Statistical optimisation of the exopolysaccharide production by *Lactobacillus fermentum* Lf2 and analysis of its chemical composition

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Running headline: Optimisation of EPS production and chemical analysis

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ABSTRACT

Lactobacillus fermentum Lf2 produces high amounts of exopolysaccharides (EPS) (~1 g/L) with demonstrated functional and technological roles when applied as a food ingredient in dairy matrices, properties that made these EPS interesting in comparison with other similar molecules from lactic acid bacteria (LAB). Those characteristics encouraged us to optimise the production. The EPS extract is composed of a high molecular mass β-glucan and a medium molecular mass heteroglycan. In the present work, the optimal conditions that doubled the EPS yield using a semi-defined medium (SDM, 0.63% yeast nitrogen base, 0.53% bacto casitone, 0.53% ammonium citrate, 6.25% sucrose, pH 6.5) were found by means of response surface methodology (RSM). The chemical characterization indicated that under optimised conditions the synthesis of the heteroglycan was favoured compared with that of the β-glucan.

Keywords: exopolysaccharides; statistical optimisation; chemical composition, *L. fermentum;* response surface methodology.

INTRODUCTION

Lactic acid bacteria (LAB) are generally recognised as safe (GRAS) microorganisms that are widely used in the food industry. Apart from their technological properties, they have been associated with several functional effects such as the inhibition of pathogens (Adriana *et al*. 2016; de Sant'Anna *et al*. 2017) and immunomodulatory properties (Pérez-Cano *et al*. 2010; Gao *et al*. 2017). Besides, they were successfully included in several dairy matrices as well (Cordeiro *et al*. 2019; Zhang *et al*. 2019).

Some LAB are able to produce exopolysaccharides (EPS) and have been frequently used in the dairy industry, mainly with the aim to improve the texture and rheology of dairy products, such as fermented milk and cheese, and reduce syneresis in yogurt (Zisu and Shah 2005; Dabour *et al.* 2006). They can play the role of natural texturisers, and can function as thickeners, stabilisers and gelling agents in several food matrices (Ahmed and Ahmad 2017; Rehman *et al.* 2018). More recently, these molecules have been also associated with beneficial roles for health, since they presented anticancer properties (Deepak *et al.* 2016), cholesterol-lowering effects (Korcz *et al.* 2018), immunomodulatory (Hidalgo-Cantabrana *et al.* 2012; Ale *et al.* 2016a) and prebiotic (Hamet *et al.* 2016; Ale *et al.* 2019) properties. Besides, EPS from LAB have presented protective roles against pathogenic microorganisms (Nagai *et al.* 2011; Maruo *et al.* 2012; Ale *et al.* 2016a), have been related to the prevention of oxalate stone disease (Sönmez *et al.* 2018), as well as to oxidative damage protection (Chen *et al.* 2016). Due to these health-promoting properties, these molecules represent part of a wider group called "postbiotics". This term refers to microbial metabolites (enzymes, proteins, peptides, polysaccharides, organic acids or lipids) and components (lipoteichoic and teichoic acids, peptidoglycans, cell-surface proteins and polysaccharides) that exert local and/or systemic positive effects in the host (Aguilar-Toalá *et al.* 2018). These technological and health promoting properties highlight the relevance of finding new EPS-producing LAB for the design of novel techno-functional products. Despite all these advantages, the low EPS yield by LAB (Ryan *et al.* 2015) limits its use for commercial purposes.

The medium MRS (Man, Rogosa and Sharpe broth, De Man *et al.* 1960), routinely used for the development of lactobacilli, contains components (meat extract, yeast extract and proteose peptone) that interfere with the quantification and analysis of EPS, mainly due to the presence of mannans from the yeast extract, among other compounds of carbohydrate nature (Cerning *et al.* 1992; Kimmel and Roberts 1998) that co-precipitate with EPS. For this reason, a semi-defined medium was selected (SDM, Kimmel and Roberts 1998), which was suitable for evaluating the influence of its composition on EPS production (Ale *et al.* 2016a;b). According to former studies (Shi *et al.* 2014), some components have more influence on EPS yield and composition than others, such as the carbon and nitrogen sources used. In order to evaluate their impact on the EPS yield, it is necessary to look for the optimal experimental conditions through proper statistical approaches.

Lactobacillus fermentum Lf2 is an autochthonous strain that was isolated from semi-hard Tybo cheese as non-starter culture, and its EPS extract was related to interesting functional and technological properties. This extract, in small doses, was able to protect mice against a *Salmonella* infection and increase intestinal IgA levels when added in yogurt (Ale *et al.* 2016a). Furthermore, it was able to modify beneficially the microbiota of mice when added solely or combined with a probiotic strain, reinforcing its ability to act as functional food ingredient (Ale *et al.* 2019). In the future, clinical trials could be performed to verify the health-promoting properties of this postbiotic to suggest its future application as a food ingredient. On the other hand, it provided yogurts with increased consistency and hardness, together with improved water holding capacity, with no detectable sensory defects (Ale *et al.* 2016b). The EPS extract is mainly composed by two polysaccharides: a high molecular mass β-glucan, whose repeating unit is a trisaccharide ($1.8x10^3$ KDa), and a medium molecular weight heteropolysaccharide (HePS), with a highly complex repeating unit composed of glucose and galactose (Vitlic *et al.* 2019). Particularly, the β-glucan produced immunotolerance in peripheral blood mononuclear cells by the modulation of proinflammatory mediators, such as TNF-α (Vitlic *et al.* 2019). Considering these promising properties, and the relatively high EPS yield obtained for this strain (1 g/L, approximately) under

non-optimised conditions (Ale *et al.* 2016b), the need to optimise its production and to understand how the new conditions impact on the composition arose with the aim of making its application feasible.

MATERIALS AND METHODS

Organisms and growth conditions

L. fermentum Lf2 (from the collection of the Instituto de Lactología Industrial- INLAIN, Santa Fe, Argentina) was routinely grown in MRS (Biokar, Beauvais, France) broth at 37 °C for 18 h. Although its EPS extract demonstrated to have beneficial effects in the host, the probiotic capacity of the strain itself has not been verified yet. *In vivo* assays are being addressed to confirm this hypothesis. It was stored at -80 ºC in the same medium with the addition of 15% (v/v) glycerol.

Preliminary experimental design for selection of factors: time of fermentation, carbon source and percentage of nitrogen sources

Cultivations were performed in a 2-L fermentor (Sartorius Biostat A plus®, Goettingen, Germany) in SDM broth (Kimmel and Roberts 1998), as described by Ale *et al.* (2016b), with modifications according to the D-Optimal design applied (Table 1). The D-Optimal Mixture Design was selected to evaluate the significance of the categorical factors that are part of a mixture. In a mixture experiment, the response depends on the relative proportions of the components (the total sum of factors remains constant). This design is applied as an alternative to the General Factorial design option, which may imply designs with more runs than expected. The D-optimal design will choose an ideal subset of all possible combinations, based on the model that it is specified, obtaining a smaller number of experiments.

Table 1. Experimental points for the D-Optimal design. YNB: yeast nitrogen base; BC: Bacto Casitone; AC: ammonium citrate. The central point (N° 7) indicated in bold was done in duplicate.

Carbon source: glucose or sucrose 2% (w/v)			
N° Experimental point	% (w/v) YNB	% (w/v) BC	% (w/v) AC
1	1.133	0.283	0.283
2	0.850	0.850	0.000
3	0.850	0.000	0.850
4	0.283	1.133	0.283
5	0.283	0.283	1.133
6	0.000	0.850	0.850
7	**0.567**	**0.567**	**0.567**

For this experimental design, the proportions of the three nitrogen sources, bacto casitone (BC), yeast nitrogen base (YNB) (both from Difco, Becton, Dickinson and Company, Le Pont de Claix, France) and ammonium citrate (AC, Cicarelli, Buenos Aires, Argentina) were modified, maintaining the original total amount of these sources constant, normally 1.7% (0.5% YNB, 1% BC and 0.2% AC). Besides, the same experimental design was applied to study another carbon source, sucrose instead of glucose, at 2% (w/v), concentration normally used in the SDM medium. These sugars were selected considering preliminary studies that showed the EPS extract was composed mainly by a β-glucan and a HePS with glucose and galactose in its structure, and the experimental evidence of an important EPS production when sucrose was used. In both cases, the central points were made in duplicate. *L. fermentum* Lf2 was inoculated from an overnight culture (0.1% v/v) and incubations were made at 30 °C for 48 and 72 h, with agitation (6 ×*g*) and sparging with CO_2 (0.2 L/min). These time points and the temperature were selected according to previous studies that evidenced the highest EPS production during the post-stationary phase of growth at 30 ºC (Ale *et al.* 2016b). Samples of 200 mL were aseptically withdrawn to determine cell counts (MRS Agar, 48 h,

37 °C, aerobiosis) and EPS yield. The pH was kept automatically at 6.0 with sterile 8 M NaOH. After cultivation, the EPS extract was obtained from 200 mL of culture broth according to Ale *et al.* (2016b). Briefly, bacteria were removed by centrifugation (19,000 ×*g*, 30 min, 5 °C) and EPS was precipitated at 4 °C for 48 h with the addition of 2 volumes of chilled absolute ethanol (Cicarelli). The precipitate was then collected by centrifugation (4000 ×*g*, 30 min, 5 °C), dissolved in ultrapure water and dialyzed against distilled water, using 12-14 kDa MWCO membranes (Sigma Aldrich, St. Louis, MO, USA) for 3 days, at 4 °C with daily changes of water. Finally, the EPS solution was freeze-dried (Christ Alpha 1-4 LD Plus, Osterode am Harz, Germany), weighed and expressed as g crude EPS/L. Blanks of each experimental point (100 mL, with no inoculum) were prepared in order to subtract the interferences provided by each medium in EPS yield determination. When necessary, the EPS extract was purified according to Ale *et al.* (2016b).

CCD to study the influence of the concentration of carbon source and pH on EPS production

Once the optimal percentages of each nitrogen source, the time of growth, as well as the type of carbon source were determined by D-optimal design, a CCD was applied to study the effect of the pH (a range from 5 to 7) and percentage of the carbon source chosen (from 1 to 8% w/v) on the EPS yield. A CCD is an experimental design useful in RSM for building a polynomial model for the variable without the need to use a complete three-level factorial experiment. This design was selected because a small number of experiments could be performed, using five levels of each numerical factor. The compositions of the different experimental points are detailed in Table 2. In this case, the central point was made in triplicate. The final validation of the model was done with fermentations of 700 mL as described in section 2.2, in triplicate.

Table 2. Experimental points for the Central composite model. YNB: yeast nitrogen base; BC: Bacto Casitone; AC: ammonium citrate. The concentrations of BC, YNB and AC were 0.53, 0.63 and 0.53% (w/v), respectively. The central point (N° 5, in bold) was done in triplicate.

N° Experimental point	% (w/v) sucrose	pH
1	4.50	7.0
2	2.75	6.5
3	1.00	6.0
4	6.25	5.5
5	**4.50**	**6.0**
6	2.75	5.5
7	8.00	6.0
8	6.25	6.5
9	4.50	5.0

154

155 **Chemical characterization of the EPS purified extract**

156 Samples of the EPS extracts produced under non-optimised and optimised conditions were
157 analysed by ^{1}H-NMR on a Bruker 600MHz NMR spectrometer fitted with a liquid nitrogen cold
158 probe. Samples (approximately 10 mg) were dissolved in D_2O (650 μl) and spectra were recorded at
159 70 °C. The elevated temperature increased the resolution of the NMR spectra and moved the
160 residual H_2O signal out of the anomeric region (from 4.69 to 4.29 ppm). Details of the experiments
161 used to separate and characterise the structures of the high molecular mass EPS have been reported
162 elsewhere (Vitlic *et al.* 2019). The ratio of the two EPS in the standard and optimised samples was
163 determined by measurement of the peak integration of the appropriate anomeric resonances.

164

165 **Statistical analysis**

Experimental designs and statistical analysis were done with the Design-Expert software version 11 (free trial). Figure 1 summarises the statistical strategies applied in the present work.

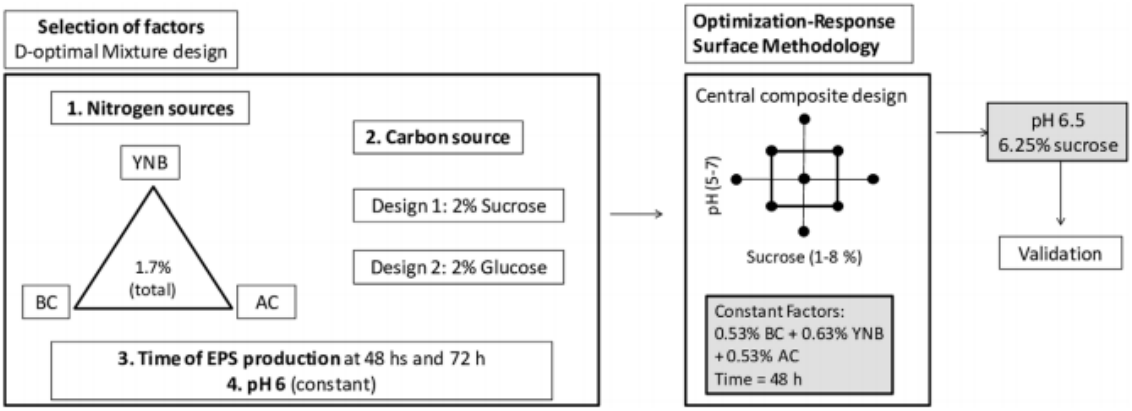


Figure 1 Flowchart summarises of the main stages of the statistical optimization. AC, ammonium citrate; BC Bacto Casitone; YNB, Yeast Nitrogen Base.

RESULTS

Preliminary experimental design for selection of factors: glucose as carbon source

In Figure 2a the cell counts of the different D-optimal experimental points when glucose was used as a carbon source are shown. The initial level was approximately 6 log (CFU/mL) in all cases, as expected. For experimental conditions 1, 2 and 7, the maximum development of the strain was achieved at 48 h, reaching values higher than 9 log (CFU/mL), and after 72 h of incubation, the cell viability decreased approximately one logarithmic order. For point 3, it can be observed that there was no cell development, keeping the final cell count similar to the initial, indicating that BC is an essential component for cell growth under these conditions. On the other hand, for points 4, 5 and 6, cell counts at 72 h were higher than at 48 h of incubation, being point 4 the one that showed the most important growth (9.2 in log scale).

In Figure 2b, the EPS yield obtained for each experimental point using glucose as carbon source can be observed. In all the cases, the levels of total protein were lower than 0.9%. The highest production of EPS was obtained for the combination of the three nitrogen sources proposed

186 by point 1 (1.13 % w/v YNB; 0.28 % w/v BC and 0.28 % w/v AC), reaching a yield of
187 approximately 1.2 g/L. The fermentation conditions indicated by the central point 7 (equal
188 proportions of the three nitrogen sources) made also possible an interesting EPS yield too, 1.11 and
189 0.82 g/L at 48 and 72 h, respectively. According to a paired t-test (confidence level of 95%), no
190 significant differences existed between the EPS amount obtained at each time evaluated. For the
191 experimental point 3, EPS production was not significant, due to the absence of growth of the
192 strain. Besides, since no BC was added in this point, it seems that this component would play a
193 crucial and limiting role in bacterial growth and, consequently, in the EPS production.
194 .

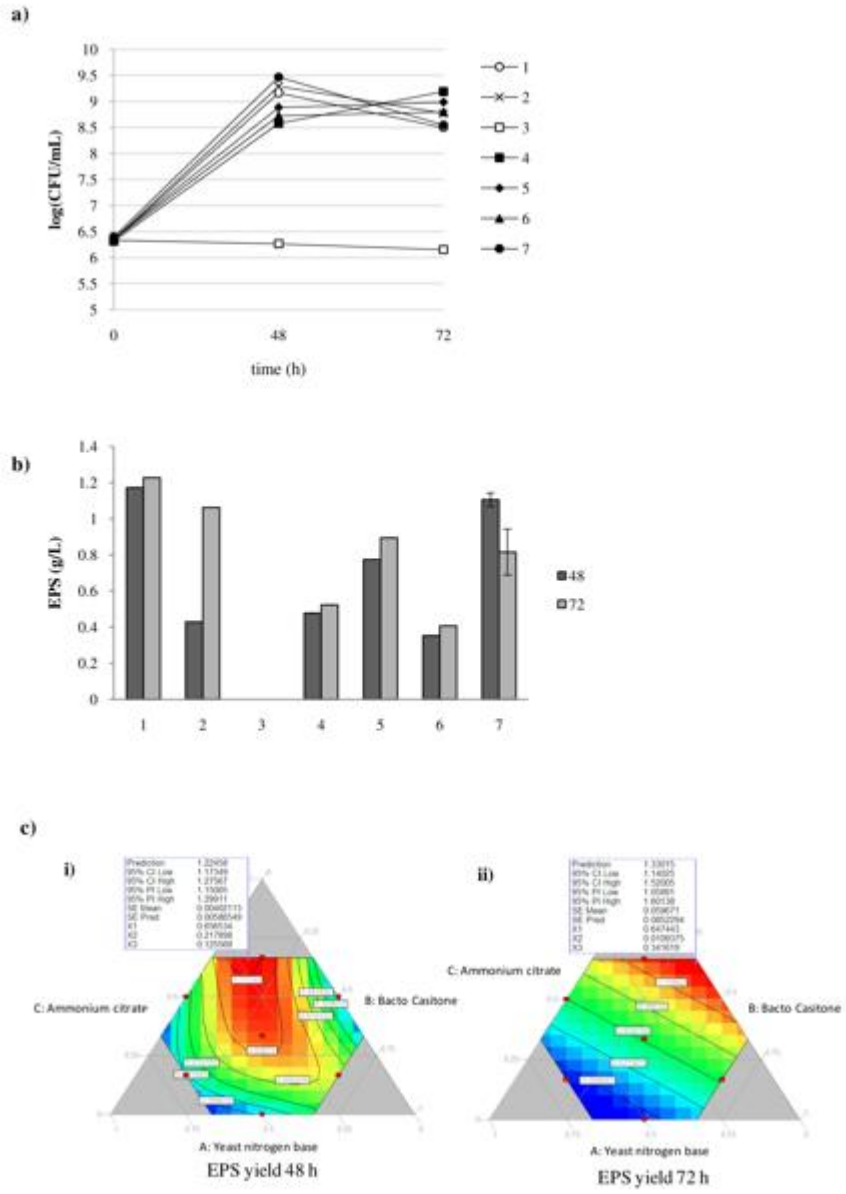


Figure 2. a) Cell development using glucose 2% (w/v) as C source; b) EPS yields obtained for each experimental point described in Table 1, at 48 and 72 h at 30 ºC and pH 6.0. The central point (7) was done in duplicate; in this case $\overline{x}$ ± SEM is shown; c) Contour plots for the EPS yields obtained at 48 h (i) and 72 h (ii) of fermentation at 30ºC, pH 6.0, using glucose 2% (w/v) as C source. The optimal conditions for each case are indicated in the boxes above the figures. X1: yeast nitrogen base, X2: Bacto Casitone and X3: ammonium citrate

For the experimental data corresponding to the EPS production at 48 (R1) and 72 h (R2), using glucose as carbon source, polynomial models were applied. The coefficients were obtained by multiple regression with backward elimination and were validated by ANOVA. The production of EPS at 48 h was adjusted with a linear model which included double and triple interactions, while the production of EPS at 72 h was adjusted with a linear model, according to the equations which are shown below. The *p*-value for these models were 0.007 and 0.004, and the adjusted R^2 0.999 and 0.965, for R1 and R2, respectively. These R^2 values indicate that 99.9% and 96.5% of the variability in the response could be explained by the model applied in each case.

$$EPS\ production\ (48\ h,\ glucose) = 1.19YNB - 0.25BC + 0.64AC - 0.27YNB\ BC - 2.14YNB\ AC + 5.37YNB\ BC\ AC \quad (1)$$

$$EPS\ production\ (72\ h,\ glucose) = 0.94YNB - 0.04BC + 0.51AC \quad (2)$$

Where *YNB* means yeast nitrogen base; *BC*, bacto casitone and *AC* ammonium citrate (% w/v). From these equations, the influence of the factors on each response (EPS yield at 48 and 72 h) can be analysed. Considering the coefficient values from both equations, the concentration of YNB is the factor that most impacts on the production of EPS, since its concentration affects proportionally the final yield (factor affected by a positive sign with the higher coefficient). In decreasing order of importance, the concentration of AC and BC can be mentioned.

Equation 1 shows that a high proportion of BC would produce a decrease in EPS production, due to the negative sign (-0.25) that affects the coefficient of this factor. But, in the case of equation 2, this coefficient has little influence (-0.04), affecting in a lower proportion the EPS yield. Besides, in equation 1, the interaction between the three factors is affected by the highest positive coefficient (5.37), indicating that there is a beneficial interaction among the three components (BC, YNB and AC) that significantly impacts on the EPS synthesis under the conditions studied. Therefore, the

presence of BC seemed to be critical, at least at low concentration, for the growth of *L. fermentum* Lf2, in agreement with the results observed for the experimental point 3, in which the only nitrogen sources were the YNB and the AC.

Finally, the combinations of factors that maximise the production of EPS at 48 h were: 0.66% of BN, 0.22% of BC and 0.13% of AC, predicting a yield of 1.22 g/L of EPS. Additionally, to maximise EPS production at 72 h, the best combination of factors would be: 0.65% BN, 0.01% BC and 0.34% AC (all in % w/v), obtaining in this case a predicted yield of 1.33 g/L of EPS. Figure 2c shows the contour plot of the response surface obtained for EPS production at 48 h and 72 h. It has been described that a circular contour plot of response surfaces indicates that the interaction between the corresponding variables can be ignored, while an elliptical or saddle nature of the contour plot (as the one observed in Figure 2c at 48h) suggests that the interaction between the corresponding variables is significant, fact that is verified in equation 1 (Xu *et al.* 2010).

Preliminary experimental design for selection of factors: sucrose as carbon source

Regarding EPS production with sucrose as carbon source, the highest cell count was reached at 48 h for experimental point 2 (9.8 log, Figure 3a), which decreased approximately a logarithmic order towards the end of the growth curve. As the composition of this point lacked AC and presented YNB and BC in equal amounts, it could be suggested that this component is not essential for the growth of the strain with sucrose. Cell counts were similar between points 5 and 6 at 48 h (around 9 log), and points 1, 4 and 7 (8.7 log, approximately). Experimental point 3 (with no BC added) did not provide the necessary nutrients to permit bacteria growing properly, a result that is in accordance to those obtained with glucose as carbon source.

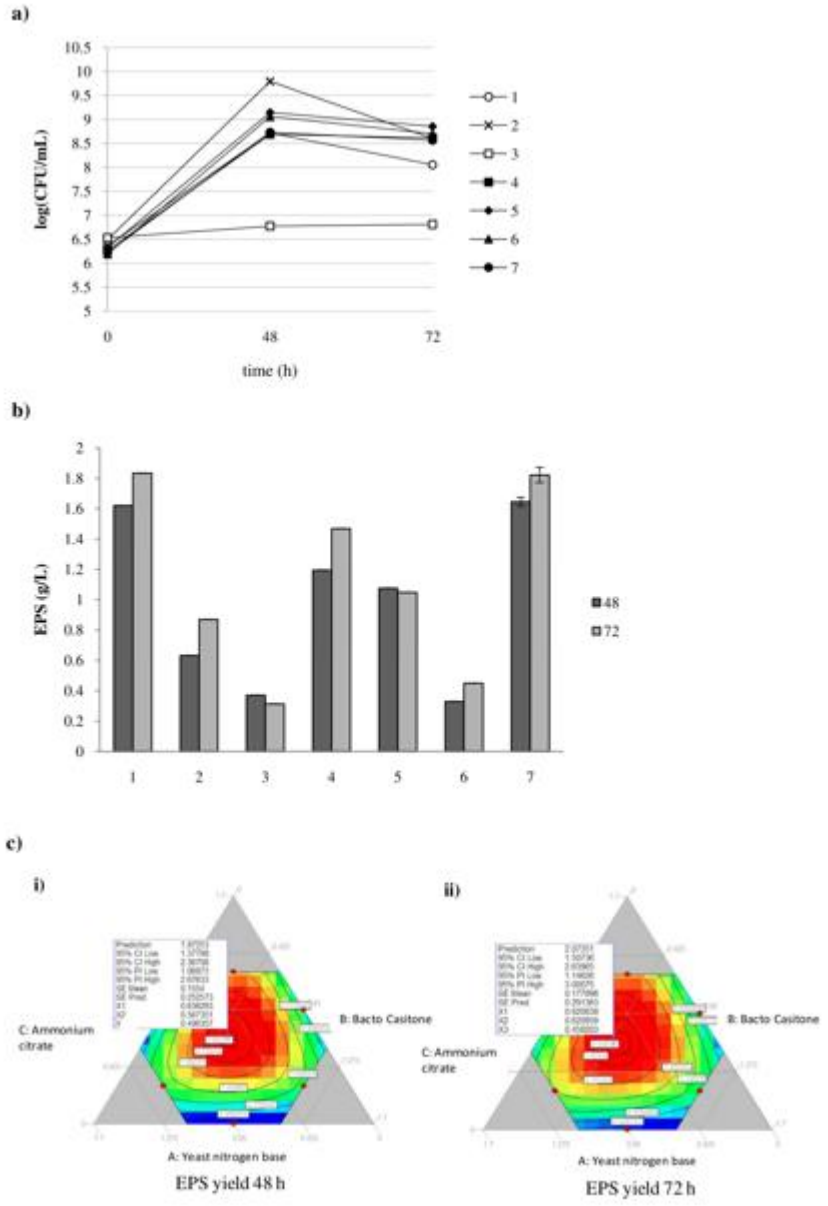


Figure 3. a) Cell development using sucrose as C source and different pH values for the experimental points shown in Table 2 (central composite model, CCM) at 0 and 48 h fermentation; b) EPS yields obtained for each experimental point described in Table 2, at 48 h. The central point (5) was done in triplicate; in this case $\overline{x}$ ± SEM is shown; c) Response surface obtained for the central composite model. Red dots represent the experimental values.

When sucrose was used, higher amounts of EPS were obtained than with glucose as carbon source. In all the cases, the levels of total protein were lower than 0.9%. Figure 3b shows that, similarly to the results achieved with the former one, the maximum production of EPS was observed for experimental points 1 and 7, both at 48 (1.6 g/L, approximately) and at 72 h (1.8 g/L) of fermentation. Points 3 and 6 were those that presented the lowest yields and, since they did not have BC or YNB, the presence of both components was relevant for EPS production. This observation was not reproduced when AC was absent (experimental point 2), achieving a performance that, although was not optimal, exceeded that of points 3 and 6. On the other hand, points 4 and 5 presented an intermediate behaviour, with yields <1.5 g/L.

The experimental data corresponding to the production of EPS with sucrose 2% (w/v) at 48 (R1) and 72 h (R2) were adjusted as described for glucose. The yields of EPS at 48 h and 72 h were adjusted with linear models according to the equations which are shown below, both including triple interactions:

$$EPS\ production\ (48\ h,\ sucrose) = 2.69YNB + 1.46BC + 1.37AC + 46.04YNB\ BC\ AC \quad (3)$$

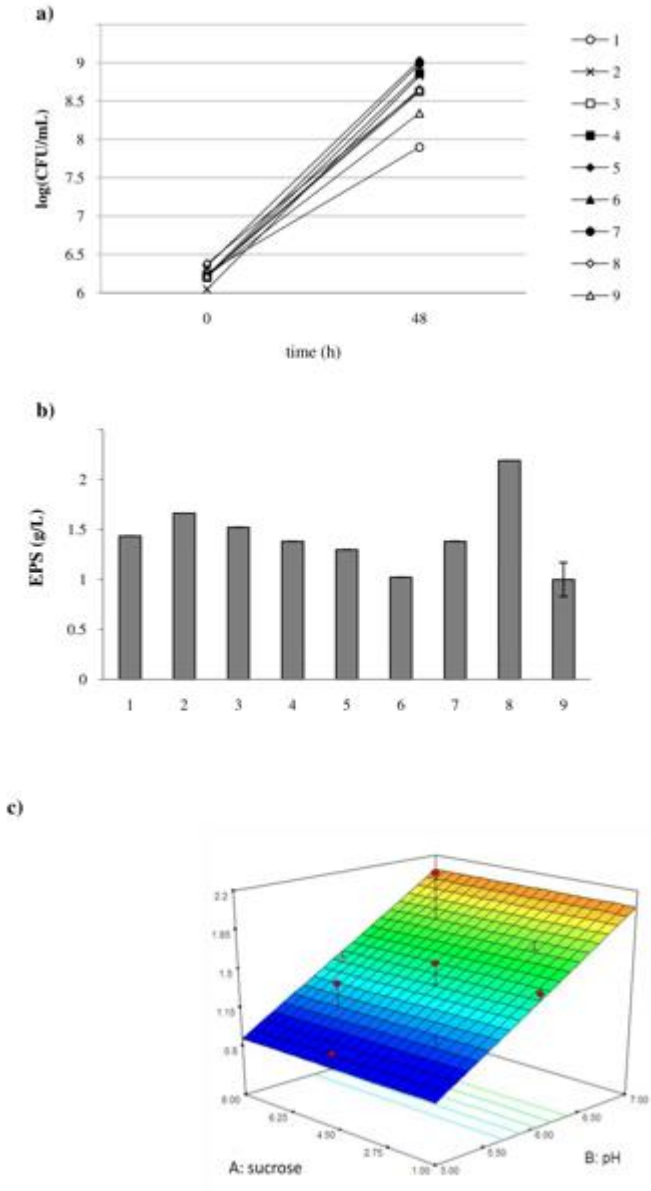
$$EPS\ production\ (72\ h,\ sucrose) = 3.09YNB + 1.86BC + 1.15AC + 48.91YNB\ BC\ AC \quad (4)$$

These models were the ones that best explained the behaviour of the data, obtaining a *p*-value of 0.03 for R1 and R2. The adjusted R^2 was 0.937 and 0.932, respectively.

From equations 3 and 4 the influence of the coefficients corresponding to the triple interaction (46.04 and 48.91 for R1 and R2, respectively) can be appreciated. When the response surfaces were analysed, it was concluded that the combination of factors that maximise the

283 production of EPS at 48 h was: 0.64% of YBN, 0.57% of BC and 0.50% of AC, with a predicted
284 yield of 1.87 g/L of EPS. In order to maximise the production of EPS at 72 h, the best combination
285 of factors found was: 0.62% of YBN, 0.62% of BC and 0.46% of AC (all in% w/v), with a
286 predicted yield of 2.07 g/L of EPS. Figure 3c shows the contour plot for the response surfaces
287 obtained at 48 h and 72 h of growth. Since the aim of this work was not only to maximise the EPS
288 production but also to minimise costs, and considering that the yields obtained at both times, 48 and
289 72 h, were quite similar (differences <10%), the time selected to continue with the optimisation was
290 48 h.
291
292 **CCD to study the influence of the concentration of carbon source and pH on EPS**
293 **production**
294 Finally, once the proportions of the nitrogen sources were chosen according the results
295 obtained, together with the type of carbon source and time of growth, a CCD was applied for the
296 optimisation of the pH (5 to 7) and percentages of sucrose (1 to 8% w/v). The model that best
297 explained the response behaviour was linear and it is shown below:
298
299 $$EPS\ production\ (optimised\ conditions,\ 48\ h) = -2.35 + 0.62pH + 0.03Sucrose \quad (5)$$
300
301 This model was significant with a *p*-value of 0.02, while the R^2 obtained was 0.728 in this
302 case. Although the sucrose concentration was not significant, this parameter was included in the
303 equation 5 to improve the regression.
304 In Figure 4a the cell counts for each experimental point at 0 and 48 h are shown. It can be
305 observed that, in general, a difference of around 2 log scales is found between initial and final time
306 of fermentation, with the exception of experimental point 1 (developed at pH 7), which did not
307 reach 8 log. Regarding EPS yields, the experimental point 8 (pH 6.5 and 6.25% sucrose), presented
308 the highest value, exceeding 2 g/L EPS extract (Figure 4b). Despite the fact that points 2 and 8 were

developed at the same pH, an important difference in the EPS yield was obtained (1.7 vs. 2.2 g/L, respectively), suggesting that the amount of carbon source used could be a significant factor for the model. Surprisingly, no significant effect was observed for the percentage of sucrose, being the pH the variable that only affected significantly the response obtained. Due to the poor development of the strain at constant pH 7, pH 6.5 was chosen as the superior level of this factor.



315 **Figure 4.** a) Cell development using sucrose as C source and different pH values for the
316 experimental points shown in Table 2 (central composite model, CCM) at 0 and 48 h fermentation;
317 b) EPS yields obtained for each experimental point described in Table 2, at 48 h. The central point
318 (5) was done in triplicate; in this case $\overline{x}$ ± SEM is shown; c) Response surface obtained for the
319 central composite model. Red dots represent the experimental values.
320
321 The response surface obtained (Figure 4c) indicates that the EPS yield increases
322 proportionally with the pH, so the final conditions chosen to validate the model were the following
323 (expressed as %w/v): 0.63% YNB, 0.53% BC, 0.53% AC, pH 6.5, 6.25% sucrose (amount
324 previously used for the experimental point 8 which presented the highest EPS yield), and the time
325 of fermentation selected was 48 h, as explained in section 3.2. The yield obtained was 2 g/L
326 approximately, when the interferences provided by the medium were subtracted. This model was
327 validated by repeating the conditions three times, and a yield of 1.8 ± 0.2 g/L ($\overline{x}$ ± SD) was reached,
328 doubling the amount frequently obtained under not optimised conditions (1 g/L, Ale *et al.* 2016b)
329 (SDM with 2% glucose, pH 6.0, 72 h). All these results showed a good agreement between the
330 experimental and predicted values and implied that the mathematical models were suitable for the
331 simulation of EPS production in the present study. These results are important to make the SDM
332 broth originally proposed by Kimmel and Roberts (1998) more economical, since lower amounts of
333 BC (one of the most expensive components) are required.
334
335 **Chemical characterization of the EPS extract and comparison with the one obtained**
336 **under non-optimised conditions**
337 The anomeric region of the ^{1}H-NMR spectra for the EPS spectra contains unique resonances
338 corresponding to a single proton belonging to both the medium molecular mass (5.34 ppm) and the
339 high molecular mass (4.85 ppm) polysaccharides (Figure 5). In the spectra for the non-optimised
340 sample the ratio of the integrals for the two protons was 1:5.87 in favour of the high molecular mass

homoglucan, and the ratio in the optimised sample was 1:1.66 again in favour of the high molecular mass homoglucan. As the repeat unit for the medium molecular mass EPS contains ten monosaccharides whilst that for the high molecular mass EPS has only three, in order to determine the absolute amounts of each EPS present it is necessary to multiply the different integrals by the respective number of monomers. For the non-optimised samples this gives a ratio for the medium to high of 1:1.76 whilst for the optimised sample the ratio is 1:0.5.

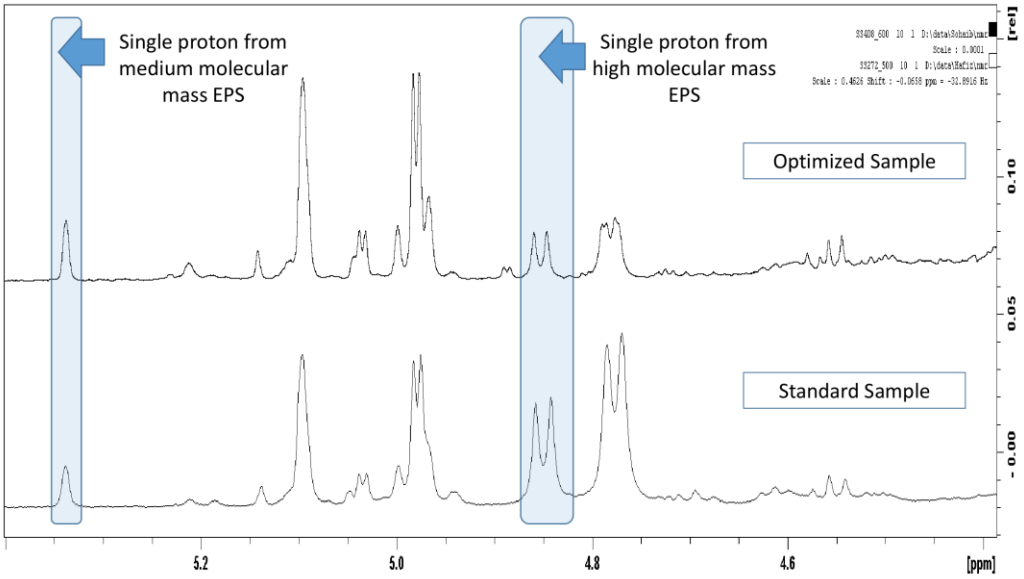


Figure 5. ^{1}H-NMR spectra of the purified EPS samples obtained under standard and optimized conditions.

DISCUSSION

In general, the production of EPS by BAL of different strains under non-optimised conditions is very variable (between 0.045 and 0.350 g/L) (De Vuyst and Degeest 1999), and strongly depends on the chemical structure of the polysaccharides. When our results were compared with the EPS yields previously described for this species (Fukuda *et al.* 2010; Shi *et al.* 2014), a considerably higher production was appreciated for *L. fermentum* Lf2. According to our results, there are some

key components of the culture broth that highly influence on the EPS yield of this strain: the carbon source and the proportions of the different nitrogen sources. As the EPS synthesis depends on the cell growth (as observed for point 3 with glucose as carbon source), the role of the nitrogen sources may contribute indirectly with the production of this postbiotic by the enhancement of cell development. In our study, the development of *L. fermentum* Lf2 was restricted to the presence of BC, indicating that some peptides of this pancreatic digest of casein may be necessary for their growth. Concerning the EPS synthesis, although the YNB seems to be the most important, a strong interaction among the three nitrogen sources exists, indicating that all of them should be present in the medium for an optimal EPS production. Considering the high diversity of vitamins (biotin, calcium pantothenate, folic acid, inositol, niacin, p-aminobenzoic acid, riboflavin, among others) and the trace elements (boric acid, copper sulfate, sodium molybdate, zinc sulfate, etc.) present in YNB, as well as the casein peptides from the BC, it is probable that some of these components are essential for the EPS biosynthesis, together with an inorganic nitrogen source as the AC. More studies about the different metabolic pathways of biosynthesis will be mandatory to understand the role of each component on the EPS production. Besides, sucrose presented better results than glucose when the EPS yield was evaluated, suggesting that this sugar should be considered when new media are designed for this objective, at least for this strain. Another factor of great importance when the EPS yield optimisation was addressed was the pH. Constant pH values during fermentation may inhibit the enzymatic hydrolysis of these molecules since the action of hydrolases is favoured below pH 5 (Degeest *et al.* 2002). Several reports have indicated better results with pH control than with free-pH fermentations (Ale *et al.* 2016b; Cheirsilp *et al.* 2017).

A study was reported by Imran *et al.* (2016), who chose two out of 27 strains of LAB based on their ability to produce EPS, *L. plantarum* NTMI05 and *L. plantarum* NTMI20. The EPS yield was optimised considering some components of the medium, like glucose (2% w/v), yeast extract (2.5% w/v) and ammonium sulfate (0.2 % w/v), by the application of CCD and RSM. The yields achieved were 0.96 g/L and 0.83 g/L for *L. plantarum* NTMI05 and NTMI20, respectively. The

high production of EPS was also observed at 72 h, in accordance to our results. The authors suggested that this incubation period probably favoured the enzyme activity and the metabolism rate of the polysaccharide synthesis. Xu *et al.* (2010) studied the EPS yield of the strain *L. paracasei* HCT in a chemically defined medium, and found that the factors that most influenced on the EPS production were the C/N ratio, cultivation time and temperature. They could evaluate the interactions among these three variables by Box–Behnken experimental design and RSM. The optimal culture conditions found were: C/N ratio 9.09 (using glucose as the carbon source), a cultivation time of 60.67 h and a temperature of 29.2°C, obtaining an EPS yield of 39.07 mg/mL, 4 times higher than the original yield. These authors found similar EPS yields between 60 and 72 h of cultivation. Previous studies about *L. fermentum* Lf2 (Ale *et al.* 2016b) demonstrated that EPS yield increased during the late stationary phase, as described by Xu *et al.* (2010).

Another aspect to consider is the possible change in the composition and/or proportion of polysaccharides in the total EPS extract after the optimisation. For example, Li *et al.* (2016) optimised the production of EPS from *S. thermophilus* 05-34, evidencing that, although the monosaccharide composition did not change, the optimised EPS presented a molecular mass of $4.7x10^2$ KDa, which was increased by 9 times compared with that obtained under the non-optimal fermentation condition. They explained this fact by showing an increased transcription level of *epsC*, responsible for chain length determination. Considering that the non-optimised EPS extract was composed of two different exopolysaccharides (Vitlic *et al.* 2019), our results indicate that the purified EPS produced under non-optimised conditions (pH 6, SDM, 72h, 30 ºC) had significantly more high molecular mass β-glucan compared to the heteroglycan (ratio of medium to high 1:1.76). In contrast, the ratio in the optimised sample changed, presenting twice as much of the heteroglycan compared to that of the β-glucan (ratio of medium to high 1:0.5). As sucrose was the carbon source, an increase in the proportion of the HoPS was initially expected according to the classical criterion that considers most HoPS are synthesised using sucrose as the glucosyl donor, by using the energy of the sucrose osidic bond to catalyse the transfer of a glycosyl moiety (Ryan *et al.* 2015). But,

according to recent studies (Fraunhofer *et al.* 2018), the synthesis of β-glucans resembles more the mechanisms of HePS biosynthesis than those of HoPS, which are formed from the hydrolysis of energy-rich disaccharides, such as sucrose. It is not the first time that this species was associated with sucrose consumption (Hammes and Hertel. 2006; Wayah and Philip 2018). Although additional studies are necessary, and from this metabolic characteristic, it could be proposed that the UDP-glucose necessary to promote the HePS and β-glucan synthesis comes from the hydrolysis of this disaccharide.

CONCLUSION

Our results evidenced that the modification of the growth conditions can significantly improve the EPS yield of *L. fermentum* Lf2. The nitrogen sources play a key role in the production of EPS of this strain, as well as the type of sugar used, being the concentration of the last a not significant factor, according to the results obtained. Besides, pH was also a very important variable that influenced on the yield reached, indicating that the control of pH during fermentation is desirable when EPS production is optimised. Under these conditions, the medium molecular mass HePS was favoured, on the contrary to the composition described for the EPS obtained under non-optimised conditions, which presented significantly more amount of the high molecular mass β-glucan. The statistical optimisation made it possible to achieve an important increase in the EPS production, doubling the yield frequently obtained, and making the future application of EPS from *L. fermentum* Lf2 as a new techno-functional food ingredient feasible. An important economic impact could be appreciated from our results when a possible industrial application of this postbiotic is proposed, since a significant reduction on the cost of production (45%) was obtained per gram of EPS obtained.

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Conflict of Interest

The authors declare that no conflicts of interest exist.

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