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SHORT COMMUNICATION

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Effect of salt nutrients on mannitol production by *Lactobacillus intermedius* NRRL B-3693

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Abstract The effects of four salt nutrients (ammonium citrate, sodium phosphate, magnesium sulfate, and manganese sulfate) on the production of mannitol by *Lactobacillus intermedius* NRRL B-3693 in a simplified medium containing 300 g fructose, 5 g soy peptone, and 50 g corn steep liquor per liter in pH-controlled fermentation at 5.0 at 37°C were evaluated using a fractional factorial design. Only manganese sulfate was found to be essential for mannitol production. Added manganese sulfate concentration of 0.033 g/l was found to support maximum production. The bacterium produced 200.6±0.2 g mannitol, 61.9±0.1 g lactic acid, and 40.4±0.3 g acetic acid from 300 g fructose per liter in 67 h.

Keywords *Lactobacillus intermedius* · Mannitol production · Fructose fermentation · Salt nutrients · pH-controlled fermentation · Lactic acid production

Introduction

Mannitol, a naturally occurring polyol, is widely used in the food, pharmaceutical, medicine, and chemical industries [5]. It (US $3.32/lb; global market, 30 million lb/year) is currently produced industrially by high-pressure hydrogenation of fructose/glucose mixtures in an aqueous solution at high temperature (120–160°C) with Raney nickel as catalyst [3]. Typically, the hydrogenation of a 50/50 fructose/glucose mixture results in an approximately 25/75 mixture of mannitol and sorbitol. This means that about half of the fructose is converted to mannitol and half of it to sorbitol. The glucose is hydrogenated exclusively to sorbitol. As a consequence, the commercial production of mannitol is always accompanied by the production of sorbitol thus resulting in an inefficient process [9]. Moreover, it is relatively difficult to separate sorbitol and mannitol, which results in even higher production costs and decreased yields [2].

Several heterofermentative lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Leuconostoc*, and *Oenococcus* have been reported to produce mannitol from fructose [4, 9, 12]. These LAB have the capability to utilize fructose as an alternative electron acceptor, thereby reducing it to mannitol with the enzyme mannitol dehydrogenase (EC. 1.1.1.67) [7]. In this method, the reducing equivalents are generated by conversion of about one-third of the fructose to lactic acid and acetic acid.

Manganese and magnesium ions are essential cofactors for enzymes in the primary sugar metabolism of LAB [10]. Magnesium functions as a cofactor for enzymes, such as fructokinase, phosphoketolase, and acetate kinase, whereas manganese functions as a cofactor for some enzymes in the pathway from glyceraldehyde-3-phosphate to pyruvate, and for lactate dehydrogenase. Thus, these two metal ions play a central role in the production of reducing power NAD (P)H and ATP and are, therefore, essential for many cellular functions and more importantly for transport and reduction of fructose [10]. Mannitol dehydrogenase, on the other hand, does not require any cofactors [7].

Citrate is a typical component in LAB growth medium [1]. In the cell, citrate is cleaved to acetate and oxaloacetate. Oxaloacetate is then decarboxylated to pyruvate, which can be further reduced to metabolic end products such as lactate. The generation of metabolic energy by citrate metabolism contributes to the growth

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advantages during co-metabolism of citrate and glucose or fructose [8].

In a previous paper, we reported the production of mannitol and D-lactic acid by *Lactobacillus intermedius* NRRL B-3693 from fructose using a simplified MRS medium [6]. This LAB was selected after screening 72 bacterial cultures from the ARS Culture Collection, Peoria, IL, USA. In this paper, the author describes the effects of salt nutrients generally present in MRS medium [1] on the production of mannitol and lactic acid from fructose by *L. intermedius* NRRL B-3693 replacing expensive Bacto-peptone and Bacto-yeast extract with soy peptone and corn steep liquor, respectively.

Materials and methods

Materials

Fructose syrup (Kryster liquid fructose), Soy peptone type D, and corn steep liquor were supplied by A. E. Staley Manufacturing Co., Decatur, IL, USA; Marcor Development Corp., Carlstadt, NJ, USA; and Cargill, Minneapolis, MN, USA, respectively. Soy peptone D contains 7.8% total nitrogen and 2.0% amino nitrogen (w/w) according to the product specification (#1203) supplied by the manufacturer. The supplied corn steep liquor contains 3.7% total nitrogen, 1.25% amino nitrogen, 1.75% phosphorus and 0.002% manganese (w/w). Aminex HPX-87P and Aminex HPX-87H columns (300×7.8 mm each) and carbo P and Cation H guard columns (30×4.6 mm each) were purchased from Bio-Rad Laboratories, Hercules, CA, USA. All other chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Bacterial strain

Stock cultures of *L. intermedius* NRRL B-3693 (obtained from the ARS Culture Collection) were maintained in 70% glycerol vials at –80°C. It was transferred and maintained in agar (1.5%, w/v) slants made with simplified MRS medium containing 10 g peptone, 5 g yeast extract, 2 g ammonium citrate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, and 2 g disodium phosphate per liter (final pH 6.5) at 4°C for use in seed culture preparation [1]. Glucose (10 g/l) was used as carbon source.

Preparation of seed culture

The simplified MRS medium described above (pH 6.5) was used for the preparation of seed culture. The medium and the substrate were sterilized separately at 121°C for 15 min. A 250-ml Erlenmeyer flask containing 100 ml of the medium with 2% (w/v) fructose was inoculated with a loopful of cells taken from a stock slant and incubated at 37°C on a rotary shaker (130 rpm) for 24 h. This culture was used as seed culture.

Fermentation conditions

Batch culture experiments were performed in pH-controlled 500 ml fleakers with an initial medium volume of 300 ml at 37°C as described previously [6]. Unless otherwise stated, the medium contained 300 g fructose, 5 g soy peptone D, 50 g corn steep liquor (~50% solids, w/v; as is basis), 2 g ammonium citrate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, and 2 g disodium phosphate per liter (final pH 5.0). The pH was maintained at 5.0 throughout the fermentation period by adding 2 N NaOH. Cultures were stirred magnetically using 1.5 in. stir-bars at 130 rpm. Samples were withdrawn periodically to determine sugar utilization and product yields.

Analytical methods

Sugar utilization and product analysis were performed by high-pressure liquid chromatography (HPLC) (Thermo Separation Products, Inc., San Jose, CA, USA). For quantification of fructose and mannitol, an Aminex HPX-87P column was used. The column was maintained at 85°C, and the compounds were eluted with deionized water (Milli-Q water, Millipore Corp., Bedford, MA, USA) at a flow rate of 0.6 ml/min. For lactic acid and acetic acid analyses, an Aminex HPX-87H column was used. The column was maintained at 65°C, and the organic acids were eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Peaks were detected by refractive index and identified and quantified by comparison to retention times of authentic standards.

Results and discussion

The effects of four salt nutrients (ammonium citrate, sodium phosphate dibasic anhydrous, magnesium sulfate, and manganese sulfate) on the production of mannitol from fructose at a high concentration (300 g/l) by *L. intermedius* in a simplified medium containing soy peptone (5 g/l) and corn steep liquor (50 g/l) were studied using a fractional factorial design. The results are presented in Table 1. The mannitol production increased when both magnesium sulfate and manganese sulfate were added to the medium separately. However, the relative increase in mannitol yield when magnesium sulfate was added in the absence of manganese sulfate was less than when manganese sulfate was added in the absence of magnesium sulfate in the medium. Also, there appears to be no synergistic effect when both magnesium sulfate and manganese sulfate were supplied in the medium at the concentration levels used. It is clear that magnesium sulfate, ammonium citrate, and sodium phosphate did not have any significant influence on the utilization of fructose and production of mannitol in the medium containing manganese sulfate. Therefore, added manganese sulfate (0.05 g/l), as is evident from the data in Table 1, was essential for *L. intermedius* B-3693 to utilize fructose

Table 1 Effect of ammonium citrate, sodium phosphate, $MgSO_4$, and $MnSO_4$ on the production of mannitol, lactic acid and acetic acid from fructose by *Lactobacillus intermedius* NRRL B-3693 in pH-controlled batch fermentation at 37°C

No.	Ammonium citrate (g/l)	$NaHPO_4$ (g/l)	$MgSO_4$ (g/l)	$MnSO_4$ (g/l)	Mannitol yield (g/l)	Lactic acid yield (g/l)	Acetic acid yield (g/l)
1	0	0	0	0	159.2±6.1	57.1±2.0	37.8±2.0
2	2	2	0	0	145.6±6.0	50.7±9.9	31.1±3.4
3	2	2	0.1	0	176.7±5.7	57.7±0.6	35.1±2.8
4	2	0	0.1	0	177.9±13.2	64.5±1.5	43.6±0.2
5	0	2	0	0.05	194.3±0.6	68.1±0.4	46.1±0.5
6	2	0	0	0.05	192.5±2.9	63.5±2.8	37.8±1.5
7	0	0	0.1	0.05	192.7±3.4	68.3±1.3	46.6±1.3
8	2	2	0.1	0.05	190.0±0.6	61.6±2.1	43.3±3.0

The medium contained fructose (300 g/l), soy peptone (5 g/l), and corn steep liquor (50 g/l). Initial pH was 5.0 and it was maintained at 5.0 during the entire fermentation period with 2 M NaOH. The fermentation time was 67 h in all cases. Values reported are averages from duplicate experiments

and metabolite production in a medium containing corn steep liquor and soy peptone. von Weymarn et al. [10] reported that the volumetric mannitol productivities were affected by 32, 17, 9, and 6% by the removal of Mn^{2+} from the simplified growth medium (tryptone, 10 g; yeast extract, 5 g; K_2HPO_4, 2 g; $MgSO_4$, 0.2 g; $MnSO_4$, 0.01 g; total sugars, 30 g/l) of the *Leu. pseudomesenteroides*, *L. fermentum*, *L. brevis*, and *Leu. mesenteroides*, respectively. They also reported that the removal of Mg^{2+} from the medium caused 2–4% decrease in volumetric productivities.

The effects of both corn steep liquor (17, 33, or 50 g/l) and manganese sulfate (0.017, 0.033, or 0.050 g/l) each at three concentration levels using a factorial design on the production of mannitol from fructose (300 g/l) by *L. intermedius* are presented in Table 2. Manganese sulfate at 0.033 g/l and corn steep liquor at 50 g/l gave the best result with respect to mannitol production. The bacterium was able to utilize fructose well and produce mannitol at 200 g/l level which is a desirable concentration because mannitol at over 180 g/l concentration precipitates out by simply removing the cells from the fermentation broth and cooling slowly to 4°C [6].

The data presented in this paper shows that it is possible to produce mannitol from fructose at a concentration high enough for easy downstream processing using *L. intermedius* NRRL B-3693, and the medium components can be simplified by using only soy peptone (5 g/l), corn steep liquor (50 g/l), and manganese sulfate (0.033 g/l). Yun and Kim [12] reported that *Lactobacillus* sp. Y-107 and *Leuconostoc* sp. (isolated during fermentation of kimchi, a Korean pickled vegetable) were not able to use high concentrations of fructose above 100 g/l due to low osmotolerance. von Weymarn et al. [11] reported that increasing the initial fructose concentration from 100 to 120–140 g/l resulted in decreased productivities due to both substrate and end-product inhibition of the enzyme mannitol dehydrogenase for production of mannitol by *Leu. mesenteroides* ATCC-9135 in high cell density membrane recycle cultures. This *Leu. mesenteroides* strain was selected as the best producer of mannitol among ten heterofermentative LAB by comparing their ability to produce mannitol from fructose in high cell density membrane cell-recycle cultures. To my knowledge, only *L. intermedius* B-3693 strain has the unique capability to utilize very high concentration of fructose (300 g/l) at a relatively rapid rate and produce mannitol at above 200 g/l level among the mannitol producing heterofermentative LAB reported to date.

Table 2 Effects of corn steep liquor and manganese sulfate on the production of mannitol, lactic acid and acetic acid in pH-controlled batch fermentation by *Lactobacillus intermedius* NRRL B-3693 at 37°C

Corn steep liquor (g/l)	$MnSO_4$ (g/l)	Mannitol yield (g/l)	Lactic acid yield (g/l)	Acetic acid yield (g/l)
17	0.017	80.2±2.8	28.0±0.5	18.3±0.5
33	0.017	152.8±7.0	50.8±4.0	32.1±2.1
50	0.017	190.8±12	62.8±2.1	39.3±1.3
17	0.033	79.5±4.0	26.9±1.3	17.3±0.8
33	0.033	141.9±8.1	45.9±2.8	29.2±1.7
50	0.033	200.6±0.2	64.3±0.1	40.4±0.3
17	0.050	64.0±2.2	22.0±0.8	14.0±0.5
33	0.050	164.8±6.7	52.7±2.3	33.8±1.7
50	0.050	183.8±8.9	62.6±1.0	38.7±1.0

The medium contained fructose (300 g/l), soy peptone (5 g/l), corn steep liquor (17, 33, or 50 g/l), and $MnSO_4$ (0.017, 0.033, or 0.050 g/l). Initial pH was 5.0 and it was maintained at 5.0 during the entire fermentation period using 2 M NaOH. The fermentation time was 67 h in all cases. Values reported are averages from duplicate experiments

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