Original Scientific paper
10.7251/AGRENG2203055E
UDC 579.864

IRANIAN KISHK AS A SOURCE OF LACTIC ACID BACTERIA PRODUCING EXOPOLYSACCHARIDE

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ABSTRACT

Exopolysaccharides are high molecular weight polymers composed of sugar subunits. Produced exopolysaccharides by lactic acid bacteria (LAB) play a significant role in improvement of organoleptic properties of fermented dairy products such as yogurt. Diversely, the probiotic function of these bacteria and the prebiotic properties of their produced biopolymers promote consumer's health. For this purpose, a traditional dairy product known as "Kishk" was selected. 143 strains of lactic acid bacteria were isolated from Iranian Kishk in Khorasan Province and cultured in formulated MRS mediums with different sugars such as glucose, fructose, sucrose and, lactose (40 g/L) and incubated in anaerobic conditions at 30 and 37°C for 48 hours. The microscopic features of the isolates were assessed and the production of exopolysaccharide in the culture medium was evaluated by disk and ruthenium red methods. The phenol-sulfuric and weight method were used to quantify exopolysaccharide production. Results showed pH of Kishk samples ranged from 3.60 to 4.08 and the average of total mesophilic count and LAB count of samples were 6.50 and 5.89 log CFU/g, respectively. Analysis of data exhibited 79 out of 143 lactic acid bacteria isolates were exopolysaccharide producer and 70% of them were cocci. The average of maximum and minimum production by weight method were 2.61 g/L and 0.08 g/L, respectively. The average of highest and the lowest amount of exopolysaccharide by phenol sulfuric method were measured 1.87 g/L and 0.06 g/L, respectively. This study indicates the potential of exopolysaccharide production by Iranian native species from dairy products.

Keywords: *Exopolysaccharide, Lactic acid bacteria, Kishk.*

INTRODUCTION

The increased demand for natural polymers in various industrial applications during recent years has led to a renewed interest in exopolysaccharide (EPS) production by microorganisms. Many microorganisms have an ability to synthesize extracellular polysaccharides and excrete them out of cell (Suresh Kumar *et al*., 2007). New microbial polysaccharides might have innovative uses as gelling agent, emulsifier, stabilizer or texture enhancing agent (Sutherland, 2001).

E orts have been made to use LAB as microbial cell factories for the production of industrially interesting metabolites either to be used as purified compounds or to be produced *in situ* in fermented foods (Boguta *et al*., 2014). As a traditional dairy product, Kishk is mostly produced from sheep's milk. Firstly, raw milk is boiled and then cooled and inoculated with traditional yoghurt made earlier as starter culture. The butter is separated from sour yoghurt by Mashk, which is made from hide (sheepskin) and is used for butter making. Then buttermilk is boiled and sieved by cloth bag. Finally the thick whitish semi-solid part of buttermilk, which is sieved, is shaped in form of conic or cubic balls and then sun-dried for 3–4 days (Iranmanesh *et al*., 2018). A wide variety of carbon sources, used to produce microbial exopolysaccharides, include sucrose, glucose, lactose, maltose, mannitol, sorbitol, whey, starch, sugar concentrates. The type of carbon source influences the yield of exopolysaccharide. The size of the exopolysaccharide may also vary with the carbon source (Suresh Kumar *et al*., 2007). Exopolysaccharide concentration is estimated as neutral carbohydrate content usually determined by the phenol sulfuric acid method or by weighting the polymer dry matter (Vaningelgem *et al*., 2004, Dubois *et al.*, 1956).

This study demonstrates the effect of type of carbon source on exopolysaccharide yield in EPS producing lactic acid bacteria isolated from Iranian Kishk.

MATERIALS AND METHODS

Chemical and Microbiological analyses:

The pH value of the Kishk samples was measured using a pH meter. The total number of mesophilic aerobic bacteria were enumerated on Nutrient Agar (NA) incubated at 35±2°C for 24 h. MRS agar was used for counting Lactic acid bacteria (Kirdar and Advances, 2012).

Isolation of Lactic acid bacteria:

Kishk samples were taken from eight regions in Khorasan province according to Iranian National Standard No. 326. For the isolation of LAB strains from Kishk samples, 10 gr of samples were taken aseptically and transferred to 90 ml skim milk followed by the preparation of serial dilutions. Streak culture method on MRS (de Man, Rogosa and Sharpe) agar was done and the plates incubated at 30°C and 37°C for 24 h. Colonies with typical characteristics of lactic acid bacteria were selected from MRS and tested for Gram stain, cell morphology, oxidase and catalase reaction (Ispirli and Dertli, 2017).

Screening of EPS producer isolates:

EPS synthesis was performed in MRS agar medium with disc and ruthenium red methods. In the disc method, MRS culture medium was formulated with 40% of 4 kinds of sugars (sucrose, fructose, lactose and glucose) instead of glucose in the main formula and the paper discs were inoculated with inoculum, incubated at 30 and 37°C for 48 h (Paulo *et al*., 2012). In ruthenium red method, streak culture method was performed by overnight cultures on MRS-RR medium (0.08 g/l ruthenium red). After 48 h of incubation at 30 and 37°C, ruthenium red stains the bacterial cell wall, producing pink colonies for non-EPS producers and white colonies for producers(Hongpattarakere *et al*., 2012).

Isolation of EPS and determination of EPS:

For the isolation of EPS, all strains were grown in formulated MRS broth, inoculated at 1% (v/v) with an overnight culture then incubated at 30 and 37°C for 2 d anaerobically. Then centrifuged at 10,000 ×g for 10 min to remove the cells. Three volumes of 96% (v/v) cold ethanol were added to the supernatant and stored overnight in 4°C to precipitate. The culture was then centrifuged again (10,000× g for 10 minutes). After removing the supernatant, the precipitate was mixed with a sevag reagent (chloroform: n-butanol = 4: 1) and then centrifuged. The dialysis (cut-off 8000-12000Da) of the resulting supernatant with distilled water was performed at 4°C for 48 hours. The produced EPS was stored for 24 hours in -80°C freezer and then lyophilized. Determination of EPS was performed via phenol-sulfuric acid method using glucose as standard and weight method by measuring the dry weight of EPS at 60°C in an oven (Lin and Chien, 2007; Jeong *et al*., 2017; Dubois *et al*., 1956).

Statistical analysis:

Each test was performed in duplicate. Data from each test were subjected to SPSS (version 22.0.0.0) for analysis of variance. Duncan's multiple range test was used to determine any significant difference ($p<0.05$) among treatments.

RESULTS AND DISCUSSION

Chemical and Microbiological characteristic:

The results concerning chemical and microbiological properties (mean values and standard deviation) of the Kishk samples were shown in Table 1. The mean value of pH ranged from 3.62-4.08 in Kishk samples from different region of Khorasan province in Iran. Noori *et al* (2013) reported the pH of Kishk samples were in a range of 3.85-4.11 (Noori *et al*., 2013). According to Gadallah *et al* (2019) in Kishk samples pH ranged between 4.39–4.84 (Gadallah and Hassan, 2019). The average value of total aerobic mesophilic bacteria and lactic acid bacteria were 6.50 and 5.89 log CFU/g in Kishk samples, respectively. The results of Kirdar *et al* (2012) showed the average values of total bacterial count, *Lactobacillus* sp., *Lactococcus* sp. and *Enterobacteria* in Kishk samples were 8.24±0.95, 7.63±0.99, 7.52±0.96 and 2.62±1.13, respectively. These findings were higher than that of this study. A high number of TAMB can be explained by sufficient change in the environmental conditions which occur during Kishk storage and which allows for

the growth of microorganisms (Kirdar and Advances, 2012). The total count and Lactic acid bacterial count of Kishk samples in different researches have been reported in a range of 3.46-7.4 and 3.65-4.89 log cfu/g, respectively (Tamime and Robinson, 2007).The total bacterial counts of different Kishk samples in work of Gadallah *et al* (2019) were ranged from 5.15 to 7.50 log cfu/g and Lactic acid bacterial counts were ranged from 6.04 to 7.88 log cfu/g (Gadallah and Hassan, 2019). There was a significant positive correlation between total aerobic mesophilic bacteria and lactic acid bacteria ($r=0.93$, $P\leq0.05$) (Figure 1). These results were in agreement with the findings of Kirdar and Advances, 2012.

Table1- pH and microbiological properties of Kishk samples

Code of Samples	**pH** *	**The total lactic acid bacteria*(TLAB)**	**The total aerobic mesophilic bacteria (TAMB)***
Q	3.66±0.014^{c**}	6.08±0.38^{c}	7.02±0.4^{b}
K	3.675±0.007^{c}	5.45±0.35^{e}	5.85±0.27^{e}
T	4.04±0.056^{a}	7.2±0.41^{a}	7.8±0.42^{a}
M	3.62±0.028^{d}	5.36±0.22^{f}	6.03±0.32^{d}
S	3.85±0.07^{b}	5.64±0.41^{d}	5.8±0.2^{e}
N	4±0.035 ^{ab}	5.38±0.3^{ef}	5.68±0.41^{f}
R	3.85±0.07^{b}	5.9±0.3^{c}	6.75±0.43^{c}
F	4.08±0.042^{a}	6.14±0.34^{b}	7.1±0.35^{b}

Q: Sarab Region, K: Nasr Region, T: Suran Region, M: Darsoufian Region, S: Rayab Region, N: Chenesh Region, R: Akhlamad, F: Azghad Region.

*Values are reported as (mean ± sd)

**The letters in each column show statistical differences

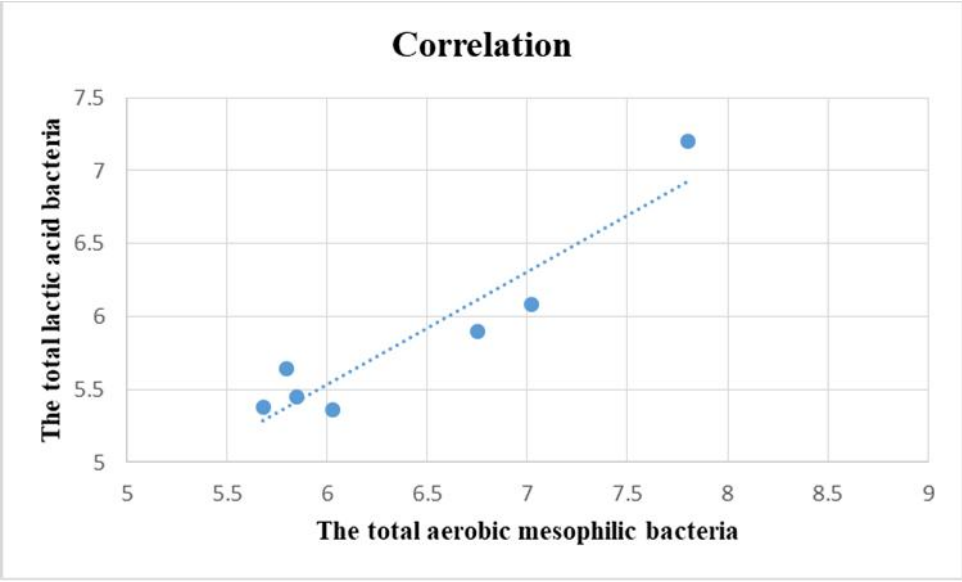

Figure 1-Correlation between the number of total aerobic mesophilic bacteria and total lactic acid bacteria

Isolation and identification of EPS producers and quantification of EPS:

The findings revealed that 79 out of 143 Gram-positive, non-spore forming, catalase and oxidase negative isolates were detected as putative EPS positive with both disk and ruthenium red methods. Amount of EPS was determined by phenol-sulfuric method and weight method. These isolates had a mucoid character. Ruas-Madiedo *et al* (2007) demonstrated 92% of their isolates were mucoid. It was in accordance with our results which states mucoid phenotype is dominant phenotype (Ruas-Madiedo *et al*., 2007). According to our findings, the average of maximum EPS production in 4 culture media (containing 4 kinds of sugars) by weight method was, 2.61 g/L (*E. faecium* R114) and minimum production 0.08 g/L (isolate T229 with cocci phenotype), and the greatest amount of exopolysaccharide by phenol sulfuric method was 1.87 g/L (*E. faecium* R114) and the lowest amount was 0.06 g/L (isolate T229). In this work, 3 isolates were selected and identified for high EPS production. They were identified as *E. faecium* (code T52), *E. faecium* (code R114) and *E. durans* (code K48). The partial 16S rRNA sequences of the identified strains in this study were deposited in GenBank under accession numbers MT437248- MT437250 (Table 2). The amount of EPS by weight and phenol sulfuric methods in them measured, 2.39; 1.70, 2.61; 1.87 and 2.17; 1.55 g/l, respectively (Table 2). The amount of EPS production in different references is different. *Streptococcus thermophilus* CC30 produce 1.95 g/L of EPS when grown in skim milk lactose medium at 30°C (Kanamarlapudi and Muddada, 2017). The EPS produced by the wild and mutant *L. delbrueckii* ranged from 5570.34 – 5910.62 mg/L (Adebayo-Tayo and Fashogbon, 2020). A high molecular weight EPS was recovered and purified to a yield of 2.8 ± 0.5 g/L from *Lb. plantarum* BR2 (Sasikumar *et al*., 2017). The highest EPS producing strains isolated from boza, yielding 2.39 ± 0.49 and 1.98 ± 0.23 g/L of EPS, respectively (Heperkan *et al*., 2014). *Lactobacillus kefiranofaciens* DN1 produced EPS, using glucose and lactose, and EPS yield rose to 2.2 g/L in modified MRS broth (60 g/L glucose) (Jeong *et al*., 2017).

The reported yields of HePS range from 50 to 350 mg/L for *Strep. thermophilus*, 60 to 150 mg/L for *Lb. delbrueckii* spp. *bulgaricus*, 25 to 600 mg/L for *Lc. lactis* spp. *cremoris*, and from 50 to 60 mg/L for *Lb. casei* (Ruas-Madiedo and De Los Reyes-Gavilán, 2005).

Table 2. Results of 16S rRNA sequencing of three highest EPS producer and GenBank accession number(s).

Code	16S rRNA sequencing results	Gen Bank accession number(s)	EPS amount by weight Method (g/L)	EPS amount by phenol sulfuric methods (g/L)
K48	*Ent. Durans*	MT437248	2.17	1.55
R114	*Ent.faecium*	MT437249	2.61	1.87
T52	*Ent.faecium*	MT437250	2.39	1.7

a

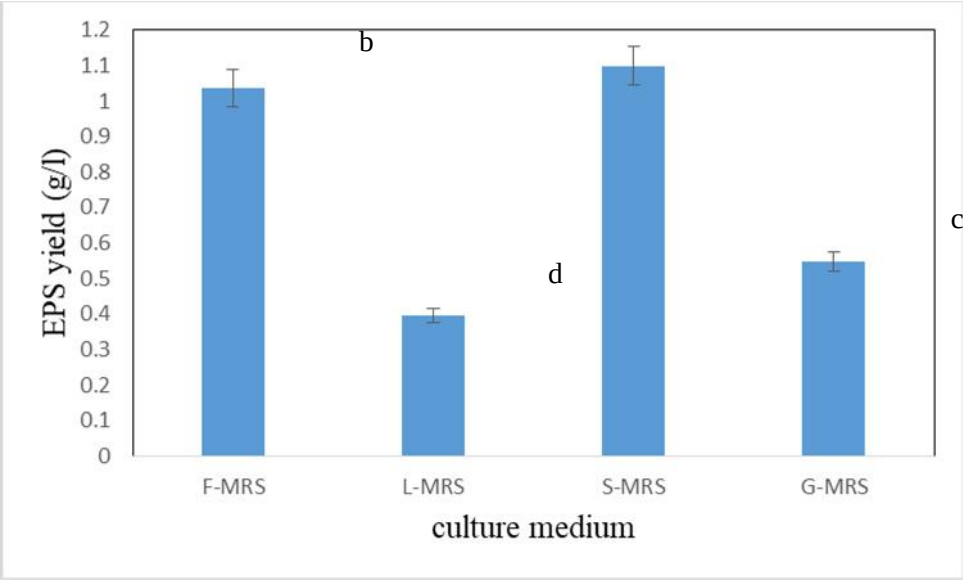

Figure 2- effect of carbon source on EPS yield

Analysis of data showed MRS broth medium formulated with sucrose had great effect on EPS production, but MRS broth formulated with lactose had a low impact on EPS synthesis. Kanmani *et al* (2013) showed that the production of EPS from *E. faecium* MC13 in the sucrose medium was higher than in lactose, glucose and fructose media which was 11.33 g/l (Kanmani *et al*., 2013). In other work Kanmani *et al* (2011) expressed the maximum yield of EPS from *Streptococcus phocae* PI80 (11.75 and 12.14 g/L) was obtained in the presence of lactose and yeast extract at a concentration of 20 g/l (Kanmani *et al*., 2011). Knowledge of the effect of the sugar source on EPS production and the activities of biosynthetic enzymes provides information about the mechanisms of regulation of the synthesis of EPS which can contribute to improve polymer production (Mozzi *et al*., 2001).

CONCLUSION

Food industry is looking for the multifunctional strains of LAB that contribute to the organoleptic, technological, nutritional and health properties of fermented dairy products. EPS producing lactic cultures have tremendous potential as functional starters, which can be better substituted to many commercial additives in use. However, EPS producing character is plasmid associated in LAB and may be lost over generations. Further it varies from strain to strain. Hence, selection of promising strains that retain EPS producing characteristic over a long period, may give opportunities to food manufacturer to produce various low-fat products. It is concluded that among these three enterococcus isolates, strain R114 identified as *Ent. faecium* produced the highest EPS amount in both methods of EPS measurement including weight and phenol sulfuric methods 2.61 and 1.87 g/L,

respectively. Fermented dairy products, especially made by traditional method in rural areas, are the potential reservoir for isolation of EPS producing cultures.

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