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Partial characterization of exopolysaccharides produced by *Leuconostoc* sp. Isolated from intestine of Animals

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Abstract

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) have gained increasing attention over the last few years because of their contribution to the rheology and texture of food products. For this purpose, a total of twelve LAB strains were isolated from intestinal content of coq and shrimp. Using a red ruthenium containing milk agar, four exopolysaccharides producing strains were selected and identified as *Leuconostoc* sp. on the basis of morphological characteristics and molecular identification using a genus specific primer which amplified an DNA fragment of 1200 pb. For EPS production and preparation, a selective sucrose media was used and show a highly viscous growth. Other work including the precipitation, hydrolysis of EPS and estimation of a total carbohydrate using a phenol sulfuric method were determined. The EPS production was varied from 654mg/l to 1254mg/l for the highest production for LGM 14 strain. Componential analysis of hydrolyzed EPS by thin layer chromatography indicated that it is a dextran, consisting of glucose monomer. The isolated strains of *Leuconostoc* sp. are a potent producer of dextran, which find its applications in various industries; it can be used as thickening or gelling agent in food.

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Introduction

Lactic acid bacteria produce a wide variety of food grade exopolysaccharides. Their synthesis has been mainly studied in *Leuconostoc* (Majumder *et al.*, 2009; Purama *et al.*, 2009). *Leuconostoc* species are the predominant group of exopolysaccharide producing mesophilic LAB; they are also exploited commercially for the production of dextran, mutan and alternan from sucrose metabolism (Park *et al* 2013).

One of the most important source of polymeric materials are microbial polymers which have great potentials for use in many applications include food, pharmaceutical, agricultural, industries and in medicine. Dextran is an example of these biopolymers which is homopolysaccharide chain linked by α-(1-6) glycosidic bonds with different amount of branched linkages such as α-(1-2), α-(1-3) and α-(1-4) (Galle *et al.*, 2010,Tsuchiya *et al.* 1952; Santos *et al.* 2000), mostly produce by *Leuconostoc* spp. especially *Leuconostoc mesenteroides* (Onilude *et al.*, 2013).

Besides having potential therapeutic activity, this polymer have received a special attention as valuable compounds because of their potential applications as viscosifying, stabilizing, emulsifying, sweetening, gelling and water-binding agent in the food as well as in the nonfood industries (Monsan *et al.*, 2001), increasing the possibility to replace or reduce the use of external hydrocolloids (Giraffa, 2004; Tieking *et al.*, 2005a; Leemhuis *et al.*, 2013a).

Most of the polysaccharides used in various industries have been derived from plants and seaweeds. With the advancement in fermentation technology, various EPSs of bacterial origin are now produced on commercial scales to replace the synthetic polysaccharides.

The aim of this study was to isolate *Leuconostoc* strains produces dextran from different intestine's animal, in order to evaluate their capacity to produce these biopolymers and select the most performing strains, to determine a monosaccharide composition of this exopolysaccharide and to characterize the isolates using the genus specific PCR approach.

Material and methods

Sampling and growth conditions of isolates

Several samples of intestinal contents of two native's cocks and shrimp was collected from various parts of west Algeria were used for the isolation of EPS producing lactic cultures. Fresh intestinal contents weighing 1g, obtained after dissection of each digestive tract was collected and homogenized with 0.9% NaCl in sterilized deionized water.

Suitable dilutions of 100 µl were spread in modified M-MRS Agar (De Man Rogosa and Sharp) containing sucrose (5%, w/v) adjusted to pH 6.5 and incubated at 30°C in anaerobic conditions for 48h to 72h. Plates that had mucoid colonies were picked up and they were purified by following the streaking method. Selected strains were stored at -80°C in MRS broth supplemented with 20% glycerol until further study.

Genomic DNA extraction of selected bacterial strains

The genomic DNA of selected bacteria was extracted using the modified method of Gevers *et al.* (2001) cells from one plate were harvested into 1 ml of sterile distilled water, centrifuged and frozen for at least 1h at -20°C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50mm Tris-HCl, pH 08.0, 1mm EDTA) and re-suspended in 300µl STET buffer (8% sucrose, 5% Triton X-100, 50mm Tris-HCl, pH 8.0, 50mm EDTA). Lysis buffer 75µl (TES containing 40mg/ml lysozyme) was added and the suspension was incubated at 37°C. After addition of 40µl SDS 20% in TE buffer (10 mm Tris- HCl, 1 mm EDTA, pH 8.0), cells were vortexed for 60 s and incubated at 37°C for 10 min, followed by 10min incubation at 65°C. TE buffer 100µl was added and the lysate was extracted with 1v phenol/chloroform/ isoamylalcohol.

The phases were separated by centrifugation (12,000xg, 10min). The aqueous phase was carefully mixed with 70µl 5M NaCl and 1ml isopropanol, and DNA was precipitated on ice for at least 15min. DNA was collected by centrifugation (12,000xg, 10min) and the pellet washed in cold 70% ethanol. The pellet was resuspended in 250µl of distilled water. The DNA was verified by a 1% agarose gel electrophoresis in TBE.

Genus-specific PCR

The specific primers designed from 16S rRNA gene sequences: Leucgrp F (5'-GCG GCT GCG GCG TCA CCT AG-3') and Leucg R (5'-GGN TAC CTT GTTACG ACT TC-3') was used for characterization of the *Leuconostoc* genera (Schillinger U. 2008). The amplification was carried out in a total volume of 25µL with 100ng chromosomal DNA, 200mm dNTPs, 0.5mm of each primer, 1U Taq DNA polymerase (TaKaRa biotechnology BIO INC. Japan) and 2.5µl 10x PCR buffer. PCR was performed on a PCR TECHNE TC-312 (Barloworld Scientific Ltd, stone ST 15 OSA, UK), programmed as follows: one cycle of denaturation for 2min at 94°C and 33 cycles of denaturation at 94°C for 1min, primer annealing at 53°C and extension at 72°C for 1.5min. 5µl of the PCR products were examined in a 1.2% agarose gel at 100V using 200 pb DNA Smart Ladder (Eurogentec).

The expected size of the amplicon was 1200 pb. Gels were stained with ethidium bromide, visualized under UV light and photographed.

Screening of exopolysaccharides producing lactic acid bacteria

To distinguish between EPS-producing and non-producing cells, milk agar medium containing 0.08% ruthenium red was used. One hundred microliter of each dilution was then spread on ruthenium red containing milk agar.

After incubation at 30°C for 24–48 h, EPS-producing colonies were selected based on their ropy appearance by touching them with a sterile inoculation loop and their ability to resist the penetration of ruthenium red, which appeared as white colonies on pink background of the milk-RR agar plate (Stingle *et al.* 1996).

Exopolysaccharides production and preparation

For the production of dextran, a white mucoid colony was inoculated in a broth medium containing: (g l–1): sucrose, 100.0; yeast extract 2.5; K2HPO4 5; MgSO4 0.2; NH4SO4 0.2; NaCl 0.6; the pH was adjusted at 7.0 and autoclaved at 121°C for 15 minutes. Inoculated broth was incubated for 24 hrs. at 25°C and streaked on the agar plates containing the above medium to isolate pure culture (Gordana R. Dimić, 2006). The colonies were showing highly viscous slimy growth on sucrose agar plate.

Exopolysaccharides were extracted by the modified method of Evans and Linker (1973). The bacterial culture on agar plate was scraped into 25ml of sterilized water and stirred to get a uniform mixture. The suspension was centrifuged at 10.000g for 30 minutes, the pellet was discarded. The EPS was then precipitated from supernatant by addition of three volume of ethanol 95% and left over night at 4°C before centrifuged. The precipitate was collected in petri plates and dried at 70°C to remove ethanol vapors. Dried pellet was lyophilized with (CHRIST, ALPHA 1-2 MODEL, Germany) and used as sample.

Total carbohydrates assay

Carbohydrates in crude EPS were determined by the phenol-sulfuric acid method according to Dubois *et al.* (1956), using D-glucose as standard. For the assay, 1ml of 5% (w/v) phenol solution and 5ml of H2SO4 96% were added to 1ml of an appropriate dilution of the EPS. Samples were incubated at room temperature for 20 min. The absorbance of each sample was spectrophoto metrically determined at 490 nm. Total carbohydrates content was expressed as mg per ml of substrate.

Exopolysaccharides hydrolysis and thin layer chromatography analysis of the monosaccharide

After acid hydrolysis of lyophilized EPS (20mg) at 100°C for 4h with 2N of sulfuric acid (Graber and coll, 1988), the determination of monosaccharide component was performed on TLC silica gel 60 F254 plates (Merck, Darmstadt, Germany) with chloroform: acetic acid: water (30:35:5) as a developing solvent system (Souly F., 1978). After the development process was performed twice, the developed spots were visualized by spraying the plates with potassium permanganate/sodium carbonate (V: V) solution following heating of the plates at 120°C. The Rf values of spot were measured and compared with those reported for some available standard sugar (glucose, galactose and Raffinose).

Results and discussion

Phenotypic characteristic of isolates

A total of 12 strains were obtained from the isolation performed at anaerobic condition on M-MRS at 30°C for 48h. A selected strains were presumptively identified in LAB *cocci* group since they displayed the following properties: Gram-positive, catalase negative, *cocci* or ovoid cells occurring in short and medium chains (Fig. 1) able to grow at 6,5% NaCl and to produce mucoid colonies .

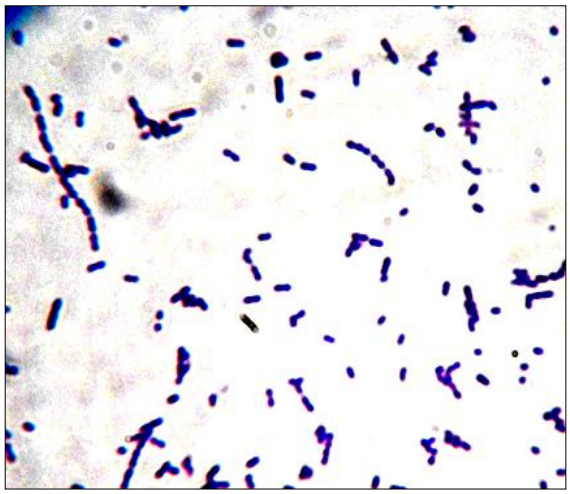

Fig. 1. Microscopic cell morphology of wild *Leuconostoc* LGM 14 (×1000).

Identification of amplified DNA genus products (Genus-specific PCR)

The specificity of the genus was determined by testing all isolates presumptively identified as LAB. Only 04 strains were amplified with the *Leuconostoc* genus primers, producing a fragment of 1200 pb (Fig. 2 and 3), indicating that they members of *Leuconostoc* sp.

The presence of any significant band was observed for the strain *Lactococcus lactis* IL1403 used as negative control. Previously, Ulrich Schillinger *et al*. (2008) reported that a total of 74 of 106 strains generated an amplification product of about 1200 bp with the Leucgrp primer, indicating that these belong to the genus *Leuconostoc*.

In this study, all of the four *Leuconostoc* isolates could be correctly classified as *Leuconostoc* sp. with the specific primer designed for this genus.

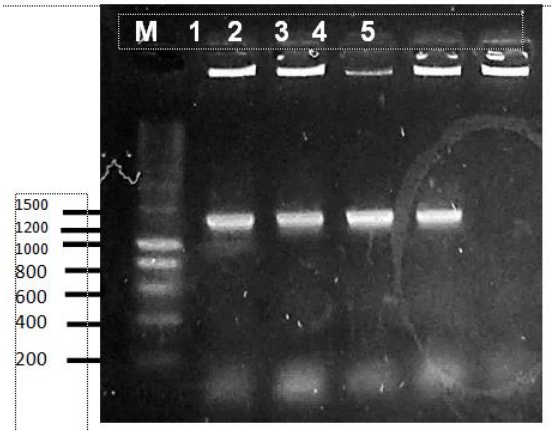

Fig. 2. Gel electrophoresis of Genus-specific PCR products.

Lane 1-4: LGM's strains LGM4, LGM5, LGM14, LGM15.

Lane 5: *Lactococcus lactis* IL1403 as negative control.

Lane M: Smart Ladder 200 bp DNA.

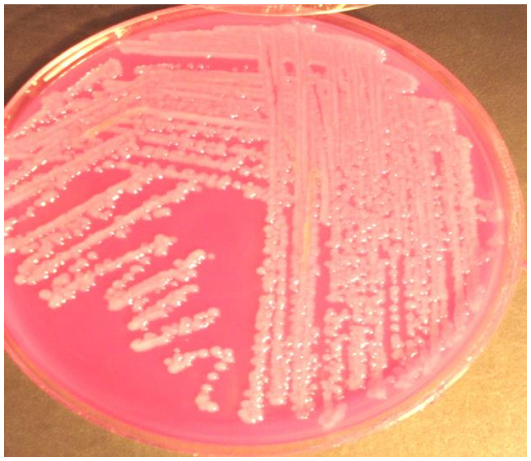

Fig. 3. Ruthenium red plate assay showing white colonies of LGM 14.

Screening of EPS producing LAB

Regarding screening procedures on agar plates, slimy appearance and white colony on milk contained ruthenium (RR) were used to detect EPSs production. A total of four white colonies of LAB (Fig. 4.), designed as LGM4, LGM5, LGM14 and LGM15 were isolated and selected for further studying of dextran production and characterization. It was reported that *Bouzar et al.* (1995) used the same technical for selection of Lb. *delbruckii* spp. *Bulgaricus* producing different exopolysaccharides. Furthermore, Stingle *et al.* (1996) were selected an exopolysaccharide producing strains *Streptococcus thermophilus* Sfi6 using a same protocol.

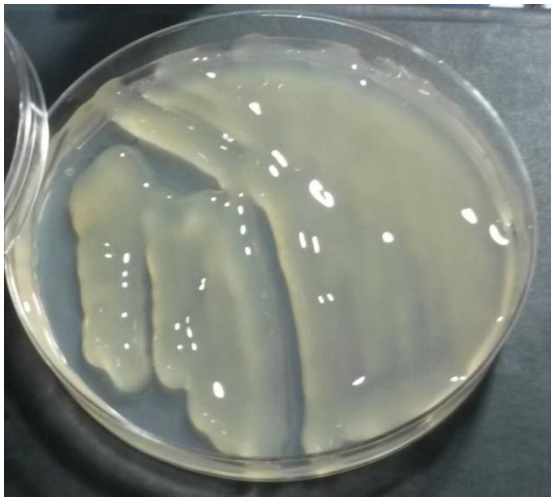

Fig. 4. EPS production by LGM14 on sucrose plate.

Production of EPS

All four *Leuconostoc* strains LGM4, LGM5, LGM14 and LGM15 were able to produce shiny, mucoid or viscous colonies on sucrose agar (Fig. 5.). The presence of mucoid colony is indicative of exopolysaccharide production potential (Paulo *et al.*, 2012b), also Torres-Rodríguez *et al.* (2014) used the same method for EPS-producing colonies detection by visual analysis to recognized between compact colony (not EPS producer) and creamy colony (EPS producer), while Rühmann *et al.* (2015) used visual analysis (mocoidy) of colonies as method of choice for detected EPS producing microbes.

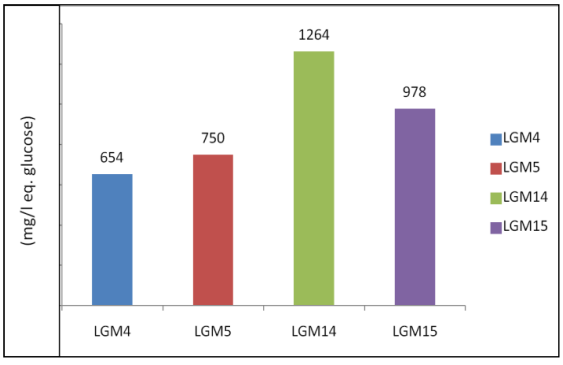

Fig. 5. Total carbohydrates concentration of EPS produced by various LGM's strains.

Total carbohydrate estimation

The carbohydrates concentrations of EPS were between 1264 mg/l for strain LGM 14 and 654mg/l for strain LGM4, as shown in table 1. Polak-Berecka *et al.* (2014) optimized medium for produced 210.28mg/L of EPS from *Lactobacillus rhamnosus* at optimum conditions while Sanhueza *et al.* (2015) reported that the amount of EPS produced by *Lactobacillus salivarius* was 450mg/L. Furthermore, Cerning *et al.* (1992) reported that the amount of EPS produced by *L. lactis* sub sp. *Cremoris* was between 80 and 600mg/L. Also, Joshi and Koijam (2014) estimated 340.82mg/L of EPS produced by *Leuconostoc lactis* isolated from fermented beverage. Our results show interesting strain LGM 14 identified as *Leuconostoc* sp. which yields of dextran production is 1264 mg/l (Fig. 6.). Moreover Yang *et al.* (2015) mentioned that the amount of dextran produced by *Leuconostoc citreum* which isolated from sauerkraut was 2300 mg/l.

Table 1. total carbohydrates concentrations.

Strains	LGM 4	LGM 5	LGM 14	LGM 15
Total carbohydrates (mg/l)	625	750	1264	978

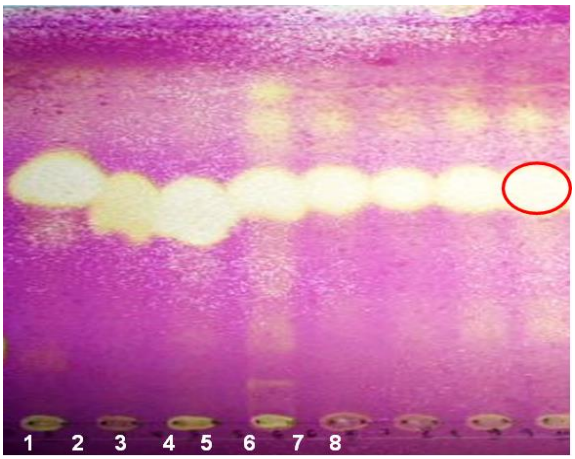

Fig. 6. TLC analysis of the monosaccharide composition of hydrolyzed EPS produced by LGM's strains.
Lane 1: Raffinose, lane 2: Glucose, lane 3: Galactose. Lane 4, 5, 6, 7, 8: hydrolyzed EPS4, EPS5, EPS14, EPS 15 and commercial Dextran.

Monosaccharides composition by TLC

The TLC analysis of the monosaccharides obtained from acid hydrolysis of four polysaccharides is shown in Fig. 5. A major apparent spot were found on TLC (Rf = 0.545) by all tested EPS, indicating the identical Rf value to glucose (Rf =0.548). It seems that the EPS from LGM'S *Leuconostoc* may be a dextran comprising the glucose monomer only.

Previously, it was reported that the EPS synthesized by a *Leuconostoc* strain were identified as dextran having only a single constituent glucose (Santos, Teixeira, & Rodrigues, 2000).

Conclusion

View the results obtained in the context of our work, we can conclude that the dextran produced by the LGM's *Leuconostoc* sp. has remarkable potential to be used for several industrial purposes and these strains can be used as natural, safe additive to enhance the rheology and food texture of novel food product. The present study further suggests the characterization of dextran including structure and branch linkage as well as investigation of their biological properties.

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