Phenotypic and Genotypic Identification of Exopolysaccharide-Producing Lactic Acid Bacteria from Traditional Dairy Products of Guilan Province

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ABSTRACT

Introduction: Numerous lactic acid bacteria (LAB) possess the ability to synthesize exopolysaccharides (EPS), which may be either cell-associated or liberated into the culture medium. This chemical has significant therapeutic qualities and is regarded as a crucial addition in the food sector. This research aimed to isolate and identify lactic acid bacteria strains capable of producing exopolysaccharides from typical dairy products.

Method: LABs were isolated with MRS broth medium. Screening for EPS-producing bacteria was conducted on the identical medium subsequent to the incorporation of 10% (w/v) sucrose and 0.5% (w/v) calcium carbonate. The bacteria responsible for production were identified by molecular techniques, specifically *16SrRNA*, while the quantitative examination of polysaccharides was conducted by assessing their dry weight.

Results: Seven of the 19 isolates from traditional dairy products in Gilan province were identified as EPS producers. The strain exhibiting the highest production rate, 1.832±12.881 g/L, was identified as the most effective producer. Within 24 hours, *Lactobacillus plantarum* generated 923 mg of polysaccharide per liter of culture, marking the highest yield.

Conclusion: The *L. plantarum* isolate obtained in this work is proposed as an effective and appropriate source for semi-industrial and industrial exopolysaccharide production.

KEYWORD: Exopolysaccharide, Lactic Acid Bacteria, MRS broth

1. INTRODUCTION

Exopolysaccharides (EPS) are valuable compounds composed of various monosaccharides and are produced by all living cells, including plants, algae, fungi, and bacteria (Dilna et al., 2015). Among EPS-producing bacteria, lactic acid bacteria (LAB) have attracted particular attention because they are generally recognized as safe (GRAS) and have a high capacity for producing EPS without any health risks (Surayot et al., 2014).

Exopolysaccharides have significant applications and benefits in various fields, such as the pharmaceutical and food industries. They help maintain the consistency, structure, and desirable flavor of dairy products like yogurt and milk. Many types of EPS also exhibit anti-tumor, immunomodulatory, cholesterol-lowering, antioxidant, and anti-diabetic properties (Chen et al., 2014; X. Liu, Luo, Guan, Chen, & Xu, 2022; Wang, Zhao, Tian, et al., 2015).

Gilan province, like many other regions in Iran, is a hub for traditional dairy products. Various traditional dairy products have been produced and supplied in rural areas of the province for over a century, and with the community's growing public awareness of hygiene, there is an increasing trend toward using these products. Considering the high potential of these products to yield new LAB strains with industrial capabilities, this study was conducted to isolate and identify EPS-producing LAB from traditional dairy products of Gilan province and to evaluate the feasibility of their semi-industrial and industrial production.

2. MATERIALS AND METHODS

This research was conducted using laboratory (in vitro) methods. The first step involved the isolation and identification of EPS-producing microorganisms from traditional dairy products of Gilan. All experiments were performed in triplicate.

2.1. Microorganism Isolation

Fifty samples of traditional Gilan province dairy products—such as yogurt, cream, clotted cream, milk, and butter—were aseptically transported to the lab in order to isolate microorganisms. The plates were incubated for 48 hours at 37°C

in an anaerobic environment (in a jar) with the samples cultivated in sterile MRS broth. The cultures were then subcultured onto MRS agar supplemented with 50 mg/L of nystatin (to prevent yeast growth) after this time. Growth was then assessed after the plates were incubated for 48 hours at 37°C in anaerobic conditions.

2.2. Microorganism Identification

Gram staining, catalase, and oxidase tests were conducted for identification purposes. The catalase test required the application of one drop of 3% hydrogen peroxide onto a slide, the addition of a solitary pure colony from the solid culture, and the observation of gas evolution. The oxidase test was conducted with specialized test strips, and a color alteration on the strip signified a positive or negative outcome. Colonial attributes like color, shape, and distinguishing traits, as well as microscopic parameters (Gram reaction, cell morphology, and organization), were documented. Subsequent to the preliminary evaluations, all bacteria tentatively classified within the extensive family of lactic acid bacteria were isolated from different specimens and re-cultured in MRS agar and broth. The samples were subsequently incubated for 48 hours at 30°C (Patel, Majumder, & Goyal, 2012).

2.3. Screening for EPS-Producing Isolates

All isolates identified phenotypically as lactic acid were cultured on MRS agar medium supplemented with 10% sucrose and 0.5% calcium carbonate. The emergence of a distinct halo surrounding the colonies or a comprehensive clearing of the medium post-incubation signified EPS production (Patil, Wadehra, Munjal, & Behare, 2015).

2.4. Exopolysaccharide Production

A culture medium containing 25 mL of MRS broth was prepared and inoculated with a pure colony from the screened cultures. The inoculated broth was incubated for 18 hours at 30°C. Once the turbidity (OD) reached 0.5 at 600 nm (equivalent to 10^9 CFU/mL), 10% (v/v) of this culture was used as an inoculum

for fresh sterile MRS broth containing 10% (w/v) sucrose. The cultures were then incubated for 24 hours at 30˚C. Following incubation, the cultures were centrifuged at 600 rpm and 4˚C for 15 minutes. The supernatant was carefully separated, and 95% ethanol was added. This mixture was kept at 4˚C for 24 hours, after which the purified EPS was isolated as a dry powder using a freeze-dryer (Wang, Zhao, Yang, Zhao, & Yang, 2015).

2.5. FT-IR Analysis of Polysaccharides

To determine the components of the polysaccharide, FT-IR analysis was performed (DuboisGilles, 1952). The phenol-sulfuric acid method, with a glucose standard, was applied to quantify the total sugar content of the exopolysaccharide. A 0.01 p sample of the synthesized EPS was dissolved in 250 mL of pure water. To 2 mL of this solution, 1 mL of 5% phenol and 5 mL of 96% sulfuric acid (v/v) were included. The solution was subsequently subjected to a boiling water bath for 30 minutes, after which its optical absorbance was assessed at a wavelength of 490 nm. The total glucose concentration was ascertained utilizing a standard curve.

2.6. Viscosity Measurement

The viscosity of a 2% (w/v) exopolysaccharide solution was determined at 25°C using a viscometer at two distinct pH values, 4.6 and 7. The viscosity of the liquid culture media was concurrently evaluated at various time intervals. The properties of the exopolysaccharide were analyzed between 4000-400 cm^{-1} (Shao et al., 2014).

2.7. Molecular Identification of Isolates

Following the confirmation of EPS generation through phenotypic and chemical investigation, the highest-yielding isolates were subjected to molecular identification. Initially, isolates were cultured on MRS agar for 48 hours at 30°C. DNA was extracted utilizing a DNA extraction kit (yektatajhiz, Iran). Following the verification of the extraction by 0.7% (w/v) agarose gel electrophoresis, a PCR assay was conducted to amplify the *16SrRNA* gene with a pair of primers:

Forward (5'- AGAGTTTGATCCTGCCTCAG -3') and Reverse (5'- GGTTACCTTACGACTT-3'). The PCR thermal cycling protocol comprised a pre-denaturation step at 94°C for 30 seconds, denaturation at 55°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1.5 minutes, totaling 35 cycles. The resultant amplicon was subsequently subjected to electrophoresis on a 1.5% (w/v) agarose gel. Subsequent to sequencing, the sequences were compared to reference sequences in the NCBI database with MEGA7 software.

2.8. Genotypic Analysis of Exopolysaccharide Production

To investigate the genotypic basis of EPS production in the isolates from dairy products, relevant primers were obtained from Pishgam Biotechnology Company (Table 1). A standard strain of *Lactobacillus casei* was used as a positive control.

Table 1. Primers for Exopolysaccharide Genes.

Primer name	Primer sequence	Reference
esp A-F	5'- TAGTGACAACGGTTGTACTG - 3'	(Van der Meulen et al., 2007)
esp A-R	5'- GATCATTATGGACTGTCAC - 3'	
espB-F	5'-CGTACGATTCGTACGACCAT- 3'	(Van der Meulen et al., 2007)
espB-R	5'-TGACCAGTGACACTTGAAGC- 3'	
Glucansucrase genes-F	5'- GAYAAYWSIAAYCCIRYIGTIC - 3'	(Van der Meulen et al., 2007)
Glucansucrase genes-R	5'- ADRTCICCRTARTAIAVIYKIG - 3'	
Fructansucrasr genes-F	5'- GAYGTITGGGAYWSITGGC - 3'	(Van der Meulen et al., 2007)
Fructansucrasr genes-R	5'- TCITYYTCRTCISWIRMCAT - 3'	

2.9. Hemolysis Test

In order to conduct this experiment, 2.8 grams of Nutrient agar medium were weighed and dissolved in 100 mL of distilled water. The medium was heated until transparent, thereafter autoclaved and sterilized at 121°C for 20 minutes. Subsequent to cooling and prior to the solidification of the agar, 5 mL of sheep's blood was included into the medium, which was subsequently dispensed into plates. A brief streak culture of bacterial broth with suitable turbidity was prepared on solidified agar plates using a sterile loop and incubated at 37.5°C for

24 hours. The absence of hemolysis was anticipated, given probiotic lactic acid isolates were recognized as non-hemolytic (Ramos, Thorsen, Schwan, & Jespersen, 2013).

2.10. Pathogen Growth Inhibition Test

The pathogen growth was assessed using the agar well diffusion method as described by Koohi *et al.* in 2020 . Fresh 24-hour MRS broth cultures of the lactic acid isolates were prepared and centrifuged at 3500 rpm for 5 minutes. The cultures of the gram-negative bacteria, included *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii*, as well as the gram-positive bacterium *Staphylococcus aureus*, were prepared on Nutrient agar plates. A microbial suspension, 0.5 McFarland (0.5×10^8 CFU/mL), was prepared from pure colonies and streaked onto the MRS agar plates using a sterile swab to create a lawn culture. Then wells with a depth of 5 mm were created in the agar. Fifty microliters of the centrifuged extract were added to each well, and the plates were incubated at 37°C for 24 hours. The formation of a clear zone around the wells was considered an indication of the inhibitory or suppressive effect of the lactic acid isolates on pathogen growth (Ramos et al., 2013)(Rojo-Bezares et al., 2006).

3. Results

3.1. Isolation and Screening of EPS-Producing Strains

Out of a total of 19 dairy isolates, 7 were found to be EPS producers. They were numbered from D1 to D7, respectively. All isolates were Gram-positive, non-spore-forming, and catalase-negative, and the best producing isolate was D6. Tables 2 show the biochemical analyses performed on the isolates, and Figures 1 show the colony morphology of the EPS-producing bacteria on MRS agar and the transparent halo around the colonies on MRS agar containing sucrose, respectively. The formation of a transparent halo confirmed EPS production.

Table 1. The biochemical analyses performed on the isolates.

Probiotic Isolates	Biochemical properties									
	Arabinose	Raffinose	Galactose	Melibiose	Sucrose	Glucose	Mannose	Lactose	Sorbitol	Xylose
D.1	+	+	+	-	+	+	+	+	+	+
D.2	-	-	-	-	+	+	-	+	-	-
D.3	+	+	+	+	+	+	+	+	+	+
D.4	-	+	-	-	+	+	+	+	-	+
D.5	-	-	-	-	-	+	-	-	-	-
D.6	-	+	+	-	+	+	+	+	-	+
D.7	+	+	+	-	+	+	+	+	+	+

A "+" indicates fermentation, while a "-" indicates that the sugar is typically not fermented by that species.

Figure 1. Colony morphology of the EPS-producing bacteria on MRS agar.

3.2. Viscosity

The viscosity of the exopolysaccharide solution was measured at approximately 6 cps. Also, the viscosity of the culture media increased from 10 to 38 within 24 hours, and a significant relationship was observed between EPS production and this increase.

3.3. Molecular Identification of Isolates

Based on *16SrDNA* identification, the size of the replicon was determined, and the genus and species of the strains were identified. The results showed that the best-producing isolate belonged to the genus and species *Lactobacillus plantarum*.

3.4. Exopolysaccharide Production

All producing isolates showed EPS production between 3.102 to 8.771 g/L. The highest production was from isolate D6, and the lowest was from isolate D2.

3.5. Molecular Identification of the Best Isolate

The results of the electrophoresis of lactic acid isoltes with *16SrRNA* primers on an electrophoresis gel are shown in Figure 2.

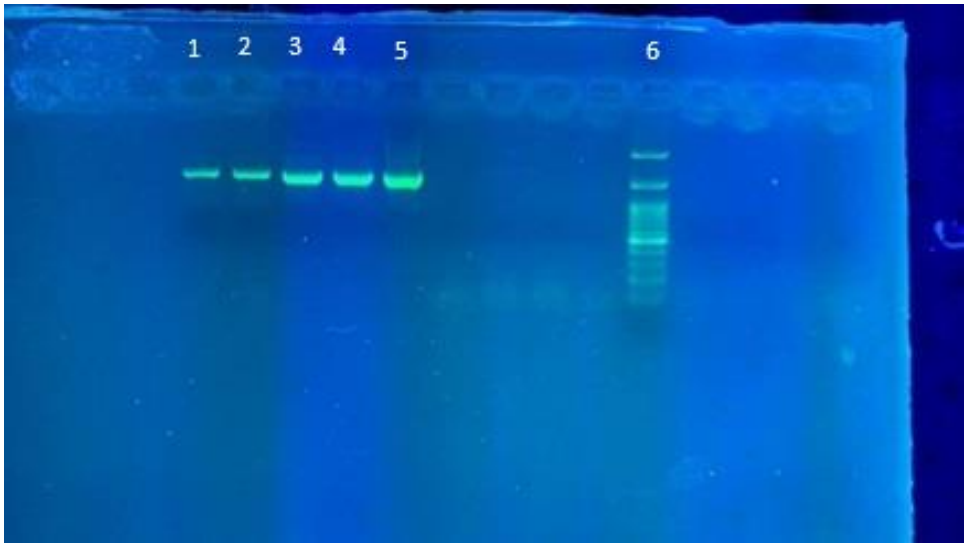

Figure 2. Samples 1 to 5 in the 1500 bp, with a 6-marker 100-2000 bp.

Figure 3 illustrated the resultant phylogenetic tree derived from sequencing. The evolutionary history was deduced via the Neighbor-Joining approach. The ideal tree is presented with a total branch length of 1.48199667. The proportion of replicate trees in which the corresponding taxa clustered together during the bootstrap test (1000 repetitions) is indicated adjacent to the branches. Evolutionary distances were calculated with the Kimura 2-parameter approach, expressed in terms of base substitutions per site. The variance among sites was modeled using a gamma distribution with a shape value of 1. The ratio of sites with at least one unambiguous base in at least one sequence for each descendant class is indicated adjacent to each internal node of the tree. The analysis encompassed 57 nucleotide sequences. All entries with gaps and absent data were removed. The final dataset comprised 202 locations. Evolutionary analyses were performed using MEGA X.

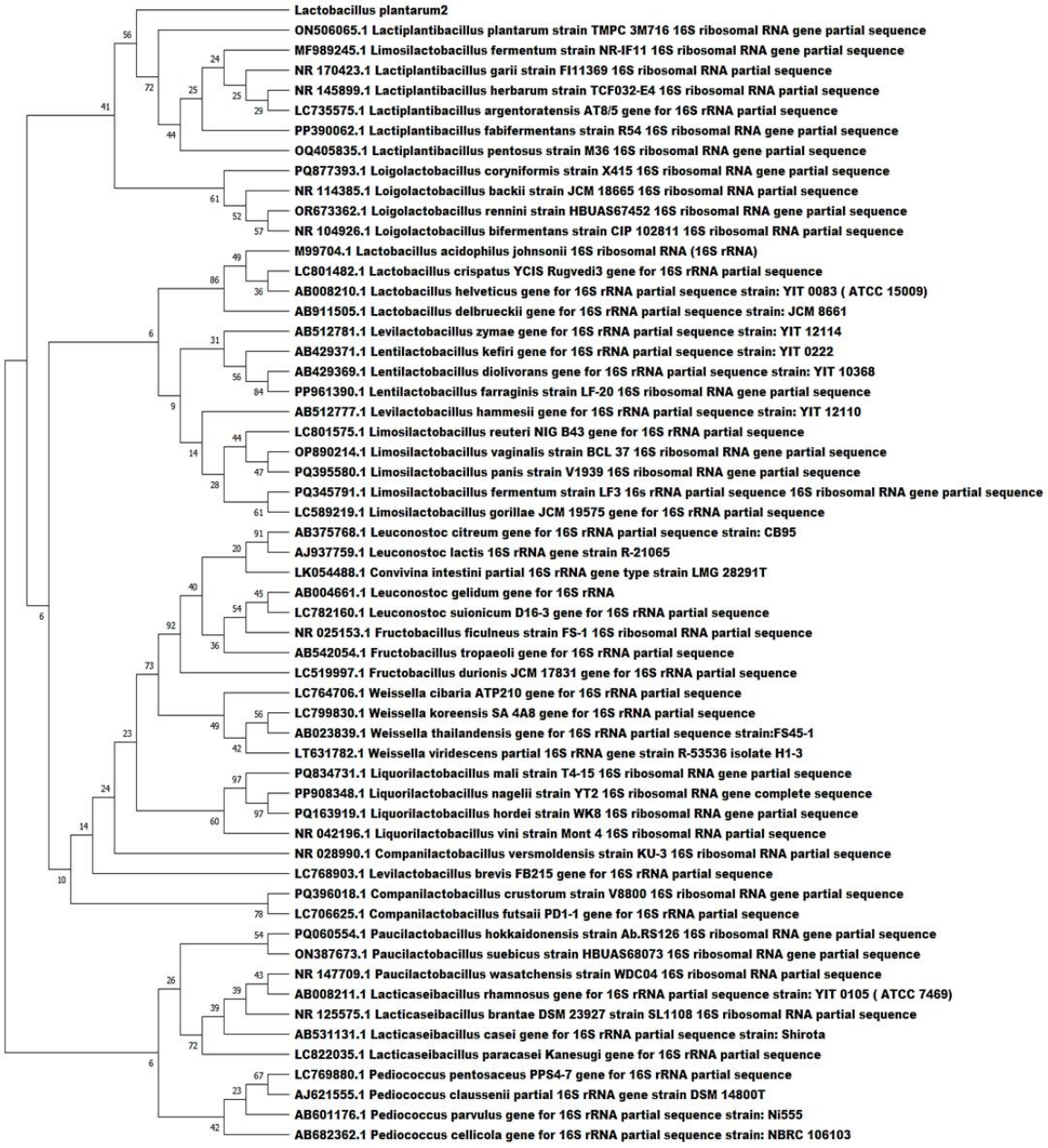

Figure 3. The phylogenetic tree from sequencing.

3.6. Genotypic Analysis of Exopolysaccharide Production

The results of the genotypic analysis of the EPS genes showed that all isolates contained one of the four EPS production genes when compared to the positive control *Lactobacillus casei* PTCC 1601. The gel images show the results of the genotypic analysis (Figure 4).

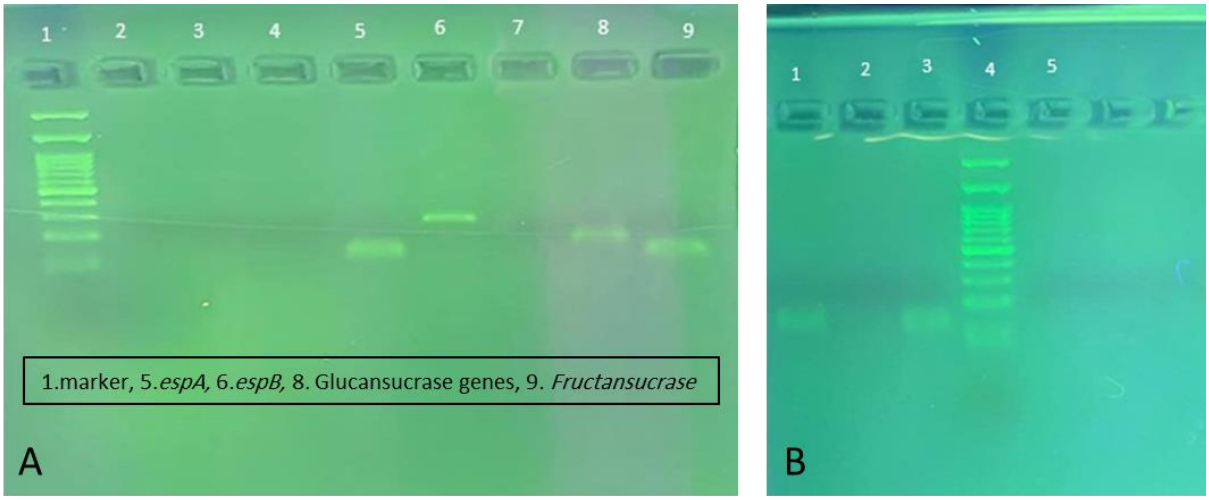

Figure 4. A. Genetic analysis of exopolysaccharide genes (Positive control: *Lactobacillus casei*). **B.** Gene related to the best isolate.

3.7. FT-IR Measurement Results

The FT-IR spectrum of the analyzed sample was recorded between 4000 to 500 cm^{-1} and showed specific absorption peaks, indicating the presence of various functional groups in the sample's structure. Peak at 3417.63 cm^{-1} corresponds to the stretching vibration of the hydroxyl (O-H) group. The presence of this group indicates the existence of alcohols or hydroxyl groups in the sample's structure. The intensity of this peak can also indicate hydrogen bonds. In addition, peaks at 2927.74 and 2801.18 cm^{-1} are related to the stretching vibration of C-H bonds in alkane groups. They suggest the presence of methyl (CH_3) or methylene (CH_2) groups in the sample. Furthermore, peak at 1654.81 cm^{-1} corresponds to the stretching vibration of the carbonyl (C=O) bond, likely confirming the presence of functional groups such as ketones, esters, or amides. Peak at 1407.94 cm^{-1} is related to the bending vibration of CH_2 or CH_3 groups, pointing to the presence of alkane compounds or alkyl groups. Additionally, peaks at 1122.49, 1071.39, and 1039.56 cm^{-1} are associated with the stretching vibration of C-O bonds, indicating the presence of alcohols, esters, or ethers in the sample. Peak at 778.9 cm^{-1} is related to the bending vibration of C-H bonds in aromatics, suggesting the likely presence of aromatic compounds in the sample. Peaks at 621.04 and 549.67

cm^{-1} typically refer to the bending vibrations of C-Cl or C-Br bonds and may confirm the presence of halogenated compounds in the sample. The FT-IR spectrum indicated the presence of various functional groups, including hydroxyl (O-H), carbonyl (C=O), alkanes (C-H), and C-O bonds. Additionally, the presence of aromatic and halogenated compounds was also observed.

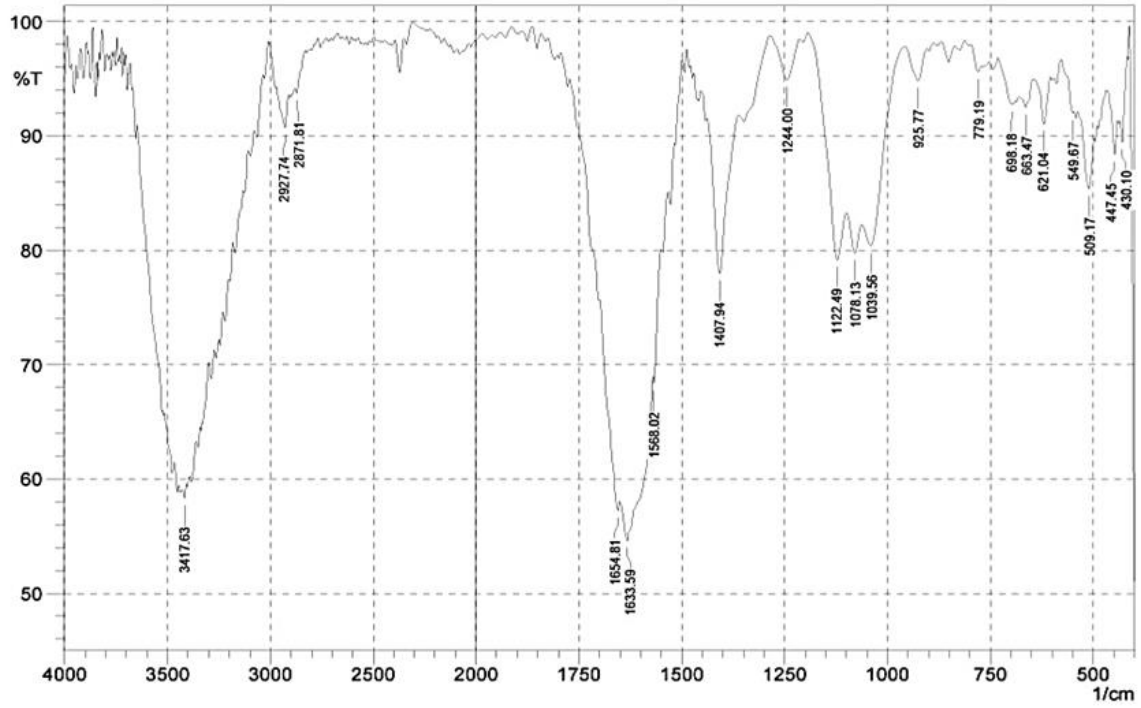

Figure 5. FT-IR spectrum of isolate 3 (D3).

4. DISCUSSION

The use of EPS-producing *Lactobacillus* has recently gained attention due to their role as starter cultures in the production of fermented foods like yogurt and cheese. Although genetic modifications can increase the production of such products, most countries prefer not to use genetically modified microorganisms (Kodali, Das, & Sen, 2009). Therefore, finding naturally occurring strains with the ability to produce these productions is of great interest. Consequently, it is crucial to develop rapid screening techniques for isolating these bacteria with suitable qualitative and quantitative production. According to our phenotypic findings, 7 out of 10 isolates were able to produce EPS, characterized by shiny

and mucoid colonies on MRS agar containing sucrose. All these isolates belonged to the genus *Lactobacillus*. Molecular analyses also confirmed the presence of one of the four main EPS-producing genes in these bacteria. Additionally, genetic screening can serve as a useful tool for selecting producing isolates.

Exopolysaccharides are high-molecular-weight extracellular polysaccharides composed of monosaccharides and non-sugar substrates like acetate, phosphate, pyruvate, and succinate. They can exist in two forms: a capsule tightly bound to the bacterial cell surface or a slime layer that is loosely bound or even secreted (Kodali et al., 2009; Zhou, Cui, & Qu, 2019).

Our phenotypic studies showed that the isolates from traditional Gilan diary products were identified as EPS producers. They were characterized by shiny, mucoid colonies on MRS agar and a transparent halo around the colonies on MRS agar containing sucrose. The EPS production based on dry weight was between 3.102 to 8.771 g/L. The highest production was observed in the *L. plantarum* isolate. Rajoka *et al.* reported a similar study on *Lactobacillus* species with production between 50 to 500 mg/L (Rajoka et al., 2018). The different production capacities of various *Lactobacillus* species and even isolates within a single species indicate that production is specific.

To evaluate the chemical structure of the EPS, the FT-IR spectrum of the best producing isolate was examined. A strong peak in the 1654 cm^{-1} region indicated a strong vibration of the carboxyl group, while the 2927 cm^{-1} peak and its vicinity usually suggest the presence of hexoses like glucose, galactose, or deoxyhexoses like rhamnose or fructose. As the spectrum showed, the current spectrum has a high similarity to standard exopolysaccharide. This findings were consistent with reports from Siddiqui *et al.* and Vettori *et al.*(Siddiqui, Aman, Silipo, Qader, & Molinaro, 2014; Vettori, Franchetti, & Contiero, 2012). Overall, this spectrum suggests a similar pattern of chemical groups, such as carboxyl groups, methyl groups, and hydrogen bonds, as other reported exopolysaccharides.

In the study of Van der Meulen *et al.*, out of 174 LAB isolates, only 10 were EPS producers. Furthermore, 7 isolates possessed all four main EPS-producing genes, including glucansucrase, fructansucrase, exopolysaccharide A, and exopolysaccharide B, and glycosyltransferase (Van der Meulen et al., 2007). In the present study, all tested isolates contained only one of the targeted genes, glycosyltransferase. This plays an important role in the biosynthesis of exopolysaccharides in most *Lactobacillus* species. This enzyme also catalyzes the formation of glycosidic bonds for the polymerization of monosaccharides (Krajl, van Geel-Schutten, Van Der Maarel, & Dijkhuizen, 2003).

Exopolysaccharide biosynthesis is a complex process involving more than one enzyme. In the first stage, simple sugars such as UDP-glucose, dTDP-glucose, UDP-galactose, and dTDP-rhamnose are synthesized. A monosaccharide chain is then built from these sugars by several glycosyltransferases (Patel et al., 2012). This chain is then attached to a 55-carbon lipid carrier molecule and transferred to the cytoplasmic membrane (Kumari et al., 2025; Tieking, Korakli, Ehrmann, Gänzle, & Vogel, 2003). In the final step, the sugar chains are secreted outside the cell. The genes of *eps* type A, B, and E create long exopolysaccharide chains, and their deletion or mutation often inhibits EPS formation in the early stages. Total carbohydrate analysis of the EPS structure using the phenol-sulfuric acid method indicated a total carbohydrate between 73% and 70%, which is consistent with the results of other researchers (Madhuri & Vidya Prabhakar, 2014).

The study of EPS production at different temperatures showed that the highest production by the superior strain occurred at 25°C under aerobic conditions(L. Liu et al., 2025; Naveena, Altaf, Bhadriah, & Reddy, 2005). This is notable since most lactic acid isolates grow better at temperatures above 30°C and under microaerophilic conditions, and the results of this study are consistent with those of other researchers. The viscosity of the produced EPS solution was 63 cps, indicating that the EPS had sufficient thickness and suggesting high production. However, a fully scientific and logical correlation between the number of EPS

produced and viscosity has not been established (Badel, Bernardi, & Michaud, 2011).

Based on morphological and biochemical characteristics, seven isolates were successfully identified as EPS producers. The exopolysaccharide was confirmed as a visible, shiny, mucoid mass on MRS agar containing sucrose. The highest production, approximately 8 g/L, was attributed to isolate D6. FT-IR analysis specified the functional groups and present peaks in the EPS structure of the best-producing bacterium, and polysaccharide production were observed in the 1250, 1400, and 1650 cm^{-1}. Other researchers have shown production between 916 to 11590 cm^{-1}. Lactobacillus species typically synthesize biopolymers primarily composed of glucose and galactose, and this can vary depending on the isolates species. Maximum EPS production occurs at the end of the stationary phase and the beginning of the death phase, and it decreases over time, which may be due to the degradation of EPS by lactic acid (Tan et al., 2024).

When dissolved in water, the resulting polysaccharide did not show high viscosity, which might be due to low hindrance. However, in contrast to the solution's specific viscosity, the viscosity of the cultures was much higher. EPS was produced in the MRS medium by seven lactic acid isolates from yogurt. The production was between 3.102 and 8.771 g/L. The extracted exopolysaccharides showed a significant thickening property when dissolved in water and contributed to increased viscosity in the fermentation media. Further research on the interaction of proteins with exopolysaccharides may lead to improved functional properties of lactic acid bacteria EPS. Such an exopolysaccharide has the potential to replace gums used in the food industry.

5. CONCLUSION

This study successfully isolated and characterized a high-yield exopolysaccharide (EPS)-producing strain of *L. plantarum* from traditional Gilan dairy products. The superior isolate, D6, produced an impressive 8.771 g/L of EPS, with optimal

production occurring at aerobic conditions. The study confirmed the presence of the essential glycosyltransferase gene and identified the EPS through FT-IR analysis. The EPS was also shown to significantly increase the viscosity of the culture medium. These findings suggest that the D6 isolate is a promising and safe natural source for the industrial production of EPS, offering a potential alternative to conventional food additives.

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Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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