

Optimization of Culture Conditions for Exopolysaccharide Production by a Probiotic Strain of *Lactobacillus rhamnosus* E/N

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Abstract

The effects of culture conditions on exopolysaccharides (EPS) production by a probiotic *Lb. rhamnosus* E/N have been studied using the Plackett-Burman design. Process optimization was performed in stationary cultures to maximize the production of EPS. In order to verify the optimal conditions, an analysis was performed of EPS production in fermentation culture. Batch fermentation was carried out at working volume of 2.5 l. The optimal temperature, pH, carbon source, and nitrogen source conditions were 37°C, pH 5.0, galactose, and yeast extract, respectively. EPS production was improved by 210.28 mg/l in stationary cultures compared to 134.2 mg/l in a control grown on commercial MRS medium. The fermentor experiment showed the possibility of increasing EPS biosynthesis by 175.8%. Our results clearly demonstrate that in the case of *Lb. rhamnosus* E/N specific culture conditions can enhance EPS production for possible application in the industry.

Key words: *Lactobacillus rhamnosus*, exopolysaccharide, Plackett-Burman design

Exopolysaccharides (EPS), one of the primary metabolic products of food-grade lactic acid bacteria (LAB) with GRAS (Generally Recognized as Safe) status, are an important source of natural alternatives to commercial additives of plant and animal origin. Because a greater part of those additives are chemically modified to improve their structure and the rheological properties of the product, they are not allowed in most European Union countries (Gibson and Roberfroid, 1995; Tomb and Harding, 1998). By contrast, the use of LAB producing EPS could result in safe, natural products which could be widely applied in the industry. Such products (*e.g.*, fermented milk products) would not only be characterized by improved physical characteristics (*e.g.* stability and mouth-feel) (De Vuyst and Degeest, 1999) but would also have health-promoting properties since EPS produced by LAB have various beneficial physiological effects on humans, such as anti-ulcer action (Nagaoka *et al.*, 1994), modulation of the immune response (Kitazawa *et al.*, 1998), and improvement of intestinal adhesion of probiotics (Ouweland *et al.*, 1999). Unfortunately, so far commercial use of EPS has been limited by low production levels dur-

ing fermentation (Torino *et al.*, 2000). EPS production largely depends on culture conditions (Kimmel and Roberts, 1998; Degeest and De Vuyst, 1999; Grobgen *et al.*, 2000; Macedo *et al.*, 2002). Usually, an excess of carbohydrates in combination with nutrient (*e.g.*, nitrogen) limitation, stimulates EPS production (Sutherland, 1990). The effect of nitrogen on EPS production by LAB has not been examined in detail in papers published to date, but our preliminary studies indicate that limiting of the concentration of a nitrogen source in the growth medium does not decrease EPS yield (data not published). Medium constituents and other critical variables responsible for the production of biomolecules can be optimized using statistical experimental design (Chaari *et al.*, 2012; Dominguez *et al.*, 2012; Raza *et al.*, 2012). Until now, however, there have been no reports on the statistical optimization of culture conditions for the production of EPS by *Lb. rhamnosus* strains.

In the present study, an attempt was made to optimize the production of exopolysaccharides by a probiotic *Lb. rhamnosus* E/N using Plackett-Burman design. Process optimization was performed to maximize the

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Table I
Plackett-Burman design for seven variables

Run	Glucose	Lactose	Galactose	Yeast extract	Pepton K	Meat extract	Temperature	EPS (mg/l)
1	-1	-1	-1	1	1	1	-1	87.33
2	1	-1	-1	-1	-1	1	1	42.90
3	-1	1	-1	-1	1	-1	1	34.32
4	1	1	-1	1	-1	-1	-1	111.32
5	-1	-1	1	1	-1	-1	1	210.28
6	1	-1	1	-1	1	-1	-1	18.80
7	-1	1	1	-1	-1	1	-1	74.88
8	1	1	1	1	1	1	1	237.12
	-1 = 0 g/l 1 = 20 g/l	-1 = 0 g/l 1 = 20 g/l	-1 = 0 g/l 1 = 20 g/l	-1 = 0 g/l 1 = 10 g/l	-1 = 0 g/l 1 = 10 g/l	-1 = 0 g/l 1 = 10 g/l	-1 = 30°C 1 = 37°C	

production of EPS. For verification, an analysis of EPS production in fermentation culture was performed.

Lactobacillus rhamnosus E/N, one of the components of Lakcid[®], a pharmaceutical product containing viable probiotic bacteria, was obtained from Biomed Serum and Vaccine Production Plant Ltd., Lublin, Poland. The strain is deposited at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences under number 2594. The bacteria were stored at -80°C in MRS (de Man *et al.*, 1060) medium supplemented with 20% (v/v) of glycerol and were revitalized for 24 h in MRS broth at 37°C before use.

The influence of the following sources of carbon on the production of EPS by *Lb. rhamnosus* E/N was tested in one-factor-at-a-time experiments: glucose, galactose, fructose, lactose, sucrose, and maltose. All the saccharides were purchased from POCH, Warsaw, Poland. MRS medium containing each carbon source separately at a concentration of 20 g/l and pH 6.3 was distributed into flasks at a volume of 100 ml and sterilized at 121°C for 15 min. The effect of the optimal concentration of a complex nitrogen sources was examined in modified MRS broth in which yeast extract, meat extract, and peptone K (BTL, Poland) were used at different concentrations. The effect of temperature and pH on EPS production was also investigated in bioreactor conditions.

A revitalized culture of *L. rhamnosus* E/N was used to prepare 24-h inocula in MRS medium, pH 6.3 ± 0.1. Each kind of medium in flasks was inoculated with bacteria at a concentration of 2.5% (v/v), and the cultures were incubated at 37 or 30°C for 72 h in relatively anaerobic conditions.

Plackett-Burman design is a very useful tool which makes it possible to screen n variables using only $n+1$ experiments (Macedo *et al.*, 2002). In the present study, it was applied to evaluate the relative importance of various nutrients for biomass production (Ghanem *et al.*, 2000) and to limit their number. The effect of seven variables (nutrients and growth conditions) on the

production of EPS by *Lb. rhamnosus* E/N was studied. Each variable was tested at two levels, namely, a high level denoted by (1) and a low level denoted by (-1) as shown in Table I. All experiments were conducted in triplicate, and the average value of EPS yield was used for statistical analysis.

In order to verify the optimized factors, batch fermentation experiments were performed at 2.5 l working volumes using the optimal culture conditions obtained from the lab-scale tests. *Lb. rhamnosus* E/N was cultivated in a 5-L fermentor (Biostat, Sartorius, Goettingen, Germany). The fermentor was controlled automatically for temperature, at 37°C; pH, at a constant value of 6.3 or 5.0; and stirring, at 25 rpm, to keep the fermentation broth homogeneous. Fermentation was carried out for 72 h.

After incubation, the cultures were heated at 100°C for 15 min, and the cells were removed by centrifugation (4,000 × g, 30 min, rt). To reduce the volumes, the supernatants were evaporated using a vacuum rotary evaporator (Büchi R210: 0.093 MPa, 40°C). EPS were precipitated by adding three volumes of chilled 96% ethanol. The precipitating mixtures were left for 24 h at 4°C. EPS were collected by centrifugation at 20,000 × g for 30 min at 4°C, dissolved in distilled water, and dialyzed (4,000 Da molecular weight cutoff membranes, Roth, Karlsruhe, Germany) against distilled water for 48 h at 4°C. The water was changed twice. Crude freeze-dried EPS were purified by dissolving in 15% trichloroacetic acid (TCA), and the protein precipitates were removed by centrifugation (20,000 × g). Purified EPS were exhaustively dialyzed and, finally, lyophilized. Samples were frozen at -80°C for one hour and then freeze-dried in a freeze-dryer (Labconco, Kansas City, USA) at -50°C and 0.024 mBar for 18 h.

EPS production was determined by measuring protein and carbohydrate concentrations using colorimetric methods. The total sugar content was determined by the method of Dubois *et al.* (1956) with glucose used as

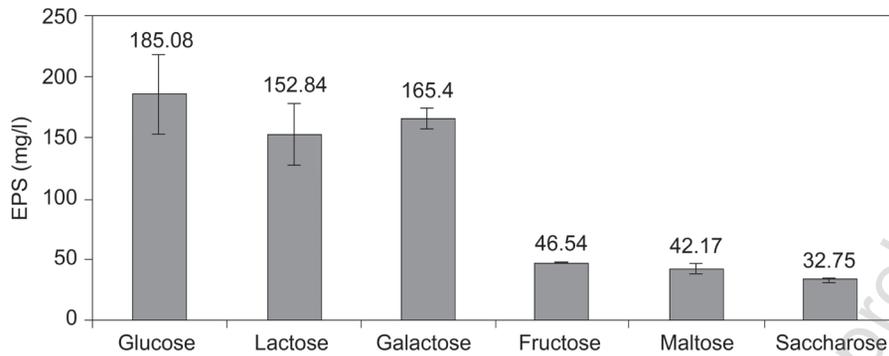


Fig. 1. Effect of various carbon sources at a concentration of 20 g/l on exopolysaccharide production by *Lactobacillus rhamnosus* E/N.

a standard. Protein concentrations were estimated using the Bradford Assay Kit (Bradford, 1976).

In order to exclude contamination of EPS by sugars derived from MRS medium, the same procedure was used to precipitate polysaccharides from an uninoculated medium.

Different carbon sources were evaluated for their effect on EPS production by *Lb. rhamnosus* E/N. It was observed that the type of carbon source had a huge influence on EPS productivity. The results revealed that glucose, followed by lactose and galactose, gave maximum EPS production (Fig. 1). These sugars are used in lactic acid bacteria to form polysaccharides in the cell with use of the “housekeeping enzymes” (Welman and Maddox, 2003). *Lb. rhamnosus* E/N produced three times more EPS with those saccharides than with fructose, maltose, or saccharose as a sugar source. Our findings are consistent with Gamar *et al.* (1997), who have reported that EPS production and yield in *Lb. rhamnosus* cultures were influenced by the type and concentration of the carbon source. Similar conclusions have been reached for other lactobacilli. In agreement with our results, Macedo *et al.* (2002) and Sánchez *et al.* (2006) have found that EPS production

by lactobacilli was higher when glucose was used as a carbon source. Degeest *et al.* (2001), observed that the amount of EPS produced by *Lactobacillus sakei* was the highest for glucose. Also Petry *et al.* (2000) have reported that glucose was a better substrate than lactose for high EPS production by *Lb. delbrueckii* subsp. *bulgaricus*. All these results suggests that regulation of the biosynthetic pathway of EPS may be dependent on the carbohydrate source. On the other hand, however, also galactose has been noted as a preferred carbon source for EPS production (Fukuda *et al.*, 2010). Because there is no consistency in the reported preference for carbohydrates regarding EPS production (Torino *et al.*, 2005), this preference seems to be strain-dependent.

Medium components such as carbon source (chosen in the first screening experiments), nitrogen source, and incubation temperature were further screened by a Plackett-Burman design consisting of eight experiments. The results of the Plackett-Burman design analysis are presented in Table I together with factor effect estimates (Fig. 2). The factors that had a profound influence on EPS production by *Lb. rhamnosus* E/N were galactose, yeast extract, and incubation temperature. It was noted that EPS production increased

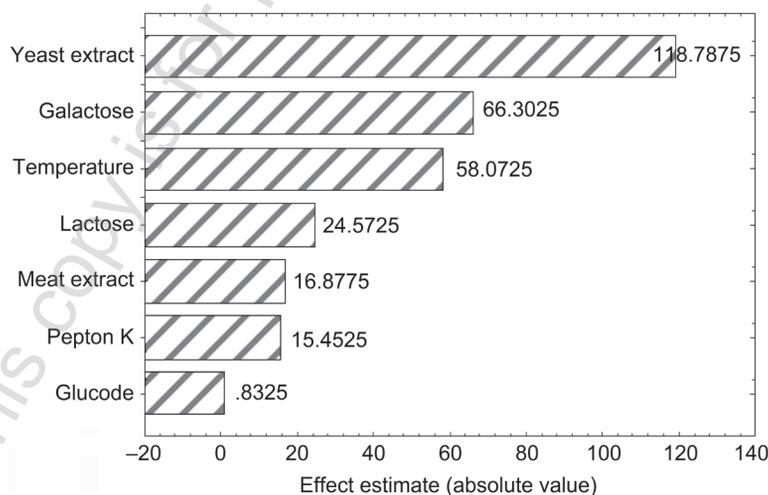


Fig. 2. Pareto chart – a ranking of variables investigated in the Plackett-Burman design.

with an increase in the concentration of galactose and yeast extract. The production level was maximum when both of the components were added at their maximum levels. It has been shown for *Lb. casei* (Cerning *et al.*, 1994) and *Lb. rhamnosus* (Gammar *et al.*, 1997) that the presence of excess sugar in the medium (at concentrations between 10 and 20 g/l) has a stimulating effect on EPS production. Ismail and Nampoothiri (2010) have reported the highest EPS production by probiotic *Lb. plantarum* when the carbon source (lactose) and the nitrogen source (yeast extract) were at their maximum levels. It was shown in our experiments that yeast extract could be used to improve the production of EPS by *Lb. rhamnosus* E/N; this may be explained by the composition of this organic nitrogen source which includes amino acids, peptides, carbohydrates, and salts. On the negative side, yeast extract supplementation may result in contamination of EPS samples after extraction. In our study, however, contamination of EPS with carbohydrates present in yeast extract was excluded, because the EPS yields obtained were corrected by subtraction of the amounts of sugar precipitated from uninoculated MRS medium (data not shown). Because lactobacilli require various vitamins for their growth and the production of EPS (Grobben *et al.*, 1998), yeast extract is advantageous as a source of nitrogen since it also provides vitamins and nucleic acid base components (Amrane and Pringent, 1998). Our results are in agreement with Macedo *et al.* (2002), who have reported that the addition of yeast extract to the production medium increased both cell growth and EPS levels in a *Lb. rhamnosus* culture.

The effect of temperature on EPS production by *Lactobacillus rhamnosus* E/N indicated that the maximum EPS yield 210.37 mg/l was obtained in cultures incubated at 37°C whereas in 30°C EPS yield was 94.66 mg/l. Numerous researchers describe a direct relationship between EPS production and growth temperature in lactic acid bacteria strains (Degeest and De Vuyst, 1999; Aslim *et al.*, 2005; Chen *et al.*, 2006; Kim *et al.*, 2008). Our results are similar to the results of Chen *et al.* (2006) and Kim *et al.* (2008), who have reported the highest production of EPS by *Lactobacillus* strains at 37°C. In other reports, mesophilic bacteria have shown higher EPS production at temperatures equal to or lower than 30°C (Cerning *et al.*, 1994; Degeest *et al.*, 2001). These reports indicate that the optimal temperature for EPS production is dependent on the strain. Differences in optimal carbon source and incubation temperature for EPS yield highlight variations in EPS synthesis and regulation pathways in different lactobacilli. The higher production of EPS by *Lactobacillus rhamnosus* E/N at 37°C may have been caused by the fact that this temperature was most suitable for the enzyme activity and that the metabolism rate of the polysaccharide was faster under those conditions.

Batch fermentation experiments were performed using the optimal culture conditions obtained from the Plackett-Burman design tests. The fermentation medium was modified MRS with galactose (20 g/l) and yeast extract (10 g/l) as the only carbon and nitrogen sources. The culture of *Lactobacillus rhamnosus* E/N was run in a bioreactor at a constant temperature of 37°C. Two pH values were tested: 6.3 and 5.0. Our results show that the maintenance of pH at a constant level is important for EPS production by *L. rhamnosus* E/N. The highest yield of EPS 580.38 mg/l was obtained on the optimal medium when the pH was maintained at the level of 5, in pH 6.3 EPS yield was 485.15 mg/l. These results are in agreement with Kim *et al.* (2008), who have reported profound EPS production in *Lb. rhamnosus* ATCC 9595 at pH 5.5. Constant pH of a bioreactor culture is important as it may affect cell growth, EPS production, and uptake of different nutrients (Raza *et al.*, 2012). The effect of pH on EPS production depends on the experimental conditions and the strains used. Nevertheless, it has been observed that pH-controlled conditions have the advantage of considerably increasing EPS yield (Petry *et al.*, 2000). For instance, Aslim *et al.* (2005) have reported that the amount of EPS was more than three times higher in fermentations with pH control than in those without pH control.

In conclusion, our results show that culture conditions have a clear impact on EPS production by *Lb. rhamnosus* E/N. The optimal temperature, pH, carbon source, and nitrogen source conditions were 37°C, pH 5.0, galactose, and yeast extract, respectively. EPS production was improved by 210.28 mg/l in stationary cultures compared to 134.2 mg/l in a control grown on commercial MRS medium. The scale up from flask to fermentor showed the possibility of increasing EPS biosynthesis by 175.8% in commercial production processes. In batch cultures, EPS yield was 580.38 mg/l, which was a relatively high value compared to those generally described for heteropolysaccharides produced by lactic acid bacteria (Petry *et al.*, 2000; Aslim *et al.*, 2005; Sánchez *et al.*, 2006). So far, only three studies have been published concerning the optimization of EPS from other *Lactobacillus rhamnosus* strains (Gamar *et al.*, 1997; Macedo *et al.*, 2002; Kim *et al.*, 2008). Our results clearly demonstrate that in the case of *Lb. rhamnosus* E/N specific culture conditions can enhance EPS production for possible application in the industry.

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