose, fructose, and mannitol concentrations were directly measured with HPLC (YOUNG-LIN, Acme 9000, South Korea) using a Shodex Asahipak NH2P-50 4E column (Tokyo, Japan), and a refractive index detector (YOUNG-LIN). Acetonitrile and water [75:25 (v/v)] were used as the mobile phase at 1.0 ml/min.

The results of the mannitol production in the tested strains in MRS and simplified media are presented in Table 1. In MRS medium, high mannitol concentrations, 30.4, 27.3, and 29.4 g/l, were achieved by *L. citreum* KACC 91348P, *L. mesenteroides* D1, and *L. mesenteroides* B-742C, respectively. In simplified medium, high mannitol concentrations, 26.1, 18.9, and 18.9 g/l, were obtained by *L. citreum* KACC 91348P, *L. mesenteroides* B-512F, and *L. mesenteroides* MU3, respectively. *L. citreum* KACC 91348P grew notably faster (0.31 h^{-1} in MRS and 0.28 h^{-1} in simplified medium) than the other species in both media, and it showed the highest mannitol production rate of 1.27 $g{\cdot}l^{-1}h^{-1}$ in MRS and 1.09 $g{\cdot}l^{-1}h^{-1}$ in simplified medium. Compared with MRS medium, the simplified medium resulted in lower values in specific growth rate, mannitol production rate, and mannitol yield (except ATCC8293, B-742C, and ATCC9135). Accordingly, *L. citreum* KACC 91348P, *L. mesenteroides* D1, and *L. mesenteroides* MU3, which showed higher mannitol production rate and concentrations among the tested strains in both media, were selected for the next experiment.

The effects of temperature and pH on biomass and mannitol productivity were investigated to find the optimal culture condition for mannitol production. *L. mesenteroides* MU3, *L. mesenteroides* D1, and *L. citreum* KACC 91348P were cultivated in MRS broth (pH 6.5) containing 4% fructose and 2% glucose, and their biomass and mannitol concentrations were measured at the middle of the stationary phase. For this, samples incubated at 20°C and 30°C were analyzed after 12 h, and a sample incubated at 10°C was analyzed after 18 h. As a result (Fig. 1A), the highest biomass and mannitol concentration values were obtained at 30°C for the tested strains, and the mannitol concentration was approximately 2-fold higher than those at 20°C and 10°C. Beside temperature, pH also affected mannitol production and biomass, and the best result was obtained at the initial pH of 6.5 (Fig. 1B). These results show that, for these three strains, temperature and pH conditions are important environmental factors affecting the mannitol productivity, and for the best productivity the fermentation condition should be maintained at 30°C and pH 6.5.

From the results demonstrated in Table 1 and Fig. 1, *L. citreum* KACC 91348P was chosen as a superior strain for maximum mannitol yield. For the synthesis of mannitol using this strain, batch fermentation was performed in 2 l of medium containing 80 g/l fructose and 40 g/l glucose at an initial pH 6.5 at 30°C. The bioreactor (Fermentec Inc., Cheongwon, Korea) was operated with a disk impeller speed of 100 rpm, and the pH was controlled automatically by addition of 5 M NaOH and 2 M HCl for a constant pH of 6.5. As shown in Fig. 2, after cultivating for 12 h, 80 g/l of fructose was converted to 66.5 g/l of mannitol, and 40 g/l of glucose was used to grow cells up to 13.3 optical density (OD600). The mannitol production rate was 8.3 $g{\cdot}l^{-1}h^{-1}$ and the conversion yield was 86.7 mol%. The maximum volumetric mannitol productivity, 14.8 $g{\cdot}l^{-1}h^{-1}$, was achieved between 4 and 6 h. Noticeably, a small

Table 1. Results of mannitol production by 9 strains of *Leuconostoc* species in MRS and simplified media.

Microorganisms	MRS medium[a]				Simplified medium[b]			
	μ (1/h)	Qmtl ($g{\cdot}l^{-1}h^{-1}$)	Cmtl. (g/l)	Ymtl %(mol/mol)	μ (1/h)	Qmtl ($g{\cdot}l^{-1}h^{-1}$)	Cmtl. (g/l)	Ymtl %(mol/mol)
Leuconostoc mesenteroides ATCC 8293	0.25	1.13	27.1	93.4	0.31	0.71	17.0	73.6
L. mesenteroides ATCC 9135	0.22	0.98	23.5	90.9	0.31	0.67	16.0	68.7
L. mesenteroides KCTC 3719	0.40	1.10	26.4	93.9	0.20	0.73	17.5	72.1
L. mesenteroides NRRL B-512F	0.25	1.08	25.9	92.0	0.25	0.79	18.9	80.9
L. mesenteroides NRRL B-742C	0.26	1.21	29.4	84.3	0.34	0.63	15.1	69.5
L. citreum KACC 91348P	0.31	1.27	30.4	89.3	0.28	1.09	26.1	82.0
L. mesenteroides D1	0.28	1.14	27.3	93.7	0.22	0.75	18.0	72.8
L. mesenteroides MU3	0.19	1.11	26.6	95.6	0.27	0.79	18.9	86.2
L. pseudomesenteroides AJ	0.27	1.06	25.4	89.1	0.18	0.68	16.3	66.7

μ, cell growth rate; Q_{mtl}, mannitol production rate (productivity); C_{mtl}, mannitol concentration accumulated; Y_{mtl}, mannitol yield from fructose.
[a,b]Compositions of MRS and simplified media are listed in the text.

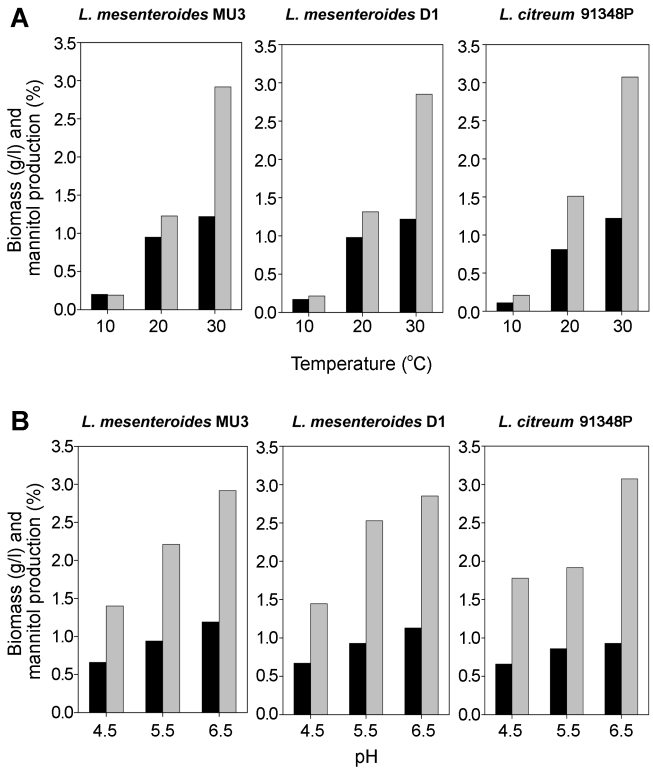

Fig. 1. Effects of temperature (**A**) and pH (**B**) on biomass and mannitol production by *L. mesenteroides* MU3, *L. mesenteroides* D1, and *L. citreum* KACC 91348P.
Columns: *grey*, mannitol production (%, w/v); *dark*, biomass (g/l). **A**. Cells were cultivated in a MRS medium containing 4% fructose and 2% glucose at the initial pH 6.5 for 12 h (at 20°C and 30°C) and 18 h (at 10°C). **B**. Cells were cultivated for 12 h at 30°C at various initial pH conditions (4.5, 5.5, and 6.5).

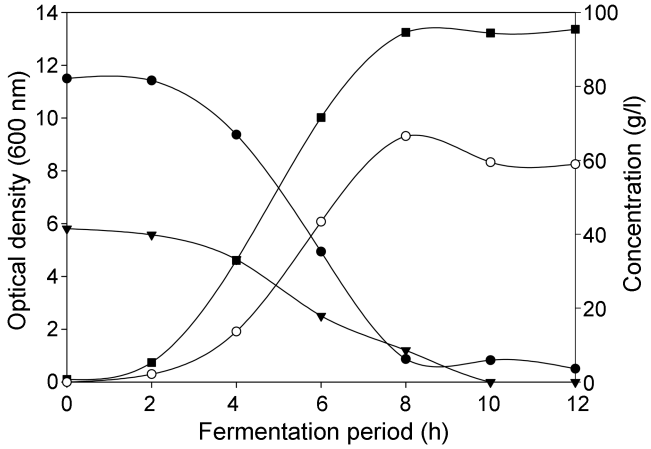

Fig. 2. Mannitol production by *Leuconostoc citreum* KACC 91348P in a 2 l batch fermentation.
Legends: (●), fructose (g/l); (▼), glucose (g/l); (○), mannitol (g/l); and (■), optical density at 600 nm.

amount of mannitol was consumed after depletion of glucose and fructose from the medium.

For microbial production of mannitol, yeast, filamentous fungi, and bacteria have been used for the advantage of cofactor regeneration. However, yeast and fungi produce mannitol in too low volumetric productivities and it makes the process less useful. Purification, especially with yeasts, is complicated by the high concentrations of glycerol present in the culture media [18]. Meanwhile, bacteria, namely lactic acid bacteria, seem to be efficient producers of mannitol and the genera *Lactobacillus* and *Leuconostoc* have led the attention [8, 18]. Specifically, *Leuconostoc* spp. have great importance in the production of many fermented foods such as sauerkraut, pickles, meat products, and *kimchi*, where it gives a refreshing soft sweet taste by production of mannitol. Studies using different *Leuconostoc* species report various mannitol yields: 65% by *Leuconostoc* sp. Y-002 [21], 97% by *L. mesenteroides* 9135 [17], and 91% by *L. mesenteroides* 9135 [15]. Different results are often caused by different culture conditions such as media (complex *vs*. defined), substrate (glucose *vs*. corn carob syrup), carbon sources (glucose *vs*. fructose), culture methods (batch *vs*. fed-batch), and status of cells (growing *vs*. resting). Recent studies [2, 12, 20] using different *Leuconostoc* species showed various growth rates and mannitol yields depending on strains and culture conditions. Therefore, in this study, we compared the effectiveness of the newly isolated strains in mannitol productivity with the five well-known species ever studied in *Leuconostoc* genus. As shown in Table 1, even though those type strains resulted in high mannitol yields (84.3~93.9%) in complex media, their accumulated mannitol concentrations (23.5~29.4 g/l) were much lower than that (30.4 g/l) of *L. citreum* KACC 91348P. After complete consumption of glucose, those strains partly converted fructose into mannitol resulting in high mannitol yield, but with low accumulation concentration. These results emphasize that, beside mannitol yield, the accumulated mannitol concentration or mannitol production rate are also important for economical mannitol production.

Conclusively, compared with type strains of *Leuconostoc* species tested in this study, *L. citreum* KACC 91348P shows very fast growth rates (0.31 and 0.28 h^{-1}) with relatively higher mannitol productivity (1.27 and 1.09 $g \cdot l^{-1} h^{-1}$) in both MRS and simplified media, respectively. This result reveals that, *L. citreum* KACC 91348P has not only an efficient fructose uptake system such as fructose-PTS or permease, but also a fast reduction system with high fructokinase activity and low phosphoglucoisomerase activity. Further enzymatic analyses will provide more detailed information about the metabolic characteristics of this strain. *L. citreum* KACC 91348P can be used as a useful starter in the single or mixed fermentation of fructose-containing foods such as *kimchi* and sauerkraut to overproduce mannitol.

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