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Selection of exopolysaccharide-producing lactic acid bacteria isolates from Inner Mongolian traditional yoghurt

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Abstract

Lactic acid bacteria (LAB) isolated from Inner Mongolian traditional yoghurt were evaluated for the production of exopolysaccharides (EPS) by phenol-sulphuric acid method after ethanol precipitation and dialysis. Total polysaccharide was extracted from sucrose-containing MRS broth cultures of the selected LAB strains. Comparison of the EPS yields revealed that among tested LAB, strain 37 exhibited the highest production of 536.904 mg/L. The strain was identified as *Leuconostoc citreum* with carbohydrate assimilation profiling, 16S rRNA and *phe*S gene sequencing. The *Ln. citreum* 37 was found to be a novel EPS producing strain. It was found that there was no direct linear relation between the colony size and EPS yield, so the colony size could not to be used to screen EPS-producing strains.

Key words: lactic acid bacteria, exopolysaccharides, *Leuconostoc* spp., identification, yoghurt

Introduction

Polysaccharides of plant, microbial and animal origin with thickening and gelling properties play an important role in food formulation. They are used as industrial thickeners, gels and stabilizers (Welman and Maddox, 2003). Some polysaccharides, such as starch, xanthan or agar, were used in dairy products as thickening agents in China. However, these polysaccharides represent a small fraction of the current biopolymer market. One of the limiting factors was the high cost of their recovery and the non-food bacterial origin. For industrial practice, most are chemically modified, so their use is restricted. Alternative bio-thickeners are the microbial polysaccharides. Because microbes have abundant species and produce polysaccharide without geographical and seasonal restrictions, microbial polysaccharides show better characters than polysaccharides from animals and plants. Consequently, microbial polysaccharides gradually became a research focus with great development potential in food industries.

Exopolysaccharides (EPS) produced by LAB contribute to the quality of fermented milk, especially to the texture, flavour and viscosity. These molecules can maintain a certain viscosity of the fermented milk and a good taste while reducing the use of food additives. It is clearly prohibited by law to add thickener and stabilizer to the ordinary yogurt and other fermented milk in France, the Netherlands and other countries, where high quality fermented milk is produced and consumed. EPS of LAB have the bioactivity of anti-tumor, immune activation, and lowering serum cholesterol and the role of prebiotics. Therefore, EPS of LAB could be used as an additive in food industry, and even have great potential to be applied in the pharmaceutical industry. In this paper, LAB isolated from the traditional dairy products in the Inner Mongolia region were used to screen the EPS-producing strains. After the centrifugation, ethanol precipitation and dialysis, the EPS producing ability was evaluated by phenol-sulphuric acid method. Subsequently, the strain with the highest yield of EPS was studied for the further

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taxonomic identification. The aim of the study was to select potential EPS-producing LAB, which could be used as functional starter cultures to enhance the quality and bring functional attribute to fermented dairy products.

Materials and methods

Bacterial strains and growth conditions

The 11 strains of LAB were used for determination of EPS-production. The strains were isolated from Inner Mongolia traditional yoghurt, as described previously by in Xu (2006). Bacterial cultures were stored in 70 % glycerol (v/v) at -80 °C and activated in MRS broth (Beijing Land Bridge Technology Co., Ltd.) which was sterilized at 121 °C for 20 min.

MRS agar containing 2 % sucrose was used for the determination of LAB colony diameter. MRS liquid medium containing 2 % sucrose was used for the cultivation of facultative anaerobic strains to detect the EPS yield. The anaerobic conditions were achieved by AnaeroGen™ Anaerobic generation system (OXOID, United Kingdom).

Determination of colony diameter of lactic acid bacteria

Facultative anaerobic strains of LAB were subsequently inoculated in MRS broth at 37 °C for 24 h. The strain inoculums were streaked on MRS agar containing 2 % sucrose and incubated at 37 °C for 72 h. The colony changes were observed at intervals and the colony diameter was finally measured by Vernier calliper (Taizhou Hangyu Measure And Cutting Tool Co., Ltd., China). Among them, the slime colonies were drawn by sterilized swab to measure the length of wire. In addition, all isolates were gram stained and observed under oil lens of biological microscope (Carl Zeiss Microimaging GmbH, Germany).

Extraction of exopolysaccharides

As not every EPS-producing LAB showed slime colony, crude EPS was extracted using centrifugation, ethanol precipitation, and dialysis to avoid from failing to choose less-EPS-yield strains with non-sticky colonies. Before mentioned colonies were inoculated in 100 mL MRS broth containing 2 % sucrose. 24 h grown cultures were centrifuged at 8000 g for 10 min in Allegra X-15R centrifuge (Beckman Coulter, Inc. USA) to remove cells. EPS were precipitated from supernatants by adding 3 volumes of anhydrous ethanol, and the mixture was stored overnight at 4 °C. After ethanol precipitation and another centrifugation at 8000 g for 10 min, precipitates were re-suspended in distilled water and filtered using dialysis tubes (molecular weight cut-off 8 to 14 kDa, Beijing Solarbio Science & Technology Co., Ltd., China). Solutions were prepared for the further yield determination after dialysis for 48 h against water which was removed each 8 h (Xu et al., 2010).

Determination of exopolysaccharide yield

The crude EPS obtained above was diluted with distilled water to 200.0 mL in order to determine the yield by phenol-sulfuric acid method. The glucose standard curve was prepared for the quantitative determination, according Dubios et al. (1956) with some modifications. Briefly, 4.00 mg glucose was dissolved in water to make 100mL. Then 4.0, 8.0, 12.0, 16.0 mg/L glucose solution were made by diluting with distilled water to 2.0 mL, respectively. 1.0 mL of 6 % phenol and 5.0 mL sulfuric acid 95 % (v/v) were added quickly and shake up after 10 min standing, then absorbance at 490 nm was measured using YU-1810 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China). 2.0 mL of distilled water used as a blank in the same conduct. The standard curve was drawn with concentration of EPS as the horizontal axis and absorbance values as the vertical axis.

For EPS yield determination, accurately 0.1 mL of EPS samples were weighed and diluted to 2.0 mL with distilled water, then added quickly with 1.0 mL of 6 % phenol and 5.0 mL of sulfuric acid 95 % (v/v) and shake up after 10 min standing, and then absorbance at 490 nm was measured. The concentration of EPS was determined in triplicate and 2.0 mL distilled water used as blank. The EPS content of each sample was calculated by the standard curve.

After screening, EPS-producing strains were inoculated on MRS agar of inclined plane and temporarily stored at 4 °C after cultured at 37 °C for 24 h. Meanwhile, strains cultured in MRS broth were stored in 30 % glycerol at -80 °C.

Identification of high-yield EPS-producing strain

Determination of physiological and biochemical characteristics

The physiological and biochemical characteristics of the highest yield-producing strain were examined using standard procedures (Dong and Cai, 2001). Gram staining, bacterial morphology, catalase-activities, oxidase reaction and other characteristics were investigated. The utilization of some carbon sources, such as laetrile, glucose, melibiose, raffinose, by the strain was performed based on Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

PCR amplification and sequence analysis

The highest EPS yielding strain was subjected the 16S rRNA sequencing. The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3', and the reverse primer was 5'-CTACGGCTACCTTGTTACGA-3' (Mauros et al., 2002). PCR reaction mixture was of 50 μL and the amplifying conditions were as follows: denatured at 94 °C for 5 min, amplified for 30 cycles under the following conditions: 94 °C for 1min, 52 °C for 1 min, 72 °C for 3 min; then finally extended at 72 °C for 10 min. The PCR products were sent to a Biotechnology Company (Beijing Haocheng Mingtai Technology Co., Ltd., China) for sequencing.

The *phe*S sequencing was applied for identification of the EPS highest-yield strain. The forward primer was 5'-CAYCCNGCHCGYGAYATGC-3', and the reverse primer was 5'-GGRTGRACCATVCCNGCHCC-3' (Naser et al., 2007), PCR reaction system was 50 μL and the amplifying conditions were as follows: denaturized at 94 °C for 5 min, amplified for 30 cycles under the following conditions (94 °C for 1 min, 46 °C for 1 min and 15 s, 72 °C for 1 min and 15 s), then finally extended at 72 °C for 7 min. The PCR products were also sent to the Biotechnology Company for sequencing.

The obtained 16S rRNA sequence and *phe*S sequence, respectively, were manually corrected and subsequently aligned to sequences in GenBank Database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The aligned 16S rRNA sequences and *phe*S sequence of related species were retrieved from the NCBI nucleotide database. Phylogenetic and distance analysis of the aligned sequences was performed by the program MEGA (version 3.1) (Kumar et al., 2004). The resulting unrooted tree topologies were evaluated by bootstrap analysis of un-weighted pair group method arithmetic averages clustering algorithm (UPGMA).

Results and analysis

Microbiological characteristics

11 strains of LAB were examined and the consistence of morphology of each strain was ensured by gram stain and microscopic examination. The EPS-producing strains were initially estimated by

Table 1. Colony size and exopolysaccharide (EPS) production of different strains of lactic acid bacteria

Strains	Colony size (mm)	EPS content (mg/L)
29-1	0.78	116.81
TH	0.84	29.34
39	5.07	290.25
37	4.46	536.90
28-2	0.70	35.30
9-1	1.39	36.50
30-2	1.23	34.11
1-1	1.28	22.97
21-1	1.40	23.76
35-1-1	1.38	40.68
44	1.48	0.00

measuring the size of the colony producing extracellular polysaccharide production. The results are shown in Table 1. But it was not very satisfactory to draw wires from colonies. There was no ropy phenomenon on all of strains. Even though strains 39 and 37 formed large, smooth, sticky colonies, they were also difficult to pull out the ropy wire.

Exopolysaccharide production

The visual inspection of bacterial colonies on agar plates is most probably the easiest method among many different EPS screening methods. This method does enable the detection of LAB strains that produce low amounts of EPS, unless they are very ropy. The partial purification of EPS through precipitation with ethanol and its photometrical quantification is another quick screening method. Therefore, 11 selected LAB strains were examined for EPS yields.

Phenol-sulfuric acid method was used to measure the content of exopolysaccharides using glucose as standard. There was a good linearity and the correlation coefficient was higher than 0.999. EPS content was calculated according to the regression equation based on the standard curve, and then converted with dilution ratio. EPS production of each strain was shown in Table 1.

EPS by selected LAB isolates ranged from 0.0 in for strain 44 to 536.9 mg/L for strain 37. Table 2 clearly demonstrates that the strains assigned by numbers 37, 39, and 29-1 showed higher EPS production than the rest of the strains in analysed in this study. EPS yield of strain 37 was the highest, which was more than other large-EPS-producing microbes reported in the literature (Xu et al., 2010). Therefore, strain 37 was selected for the further identification, in which classical microbiological classification methods and 16S rRNA gene sequence analysis involved.

Characterization of the highest EPS yield strain

The strain 37 was identified to genus level by classical microbiological classification methods and the fermentation profile of strain 37 was presented in Table 2. Besides, strain 37 is gram-positive, ovoid-shaped, facultative anaerobic, asporogenic and catalase negative bacterium. It could grow in air, at 10 °C but cannot grow at 45 °C. In addition, it could grow at pH 4.5 but cannot grow at pH 9.6 and in 6.5 % NaCl. The physiological and biochemical determination showed that strain 37 was belonged to *Leuconostoc* and closely related to *Leuconostoc citreum*.

16S rRNA and pheS sequencing

Total length 1455 bp nucleotide sequence of 16S rRNA was amplified from strain 37. Its phylogenetic tree based on other LAB 16S rRNA sequences from GenBank was shown in Figure 1. Phylogenetic analysis revealed that strain 37 was clustered closely with *Leuconostoc citreum*.

Overall-length of *phe*S sequence amplified from strain 37 contained 844 bp nucleotides. The *phe*S sequence was further aligned with the corresponding sequences from additional strains of species of

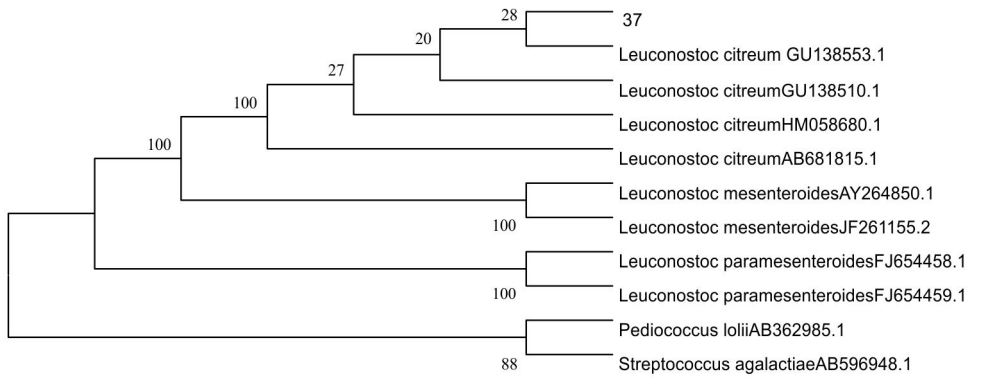

Figure 1. Phylogenetic neighbour-joining tree based on the 16S rRNA gene sequences showing the relation of strain 37 and related species

Table 2. Fermentation profile of exopolysaccharide producing strain *Leuconostoc citreum* 37

Carbohydrates	Result	Carbohydrates	Result
Amygdalin	+	Mannose	+
Arabinose	+	Melezitose	-
Cellobiose	+	Melibiose	-
Esculin	+	Raffinose	-
Fruit sugar	+	Rhamnose	-
Galactose	-	Ribose	-
Glucose	+	Salicin	-
Sodium gluconate	+	Sorbitol	-
Lactose	-	Sucrose	+
Maltose	+	Trehalose	+
Mannose	+	Xylose	+
Starch	-	Inulin	-

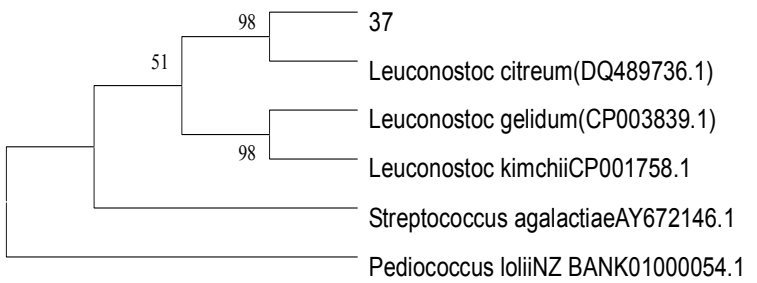

Figure 2. Phylogenetic neighbor-joining tree based on the *phe*S gene sequences showing the relation of strain 37 and related species

Leuconostoc. Phylogenetic analysis also revealed that strain 37 was clustered with *Leuconostoc citreum*. Its phylogenetic tree based on other LAB *phe*S sequences from GenBank was shown in Figure 2.

Discussion

In this study, EPS-producing ability of different strains of LAB was detected by measuring the colony size and the EPS content using phenol-sulfuric acid method. It was found there is no correlation between colony size and its EPS production. For example, strain 37 had a smaller colony but had larger EPS yield than strain 39, which showed a very slime large colony. Therefore, measuring colony size cannot be used as the only method to screen EPS-producing strains, unless the combination with other methods. Furthermore, not all of LAB was too sticky to draw ropy wire, although strain 37 and 39 presented a ropy phenotype. It may be related to the colony itself and/or media. For example, thallus may be adhered to the culture medium if the medium was too sticky, so that the drawing cannot be successful. However, the exact cause was not clear and had to be confirmed in further examinations.

16S rRNA has functional and evolutionary homology, and the mutation frequency of 16S rRNA sequence was slowly, so its overall structure is extremely conservative. Otherwise, 16S rRNA molecules has a moderate size, carrying sufficient biological information can be used for reliable system evolution (Tu et al., 2004). 16S rRNA sequence analysis can identify microbial quickly and accurately by phylogenetic tree. With the development of bioinformatics and the large number of microbial

16S rRNA gene sequencing work is completed, the role of 16S rRNA sequence analysis during microbial identification becomes irreplaceable (Liu et al., 2006). Therefore, the 16S rRNA sequence analysis and phylogenetic tree was applied in this study for the identification of selected strains.

*phe*S is a gene encoding α subunit of phenylalanyl-tRNA synthetase (Jie et al., 2009). Naser et al. (2007) and De Bruyne et al. (2009) have already proven the ability to distinguish of *phe*S is higher than 16S rRNA, and it is highly conserved. So, *phe*S sequence analysis is a reliable tool for the identification of unidentified strains. At present, there is a less application of *phe*S gene sequence alignments on identification of *Leuconostoc* species, but it is prevalent in the identification of *Lactobacillus* genus. In this paper, *phe*S gene sequencing and phylogenetic tree were used for further identification of strain 37. The results showed that this method was also appropriate for species and subspecies identification of *Leuconostoc*.

The screening method applied in this work is highly reliable and simple to operate with low requirements for equipment. It is suitable for initial screening of a large number of species. Three strains of LAB producing large EPS screened in this study needs to do the separation and purification, and further structural analysis. Furthermore, the culture conditions should be optimized to increase its EPS production by improving strains´ fermentation conditions, thus further study on function and structure of extracted EPS will be undertaken.

Conclusion

11 strains of lactic acid bacteria isolated from Inner Mongolia traditional yoghurt were used to screen EPS-producing strains. Measurement of colony diameter and determination of EPS yield after primary purification were applied and three high EPS yield strains were found. The highest EPS-producing strain was identified to be *Leuconostoc citreum* by physiological and biochemical methods, 16S rRNA and *phe*S sequencing.

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Odabir sojeva bakterija mliječne kiseline sa sposobnošću proizvodnje egzopolisaharida izoliranih iz tradicionalnog jogurta regije Inner-Mongolija

Sažetak

U ovom radu ispitivana je sposobnost proizvodnje egzoplisaharida (EPS) u bakterija mliječne kiseline (BMK) izoliranih iz tradicionalnog jogurta regije Inner-Mongolija. Pri tom je nakon taloženja i dijalize etanola korištena metoda fenol-sumporne kiseline. Ukupni polisaharidi izolirani su iz MRS hranjivog bujona u kojemu su uz dodatak saharoze uzgajani odabrani sojevi BMK. Usporedba prinosa egzopolisaharida pokazala je da je među analiziranim sojevima BMK, soj označen brojem 37 imao najvišu sposobnost proizvodnje EPS i to u koncentraciji od 536.904 mg/L. Na temelju analize asimilacije različitih ugljikohidrata te sekvenciranja regije 16S rRNA i gena pheS soj je identificiran kao *Leuconostoc citreum*. Time je otkriven novi soj (*Ln. citreum* 37) sa sposobnošću proizvodnje EPS. Također je utvrđeno kako veličina kolonija nije u linearnoj korelaciji s prinosom EPS te se stoga ne može koristiti za nadzor sojeva sa sposobnošću proizvodnje EPS.

Ključne riječi: bakterije mliječne kiseline, egzopolisaharidi, Leuconostoc spp, identifikacija, jogurt

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