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ISOLATION OF LACTIC ACID BACTERIA PRODUCING MANNITOL FROM INDONESIAN COCOA BEANS FERMENTATION

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ABSTRACT:

Mannitol is a polyol which is widely used in many industries. Lactic acid bacteria known as one of microorganism which produce mannitol in a certain condition. LABs are widely found in fermentation product. This research aims to isolate LAB producing mannitol from Indonesian cocoa beans fermentation as an alternative of comercial mannitol production. LAB isolated from 0 to 96 h of fermentation, screened by the clear zone formed in MRS medium contained $CaCO_3$ 1% and its catalase activity. Initial screening of 94 LAB isolates using TLC yield only ten LAB producing mannitol isolates. Further screening through HPLC analysis showed that LAB 4.32 produced the highest concentration of mannitol (1.873 g/L). Analysis of 16S rRNA genes revealed that LAB 4.32 was *Lactobacillus plantarum*. Cultivation of the LAB 4.32 in tofu whey with addition of $(NH_4)_2SO_4$ 1% (w/v) and fructose 5% (w/v) obtained the highest mannitol production up to 0.719 g/l.

INTRODUCTION

Mannitol constitutes a natural sugar alcohol (polyol) with six carbons, and can be produced by bacteria, yeasts, fungi, algae, lichens as well as some plants [1]. The sweetness of mannitol is about 50-72% compared to sucrose [2]. In addition, mannitol also has a low calorie value (1.6 kcal/g), which is lower than sucrose (4 kcal/g). Hence, it is often used as an artificial sweetener on low-sugar food products such as sugar-free gum products [3]. This compound has been widely applied to various industries (food industry, pharmaceutical, medical and chemical) because it has several health benefits. Since it is slightly metabolized and has anti-hyperglycemia properties (glycemic index value 0), mannitol is applied for diabetic diets [2]. As a food ingredient, mannitol is also used as a texturing agent or in breath-freshening products [3]. Most polyols are not digested in mouth, therefore mannitol compound is regarding non-cariogenic (not causing caries in teeth) [4]. The crystalline mannitol shows a low level of hygroscopicity [3].

Currently, mannitol has been commercially produced using a fructose or a fructose-glucose mixture catalytic hydrogenation method with Raney-nickel catalyst [5]. However, the method has several disadvantages since mannitol is only produced as a by-product, while the main product is sorbitol. This separation leads to increased mannitol production cost and reduced yield [6]. Additionally, the raw materials used in this method must also have a high degree of purity because the metal catalyst used is not specific to the substrate. Mannitol-making reactions also require high temperature and pressure [3]. Helanto *et al*. [7] states that the hydrogenation reaction of glucose-fructose mixture (50:50) results in mannitol 25% and sorbitol 75%. Several studies have reported alternative techniques of producing mannitol using microbes [3]. Helanto *et al*. [7] states that yeast, fungi and lactic acid bacteria are capable of producing mannitol. Lactic acid bacteria are previously reported to have an effective capability in producing mannitol [3].

Indonesia is the third largest cacao producer over the world after Ivory Coast and Ghana. Newly harvested raw cocoa beans require post-harvest processing such as fermentation, drying, and roasting to obtain desired cacao flavor and aroma characteristics [8]. Desirable spontaneous fermentation of cocoa beans requires the succession of microorganisms with microbial activity and substrate metabolism in cocoa pulp [8]. Lactic acid bacteria (LAB) are regarded as one of the important bacteria in initial stage of cocoa fermentation [9]. Fahrurrozi [10] found various levels of mannitol in fermented cocoa pulp and bean. After 24 h of fermentation, the concentration of mannitol increased and reached the maximum concentration of 8.79 mg g-1 at 48 h of fermentation. This finding suggests that cocoa bean fermentation may be a promising source of mannitol-producing LAB isolates. Therefore, valorization of cocoa fermentation in various regions of Indonesia, in this case Sukabumi, needs to be carried out in order to obtain potential local isolates as efficient mannitol producing agents.

In this study, we also tried to use an alternative LAB growth medium, *de Mann Ragosa and Sharpe* (MRS), which known as a high-priced medium. The use of tofu whey, a by-product of tofu making process, is considerable as it is high nutritious content and affordable. Thus, tofu whey is utilized as alternative growth media for mannitol-producing LAB, regarding maximum yield and biomass concentration [11]. It is also widely used as a growth medium of LAB as reported by Thi *et al*., Tripathi, Kurniasari *et al*. and Yeni [12-15].

MATERIALS AND METHOD

Isolation and identification of lactic acid bacteria

Fermented cocoa bean sample (24 g) was added to 50 ml of de Mann Ragosa and Sharpe (MRS) liquid media (Merck, Germany). Serial sample dilution was made at 10^{-1} to 10^{-4} using 0.85% sterile NaCl (Merck, Germany). Diluted sample solution (100 µl) was dispersed on MRSA media containing cycloheximide (100 mg/l) to suppress the growth of yeast. The incubation was carried out at 37 °C for 72 to 96 h in an anaerobic jar with GasPak (Merck, Germany). Single formed colony was selected and dispersed on MRSA media (Merck, Germany) containing 1% (w/v) $CaCO_3$ (Merck, Germany). The bacterial isolates forming clear zone were then purified by the quadrant method and stored for initial identification.

For catalase experiment, a colony was spread onto the slide using loop, and added with 1 drop of 3% (w/w) H_2O_2 (Merck, Germany), and then homogenized [16]. The positive catalase activity characterised by presence of air bubbles. Generally, LAB showed a negative catalase activity.

Screening of potential LAB isolate producing mannitol

Selected LAB isolates from previous stage were grown on MRSA media for 48 h. The bacterial culture was then transferred into a tube containing 5 ml MRSB media, and then incubated at 37 ºC to reach optical density at 600 nm = 0.8. The sample was then used as an inoculum.

The bacterial screening was carried out referred to a modified method of Saha and Nakamura [17]. MRSB media added with 20g/l fructose (Merck, Germany) were sterilized at 121 ºC for 15 min. Inoculum was inoculated into a 10 ml-threaded tube containing 4.95 ml of MRSB media and fructose, and then incubated at 37 ºC for 48 h at a water bath shaker incubator (130 rpm). Samples were observed at 0h and 48 h for further analysis.

Selected LAB isolate from the initial screening process was cultivated on MRSB media added with various concentrations of fructose (10, 20, and 30 g/l). Procedure at this experiment was similar to initial screening process described earlier.

Analysis of mannitol formation

Fermented sample was centrifuged at 2012,4 g for 30 min to obtain supernatant. The supernatant was analyzed for production of mannitol using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Initial analysis was performed using TLC with Silica Gel $60F_{254}$ A 2.2% (b/v) fructose and 1% (b/v) mannitol (Merck, Germany) used as standards. The product was separated twice using a mixture of acetonitrile:ethyl acteate:1-propanol:aquadest (85:20:20:15, v/v/v/v). Spot visualization was carried out through dipping of TLC plate on $AgNO_3$-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 [18]. Retention factor (Rf) was calculated using following equation:

$$\mathrm{Rf} = \frac{\text{distance traveled by sample}}{\text{distance traveled by solvent}}$$

Quantitative analysis was performed using HPLC (HPLC Agilent 1260 Infinity) with Agilent column HIPLEX Ca Duo at 80 ºC and RID temperature of 45 ºC. Milli-Q ultra water was used as eluent at speed of 0.4 ml/min. HPLC was supported by two eluent pumps A and B, autosampler, column oven, RID, DAD, and fraction collector.

Mannitol production using tofu whey media

Tofu Whey was obtained from a local tofu producer in Cibeureum, Bogor. LAB isolate was cultured in tofu whey enriched with two carbon sources, including 5% glucose (Merck, Germany) and 5% fructose (Merck, Germany). As a nitrogen source, 1% $(NH_4)_2SO_4$ (Merck, Germany) was added.

Both media and carbon sources were sterilized at 121 ºC for 15 min. Inoculum (500 µl) was inoculated in a 10 ml-threaded tube containing 4.95 ml media and carbon source, then incubated at 37 ºC for 48 h in water bath shaker incubator (130 rpm). Sample was harvested at 0 and 48 h, analyzed for mannitol production using HPLC as previously described.

Molecular identification of potential LAB isolate

Potential LAB isolate was molecular identified using several stages. First stage, bacterial DNA was extracted using Presto Mini gDNA Bacteria Kit (Geneaid GBB100). The DNA was amplified using Applied Biosystems Thermal Cycler 2720 (Life Tech) with universal primer 63f (5'- CAG GCC TAA CAC ATG CAA GTC-3) and 1387r (5'- GGG CGG WGT GTA CAA GGC-3) [19]. PCR volume reaction was 50 µl consisting of 25 µl Go Taq Green Master Mix 2× (Promega, USA), 2 µl of each primer, 4 µl DNA template, and 17 µl nuclease free water (NFW). Amplification was performed 30 cycles consisting of pre-denaturation (94 ºC, 4 min), denaturation (94 ºC, 30 min), annealing (55 ºC, 30 sec), elongation (72 ºC, 1 min), post-elongation (72 ºC, 7 min), and cooling (4 ºC, 15 sec). PCR product were confirmed by running it in electrophoresis using 0.8% agarose gel in TAE buffer for 30 min on 100 V, and visualized using ethidium bromide staining on UV transiluminator.

Analysis of PCR product sequencing was performed by a professional sequencing service. Sequences then edited and aligned manually by BioEdit v 7.2.6.0 and compared with DNA sequences from GenBank using the BLASTN software at the NCBI (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using CLUSTALW. Construction of phylogenetic tree was conducted using the neighbour-joining (NJ) method by 1000 x bootstrap in Molecular Evolutionary Genetic Analysis 7 (MEGA7; The Biodesign Institute, Tempe, AZ, USA) software [20]. *Escherichia coli* was used as out-group.

RESULTS

Isolation and identification of LAB

LAB colony showed a round shape with milky white in color. The results also demonstrated that 94 of the 170 isolates obtained showed negative catalase activity and clear zone formation on MRS media added with 1% (w/v) $CaCO_3$.

Screening of potential LAB producing mannitol isolate

The result showed that retention factor (Rf) value for mannitol standard was 0.8125. Based on the analysis, we found 10 out of 94 isolates that demonstrated similar Rf value as the standard mannitol (LAB AYN0.6, AYN0.21, AYN1.17, AYN2.24, AYN3.2, AYN4.2, AYN4.3, AYN4.26, AYN4.31 and AYN4.32). Figure 1 shows the spots formed using the supernatant of ten LAB isolates.

Ten selected isolates were then cultured broth MRS media by addition of fructose with different concentrations (10, 20, and 30 g/l). The results demonstrated that 4 isolates consistently produced mannitol at 10 g/l, 20 g/l and 30 g/l fructose concentrations (LAB AYN0.21, AYN4.2, AYN4.31, and AYN 4.32). Addition of 20 g/l fructose resulted in higher color intensity compared to that of 10 g/l fructose, and showed no remarkable difference from that of 30 g/l fructose (Fig. 2). Therefore, addition of 20 g/l fructose is considered sufficient to produce mannitol with a media volume of 4.95 ml.

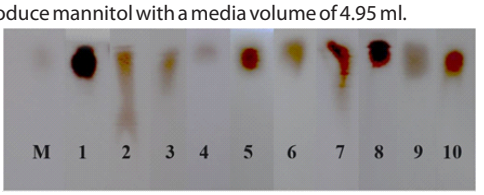

Figure 1 Thin layer chromatogram of ten selected LAB producing mannitol using 20 g/l fructose at 37°C for 48 h. (M: 1% (w/v) Mannitol, , lane 1: AYN0.6, lane 2: AYN0.21, lane 3: AYN1.17, lane 4: AYN2.24, lane 5: AYN3.2, lane 6: AYN4.2, lane 7: AYN4.3, lane 8: AYN4.26, lane 9: AYN4.31 and lane 10: AYN4.32).

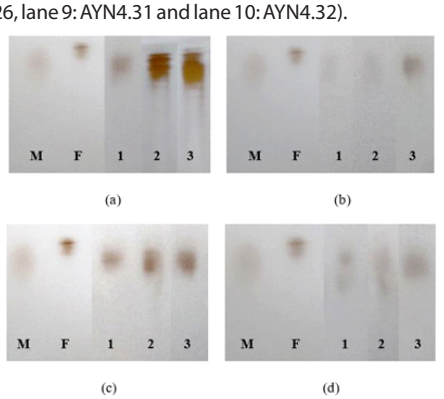

Figure 2 Thin layer chromatogram of LAB (a) AYN0.21, (b) AYN4.2, (c)

AYN4.31 and (d) AYN4.32 using 10, 20, and 30 g/l fructose at 37oC for 48 h. (M: 1% (w/v) Mannitol, F: 2.2% (w/v) Fructose, lane 1: using 10 g/l Fructose, lane 2: using 20 g/l Fructose, and lane 3: using 30 g/l Fructose.

HPLC experiment was subjected to samples produced by four selected isolates on the medium added with 20 g/l fructose. Table 1 shows carbohydrate concentration in the sample after 48 h of incubation. The results showed that isolate of LAB AYN4.32 resulted in the highest mannitol concentration (1.873 g/l) compared to other isolates. Figure 3 shows chromatogram of mannitol produced by LAB AYN4.32 isolate.

Table 1 Mannitol production profile by selected LAB isolates

LAB Isolate	Mannitol Concentration (g/l)
AYN0.21	0.796
AYN4.2	1.479
AYN4.31	0.896
AYN4.32	1.873

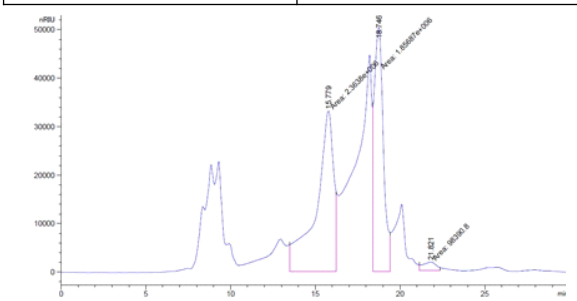

Figure3 HPLC pattern of fermentation broth analysis of LAB AYN4.32 grown on MRS medium with fructose (20 g/l). An Agilent HIPLEX Ca Dou column was used at 80°C, while the RID at 45°C. The mobile phase was deionized water (Mili-Q ultra water) at a flow rate of 0.4 mL/min.

Production of mannitol in tofu whey

Table 2 exhibits the effects of the addition of different carbon sources in tofu whey media on mannitol production. LAB AYN4.32 isolate yielded the highest mannitol concentration (0.719 g/l) in whey media added with 1% (w/v) $(NH_4)_2SO_4$ and 5% (w/v) fructose, although the value was lower than the production on MRS medium (1.873 g /l).

Table 2 Mannitol production by LAB AYN4.32 in tofu whey medium culture at pH 6.0 at 37°C for 48 h

Culture media	Glucose consumption (g/l)	Fructose consumption (g/l)	Mannitol Production (g/l)	Yp/s
Tofu Whey, $(NH4)_2SO_4$ 1% (w/v), Glucose 5% (w/v)	17.770	2.583	0.243	0
Tofu Whey, $(NH4)_2SO_4$ 1% (w/v), Fructose 5% (w/v)	2.149	40.326	0.719	0.018

Note: $Y_{p/s}$ (product yield)

Molecular identification of potential LAB isolate

Based on phylogenetic tree, LAB AYN4.32 isolate was in the first cluster, which was in the same group with Lactobacillus plantarum (Fig. 4), thus LAB AYN4.32 isolate was identified as L. plantarum. Schwan and Wheals [9] reported that L. plantarum was the most commonly found LAB strain in the early 24 h of cocoa bean fermentation.

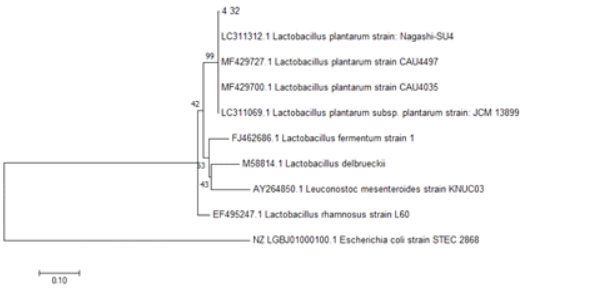

Figure 4 Phylogenetic tree of selected LAB isolate AYN4.32. The phylogenetic tree was obtained based on neighbour-joining method of 16S rRNA gene sequence.

DISCUSSION

LAB isolate was obtained from cocoa bean samples fermented for 4 days with MRS media, a selective medium for LAB growth. The formation of clear zones on the MRS media added with 1% (w/v) $CaCO_3$ indicates that selected isolates have the ability to use glucose as an energy source to produce acidic compounds, which then react with $CaCO_3$ to form Ca-Lactate, and form clear zones around the colony [21]. Holt *et al*. [22] found that LAB commonly had a negative catalase activity. The negative catalase activity was indicated by absence of air bubbles, suggesting that LAB suspected isolate was anaerobic.

A qualitative initial analysis of mannitol formation was performed using thin layer chromatography (TLC). TLC enables to separate the component mixture based on the component distribution between stationary phase and mobile phase. Each component moves at a certain rate expressed by the retention factor (Rf). Blackish-brown spots were formed with presence of carbohydrate in the sample [18]. The spots formed by mannitol standard showed pale color which might be caused by evaporation. Presence of OH chain in mannitol makes it more volatile. Mannitol harvested at 48 h of fermentation showed a higher color intensity than mannitol standard. This presumably reflects low purity of mannitol due to interference of other carbohydrates with a Rf value close to mannitol. This result was in accordance with research conducted by Kim *et al*. [23], finding that formation of mannitol spots occurred after 45 h of fermentation.

Ten selected isolates were then cultured broth MRS media by addition of fructose with different concentrations (10, 20, and 30 g/l). In screening stage, TLC experiment enabled to compare Rf values of samples and standard, and observed the intensity of formed color. Kim *et al*. [23] found that high concentration of fructose could suppress cell growth, which was associated with inhibition by substrate. This was in accordance with Saha and Nakamura [17], finding that mannitol production pattern was not significantly affected by fructose concentration. Bacteria converted fructose into mannitol at an early stage of growth. Therefore, addition of 20 g/l fructose is considered sufficient to produce mannitol with a media volume of 4.95 ml.

HPLC experiment was subjected to samples produced by four selected isolates on the medium added with 20 g/l fructose. Curve of carbohydrates standard is used to obtain an equation to calculate the carbohydrates concentration in samples. The result suggested that high color intensity in TLC qualitative analysis was not always proportionally equal to quantity of mannitol produced. Although isolate of LAB AYN0.21 and AYN4.31 showed a thick spot intensity, their mannitol production was lower than LAB AYN4.32 isolate. This finding suggests that both isolates not only produce mannitol, but also other products such as lactic acid and acetic acid. The substrates present in the media are allocated for production of other compounds, thus concentration of mannitol obtained tends to be small.

Mannitol concentration produced by LAB AYN4.32 isolate was higher than that produced by *Lactobacillus reuteri* CRL 1101 [5] cultured in media containing 7.5% (w/w) molasses and 0.5% (w/w) yeast extract or 7.5% (w/w) molasses, 0.5% (w/w) yeast extract, and 1% (w/w) soy peptone, yielding 0.11 ± 0.07 g/l and 0.09 ± 0.07 g/l mannitol, respectively. Otgonbayar *et al*. [24] found that mannitol synthesis was dependent on the bacterial strain and culture conditions.

Tofu whey is a liquid waste of tofu production. Yeni [15] reported that it is applicable for growth medium of LAB due to presence of some organic compounds required from LAB growth. In addition, sugar content in whey was less than 1%, thus additional carbon source is required to support bacterial growth. In this current work, the use of different carbon sources such as glucose and fructose aimed to select the best carbon source for mannitol production. Glucose is selected as a carbon source because MRS media contained glucose by 20 g/l, while fructose is regarded as a substrate in the synthesis of mannitol.

Tofu whey has a low pH value (about 3.8), thus pH value is adjusted to 6.0 ± 0.2. pH adjustment is carried out to make the medium conditions similar to selective MRS media. Patra *et al*. [25] suggested that pH of the medium exhibited a significant factor in the polyol fermentation. In that study, maximum mannitol production was achieved at pH 6.0 and 6.5. Yun and Kim [26] also stated that the highest mannitol concentration produced by *Leuconostoc* spp. Y-002 was found at pH 6.0, while von Weymarn *et al*. [27] reported that the maximum production of mannitol occurred at pH 5.5.

Substrate concentration in the media also closely related to concentration of mannitol from fructose [25]. In this present study, addition of fructose in in the whey medium was in accordance with the recommendation of Yun and Kim [26], who found that the highest concentration of mannitol was produced from addition of 5% fructose. Kim *et al*. [23] used batch culture fermentation at 5% fructose concentration, and resulted in 78% mannitol theoretically using *Leuconostoc mesentroides* NRRL B-1147. However, this studty only yielded the highest concentration of 3.59% mannitol theoretically, produced on whey media added with 5% (w/v) fructose and 1% (w/v) and $(NH_4)_2SO_4$. Ortiz *et al*. [5] reported that mannitol production by *Lactobacillus reuteri* CRL 1101 cultured in media enriched with soy peptone as a single nitrogen source only reached 0.09 ± 0.07 g/l. In contrast, mannitol production by *L. reuteri* CRL 1101 cultured in media containing either yeast extract or beef extract reached 26.96 ± 1.28 g/l. This suggests that animal protein is needed in the formation of polyols. This was augmented by Yun and Kim [26], finding that yeast extract demonstrated the best source of nitrogen for mannitol formation. Yun *et al*. [28] exhibited that nitrogen showed a critical role in the production of polyols. The nitrogen source affects both type and amount of polyol produced.

In this study, mannitol production by LAB provides desirable benefits regarding to financial aspect, since the culture medium (in this case tofu whey) for production is cheaper than conventional procedure. To our knowledge, this is the first mannitol production that involves cheap tofu whey as a growth medium of LAB isolated from fermented cocoa beans. In addition, difficult separated compounds were not present in this study as found in chemical process, thus reducing production costs. The substrate used did not require a high degree of purity. This work is a preliminary study to obtain isolates from fermented cocoa bean that potentially produce mannitol. Hence, mannitol value may be lower in comparison with other studies. However, the production of mannitol using this isolate can be improved by modifying production conditions and sources of nutrient.

Base sequence of 16S rRNA gene amplified with universal primer 63F and 1387R is used to identified LAB AYN4.32. LAB AYN4.32 isolate was identified as *L. plantarum*. Ardhana and Fleet [29] stated that *L. plantarum* and *Lactobacillus cellobiosus* constituted major LAB strains BAL isolated from Indonesian fermented cocoa bean. LAB AYN4.32 isolate was isolated from fermented cocoa bean at 96 h. This is consistent with the results reported by Fahrurrozi [10], finding that *L. plantarum* and *Lactobacillus fermentum* are the most dominant LAB strains and can be isolated from the beginning (0 h) to the end of cocoa beans fermentation (96 h).

Wouters *et al*. [30] fermented vegetable mixture (green tomato, carrot, and cauliflower) at 25 °C for 3 days which was stored at 16°C until the end of fermentation (from 2 weeks to 2 months). The result showed that *L. plantarum* and *Lactobacillus brevis* were the most isolated species of LAB. In some cases, both species played an important role in the production of mannitol with a concentration of 5.4 g/l.

Lactobacillus plantarum belongs to facultative heterofermentative bacteria, so this species enables to ferment sugar either through Embden-Meyerhof Parnas or phosphoketolase pathway [31]. The bacteria are regarded tolerant to high acid conditions [32]. In addition, *L. plantarum* may also utilize a variety of sugars including D-mannose, D-fructose, and mannitol [33].

LAB is recognized as a food-grade microorganism, thus involvement of LAB in mannitol production provides advantages since both microorganisms and the product can be be directly applied to food stuffs [1]. In addition, some mannitol advantages could also enrich nutritional value of food products, also known as functional food [1]. LAB AYN4.32 isolate from fermented cocoa bean potentially produced mannitol, yielding the highest mannitol concentration (1.873 g/l) in MRS media enriched with 20g/l fructose at 37 °C for 48 h. This isolate also enabled to produce mannitol (0.719 g/l) in tofu whey media added with 1% (w/v) $(NH_4)_2SO_4$ and 5% (b/v) fructose. This potential isolate is closely related to *Lactobacillus plantarum*. Further research is needed to increase mannitol production through optimization of fermentation conditions, variation of carbon and nitrogen sources.

Conflict of interest

Authors declare that there is no conflict of interest.

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