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Isolation, Identification and Taxonomic affiliation of Exopolysaccharide Producing lactic acid bacteria from Kradi cheese

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Abstract

Kradi cheese is manufactured by coagulation of naturally fermented butter milk containing different strains of lactic acid bacteria. In this study lactic acid bacterial strains were isolated from kradi cheese and screened for exopolysaccharide potential. The taxonomic group of the exopolysaccharide producing lactic acid bacterial strains was determined on the basis of their 16S rRNA sequences. Nine strains were shown to produce exopolysaccharides in MRS medium with sucrose. Four strains belonged to lactococcus lactis subsp. lactis and five strains to Lactococcus lactis subsp. cremoris species. Isolated strains showed varied yield of exopolysaccharides. The exopolysaccharides had high molecular mass of 1500 kDa and glucose was dominant in their monomer composition.

Keywords: lactic acid bacteria, isolation, taxonomic affiliation, exopolysaccharides, monomer composition

1. Introduction

Exopolysaccharides are natural polymers water soluble and have long chain high molecular mass (Deepak *et al.* 2016a; Deepak *et al.* 2016b). Lactic acid bacteria produce exopolysaccharides (EPS) which show lot of structural diversity (Bernal and Liamsa 2012; Ruas Madiedo 2002). Exopolysaccharides may be released into surrounding environment or loosely bound to cell surface (Abedfar and Hossininezhad 2016; Ram Kumar Pandian *et al.* 2016; Deepak *et al.* 2016b; Patel *et al.* 2012). Exopolysaccharides biosynthesis is controlled by many enzymes and proteins which are controlled through regulation by gene expression (Ciszek-Lenda 2011). The nature of exopolysaccharides depends on strain, culture condition and media composition. Lactic acid bacteria are present in fermented dairy products. Kradi cheese is a traditional indigenous cheese produced in Jammu and Kashmir India (Punoo *et al.* 2018a and Punoo *et al.* 2018b) by Gujjar tribes residing in green pastures. The raw material for kradi cheese production is naturally fermented raw milk which contains different strains of lactic acid bacteria (LAB). In the present study different strains of lactic acid bacteria from kradi cheese were isolated, identified and recognized for exopolysaccharide production. The taxonomic affiliation of exopolysaccharide producing bacteria was also verified.

2. Materials and Method

2.1 Lactic acid bacterial strains, growth conditions, screening for EPS production

The nine LAB used throughout this study were isolated from kradi cheese. These strains were isolated by plating on MRS agar (De Mann *et al.* 2013). Also modified MRS agar with 50 g/l of sucrose (MRS-s) and modified MRS agar with 20 g/l of fructose (MRS-f) were used as isolation media. All the strains were stored at -85°C in their corresponding isolation medium, containing 25% (v/v) of glycerol as a cryoprotectant. LAB strains were propagated twice in fresh liquid medium for obtaining fresh culture from frozen stock, before the experiments. LAB strains were grown in MRS-s while screening for EPS production. The glucomannans in the growth medium which could interfere with the EPS screening were removed (Vander Meulen *et al.* 2007). The LAB strains were screened for EPS production by Gel Permeation

Chromatography (GPC), using a Jasco HPLC System (Jasco Europe, Cremella, Italy), equipped with an Ultra-hydrogel Linear column (Waters Corp., Milford, Mass., USA), kept at 35°C, and coupled to RI-2031 refractive index detector (Jasco). Samples were prepared (Vander Meulen *et al.* 2007) prior to the injection on the GPC column. The EPS were eluted with 0.1M $NaNO_3$ at a flow rate of 0.6ml/l. Dextran standards with molecular masses ranging from 80 kDa to 1.4 MDa (Sigma-Aldrich, Switzerland) were used to calculate the molecular mass of the purified EPS.

2.2 Taxonomic identification of EPS-producing LAB strains

The taxonomic affiliation of the EPS-producing LAB strains was determined on the basis of their 16S rRNA sequence. PCR amplification of 16S rRNA gene and purification were performed (Stancu 2012). Sequencing of amplification products was performed by biotechnology division of university. DNA sequencing runs were assembled using the Bio-Edit software. The sequences were compared to those from databases using the BLAST search program.

2.3 Isolation of EPS and quantification

EPS were isolated from GPC positive LAB strains according to a two-step precipitation protocol (DeVusy *et al.* 1998). The LAB strains were cultivated in filtered MRS-s for 12 h, with no pH control or agitation. Total EPS yields was determined gravimetrically by measuring the polymer dry mass (PDM) after 48 h of drying at 42°C. The further purification of the EPS was done by ultrafiltration using a Vivaspin 6 ultrafiltration module with a 10-kDa MM cut-off (Sartorius Stedim Biotech GmbH, Goettingen, Germany).The retentate obtained after two centrifugation steps was adjusted to 2 ml with ultra pure water and used for further analysis.

2.4 Monomer analysis

The purified EPS were hydrolyzed for 6 h at 100°C with 8N HCl, evaporated in an Eppendorf AG centrifugal concentrator (Eppendorf, Hamburg, Germany) and re-suspended in ultrapure water. Monosaccharide composition of EPS was determined by automated thin-layer chromatography (TLC) (CAMAG, Muttenz, Germany) using the ascending technique with silica gel 60 F254 precoated glass sheets (Merck, Damstadt, Germany). The sugars were eluted with a mixture of 1-butanol/acetic acid/water, 6/1/2 (v/v) and the bands were visualized by spraying with p-aminobenzoic acid (Wall, 2005). Glucose, galactose, rhamnose, manose, ribose, xylose (Fluka, Sigma-Aldrich, Switzerland), fructose (Merck KGaA, Darmstadt,

Germany), arabinose (Veb Berlin Chemie, Germany), glucosamine and galactosamine (both from Calbiochem, Inc. San Diego, Calif., USA) were used as standards. In another option, HPLC was used to determine the sugar composition of the hydrolyzed EPS. A Jasco HPLC system (Jasco Europe, Cremella, Italy), equipped with a Carbo Sep Coregel 87Pcolumn (Teknokroma, Spain), kept at 85 °C, and coupled with a RI-2031 refractive index detector (Jasco) was used for separation. Elution was performed with MilliQ water, at a flowrate of 1 ml/min. Arabinose, fructose, galactose, glucose, maltose, mannose, rhamnose, ribose, sorbose, sucrose, and xylose at a concentration of 0.1 mg/ml were used as standards.

3. Results and Discussion:

In addition to plant products, indigenous dairy based products like kradi cheese play an important role in the cooking habit of people of jammu kashmir and are still unexplored food ecological place. These indigenous dairy products of jammu kashmir are known for their beneficial effect on humans, due to presence of probiotic lactic acid bacteria. The literature data available regarding microbial content of kradi cheese is limited (Punoo *et al.* 2018).

Kradi cheese is produced by coagulation of mixture of part of naturally fermented raw buttermilk and milk. The fermented raw buttermilk used in kradi cheese production is the source of LAB. In the present study LAB strains were isolated from kradi cheese and screened for EPS production. EPS were isolated, purified and monomer analysis was done.

3.1 Screening and Identification of LAB strains for Exopolysaccharide production

Nine lactic acid bacterial strains having exopolysaccharide production potential were screened by gel permeation chromatography. The results showing less number of EPS positive strains agreed with others (Grosu-tudor and Zamfir 2013a).

The nine EPS producing strains determined by the 16S rRNA gene sequencing comprised four strains of *Lactococcus lactis* subsp. *lactis* and five strain*s* of *lactococcus lactis* subsp. *cremoris* (Table1). In fermented dairy products Leuconostoc strains had been tested for EPS production at high occurrence (Grosu-tudor *et al.* 2013b).

Table 1 Taxonomic association of the EPS producing LAB strains

S. No	Strain name	Species
1	Q 14	*L. lactis* subsp. *lactis*
2	Q 20	*L. lactis* subsp. *lactis*
3	G 50	*L. lactis* subsp. *lactis*
4	C 59	*L. lactis* subsp. *lactis*
5	F 16	*L. lactis* subsp. *cremoris*
6	H 17	*L. lactis* subsp. *cremoris*
7	H 21	*L. lactis* subsp. *cremoris*
8	H 41	*L. lactis* subsp. *cremoris*
9	H 61	*L. lactis* subsp. *cremoris*

3.2 Isolation and characterization of Exopolysaccharides

Exopolysaccharides were isolated in various amounts from all the Gel Permeation chromatography positive strains. The EPS yield was high in case of *Lactococcus lactis* subsp. *lactis* Q 20 strain about 18.6 g/l. The EPS yield in case of *Lactococcus lactis* subsp. *lactis* G 50 strain was 9 g/l which was similar with the two strains of *lactococcuc lactis* subsp. *cremoris* H 17 and H 21 (Table 2). EPS yield in case of *L. lactis* subsp. *lactis* Q 14 was 4.2 g/l. The EPS yield in case of *L. lactis* subsp. *lactis* strain C 59 was 2 g/l which was similar with the two strains of *lactococcus lactis* subsp. *cremoris* F 16 and H 4. Ullrich 2009 has reported EPS yield of about 50 g/l for *Leuconostoc* species. Grosu-Tudor and Zamfir 2013 has reported EPS yield of about 10 g/l by LAB obtained from fermented milk. All the EPS eluted revealed in Gel permeation chrmatograpghy chromatogram molecular mass of higher than 1.5 MDa. Grosu-Tudor and Zamfir 2013 have reported high molecular mass of EPS produced by LAB strains from fermented milk (Grosu-Tudor *et al.* 2013). Ruas-Madiedo *et al* 2002 reported that estimation of the molecular mass of exopolysaccharides is essential for their characterization which affects rheological properties.

Table 2 EPS producing strains and EPS yield isolated from MRS-s cultures

Species	Strain name	EPS Yield (g/l)	Molecular mass	Monomer composition
L. lactis subsp. *lactis*	Q 14	4.2± 0.7	>1.5 MDa	Glucose
L. lactis subsp. *lactis*	Q 20	18.6 ±0.2		
L. lactis subsp. *lactis*	G 50	9.0 ± 0.0		
L. lactis subsp. *lactis*	C 59	2.0 ± 0.0		
L. lactis subsp. *cremoris*	F 16	2.0 ± 0.0		
L. lactis subsp. *cremoris*	H 17	9.0 ± 0.0		
L. lactis subsp. *cremoris*	H 21	9.0 ± 0.0		
L. lactis subsp. *cremoris*	H 41	2.0 ± 0.0		
L. lactis subsp. *cremoris*	H 61	1.8 ± 0.1		

By thin layer chromatography several bands of isolated monomers of EPS were observed of hydrolyzed samples. From TLC bands glucose (Rf = 0.277) was major band whereas other bands could not confirm for monosaccharide or oligosaccharides. The presence of glucose monomer was confirmed by HPLC analysis of EPS isolated from *L. lactis* subsp. *lactis* Q 20.

4. Conclusion:

This study reveals that kradi cheese manufactured from coagulation of naturally fermented raw milk contains LAB strains with exopolysaccharide production potential. These isolated exopolysaccharides can be used as viscosifiers, emulsifiers, stabilizing agents with application in improvement in rheological and texture of dairy products. These isolated exopolysaccharides can have biotechnological applications. The presence of LAB strains with exopolysaccharide production potential in kradi cheese promotes kradi cheese as functional food with medicinal properties related to exopolysaccharides.

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