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Short Communication

Highly efficient synthesis of exopolysaccharides by *Lactobacillus curvatus* DPPMA10 during growth in hydrolyzed wheat flour agar

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ABSTRACT

The aim of this study was to optimize the production of exopolysaccharides (EPS) by sourdough *Lactobacillus curvatus* DPPMA10 for industrial application. The effects of pH, temperature, planktonic or attached cells and of some food matrices as substrates were studied. Wheat flour hydrolysate (WFH), reconstituted skimmed milk (RSM) and whey milk were supplemented with fresh yeast extract, mineral salts, and/or molasses. Non-controlled pH, starting from 5.6 to 3.5, was the optimal condition for *L. curvatus* DPPMA10. Temperature of 30 °C was also found to be optimal. Solid surfaces (agar culture media) stimulated attached bacteria to synthesize EPS (≥ of two-fold, $P<0.05$) with respect to planktonic cells (broth media). The highest production of EPS (ca. 46–50 g/kg of wet medium) was found during growth as attached cells in WFH agar supplemented with glucose, sucrose or molasses, mineral salts and fresh yeast extract at 30 °C for 48 h. As shown by high-performance liquid chromatography analysis, glucose was the only hydrolysis end-product for EPS synthesized during 48 h of incubation. The EPS synthesized by *L. curvatus* DPPMA10 improved the quality of bread and was utilized as carbon course by intestinal strains of lactobacilli and bifidobacteria. The synthesis of EPS by *L. curvatus* DPPMA10 under the conditions of this study may open new perspectives for their industrial applications.

1. Introduction

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) have positive repercussions on texture, mouth feel, taste perception and stability of fermented foods (Jolly et al., 2002; Tieking and Gänzle, 2005). Prebiotic effects of some EPS have been also proved (German et al., 1999). Based on their composition, EPS are classified in homo- and hetero-polysaccharides. Homo-polysaccharides consist of three or more units of the same monosaccharide, whereas hetero-polysaccharides consist of several different monosaccharides (Sutherland, 1998). Homo-polysaccharides are either glucan (e.g.: dextran, reuteran, and mutan) or fructan (e.g.: levan and inulin) and are usually synthesized by extracellular enzymes (glycosyltransferase) using sucrose as the glycosyl donor. Fructans may be synthesized by some sourdough strains of *Lactobacillus sanfranciscensis* (levan) and of *Lactobacillus reuteri* (inulin or levan). *L. reuteri* strains may produce dextran or reuteran (Tieking and Gänzle, 2005; Korakli and Vogel, 2006). Other sourdough-related LAB are also capable to synthesize homopolysaccharides (Tieking and Gänzle, 2005; Di Cagno et al., 2006; Bounaix et al., 2009; Katina et al., 2009). Glycosyltransferase of lactobacilli accumulate up to 40 g/l of EPS (Korakli et al., 2003), but few studies aimed to enhance the amount of EPS synthesized by sourdough LAB (Kaditzky and Vogel, 2008). Besides, the use of food matrices, such as by-products or surplus from beverages and food processing, for producing microbial EPS could represent an interesting economic perspective for industry.

The aims of this study were: (i) to enhance the amount of EPS synthesized by sourdough *Lactobacillus curvatus* DPPMA10, using different conditions of pH, temperature and type of growth (planktonic or attached cells); (ii) to test low-cost food matrices as potential fermentation substrates for enhanced yields of EPS; and (iii) functional characterization of the EPS produced.

2. Materials and methods

2.1. Strains and growth conditions

L. curvatus DPPMA10 used in this study had been previously isolated from sourdough. The strain was routinely cultivated at 30 °C for 24 h in modified MRS (mMRS) broth (Oxoid, Basingstoke, Hampshire, England). Modifications consisted in the addition of fresh yeast extract (5%, v/v) and maltose (28 mM). The final pH of mMRS was 5.6. Fresh yeast extract was prepared as follows. Sixty grams of commercial baker's yeast (Lesaffre Italia s.p.a., Trecasali, Parma, Italy) was suspended in ca. 300 ml of distilled water, sterilized

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in autoclave and centrifuged at 6000×*g* for 10 min at 4 °C to recover the supernatant, mainly containing the cytoplasm extract of baker's yeast. Nine intestinal strains (*Lactobacillus plantarum* DPPMA6, *L. plantarum* 4.1, *Lactobacillus bulgaricus* DPPMA01, *Lactobacillus casei* DPPMA12, *Lactobacillus acidophilus* DPPMA13, *Lactobacillus reuteri* 3S7, *Bifidobacterium infantis* DPPMA14, *Bifidobacterium brevis* DPPMA15 and *Bifidobacterium longum* DPPMA16) and three pathogens (*Escherichia coli* ED36, *Clostridium perfringens* Type A strain 22G and *Salmonella typhimurium* ATCC 6994), were also used. Intestinal lactobacilli and bifidobacteria were routinely cultivated at 37 °C for 24 h in MRS broth, under anaerobic conditions. *S. typhimurium* ATCC 6994 and *E. coli* ED36, and *C. perfringens* Type A strain 22G were cultivated in Lactose broth (Oxoid) and fluid thioglycollate broth (Difco Laboratories, Detroit, MI, USA), respectively, under the same conditions as the lactobacilli and bifidobacteria.

2.2. Effects of pH, temperature and planktonic or attached cells

Preliminarily, 24-hour-old cultures of *L. curvatus* DPPMA10, having a cell density of ca. 9.0 log CFU/ml, were inoculated at 4% (v/v) in MRS broth (pH 5.6) (De Man et al., 1960) supplemented with sucrose at different concentrations (from 2 to 16.0%, w/w). After 48 h of incubation at 30 °C, EPS were determined as described below. The same experiment was carried out inoculating *L. curvatus* DPPMA10 in medium 1 (M1 broth). M1 was a laboratory-made MRS broth (pH 5.6) with the addition of 5.0% (v/w) fresh yeast extract, 10.0% (w/w) sucrose and 2.0% (w/w) glucose (Table 1) (Di Cagno et al., 2006).

To determine the synthesis of EPS by planktonic cells, fermentations were carried out in a 2 l bioreactor (Biocontroller ADI 1030, Applikon Dependable Instruments, Genova, Italy), filled up with 1.3 l of M1 broth. At 30 °C, the following values of initial pH were assayed: non-controlled pH 5.6 and constant pH 6.2, 5.6, 4.8 or 3.8. Under non-controlled pH 5.6, the following temperatures were assayed: 25, 30, 37 or 42 °C. Incubation was allowed for 48 h. Samples were collected aseptically every 24 h.

To determine the synthesis of EPS by attached cells, M1 agar at initial pH 4.8, 5.6 or 6.2 was also made. Eight equidistant stab-streaks were made into the agar for each plate (140 mm Petri Dish, Sterilin Limited, Bargoed, UK) containing 130 ml of medium. Inoculated plates were incubated for 48 h at the same temperatures as M1 broth.

2.3. Food matrices as substrates

Eleven different agar media (M2–M12) made of several food matrices were screened (Table 1). Briefly, media were prepared according to the following formulas. Wheat flour hydrolysate (WFH) was prepared as described elsewhere (Gobbetti, 1998), added of sucrose (10.0%, w/w), glucose (2.0%, w/w) and mineral salts at the same concentration (w/w) as those contained in the MRS formula, and sterilized by filtration on 0.22 μm membrane filters. Separately, the agar suspension (4.5%, w/v) was sterilized at 121 °C for 15 min. For plating, 500 ml of WFH and 250 ml of agar suspension were held in a water bath at 50 °C, mixed together and WFH agar (Medium 2, M2) was quickly poured into plates. Medium 3 (M3) was prepared based on the formula of M2 without mineral salts and using fresh yeast extract instead of water in the agar solution. Medium 4 (M4) differed from M2 only for using fresh yeast extract instead of water. Medium 5 (M5) was prepared based on the formula of M4 with the addition of meat and yeast extracts (Oxoid). Medium 6 (M6) was made of sterile reconstituted skimmed milk (RSM) (Oxoid) added of sucrose, glucose and with a final pH of 5.6. M6 was sterilized at 121 °C for 15 min. Medium 7 (M7) was prepared based on the formula of M6 with the addition of MRS mineral salts and fresh yeast extract. Medium 8 (M8) was made of whey milk obtained after acid coagulation of pasteurized milk (Granarolo spa, Bologna, Italy). The main composition of the whey milk was the following: lactose 4.8% (w/v), protein (0.8%, w/v) and fat (0.4%, w/v). It was sterilized at 120 °C for 15 min. Medium 9 (M9) was prepared based on the formula of M8 with the addition of MRS mineral salts and fresh yeast extract. Media 10 and 11 (M10 and M11) were prepared based on the formula of M1 without sucrose (M10 and M11) and glucose (M11), but with the addition of molasses (Somona GmbH, Dulliken, Switzerland) (sucrose concentration: 51% w/w). Medium 12 (M12) was prepared based on the formula of M4 with molasses instead of sucrose.

All agar media were inoculated by stabbing into plates containing 130 ml of medium, as described above. The plates were incubated at 30 °C for 48 h. Furthermore, a small aliquot of a 24-hour culture of *L. curvatus* DPPMA10 was streaked on each plate and incubated at 30 °C for 48 h for glycansucrase activity measurements.

At the beginning, after 24 and 48 h of incubation, inoculated agar media were diluted with 260 ml of sodium citrate (2% w/v) solution

Table 1
Culture media, cellular density and synthesis[a] of exopolysaccharides (EPS) by *Lactobacillus curvatus* DPPMA10 after 48 h at 30 °C.

Culture medium code	Basic medium	Carbohydrates added (%, w/w)		Additional ingredients	Cellular density (log CFU/g)	EPS (g/kg)
		Glucose	Sucrose			
M1 broth	MRS	2	10	Fresh yeast extract (5.0%, w/w)	9.33 ± 0.11	7.81 ± 1.84
M1 agar	MRS agar	2	10	Fresh yeast extract (5.0%, w/w)	9.37 ± 0.08	19.77 ± 0.14
M2	WFH[b] agar	2	10	Mineral salts[c]	9.19 ± 0.05	21.40 ± 1.05
M3	WFH agar	2	10	Fresh yeast extract (33.3%, w/w)	9.25 ± 0.07	29.23 ± 1.03
M4	WFH agar	2	10	Mineral salts and fresh yeast extract (33.3%, w/w)	9.16 ± 0.05	50.25 ± 1.85
M5	WFH agar	2	10	Mineral salts, meat (0.1%, w/w) and yeasts extracts (0.5%, w/w) and Fresh yeast extract (33.3%, w/w)	9.30 ± 0.02	47.81 ± 1.27
M6	RSM[d] agar	2	10	–	9.13 ± 0.08	n.d.[e]
M7	RSM agar	2	10	Mineral salts and fresh yeast extract (33.3%, w/w)	9.24 ± 0.04	n.d.
M8	Whey milk agar	2	10	–	9.08 ± 0.51	n.d.
M9	Whey milk agar	2	10	Mineral salts and fresh yeast extract (33.3%, w/w)	9.14 ± 0.25	0.66 ± 0.03
M10	MRS agar	2	–	Fresh yeast extract (33.3%, w/w) and molasses (10%, w/w)	9.25 ± 0.18	18.87 ± 0.45
M11	MRS agar	–	–	Fresh yeast extract (33.3%, w/w) and molasses (10%, w/w)	9.27 ± 0.15	30.03 ± 1.02
M12	WFH agar	2	–	Mineral salts, fresh yeast extract (33.3%, w/w) and molasses (10%, w/w)	9.13 ± 0.08	46.90 ± 0.18

[a] Data are means ± standard deviations of three independent experiments.
[b] WFH, wheat flour hydrolysate.
[c] Mineral salts contained in the MRS formula: dipotassium phosphate 2.0 g/kg, sodium acetate 5.0 g/kg, triammonium citrate 2.0 g/kg, magnesium sulphate 0.2 g/kg, and manganese sulphate 0.05 g/kg.
[d] RSM, reconstituted skimmed milk.
[e] n.d., not detectable. For details of the formulas of culture media see also Materials and methods.