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Screening of exopolysaccharide-producing coccal lactic acid bacteria isolated from camel milk and red meat of Algeria

Imène Kersani*, Halima Zadi-Karam and Nour-Eddine Karam

Laboratory of Biology of Microorganisms and Biotechnology, University of Oran1 Ahmed Ben Bella, Oran, Algeria.

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The analysis of coccal lactic acid bacteria (CLAB) strains for their competence in polysaccharide production has been given proper attention as texturing and thickening agents. The characterization of these strains has been performed from two natural environments (camel milk and red meat). All the isolates were evaluated for exopolysaccharides (EPS) production on certain solid medium and ruthenium red milk agar plate. Based on their EPS-producing colony phenotype, five strains were chosen giving an important white-color and mucoid aspect on sucrose-based media which being the best for detecting the EPS. Quantitative estimation of EPS indicated that amount of this polymer rendered more than 400 mg/L and the apparent viscosity ranged from 2.1 to 2.9 milli Pascals per second (mPa.s). Therefore, there was not found a close relationship between the amount of EPS and the apparent viscosity. Three strains were selected for their significant production of EPS. For protein assay, a low content of protein was obtained on crude polymer revealing the quality of EPS extracts.

Key words: Coccal lactic acid bacteria (CLAB), exopolysaccharides (EPS), sucrose-based medium, apparent viscosity, amount of EPS.

INTRODUCTION

Lactic acid bacteria (LAB) are generally recognized as safe organisms that have been used since ancient times in fermentations food. Besides, these bacteria are also being exploited for the production of various food-grade biomolecules such as vitamins, conjugated linoleic acid, lactic acid and bacteriocins (Leroy and De Vuyst, 2004). Certain LAB are able to produce exopolysaccharides (EPS) either attached to the cell wall (capsular EPS) or released to the extracellular environment (EPS) (Torino et al., 2015). Term of EPS as proposed by Sutherland (1972) provides a general name for all these forms of bacterial polysaccharides found outside the cell wall. EPS from LAB can be subdivided into two groups: the homopolysaccharides (HoPS) composed of one type of monosaccharide, and the heteropolysaccharides (HePS) composed of a repeating unit that contains two or more different monosaccharides, substituted monosaccharides and other organic and inorganic molecules (De Vuyst et

*Corresponding author. E-mail: kersani_imene@yahoo.fr. Tel: +213 550213535.

al., 2001; Laws and Marshall, 2001; Monsan et al., 2001).

EPS produced by LAB has been clearly demonstrated for both thermophilic (for example *Streptococcus thermophilus*) and mesophilic (for example *Lactococcus lactis*) strains (De Vuyst and Degeest, 1999). This interest is due to their useful role in improvement of physical, rheological and sensory properties of fermented milks (Behare et al., 2009a, b, 2010). Moreover, consumer concern for healthy, natural, and low calorie foods has generated more demand in the market for fat-free or reduced-fat products (Khurana and Kanawjia, 2007). The alternative approach to such commercial additives is the use of EPS-producing LAB that act as natural biothickeners (Ruas-Madiedo and de los Reyes-Gavilan, 2005). In addition, the EPS produced by microorganisms vary in their composition and properties such as anti-bacterial, anti-cancer, anti-tumoral, anti-ulcer, anti-immune stimulation along with cholesterol-lowering ability (Nagaoka et al., 1994; Pigeon et al., 2002; Yoo et al., 2004; Kumar et al., 2007; Raveendran et al., 2013; Li et al., 2014; Raposo et al., 2014). An additional physiological benefit of EPS is that, it will remain for longer time in the gastro-intestinal tract, thus enhancing the colonization of probiotic bacteria (German et al., 1999). The EPS of LAB are in a great variety, which depends on the type of LAB strains, culture conditions, and medium composition (Guzel-Seydim et al., 2005; Ismail and Nampoothiri, 2010), age of the cell, pH and temperature (De Vuyst et al., 2003; Ruas-Madiedo and de los Reyes-Gavilan, 2005).

The objectives of this study consist to isolate and screen EPS producing strains of Coccal LAB (CLAB) obtained from camel's milk and fresh red meat of Algeria, in order to evaluate their capacity to produce these exopolymers and select the most performing strains.

MATERIALS AND METHODS

Sampling and isolation of CLAB strains

CLAB were isolated from camel's milk and red fresh meat of Algeria by serial dilution plating on M17 medium (Terzaghi and Sandine, 1975) and incubated anaerobically for 24 h at 30°C. All strains were subcultured in M17 broth medium (1%) and stored at -20°C in 70% skim milk (enriched with 0.05% yeast extract and 0.05% glucose) containing 30% glycerol (Samelis et al., 1994). The strains were phenotypically identified by adapting of conventional bacteriological characterization techniques.

Detection of EPS

Qualitative analysis

Visual appearance: The simplest way to detect EPS-producing strain is to examine a colony on agar plates. The screening test was carried according to Dave and Shah (1996) and Dupont (1998) with few modifications. The selection process of EPS-producing bacterial strains was fundamentally based on the development of mucoid aspect of strain colonies, according to Ricciardi et al. (1997); Welman et al. (2003) and Ruas-Madiedo and de los Reyes-Gavilan (2005), by studying the production of EPS on several solid media. Use of suitable screening medium increases the probability of EPS-producing phenotypes (Ruas-Madiedo and de los Reyes-Gavilan, 2005; Behar et al., 2009b,c).

For this purpose, we have used M17-agar medium with 50 g of sucrose per liter (M17 hypersaccharosed or M17HS) instead of lactose, M17 (5 g of lactose per liter: LM17) and MSE medium (Mayeux et al., 1962). The strains of CLAB were incubated at 30°C for 24 to 48 h and macroscopic appearance in each medium was compared.

The mucoid colonies have a glistening and slimy appearance on agar plates (Vescovo et al., 1989; Dierksen et al., 1997). It 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-Gavilan, 2005). The colonies which have mucoid and ropy phenotype were picked up and purified by following the streaking method, then preserved at 4°C on M17 agar and selected for the next step. The symbols for mucoid polysaccharide production as observed visually were given as less mucoid (+), medium mucoid (++), highly mucoid (+++) or non-mucoid (-).

Ruthenium red staining: Ruthenium red staining method could be used for the detection of EPS producing LAB strains (Dabour and LaPointe, 2005). Pink and white colonial variants of the strain were isolated from the plate surface of a solid semi-synthetic medium containing skim milk, sucrose, yeast extract, and ruthenium red (Gancel et al., 1988). After 48 h of incubation at 30°C, ruthenium red stains the bacterial cell wall, producing pink colonies for non-ropy strains and white colonies for ropy strains.

Quantitative analysis

Apparent viscosity: Production of EPS by LAB leads to a change in texture of the medium, due to change of its viscosity. Viscosity was measured in basal minimal medium hypersaccharosed (BMMHS) (Morishita et al., 1981), at 25°C using a Thermo Scientific Haake^{TM}, and falling ball viscometer with a coaxial cylinder at a steady shear rate of 173/s along with a MK50 rotor assembly. Viscosity is expressed as the dynamic viscosity using the internationally standardized absolute unit of milli Pascals per second (mPa.s). Optical density (OD_{600nm}) of the culture was also assessed to estimate the growth of cell in order to relate to the viscosity.

Quantification of EPS

EPS were isolated from the various culture strains using the ethanol precipitation as described by Cerning et al. (1994).

Extraction and purification of polysaccharide

For each isolate, the EPS was recovered and purified from the BMMHS. Fresh cultures were prepared in M17HS broth, incubated at 30°C for 18 h. These subcultures were propagated in 50 ml BMMHS medium. After incubation, the cultures were heated at 100°C for 15 min, and the cells were removed by centrifugation (9950 *g* for 30 min at 4°C).

A cold ethanol (95%) was gradually added to the supernatant in the amount from one to two and three supernatant volumes and the mixture was centrifuged (9950 *g* for 30 min at 4°C). The supernatant was discarded and the pellet was dissolved in deionized water, dialyzed against sterile water for 24h at 4°C and lyophilized. The crude EPS was purified by 10% trichloroacetic acid

Table 1. Mucoidy character of EPS in different agar media with CLAB strains.

Strain	LM17	M17HS	MSE
L4	-	+++	++
L5	-	+++	++
L6	-	+++	++
V1	-	++	+
V2	-	++	+
V3	-	+++	++
V4	-	++	+
V5	-	+++	++
Other CLAB strains	-	-	-

(TCA) and washed three times, then centrifuged, dialyzed for 5 days at 4°C, lyophilized and dissolved in sterile distilled water.

After the isolation steps, a lyophilized powder is obtained, its weight being the simplest indication of the EPS yield (De Vuyst et al., 1998; van Geel-Schutten et al., 1999; Frengova et al., 2000; Degeest et al., 2001a).

Quantification of total proteins and polysaccharides in EPS was achieved, using assays, such as the Bradford (Bradford, 1976) and the Dubois (Dubois et al., 1956) methods. These experiments were performed in duplicate and repeated three times.

Sugar determination

A spectrophotometric procedure for the determination of sugar and related compounds is the phenol sulfuric acid method described by Dubois et al. (1956) using glucose as the standard (Torino et al., 2001). The quantities of EPS were expressed as the equivalent milligrams of glucose per liter using standard curve of glucose.

Protein determination

The protein content in each dialyzed supernatant fluid was quantified as described by Bradford (1976) using bovine serum albumin (BSA) as a standard. The weight of protein was plotted against the corresponding absorbance with a UV-Vis Optizen POP spectrophotometer (A_{595nm}) resulting in a standard curve used to determine the protein in unknown samples. This weight was expressed in micrograms of protein.

RESULTS AND DISCUSSION

Screening of EPS-producing CLAB strains

In this study, 43 strains of CLAB were used to search their ability to produce EPS and it was found that eight isolates were able to produce EPS, based on the colony morphology. Three strains isolated from camel milk (L4, L5 and L6), five strains isolated from red meat (V1, V2, V3, V4 and V5). They were screened for mucoidy appearance on agar plates. All selected strains formed big slimy and mucoidy colonies on M17HS compared to MSE (Table 1). Thus, ropiness character was detected for some strains when extended with an inoculation loop. However, no mucoidness phenotype was detected on LM17 agar.

The studies noted by Ruas-Madiedo and de los Reyes-Gavilan (2005) indicated that the carbon source added to the screening media plays an important role in the detection of the EPS phenotype in LAB. In fact, incorporation of a carbon source such as sucrose in a M17 agar medium increases the number of EPS-producing and stimulated their formation, but not the growth, suggesting that most of the sugar is employed for EPS biosynthesis and little as an energy source for growth. The opposite was observed with lactose for that mucoidness phenotype could not be detected on LM17.

Consequently, M17HS was the best medium for detecting the mucoid character. These results are in accordance with those obtained by Grosu-Tudor and Zamfir (2011) who screened 31 strains of LAB developing mucoid colonies on MRS with sucrose and less or no mucoid on media with glucose or lactose as a carbon source. On the contrary, Degeest and De Vuyst (2000) and Degeest et al. (2001b) reported that HePS production is enhanced as compared to growth in media with glucose or lactose as the sole carbohydrate sources. Also, Gancel and Novel (1994) reported that the EPS production in a defined medium by *Streptococcus thermophilus* S22 was found to be higher with glucose and fructose and lower with lactose and sucrose, although the latter supported better growth.

Detection of EPS production using ruthenium red

Ruthenium red is a carbohydrate-binding dye used to stain biofilms formed by EPS-producing bacteria (Prouty et al., 2002; Borucki et al., 2003). The EPS production was determined by evaluating the color of the colonies grown in ruthenium red milk (RRM) containing sucrose by spot-seeded.

Our strains readily produced mucoid material (Muc^+) when grown on RRM medium which is used in this investigation as the method of choice because it is ideal both for growth and high EPS production (no interfering polysaccharides). As already observed by Zhang et al.

Figure 1. White colonies formed on RRM agar.

Table 2. Growth and apparent viscosity of mucoid strains.

Strain	OD=600 nm	Apparent viscosity (mPa.s)
L4	0.694	2.9
L5	0.564	2.8
L6	0.851	2.7
V3	0.664	2.1
V5	0.21	2.2

(2001), *S. thermophilus* ST1 produced a maximal amount of EPS, when this strain was grown in skim milk supplemented with sucrose.

All selected mucoid strains showed white colonies with different capacities of production. This indicates that EPS was synthesized and secreted. Figure 1 shows EPS production in RRM agar plate with white colonies of strain L4.

The production of EPS prevents the staining, and hence ropy colonies appear white on the same plates (Stingele et al., 1996). In fact, Mora et al. (2002) found that half of the analyzed strains of *S. thermophilus* were able to grow as white or pink colonies in ruthenium red skim milk indicating their ability to produce EPS.

Five isolates (L4, L5, L6, V3 and V5) were retained and chosen in the second step of screening of EPS production by CLAB, based on their high ability to produce EPS in RRM. Therefore, this result confirmed the biothickening character of our strains.

Viscosity measurement and EPS yield

The visual inspection of bacterial colonies on agar plates is most probably the easiest method, but it is insensitive and indicative. This method is unable to detect LAB strains that produce low amounts of EPS, unless they are very ropy (van den Berg et al., 1993; Smitinont et al., 1999). In this case, quantitative detections (including viscosity) were necessary for screening of EPS produced by our isolates.

Most investigators focused their attention on viscosity measurements, since those were used traditionally as an indication for EPS production in liquid media (Cerning et al., 1986, 1988). BMMHS were chosen for this investigation, in order to facilitate EPS analysis. It contains a carbon source, amino acids, vitamins, and mineral salts.

The measurements of the growth and viscosities are shown in Table 2 which revealed that viscosities are extremely variable and are dependent on the strain tested. For the ropy strains, the apparent viscosity was elevated ranging from 2.1 to 2.9 mPa.s. Whereas the bacterial growth was significantly less than OD_{600nm}=1.0. This data confirmed our previous study of mucoid phenotype that medium supplemented with sucrose induced EPS production. However, it is difficult to make a precise correlation between the ropy/mucoid phenotype and production of exopolysaccharides (Van der Meulen et al., 2007).

Many authors studied the effect of medium composition, temperature, pH and fermentation time on

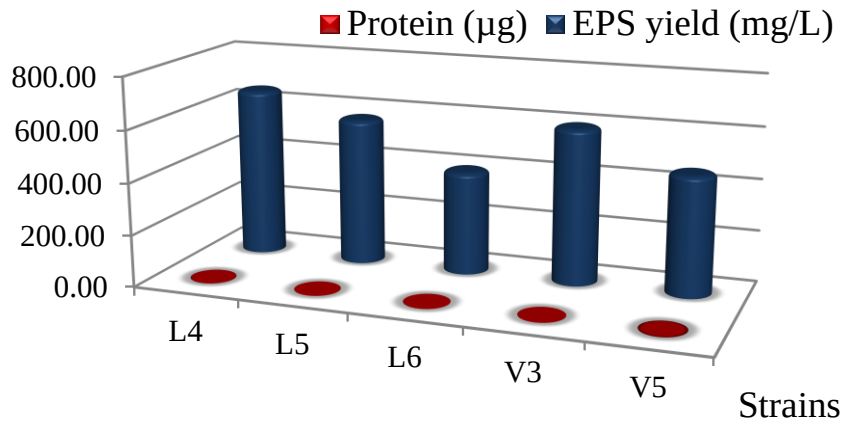

Figure 2. EPS yields and proteins content in fraction of polysaccharide.

the bacterial growth and exopolysaccharide yields (Cerning, 1990; Degeest and De Vuyst, 1999; Degeest et al., 2001a, 2002; Zisu and Shah, 2003; Vaningelgem et al., 2004). Suitable conditions for growth could be not favorable for EPS production and viscosity. For instance, L4 had a high value of viscosity (2.9 mPa.s) while cell growth reached an OD_{600nm} of over 0.69 after 72 h of incubation. These results are clearly explained by the mechanism proposed by Sutherland (1972), who postulated that there is a competition between EPS and cell-wall polymer biosynthesis. If the cells are growing slowly, wall polymer synthesis will also be slow, thereby making more isoprenoid phosphate available for exopolymer synthesis. The total yield of EPS produced by CLAB strains was monitored quantitatively by using the phenol-sulfuric acid method. Figure 2 shows the amounts of sugar and protein in EPS fraction. There was a great variation in content of EPS (mg/L) which ranged from 400.33 to 658.06 mg/L. The amounts of EPS produced by the dairy strains vary considerably (Ludbrook et al., 1997; Badel et al., 2011). In general, EPS yield among majority of *S. thermophilus* strains varies from 20 to 600 mg/L in milk-based medium under optimal conditions (De Vuyst et al., 2003; Vaningelgem et al., 2004).

Natural biothickeners with their structural diversity and functional versatility have gained commercial importance in the field of glycotechnology (Lule et al., 2016). In effect, Han et al. (2016) suggested that strains producing higher EPS might contribute to the higher viscosity of fermented milk. High-yielding of EPS was obtained by L4 (658.06 mg/L) with the highest apparent viscosity (2.9 mPa.s; Table 2). However, our results revealed no straightforward relationship between EPS concentration of most strains and the positive effect of these polymers on the rheological properties. These results are in concordance with previous studies (Cerning et al., 1986; van Marle and Zoon, 1995; Petry et al., 2003; Iwański et al., 2012).

L5 and L6 strains gave almost same viscosity (≥ 2.7 mPa.s) but the amounts of EPS were variable, 572.03 and 400.33 mg/L respectively. It appeared that EPS production depend to their structure, composition, chain stiffness, branches and side groups in the polysaccharide chain (Ruas-Madiedo et al., 2002), the average molecular mass distribution (van den Berg et al., 1995), organism used for production (Cerning, 1995) and conditions of experimentation (Badel et al., 2011). Hence, three strains (L4, L5 and V3) of CLAB were screened for their great potential of EPS production in liquid medium.

The protein content in the EPS sample was determined to give an estimate of purity. The protein was accounted for lower than 1.21 µg for samples from all studied strains (Figure 2). Our results indicate that the purification of polysaccharide with TCA was successfully performed and removed most of the initial protein content. Although according to Kimmel et al. (1998) it seems likely that the protein was carried over from medium ingredients during recovery and is not tightly bound to EPS. Consequently, the protein was not considered to interfere in the phenol-sulphuric acid method (Gentès et al., 2011).

Conclusion

This study was undertaken to screen CLAB isolated from camel milk and red meat of Algeria for their ability to produce EPS in several mediums was carried out. Eight strains conferred a highly mucoid colony on M17HS and thirty-five were not able to produce these biopolymers. Ruthenium red staining reveals the same findings. No clear-cut relationship between the slimy phenotype, the medium viscosity occurs and amount of EPS produced by a ropy strain when different strains were compared. Three strains yielded an important amount of polysaccharide. For protein contents of crude EPS, a small weight of protein contaminating these polymers was noted.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

CLAB, Coccal lactic acid bacteria; **EPS,** exopolysaccharides; **HePS,** heteropolysaccharides; **HoPS,** homopolysaccharides; **LAB,** lactic acid bacteria; **M17HS,** M17 hypersaccharosed; **LM17**, M17 lactose; **RRM,** ruthenium red milk; **TCA,** trichloroacetic acid.

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