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Screening for exopolysaccharide-producing strains of thermophilic lactic acid bacteria isolated from Algerian raw camel milk

Abdellah Mostefaoui[1]*, Ahcène Hakem[1], Benalia Yabrir[1], Saad Boutaiba[1] and Abdelmalek Badis[2]

[1]Laboratory of Exploration and Valorization of Steppic Ecosystems, University Ziane Achour of Djelfa, Algeria.
[2]Laboratory of Biochemistry and Industrial Microbiology, Department of Industrial Chemistry, University Saad Dahleb of Blida, Algeria.

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Exopolysaccharides synthesized by lactic acid bacteria play a major role in the manufacturing of fermented dairy products as thickening agents. Exploration of the biodiversity of wild lactic acid bacteria from natural environments is currently the most suitable approach to search for the desired exopolysaccharide-phenotype. A total of 82 thermophilic lactic acid bacteria strains were isolated from Algerian raw camel milk. The isolation of strains was carried on modified Chalmers agar medium, under semi anaerobic conditions at 42°C. Bacterial isolates were phenotypically characterized and grouped into four genera: *Lactobacillus* (31.7%), *Enterococcus* (30.5%), *Streptococcus* (24.4%) and *Pediococcus* (13.4%). Based on the mucous type of the colonies, thirty EPS-positive strains were selected to be screened for their ability to produce exopolysaccharides. The production of polymers was carried out on Man, Rogosa and Sharpe (MRS) broth, supplemented with lactose and glucose as carbon sources. Yields quantification of soluble exopolysaccharides using a colorimetric method, showed that the selected strains produce yields ranging between 160 and 740 mg/l for *Lactobacillus* strains, between 126 and 319 mg/l for *Streptococcus* strains, between 70 and 242 mg/l for *Enterococcus* strains and between 132 and 134 mg/l for *Pediococcus* strains. This suggests that some strains have potential to be used as new culture starters for this and possibility other dairy products.

Key words: Camel milk, thermophilic lactic acid bacteria, exopolysaccharides.

INTRODUCTION

Microbial polysaccharides have been investigated in detail during the last few decades. Today, there is an increasing demand in food industries for live microbes producing polysaccharides (Patel et al., 2010). Bacterial polysaccharides can be divided into intracellular polymers, structural polymers and extracellular polymers or exopolysaccharides (EPS) (Kumar et al., 2007). The bacterial EPS vary greatly in their composition and hence in their chemical and physical properties (Sutherland, 1999). Many lactic acid bacteria (LAB) are able to produce EPS. The dairy LAB used in the manufacture of fermented milks such as *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* subsp. *cremoris* were extensively studied in the last years (Cerning, 1995). EPS synthesized by LAB play a major role in the manufacturing of fermented

dairy products (Duboc and Mollet, 2001; Jolly et al., 2002). These molecules are economically important because they can impart functional effects on foods and confer beneficial health effects (Welman and Maddox, 2003). When suspended or dissolved in aqueous solution, EPS provide thickening and gelling properties (Marshall and Rawson, 1999; Laws and Marshall, 2001). The polymer-producing ability is an extremely unstable property; it seems to be linked to the presence of plasmids of varying size in mesophilic lactic acid bacteria, whereas most of the EPS-producing strains of thermophilic lactic acid bacteria (TLAB) do not harbor plasmids (Cerning, 1995). Some EPS confer on LAB a ropy character that can be detected in cultures that form long strands when extended with an inoculation loop. When EPS are produced *in situ* during milk fermentation, they can act as natural bio-thickeners, giving the product a suitable consistency, improving viscosity. The increasing demand by consumers of novel dairy products requires a better understanding of the effect of EPS on existing products and at the same time, the search for new EPS-producing strains with desirable properties. Therefore, exploration of the biodiversity of wild LAB strains from natural environments is currently the most suitable approach to search for the desired EPS-phenotype (Ruas-Madiedo and de los Reyes- Gavilán, 2005). The screening of ropy strains and the isolation and quantification of EPS have led to the application of a large variety of techniques (Goh et al., 2005; Ruas-Madiedo and de los Reyes-Gavilán, 2005). The amounts of EPS produced by the dairy strains vary considerably (Ludbrook et al., 1997; Laws et al., 2001; Badel et al., 2011). A wide range of bacteria are known to produce EPS. Several LAB produce exopolysaccharides that are secreted into the growth media (Cerning et al. 1986, 1988). Most bacteria produce EPS under all conditions, but the quantities and the composition of EPS are strain dependent and affected by the nutritional and environmental conditions (Garcia-Garibay and Marshall, 1991). Up to now, camel milk was not deeply investigated for the characterization of thermophilic bacteria. The purpose of this investigation was to obtain the efficient TLAB strains isolated from raw camel milk which produce high amount of EPS. In the current study, eighty two (82) strains of TLAB isolated from Algerian raw camel milk were taxonomically characterized using the phenotypic methods. On the other hand, these strains were screened according to their ability to produce EPS on solid and liquid media, and a colorimetric method was used for quantifying EPS yields.

MATERIAL AND METHODS

Sampling and isolation of TLAB strains

Twenty eight (28) samples of raw camel milk were obtained from two locations in the south of Algeria. Samples were collected in sterile bottles and immediately transported to the laboratory in ambient temperature, the pH of each sample was measured and the microbiological analysis was performed on the arrival. The strains isolation was carried out after milk acidification to retrieve a large diversity of TLAB (Khedid et al., 2009). 10 ml of each sample were mixed with 90 ml of sterile yeast water (10% w/v, Oxoid) and serial decimal dilutions were carried out. Isolation of TLAB was performed by the standard pour-plate method, using modified Chalmers-agar medium (Vanos and Cox, 1986). Plates were incubated semi anaerobically for 48 or 72 h at 42°C. The LAB colonies were picked and purified on MRS-agar plates (De Man et al., 1960), and strains were kept frozen at -20°C in MRS broth supplemented with 25% glycerol.

Preliminary identification of TLAB isolates

TLAB strains were identified according to many recommended methods (Sharpe, 1979; Samelis et al., 1994; Harrigan, 1998; Badis et al., 2004; Khedid et al., 2009). All isolates were initially Gram stained and examined for cell morphology and motility, then were examined using different kinds of tests; growth at different temperatures (10, 15, 30 and 45°C) and at different pH (4.2 and 9.6), as well as salt tolerance (6.5 and 18% of NaCl) in MRS broth (Oxoid), catalase reaction, gas production from glucose, ammonia from arginine hydrolysis, acetoin production (Voges-Proskauer test), utilization of citrate and heat resistance at 60.5°C for 30 min. Tests were repeated two times to avoid confusing results in the identification.

Screening test for mucoidy and ropiness

Screening test was carried on customized MRS-agar medium (Degeest and De Vuyst, 1999; Degeest et al., 2001, 2002). TLAB Strains were plated and incubated under semi anaerobic conditions at 42°C for 48 h. At the end of incubation, mucoidy of colonies was determined by visual appearance, and ropiness was determined by touching them with a sterile inoculation loop (Ricciardi et al., 1997; Welman et al., 2003; Ruas-Madiedo and de los Reyes-Gavilán, 2005), and confirmed by ethanol precipitation method. Colonies which have mucoid and ropy phenotype were picked up and purified by following the streaking method, then preserved at 4°C on MRS agar slants (Vijayendra et al., 2008) and selected for the next step.

Exopolysaccharides production

Customized MRS broth was used for fermentations. It contained (in grams/liter): lactose (75), glucose (25), peptone (30), yeast extract (12), Lab Lemco (8), K_2HPO_4 (2), sodium acetate (5), tri-ammonium citrate (2), $MgSO_4$-$7H_2O$ (0.2), $MnSO_4$-H_2O (0.038) and Tween

*Corresponding author. E-mail: m_abdellah2003@yahoo.fr. Tel: 0021327877763. Fax: 0021327909376.

Abbreviations: EPS, Exopolysaccharides; **LAB,** lactic acid bacteria; **TLAB,** thermophilic lactic acid bacteria; **OD,** optical density; **BSA,** bovine serum albumin.

Table 1. Phenotypic characters of TLAB strains isolated from raw camel milk.

Character	*Lactobacillus*	*Enterococcus*	*Streptococcus*	*Pediococcus*
Motility	-	-	-	-
Gram stain reaction	+	+	+	+
Cell morphology	R	C	C	Ct
Presence of spore	-	-	-	-
Catalase activity	-	-	-	-
Growth at: pH 4.2	+	v	+	+
Growth at: pH 9.6	-	+	-	-
Growth in: 2% NaCl	+	+	+	+
Growth in: 6.5% NaCl	-	+	-	-
Growth in: 18% NaCl	-	-	-	-
Growth at temperature:10°C	-	+	-	-
Growth at temperature:15°C	-	+	-	-
Growth at temperature:30°C	-	+	-	-
Growth at temperature:45°C	+	+	+	+
Survive at 60.5°C for 30min.	v	v	+	-
Glucose fermentation	+	+	+	+
Production of acetoin	-	v	-	-
CO_2 from glucose	-	-	-	-
NH_3 from arginine	-	+	-	+
Utilization of citrate	-	-	-	-
Strains number	26	25	20	11

R: Rod, C : Cocci, Ct: Cocci/tetrads, +: More than 90% of strains showed a positive result, - : More than 90% of strains showed a negative result, v: Between 10 and 90% of strains showed a positive or negative result.

80 (1 ml/L). The unfermented medium was ultra-filtered under 10 000 Da., using a tangential filtration system, in order to eliminate polysaccharides from yeast extract which would have interfered with the purification and determination of EPS composition (Ricciardi et al., 2002; Shene et al., 2008). Sterilization was performed by microfiltration under 0.22 µm using a steritop (Millipore). The selected mucoid strains were stored at -20°C in MRS broth (Oxoid), containing 25% (v/v) glycerol.

Bacterial strains and culture conditions

The bacterial inoculants were also prepared in 10 ml of customized MRS inoculated with 100 µl of freshly prepared cultures. After incubation at 40°C for 24 h, they were adjusted to OD_{600} = 1 and transferred into 500 ml Erlenmeyer flasks containing 90 ml of fermented medium. Fermentations were performed at 40°C for 24 h. Agitation was maintained at 100 rpm to provide adequate dispersion. Growth was monitored by measuring the final optical density (OD) at 600 nm, and bacterial biomass can be determined from a standard curve of absorbance. Acidification was estimated with the measurement of final pH of cultures (Gancel and Novel, 1994; Vaningelgem et al., 2004).

Isolation and quantification of exopolysaccharides

Exopolysaccharides were purified from the various culture strains using conventional method of Ruas-Madiedo and de los Reyes-Gavilán (2005), with some modifications. Grown cultures were heated in boiling water for 15 min to inactivate enzymes, and then cooled down to room temperature, centrifuged (20 min, 10 000 g) to remove cells and coagulated proteins, and the supernatant was collected. EPS were precipitated from the supernatant with three volumes of cold ethanol (96%) followed by an overnight incubation at -20°C. After centrifugation (20 min, 10 000 g, 4°C), the precipitates were re-suspended in hot ultrapure (Milli-Q) water and dialyzed (molecular weight cut-off: 10000 Da.) for 2 days against ultrapure (Milli-Q) water (changed twice each day). EPS solution was then frozen at -80°C and lyophilized. The EPS powder was determined by measuring the dry weight of the precipitate, and stored for further analysis. Total sugar content was measured according to the phenol-sulfuric acid method of Dubois et al. (1956) using glucose as standard. Proteins content was determined according to Bradford (1976) method using bovine serum albumin (BSA) as standard. Experiments of EPS production, isolation and quantification were repeated three times for each studied strain. In order to estimate the precision of the mean of a variable, the standard error of the means was calculated by using of the EXCEL program.

RESULTS

Isolation and preliminary identification of TLAB strains

After the preliminary characterization, a total of eighty two (82) Gram positive, catalase negative, no spore forming and homo-fermentative isolates, obtained from modified Chalmers medium (incubated at 42 °C for 2 or 3 days), were investigated for their phenotypic characters on the MRS medium (Table 1). The isolates were preliminary

subdivided into four (4) groups. Twenty six (26) rod shaped strains, homo-fermentative and Gram positive, catalase negative, which grew at 45°C but not at 15°C, absence of gas production from glucose, were considered as *Lactobacillus*. Twenty five (25) cocci shaped strains, homo-fermentative and Gram positive, catalase negative, which grew at 10 and 45°C, grew in the presence of 6.5% of NaCl and at pH 9.6 were considered as *Enterococcus*. Twenty (20) cocci shaped strains, in pairs or in chain cells, homo-fermentative and Gram positive, catalase negative, which grew at 45°C but not at 10°C, and resist heating at 60.5°C for 30 min, were considered as *Streptococcus*. Eleven (11) cocci shaped strains, in pairs or in tetrads, homo-fermentative and Gram positive, catalase negative, which grew at 45°C but not at 10 °C, were considered as *Pediococcus*.

Screening for EPS-producing phenotype

A set of 82 TLAB strains were screened for mucoidy and ropiness on customized MRS-agar medium, examined by the traditional pick test and confirmed by ethanol precipitation method. The results revealed the presence of thirty (30) mucoid and ropy strains in our culture collection, twelve (12) *Lactobacillus* of twenty six (26) tested strains, nine (9) *Streptococcus* of twenty (20) tested strains, seven (7) *Enterococcus* of twenty five (25) tested strains and two (2) *Pediococcus* of eleven (11) tested strains.

EPS production, isolation and determination

The investigation in the second step of screening for EPS production by TLAB isolated from raw camel milk, showed that 96.66% of selected strains produced EPS with more than 100 mg/l; *Lactobacillus* EPS yield ranged between 160-740 mg/l, *Streptococcus* EPS amount ranged between 126-319 mg/l, *Enterococcus* EPS yield ranged between 70-242 mg/l and *Pediococcus* EPS yield ranged between 132 and 134 mg/l. *Lactobacillus* strain (*L*115) had the highest EPS yield, while the *Enterococcus* strain (*E*28) had the lowest EPS yield (Figure 1a).

The total sugar content in EPS was in the range of 19.49 to 77.37% for *Enterococcus* and 50.57 to 58.69% for *Pediococcus* strains. Whereas, proteins accounted for lower than 4.86% for all studied strains (Figure 1b). For the cultures conditions and parameters, the final OD ranged between 2.85-4.55 for *Streptococcus* strains, 2.66-8.25 for *Lactobacillus* strains, 2.74 - 4.17 for *Enterococcus* strains and 3.44 - 3.61 for the *Pediococcus* strains. The final pH was estimated at a range of 4.3- 4.5 for *Streptococcus* strains, 3.7-5.4 for *Lactobacillus* strains, 4.1- 4.3 for *Enterococcus* strains and 4.2 for *Pediococcus* strains (Figure 1c).

DISCUSSION

All bacterial strains isolated from the raw camel milk samples fit the classification of lactic acid bacteria. TLAB were present in fermented raw camel milk, because of their ability to produce high levels of lactic acid as well as being able to survive under high acidic conditions. It was noted that except *Enterococcus* isolates, all TLAB strains isolated from the fermented raw camel milk were unable to grow at temperature of 30°C. The high level of TLAB in raw milk can be favored by low pH conditions (Badis et al., 2004). In this study, it was noted that the biodiversity of 82 thermophilic lactic acid bacteria isolated from fermented camel milk is limited to the four genera: *Lactobacillus* (31.7%), *Enterococcus* (30.5%), *Streptococcus* (24.4%) and *Pediococcus* (13.4%). These findings can be compared to those obtained in raw dromedary milk of Morocco (Benkerroum et al., 2003) which showed 99 isolated strains of LAB belonging to five genera: *Enterococcus* (58.6%), *Pediococcus* (28.3%), *Streptococcus* (4%), *Lactococcus* (8.1%) and *Leuconostoc* (1%). And also, our results can be compared with data obtained by Khedid et al. (2009), who have isolated 120 LAB strains from raw camel milk, grouped into six genera; they were clearly dominated by the genus *Lactobacillus* (37.5%), followed by the genus *Lactococcus* (25.8%) and *Leuconostoc* (11.7%), *Enterococcus* (10.8%), *Streptococcus* (9.2%) and *Pediococcus* (5%). In the same topic, Kacem and Karam (2006) isolated 216 LAB in camel milk from arid regions of Algeria, which were identified in four genera: *Lactobacillus* with (46.9%), followed by the genus *Lactococcus* (22%), *Enterococcus* (19.3%) and *Leuconostoc* (11.5%). Abdelgadir et al. (2008) isolated 180 LAB in the Sudanese fermented camel milk, they were clustered by rep-PCR into three genera: *Streptococcus*, *Enterococcus* and *Lactobacillus*.

The first stage of screening for EPS-producing phenoltype revealed that 36.6% of the studied thermophilic lactic acid bacteria strains show a mucous aspect of colonies. This phenotypic character can be related to the production of EPS on solid media (Gomez, 2006). Therefore, the presence of a translucent or creamy material involving a mucous colony is an indicator of EPS production potential. The production of polymers was confirmed by mixing each colony in absolute ethanol. Precipitate formation indicates the presence of EPS. The discriminatory value of the methods to test mucoidy and ropiness of bacterial colonies, were relatively low. Different EPS screening methods have been reported for LAB. The visual inspection of bacterial colonies on agar plates is most probably the easiest method, but it is insensitive. This method is unable to detect LAB strains that produce low amounts of EPS (Smitinont et al., 1999).

In the second stage of screening of various TLAB strains on the MRS broth, data showed that all the 30 selected mucous strains from 82 TLAB examined isolates, were able to produce exopolysaccharides. The amount of EPS production differs between genera and

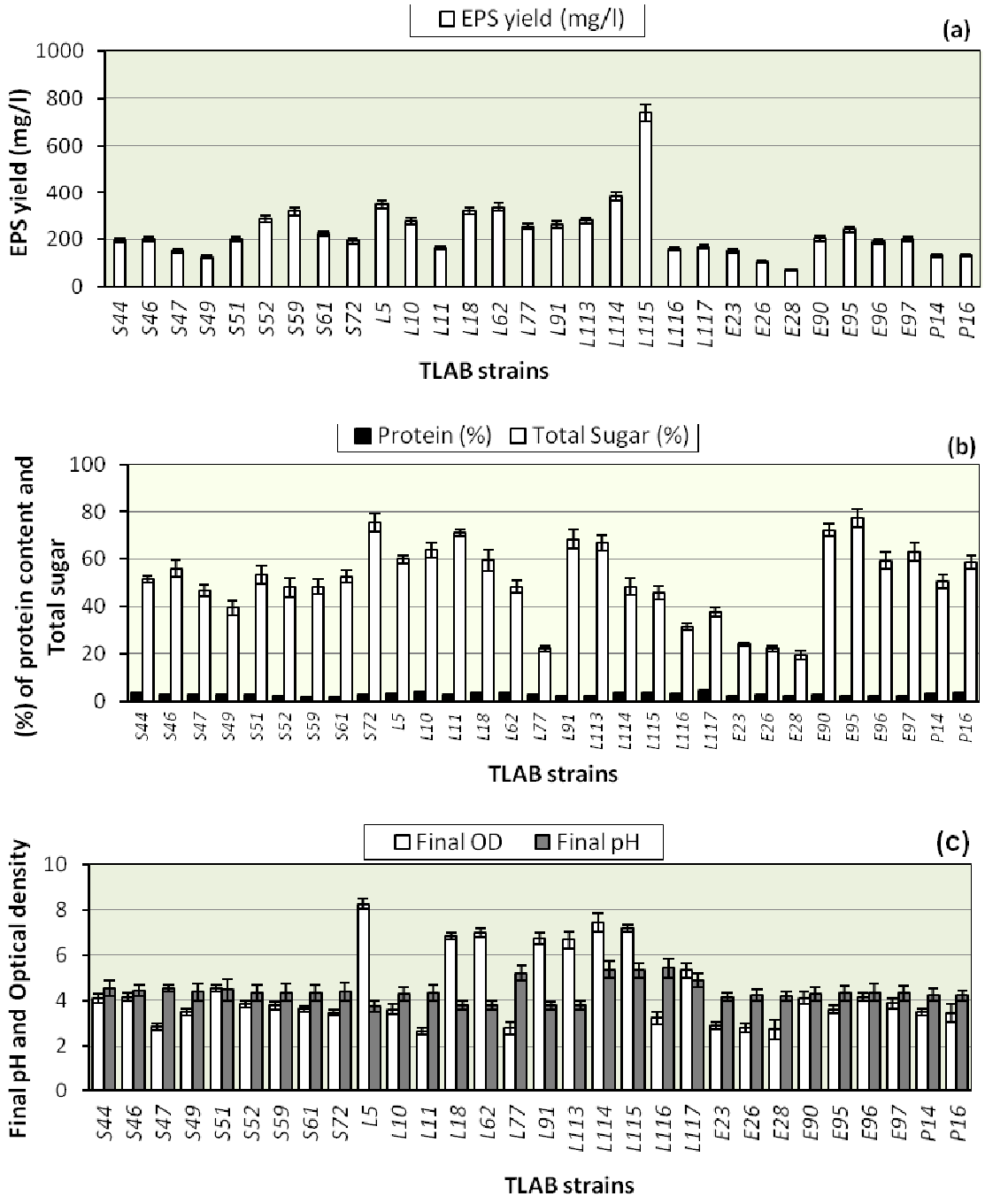

Figure 1. Screening of the TLAB strains for EPS production and partial characterization of produced exopolysaccharides, (a): EPS yields (mg/l) of TLAB screened on customized MRS broth, (b): Total sugar and proteins content of TLAB exopolysaccharides, (c): Final optical density (OD) and final pH of TLAB cultures. *L*: *Lactobacillus* strains, *S*: *Streptococcus* strains, *E*: *Enterococcus* strains, *P*: *Pediococcus* strains. Bars on the histogram represent the standard error of means.

varies within a genus. These findings approve the results on EPS from lactic acid bacteria reported by Van den Berg et al. (1993) in which 30 strains out of 607 tested showed the ability to produce exopolysaccharides. *Lactobacillus* strains produce the highest yields of exopolysaccharides, range between 160 and 740 mg/l. Our results can be compared positively with those reported by Laws and Marshall (2001), who obtained EPS yields of 175 mg/l produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* LY03 and less than 5 mg/l by *L.*

delbrueckii subsp. *bulgaricus* LY58. Similarly, Frengova et al. (2000) reported that the EPS contents were recorded between 58 and 540 mg/l for various strains of *Lactobacillus bulgaricus*. Other data reported by Xu et al. (2010) shows EPS yields ranged between 67 and 238 mg/l for *Lactobacillus paracasei* HCT. *Streptococcus* strains produce EPS yields between 126 and 319 mg/l. These data can be compared to those obtained by Laws and Marshall (2001), with EPS yields of 100 mg/l produced by *Streptococcus thermophilus* SY102 and less than 15 mg/l produced by *S. thermophilus* SY60. Similarly, Frengova et al. (2000) reported that the polysaccharide yields were recorded between 40 and 270 mg/l for various strains of *S. thermophilus*. In synthesis, we can conclude that our values of EPS yield did not differ significantly to those obtained by other authors. However, there is no report recorded for EPS production by both following genera: *Enterococcus* and *Pediococcus*. Hence, our results revealed that *Enterococcus* strains produce EPS yields ranging between 70 and 242 mg/l, and also, both *Pediococcus* strains (P14, P16) which produce EPS amounts of 132 and 134 mg/l, respectively. The isolated EPS powders had a total sugar content ranging between 22 and 71% for *Lactobacillus* strains, between 39 and 75% for *Streptococcus* strains, between 19 and 77% for *Enterococcus* strains and between 50 and 58% for both *Pediococcus* strains. Hence, protein content was negligible and ranged between 1.6 and 4.8% for various TLAB strains. These findings can be partially compared with those reported by Shene et al. (2008) for *Streptococcus* strains, having a total of sugar ranging between 20 and 60%, and protein content ranging between 0.3 and 3.6%. For the growth conditions tested, it was noted that the final pH of all cultures was decreased and ranged between 3.75 and 5.43, while the final optical density values ranged between 2.66 and 8.25. The EPS-yields of TLAB strains have shown moderate correlation with the bacterial growth, but, they were low in correlation with values of total sugar fraction. We suggest that this correlation is not necessary because the bacterial EPS may contain a non-carbohydrate moiety. These findings shows that fermented camel milk can be a potential source of thermophilic lactic acid bacteria that produce exopolysaccharides.

Conclusion

Our results demonstrate the diversity of TLAB in Algerian raw camel milk. This dairy product contains several genera of LAB, which were preliminary identified, and have a potential for EPS-producing activity with high yields. These strains can be used as starter culture with predictable characteristics and contribute to the development of fermented milk with stable consistent quality. As perspectives, three approaches are required: Firstly, genotypic characterization of isolates to determine the number of distinct strains among the described isolates of TLAB in our collection, PCR and DNA sequencing will be undertaken. The results will then be compared with other data obtained for other strains of TLAB. Secondly, optimization of our thermophilic lactic acid bacteria based on their technological properties and their use as starters (alone or in association) for dairy products. Finally, EPS-producing strains can be also examined for their ability to form biofilms, then, exopolysaccharides can be characterized, and applied according to the physicochemical characteristics.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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