Optimization of the One-Step and Two-Step Transformation Methods of Mannitol by *Lactobacillus buchneri*

Hongbin Wang, Yu Li, Yongshuai Wang, Yan Chen, Yong Zhang, Yongrui Feng, Haichao Han, Shuqi Gui and Fuping Lu

1 Introduction

Mannitol, an important functional sugar alcohol, is widely used in the pharmaceutical, food, painting and textile industry [1, 2]. The current study showed that lactic acid bacteria, yeasts, fungi and other microbial strains could synthesize mannitol [3]. However, current industrial microbial production of mannitol is not economically viable [4]. Thus, development of commercial microbial production of mannitol requires screening for more potential mannitol-producing strains.

Many studies on microbial production of mannitol have been reported, but so far no industrialization of microbial method was realized due to the problem of high cost [5–7]. Many microorganisms have been found to be able to synthesize mannitol, and the hetero-fermentative lactic acid bacterium received more attention [8–11]. Most studies focused on screening of more efficient production strains and based on the one-step batch transformation method that performing cell growth and cell catalysis at the same time [5, 6, 12]. But this method has many defects: first, it is difficult to separate mannitol from the impurities from culture medium, which increases the separation complexity and cost; second, the cells are used only one time for transformation, bringing higher cell cultivation cost [13, 14]. To solve the problem, a two-step transformation strategy was suggested. It separates the cell growth step with the biotransformation step for production of mannitol. Successful application of the two-step transformation method has been demonstrated, e.g., for the production of lactic acid [15] and propionic acid [16]. As far as we know,

H. Wang · Y. Li · Y. Wang · Y. Chen · Y. Zhang · Y. Feng · H. Han · S. Gui · F. Lu (✉)
Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education;
the College of Biotechnology, Tianjin University of Science and Technology;
National and Local United Engineering Lab of Metabolic Control Fermentation Technology,
College of Biotechnology, Tianjin University of Science and Technology,
TEDA, No. 29, Ave 13, Tianjin 300457, China
e-mail: lfp@tust.edu.cn

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no studies for mannitol production with the two-step transformation strategy were reported. In the theory, the strategy could reduce the complexity of product separation and increase the repeat times of cell use, which will help to reduce the producing cost of mannitol.

2 Materials and Methods

2.1 Materials

Peptone, beef extract and yeast extract were products of Aobox Biotechnology. Glucose and Fructose were purchased from Beijing solarbio. Ammonium citrate, Anhydrous sodium acetate, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$ and twain-80 were purchased by Tianjin north day medical chemical reagent factory, Corn steep liquor was supplied by Cargill. Mannitol standard product was purchased by Sigma.

2.2 One-Step Transformation Method of Mannitol

The activated *L. buchneri* CGMCC 7300 cells were added by 2% cell quantity to different MRS-improved fermentation medium with different total concentration of fructose (F) and glucose (G) (60, 90, 120, 150, 180 g/L) and different ratios of them (F:G = 1:2, 1:1, 3:2, 2:1) at 30 °C for 72 h.

2.3 Two-Step Transformation Method of Mannitol

The cells were added by 2% (v/v) quantity to MRS-improved medium to study the effects of different temperature (25, 30, 32, 35, 37, 40, 45 °C) and different initial pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) on the growth condition of *L. buchneri* CGMCC 7300, and then conform the best growth condition.

The cells were cultured at the best growth condition, and then collected by centrifugation at 6000 r/min for 10 min and washed twice with sterile water. Then suspended with the mixture of fructose and glucose at different concentrations (90, 120, 150 g/L) and 0.5% yeast powder for biotransformation at different pH (3.0, 4.0, 5.0, 4.0, 5.0) and different temperature (25, 28, 30, 32, 35, 37, 40 °C) for 48 h. The concentration of mannitol and residual sugar were detected by high performance liquid chromatography (HPLC).

2.4 HPLC Analysis

Sugars and mannitol were analyzed by HPLC (Agilent, USA). An aminex HPX-87P column with a Carbo-P micro-guard column was used. The column was maintained at 85 °C, and the sugars and mannitol were eluted with deionized water (Milli-Q, Millipore Corp., Bedford, MA) at a flow rate of 0.6 mL/min. Fructose, glucose, and mannitol were identified by refractive index and quantified by comparison to retention time of authentic standards.

3 Results and Discussion

3.1 One-Step Transformation Method for Mannitol

3.1.1 The Effects of the Ratios of Fructose and Glucose on the Transformation of Mannitol

In one-step transformation method, the total concentration of sugars was 90 g/L. The production of mannitol was influenced by the ratio of fructose and glucose. It is evident from the results presented in Fig. 1. When carbon source was only glucose, the production of mannitol was very low, while using fructose alone, the output of mannitol reached 35.92 g/L and the conversion rate was 39.91%. When use the mixture of fructose and glucose as carbon source, the ratio of fructose and glucose 3:2 proved to be the best proportion, and mannitol production could reach 49.98 g/L and the conversion reached 55.53%.

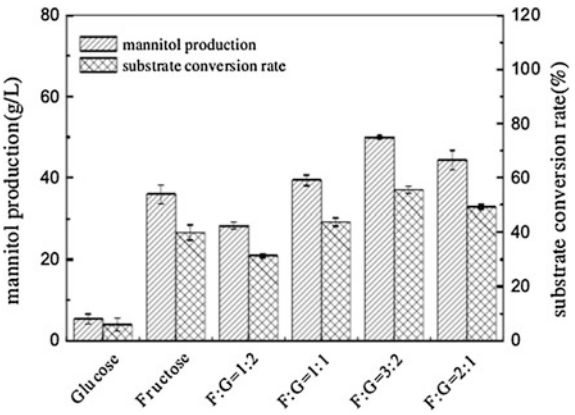

Fig. 1 Effect of different fructose/glucose ratios on the production of mannitol (n = 3)

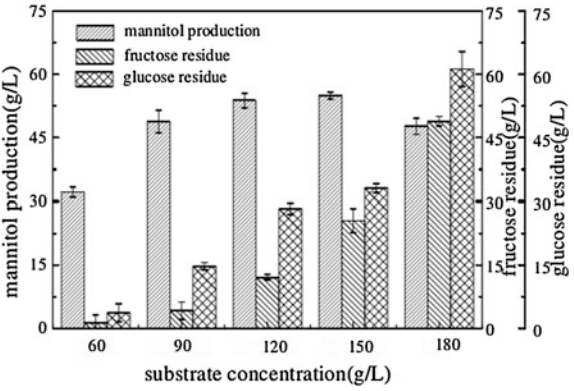

Fig. 2 Effects of different substrate concentration on the transformation of mannitol (n = 3)

3.1.2 The Effects of the Substrate Concentration on the Transformation of Mannitol

Under the condition of the ratio of fructose and glucose set to be 3:2, change the concentration of total sugar in the medium (60, 90, 120, 150, 180 g/L) and investigate their effects on the mannitol transformation. As can be seen in Fig. 2, high substrate concentration up to 180 g/L limited the transformation, while as the concentration of substrate increased from 60 to 150 g/L, the mannitol concentration also increased. When total sugar concentration was 150 g/L, the highest yield of mannitol 55.01 g/L was achieved, but the residual amount of fructose and glucose also increased to be 25.49 and 58.01 g/L respectively. Overall consideration, the substrate concentration of 90 g/L was the best, in which mannitol production reached 48.82 g/L, while fructose residual decreased to be 4.15 g/L, and conversion rate reached 50.43% and the proportion of mannitol in the total sugar reached 72.18%.

3.2 Two-Step Transformation Method for Mannitol

The two-step transformation method includes the first step of cell growth and the second step of cell catalysis. The essence of cell catalysis is the enzyme catalysis in biological system. Compared with the extraction of enzyme, whole-cell catalysis can use cellular cofactor and other enzymes for reducing cost and improving the efficiency of biological catalysis. As catalyst, the concentration of the cells determines the transformation speed of mannitol. Therefore, it is critical for transformation to optimize the growth conditions to maximize the cell concentration. In addition, it is also important to optimize the cell catalysis conditions for transformation.

3.2.1 Optimization of the Cell Growth Step

Except for the composition of medium, temperature and pH also affected the cell growth of *L. buchneri* CGMCC 7300. The effects of temperature on growth at 25, 30, 32, 35, 37, 40, and 45 °C were investigated. As can be seen in Fig. 3, cell concentration achieved maximum at 37 °C for OD600 0.4. The effects of different pH from 3.5 to 8.0 on the growth were also compared. Shown in Fig. 4, pH from 5.5 to 6.5 were suitable for the growth of L. buchneri CGMCC 7300, and the maximum cell concentration was reached at pH 6.5. The growth was obviously restrained at the pH lower than 4.0 or higher than 7.5.

3.2.2 The Optimization of the Cell Catalysis Step

The medium was removed by centrifuge followed by the catalysis step. The conditions in this step needs to satisfy the cell catalysis, but not the cell growth. So, the conditions optimized in the one-step transformation method or the first cell growth

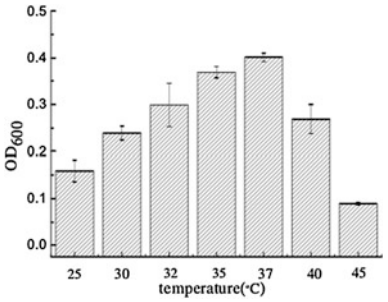

Fig. 3 Effect of temperature on growth of *L. buchneri* CGMCC 7300 (n = 3)

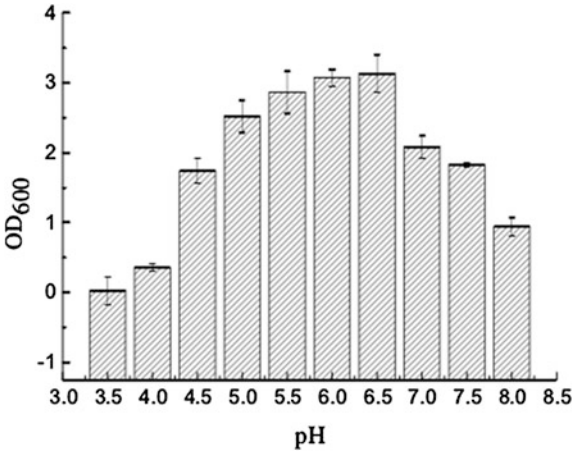

Fig. 4 Effect of different initial pH on growth of *L. buchneri* CGMCC 7300 (n = 3)

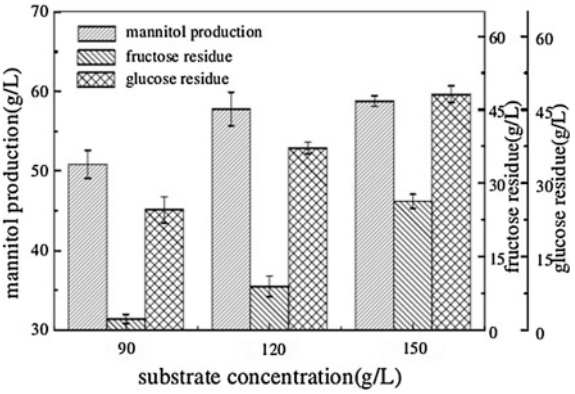

Fig. 5 Effect of substrate concentration on cell catalysis (n = 3)

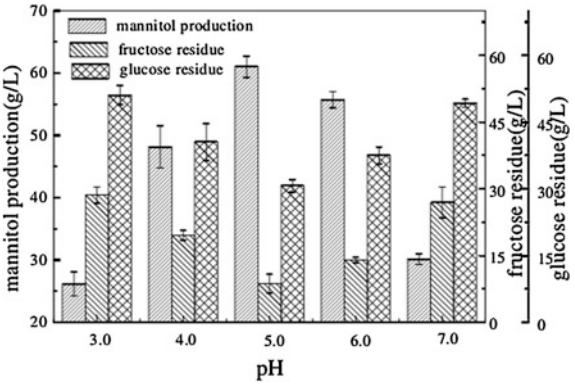

Fig. 6 Effect of pH on cell catalysis of mannitol (n = 3)

step may not suit the second cell catalysis step. The optimal substrate concentration, pH and temperature for cell catalysis were optimized in this study.

Taking both the yield and the conversion rate in account, the substrate concentration 120 g/L was considered the best, as was shown in Fig. 5. At 120 g/L, the mannitol concentration reached 57.81 g/L, and the residual fructose was only 8.93 g/L.

For the whole-cell catalysis of mannitol, the mannitol dehydrogenase plays a key role in the transformation [15]. Therefore, a suitable pH was required for the mannitol dehydrogenase to maximize the mannitol output. As shown in Fig. 6, the best pH for whole-cell catalysis by *L. buchneri* CGMCC 7300 was 5.0. Also, when pH $\geq$ 7.0 or $\leq$ 3.0, the enzyme activity of the mannitol dehydrogenase reduced greatly.

In the conditions of the ratio of fructose and glucose 3:2, total sugar concentration 120 g/L and pH 5.0, the effects of the temperature on the transformation were shown in Fig. 7. The mannitol production reached the highest 63.71 g/L at 32 °C, and meanwhile fructose was completely consumed. It indicated that the optimal temperature for transformation was different from that for cell growth.

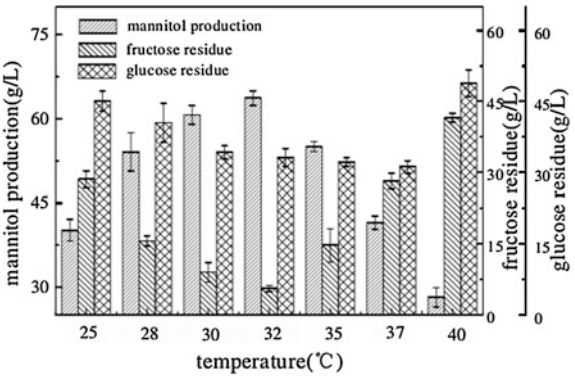

Fig. 7 Effect of different temperature on cell biocatalyst (n = 3)

4 Conclusions

In this study, both the one-step method and two-step method were used for the transformation of mannitol by *L. buchneri* CGMCC 7300 with the mixture of fructose and glucose as substrate, and their conditions were optimized respectively. The best conditions for one-step method were as follows: the ratio of fructose and glucose 3:2, substrate concentration 90 g/L. After fermentation at these conditions for 60 h, mannitol concentration reached 48.82 g/L and the conversion reached 50.43%. The best conditions of the two-step transformation method were as follows: in the first step, the initial pH was 6.5 and the temperature 37 °C; in the next cell catalysis step, substrate concentration 120 g/L, catalysis at 32 °C and pH 5.0 for 36 h. At these conditions, mannitol concentration reached 63.71 g/L with the residual fructose only 6.67 g/L and the conversion rate up to 50.24%. The yield of mannitol increased 30.5% more than that of the one-step transformation method, showing that the two-step transformation method had a great advantage in mannitol production. The results of this study will be significant for further industrial-scale production of mannitol.

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