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Authors: Ertan Ermiş, Ecem Poyraz, Enes Dertli, Mustafa Tahsin Yılmaz
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Optimization of exopolysaccharide production of *Lactobacillus brevis* E25 using RSM and characterization

Ertan Ermis[1*], Ecem Poyraz[2], Enes Dertli[3], Mustafa Tahsin Yilmaz[4]

Abstract

Response surface methodology was used to determine the exopolysaccharide production of *Lactobacillus brevis* E25. The effects of three variables, temperature (30, 36 and 42 °C), incubation time (18, 33 and 48 h) and initial pH of growth medium (4.5, 5.5, and 6.5) were studied. Under optimum growth conditions, the amount of exopolysaccharide derived from *Lactobacillus brevis* E25 ranged from 10 to 35 gL^{-1}. The size of EPS molecules ranged from 10^5 to 10^7 Da. Infra red spectrum analysis showed characteristics absorption peaks indicating the presence of -OH, C=O and C-H groups. Furthermore, only glucose was detected as monosaccharide in exopolysaccharide structure, revealing that the structure of exopolysaccharide is a homopolymeric glucan type. Based on the differential scanning calorimeter thermograms, exopolysaccharide's melting temperature was observed around 116 °C.

Keywords: Lactic acid bacteria, exopolysaccharide, response surface methodology, molecular characterization

1. INTRODUCTION

Exopolysaccharides (EPS) which are composed of monosaccharides (sugars and their derivatives) can be produced by bacteria, fungi, plants and algae. They have been widely used due to their functional effects in wide range of applications as thickeners, viscosifiers, emulsifiers and stabilizers to improve mouth feel of food products, particularly the fermented ones [1]. Several studies reported that the EPS produced by LAB species isolated from different sources could exhibit health promoting effects such as antioxidant and cholesterol lowering properties [1], [2] in addition to their immunomodulation, antiulcer, antibiofilm, antitumour, antiviral and antimicrobial activities [3], [4]. Besides these benefits of EPS, it was also reported that they

* Corresponding Author
[1] İstanbul Sabahattin Zaim University, Faculty of Engineering and Natural Sciences, Food Engineering Department, 34303, İstanbul, Turkey, ORCID: 0000-0002-1461-7357
[2] Yıldız Technical University, Chemical and Metallurgical Engineering Faculty, Food Engineering Department, 34210, İstanbul, Turkey
[3] Bayburt University, Faculty of Engineering, Food Engineering Department, 69000, Bayburt, Turkey, ORCID: 0000-0002-0421-6103
[4] Yıldız Technical University, Chemical and Metallurgical Engineering Faculty, Food Engineering Department, 34210, İstanbul, Turkey, ORCID: 0000-0002-0421-6103

demonstrated prebiotic effects [3], [5]. Furthermore EPS structures may involve in adhesion and biofilm formation [6], [7]. Therefore the EPS derived from food grade lactic acid bacteria (LAB) with a "Generally Recognised As Safe (GRAS)" [3] or a "Qualified Presumption of Safety" status are becoming economically significant in the food industry [2].

According to previous studies some LAB species can produce two types of EPS which can either encapsulate bacteria which are called as capsular polysaccharides (CPS) or be secreted into growth medium with varying composition and molecular properties [8]. Based on their structure, two types of EPS are known as homopolysaccharides (HoPS) which are composed of only one type of sugar monomer and heteropolysaccharides (HePS), composed of two or more types of sugar monomers [9]. EPSs were derived from different microbial strains isolated from varying sources and characterized by some previous researchers [3], [10]. Since the functionality of EPS is strain-dependent, researchers still screening various food materials to isolate new LABs with EPS production ability [11].

The culture conditions, growth medium (sugar composition, chain length, sugar linkages, presence of repeated units and substitutions) and the strain used may influence the EPS yield, molecular mass and composition as well as molecular structure [1]. LAB were reported to be able to produce both homopolymeric (HoPS) and heteropolymeric polysaccharides (HePS) consisting different sugar fractions such as glucose, maltose, galactose, fructose, rhamnose, mannose, glucuronic acid, N-acetylglucosamine and N-acetylgalactosamine [12].

Since the yield of EPS, their biological activities and physicochemical properties are strongly influenced by variables such as culture conditions and bacterial strains [4], it is required to optimize growth conditions and examine different strains to obtain higher EPS yields. In some of the previous works Response Surface Methodology (RSM) has been applied for optimization of culture conditions such as growth medium (carbohydrate source, nitrogen source) and process conditions (temperature, pH, oxygen tension) for production of EPS from different LAB strains [1], [13]. However there is still lack of information about the EPS production capacities of distinct LAB isolates and their characterization.

In this work *Lactobacillus brevis* E25 isolated from sourdough [14] was studied to determine the optimum incubation conditions (initial pH of the medium, incubation time and incubation temperature) in MRS broth in order to obtain highest EPS yield using RSM. The precipitated and freeze dried EPS powder was characterized to determine the molecular mass, composition, functional groups and thermal behaviour in order to evaluate it's potential use in food applications.

2. MATERIAL AND METHOD

2.1. Bacterial strains and culture conditions

Lactobacillus brevis E25 was propagated from it's glycerol stock and following it's activation in MRS broth, it was grown depending on the parameter explained in experimental design part.

2.2. EPS production

Response surface methodology (RSM) was used to determine optimum incubation conditions for EPS production of *Lb. brevis* E25. For this purpose, an overnight activated culture of *Lb. brevis* E25 was inoculated (at a rate of 0.1%) into 100 mL MRS broth (Oxoid, Wesel, Germany) (fortified with 1% sucrose). Inoculated MRS broth was incubated at selected conditions (Table 1) and an agitation speed of 50 rpm in a shaking flask. Cells were seperated by centrifugation at 4 °C, 6000g for 30 min. The supernatant was removed and 80% (w/v) TCA (trichloroacetic acid) was added at a final concentration of 4% (w/v) to precipitate the proteins by keeping 6 hours at 4 °C. Centrifugation (10000g for 30 min at 4 °C) was applied to remove the proteins. After the pH of clear supernatant was adjusted to 6.8-7.0 using NaOH and HCl solutions, double volume of absolute ethanol was added and maintained overnight at 4 °C to precipitate the

EPS. Precipitated EPS was recovered by applying centrifugation at 10000g for 30 min at 4 °C. The precipitated EPS was dissolved in ultra pure water and phenol-sulphiric acid methodology [15] was applied to determine the amount of EPS produced. The EPS solution was frozen at -80 °C and lyophilized at -60°C for up to 3 days to obtain a dried EPS powder for further analysis.

Table 1. Independent variables used to produce exopolysaccharide

Independent variables	Nomenclature	Units	Levels	Variation range
Initial pH of medium (x_1)	pH	-	4.5-5.5-6.5	(-1,0,1)
Incubation time (x_2)	t	hour	18-33-48	(-1,0,1)
Incubation temperature (x_3)	T	°C	30-36-42	(-1,0,1)

2.3. Experimental design

A Box-Behnken design was used to find the optimal values of three independent variables [initial pH of growth media (x_1), incubation time (x_2) and incubation temperature (x_3)] in EPS yield (dependent variable). In total, fifteen fermentation conditions were tested. Table 1 shows the central point and range values of these variables. Selecting these temperature levels took into account that the bacteria present higher fermentation rates at temperatures between 30 °C and 45 °C. The choice of different pH values of the growth media was because that the bacteria can work well around initial pH of 4.5. The aim was to evaluate the potential effect of starting H^+ ion concentration on EPS production capacity of *Lb. brevis* E25. Different incubation times were selected to determine the incubation time needed to get higher EPS yield. Shorter incubation times would lead to low EPS yield and similarly too long fermentation times may also result in low EPS yield. Thus, it seemed satisfactory to start from 18 hours and to increase to 33 and 48 hours in order to evaluate whether this increase is needed to get higher EPS yield.

2.4. EPS Characterization

2.4.1. Rheological properties

50 mg EPS powder was dissolved in 10 mL distilled water and vortex mixed for 30 min at 30 °C to determine the rheological behaviors. Prepared solutions were left for 24 h at room temperature before conducting esperiments. A controlled stress Anton Paar Modular Compact Rheometer MCR302 (Anton Paar GmbH, Ostfildern, Germany) coupled with a parallel-plate geometry (stainless steel, 50 mm diameter, 0.5 mm gap) was employed to determine the rheological properties. The shear rate range used was 0.1-100 s^{-1}. The Power-law model (Equation 1) was employed to determine the flow behaviors of the samples.

$$\sigma = K\,\gamma n \quad (1)$$

Where, σ is the shear stress (Pa), K is the consistency coefficient (Pa s^n), n is the flow behaviour (Power-law) index; and γ is the shear rate (s^{-1}).

2.4.2. Determination of monosaccharide composition

For the determination of the EPS repeating unit of *Lb. brevis* E25, 10 mg EPS sample was weighed and added into 0.8 mL distilled water and mixed using a vortex mixer and then treated with 218 µL perchloric acid and vortexed. The mixture was kept at 80 °C for 1 hour and 500 µL 5M KOH was added to the sample and the mixture was shaken. The mixture then centrifuged at 12000g at 4 °C for 5 minutes to precipitate the macromolecules. The supernatant was removed and passed through a 0.45 µm membrane filter (Minisart RC15, Sartorius, Goettingen, Germany) to make it ready for HPLC analysis. The monosaccharide composition of EPS sample was analyzed using Shimadzu HPLC (Shimadzu, Manchester, UK) equipped with an Aminex HPX-87C ion exchange Column (300x7,8 mm) (Bio-Rad,

Hercules, CA) and coupled with refractive index (RI) detector (10A, Shimadzu, Manchester, UK). Diluted sulphiric acid (5 mM) was used to dilute the column at 85 °C. The monosaccharides were analysed using the following parameters: injection volume: 20 µl, colon temperature: 85 °C, mobile phase: ultra pure water, flow rate: 0.4 mL/min, time: 30 min.

2.4.3. Fourier transform infrared (FT-IR) spectroscopy

EPS sample was analysed by a spectrometer (Tensor 27) (Bruker, Ettlingen, Germany) equipped with KBr beam splitter and DLaTGS detector with an ATR module attached. The EPS sample was mixed with KBr (0.1%) prior to analysis. OPUS program V.7 (Bruker, Ettlingen, Germany) was used to obtain the spectra that were recorded with a light source in the mid-range (600-4000 cm^{-1}).

2.4.4. Molecular weight (MW) determination

A Gel Permeation Chromatography/Size Exclusion Chromatography (GPC/SEC) (TDA302, Malvern Instruments, Houston, Texas, USA) integrated with a refractive index (RI) and light scattering detectors was used to determine the average molecular weight (MW) of the EPS sample. The GPC/SEC system was equipped with a Tosoh coloumn (G3000PWXL, Tosoh Bioscience, Montgomeryville, PA, USA). Regression equations obtained from Dextran standards of different molar masses (12, 25, 50, 80, 110, 150, 270, 400 and 800 kDa) with 0.1 M ammonium acetate buffer as eluent were used to quantify the EPS fractions [16]. Sample and standards were filtered through 0.2 µm filters (Chromafil Xtra, Macherey-Nagel) before injection. The column was eluted with 0.1 M PBS (pH 7.2), at 22 °C and at a flow rate of 0.8 $mL.min^{-1}$ prior to injection of 100 µL of the EPS solution which was prepared at 1.0 $mg.mL^{-1}$.

2.4.5. Analysis of the thermal properties

The thermal properties of EPS sample was analyzed by using a differential scanning calorimeter (DSC) Q100 instrument (TA Instruments Inc., New Castle, DE, USA). The heating rate was 10 °C per min from 25 to 250 °C.

2.4.6. Confocal laser scanning microscopy (CLSM) analysis

The method given by Goh et al. (2005) was applied with slight modifications. A lectin (carbohydrate-binding proteins) conjugated with a fluorescent dye [Lectin SBA From Glycine max (soybean), Alexa Fluor™ 488 Conjugate] (Thermo Fisher, Waltham, MA, USA) was used to discriminate EPS structures in the growth media after incubation. For this purpose, 15 µl fermented sample was transferred to a concave glass slide and 15 µl dye solution [0.1% (w/v)] was added. A cover slip was placed on the glass slide and left for around 30 minutes in dark at about 20 °C. The sample was examined using a laser confocal microscope Zeiss LSM-70 (Carl Zeiss AG, Oberkochen, Germany) equipped with a solid-state laser source, a 63 x PlanApo oil immersion objective lens and ZEN 2009 software. An excitation wavelength of 488 nm was used.

3. RESULT AND DISCUSSION

3.1. EPS producer LAB strains

Lb. brevis E25 was shown to produce a homopolymeric glucan type EPS consisting only glucose in its repeating unit [8] which agrees with the data reported previously [18] [3], [4]. In contrast some researchers report that some strains may produce heteropolymeric EPS with glucose and galactose in its repeating unit structure. Ai et al. [5] reported that the EPS produced by *Lactobacillus casei* LC2W was composed of glucose, rhamnose and galactose units. Di et al. [6] also reported that EPS from *Lactobacillus casei* SB27 contained galactose and glucose.

3.2. EPS production levels optimised by (RSM)

The EPS polymer precipitated could contain impurities e.g. from the salts. Furthermore, there could be impurities from growth media components. After precipitation of the proteins, there could be remaining sugars, which will distort EPS yield. The aim of this work was

to determine the optimum EPS production conditions and thus further cleaning steps were not applied to improve polymer purity in this study.

According to previous studies, the strain used in the production of EPS had an effect on the yield and its properties [19]. The effect of LAB strains selected and incubation conditions on EPS yield was investigated in 1% sucrose added MRS broth with initial pH values of 4.5, 5.5 and 6.5. The indipendent variables used for producing EPS are given in Table 1. 3D response surface graphs of EPS production can be seen in Fig. 1. As seen in Fig. 1, initial pH of the medium, temperature and time are important parameters for EPS production from *Lb. brevis* E25.

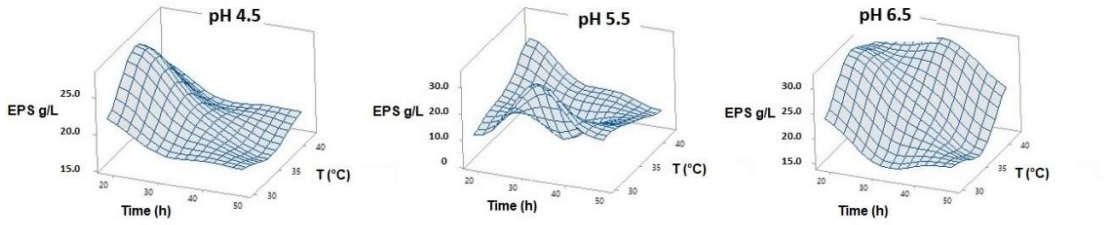

Figure 1. Response surface plots for exopolysaccharide yield

The highest EPS yields were observed at initial pH of 6.5, 18 hours of incubation time at 35 °C. The EPS yields ranged from roughly 10 to 35 gL^{-1}. The results obtained from previous studies [20] suggested that the EPS yield is strain dependent and may vary based on the incubation conditions.

Regarding the effect of pH, as can be seen from Fig. 1, higher yields were noticed at initial pH of 6.5. Increasing the initial pH of growth medium facilitated better production of EPS. These results agrees the findings of Manochai et al. [21]. The highest EPS yield they obtained was reported as a range from 99 to 110 gL^{-1} and low pH conditions resulted in lower EPS yields.

A previous study suggested that the optimal pH of the medium for exopolysaccharide production is around 5.0 to 7.0 [22]. The optimal initial pH for *Streptococcus thermophilus* S22 was found in the range from 5.5 to 7.0 depending on the carbon source [23]. Xu, Ma, Wang, Liu, & Li [33] reported the highest EPS yield (around 35 gL^{-1}) from *Lactobacillus paracasei* HCT at pH 6.70 at 37°C. Similarly, optimal initial pH of 7.0-7.5 was reported to improve the EPS yield of *Weissella confusa* NH 02 and *Weissella* sp. PSMS4-4 [25], [26]. A maximum EPS yield was obtained by *Lactobacillus casei* CRL 87 and *Lactobacillus confusus* TISTR 1498 when the pH was controlled at 6.0 and 5.5 respectively.

The EPS yields showed variations with incubation temperature. As depicted in Fig. 1, it was obvious that higher EPS yields were obtained at around 35 °C. Our results were in agreement with that of Manochai et al. [21]. They determined higher EPS yield at 30 °C than other temperatures and they reported low EPS at higher temperatures (46 °C). Similarly previous reports revealed higher yields of EPS at 30-37 °C [1], [14]. Increased EPS production at moderate temperature from mesophilic EPS-producing lactic acid bacteria suggests that the suboptimal growth conditions needed to improve EPS production [27], [28].

According to our results, it can be speculated that depending on the incubation conditions, there is optimum incubation parameters that higher EPS yield can be derived. *Lb. brevis* E25 produce higher amount of EPS at 35 °C, 18 hours and pH 6.5. However, Adesulu-Dahunsi et al. [1] report the optimal pH, temperature and cultivation time for EPS production as 7.0, 30 °C and 48 h, respectively. In this study, lower EPS yields were observed at longer incubations times which might

be attributed to consumption of byproducts by LAB at stationary phase of growth.

The EPS yields in this study ranged roughly from 10 to 35 gL^{-1} depending on the growth conditions. Some researchers studied the production of EPS in varying media and production conditions. The EPS yield from *Lb. confusus TISTR 1498* was reported as from 1 to 114 gL^{-1} depending on the substrate concentration [21]. Another study revealed that *Lactobacillus rhamnosus* C83 yielded 100-150 mg/L EPS after 40 h of fermentation at a pH range of 6-7 and subsequently decreased [29]. These results confirm that the yield depended on incubation conditions and bacterial strain [20].

According to Seesuriyachan, Kuntiya, Hanmoungjai, & Techapun 's [38] findings, up to 38.2 gL^{-1} EPS was obtained from *Lb. confusus* TISTR 1498 after a 24 h incubation period. Highest yield of EPS (17.7 gL^{-1}) could be obtained at 27°C by [31]. A maximum EPS yield of about 15.05 $g.L^{-1}$was achieved using *Klebsiella* sp. H-207 by Qiang et al. [40]. These results confirm that the yield depended on incubation conditions and bacterial strain [20].

3.3. Rheological properties

The EPS powder samples derived from different strains using varied incubation conditions were evaluated in terms of their rheological behaviour. Power-law model was used to evaluate the steady shear properties (Table 2). R^2 value was higher than 0.99, revealing good fitting of the model. As could be seen from Table 2, K (consistency index) and n (flow behavior index) values were calculated as around 0.207 $Pa.s^n$ and 0.550, respectively. Low n (flow behavior index) values indicated low stability against shear which is in agreement with the findings reported previously [20].

Table 2. Rheological properties of exopolysaccharide solution

Consistency Index (Pa s^n)	Flow indicator	R^2
0.207±0.005	0.550±0.004	0.999

3.4. FTIR spectra analysis

The organic functional groups and heteropolymeric nature of EPS samples were studied by FTIR spectroscopy (Fig. 2). EPS samples derived from five different strains presented identical FTIR spectrums except some variations in the peak intensities. As shown in Fig. 2, a broad absorption peak was observed at around 3080 cm^{-1} indicating the presence of -OH stretching vibrations (Fig. 2). The bands at 2944 cm^{-1} region assigned as C-H streching vibrations, represented a peak characteristic of polysaccharides and the existence of an intense bands in the region of 1650-1550 cm^{-1} which is also characteristic of polysaccharides and usually correspond to C=O and carboxyl group. The peaks in this region may also be due to the presence of uronic acid [33].

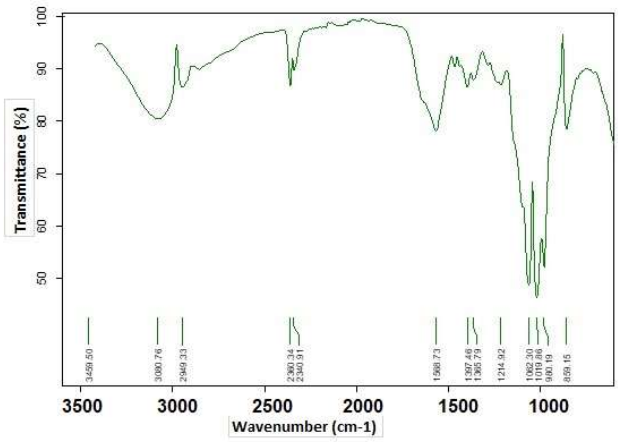

Figure 2. Fourier-transform infrared (FT-IR) spectra of exopolysaccharide

Peak at 1644 cm^{-1} was attributed to C=O stretching (Fig. 2). In the absorption regions below 1500 cm^{-1} (the fingerprint region), the absorption peak at 1465 cm^{-1} was caused by streching vibrations of the C-H group. The absorbance peak in the region of 1440-1200 cm^{-1} depicted the presence of carboxyl group as well as stretching vibration of the esters and ether groups [1] while in the peak region of 1200-1000 suggested the presence of C-O-C (glycosidic linkage) and C-O-H in addition to C-O streching vibrations. The appearance of an absorption peak at 914 cm^{-1} is usually considered to indicate the glucıside linkage in polysaccharides. The peak in

the region of 860 to 817 cm^{-1} indicate the possible presence of sulphated groups.

3.5. Molecular weight analysis

The average molecular masses of EPS from *Lb. brevis* E25 extrapolated from the dextran calibration curves were in the range from 415 to 11 600 kDa which is in agreement with the data reported by [16] previously.

3.6. Thermal properties of EPS samples by differential scanning calorimetry (DSC)

The food application of EPS in industry is highly dependent on its thermal behavior [2]. Cristallization, melting and cross-linking points attributes of EPS samples were elucidated using a DSC with heat flow from 20 to 300 °C. As can be seen from Fig. 3, the melting temperature was around 116 °C (Fig. 3). The cross-linking was started at about 200 °C. Miao et al. [42] and Y. Wang, Ahmed, Feng, Li, & Song [43] found similar melting points of EPS produced by *L. citreum* (133.9 °C) and EPS from *Lactobacillus kefiranofaciens* (93 °C) respectively.

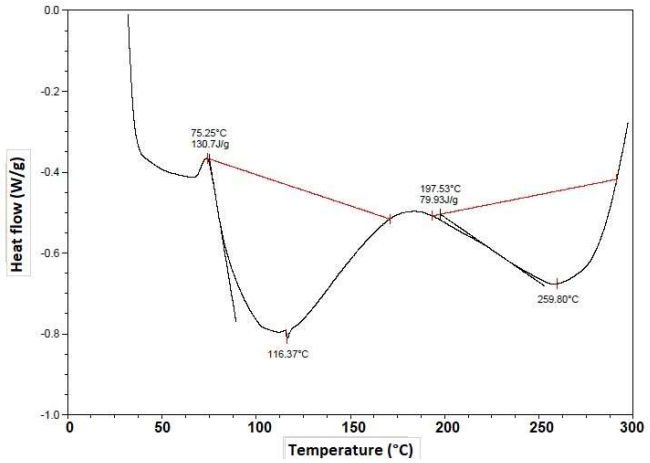

Figure 3. Dynamic scanning calorimeter (DSC) chromatogram of exopolysaccharide

3.7. CLSM visualization

CLSM images of samples stained with fluorescent-labeled lectin, allowed visualization of the EPS distribution within MRS broth (Fig. 4). Fig. 4 exhibits the distribution of EPS in the growth medium after the incubation. As can be see in Fig. 4, well distributed relatively small masses of EPS structures (small green colored dots) could easily be distinguished in MRS medium. The varying molecular characteristics those influencing the size of the polymer in solution was reported previously [36].

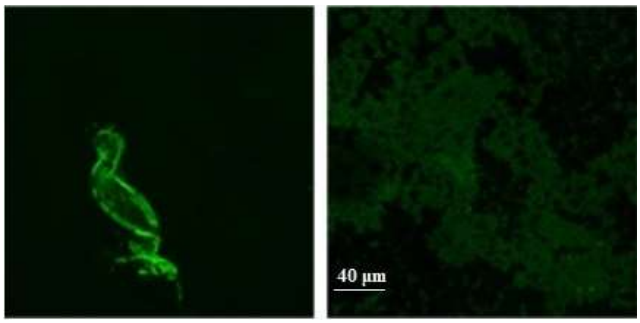

Figure 4. Confocal laser scanning microscope (CLSM) images of exopolysaccharide

4. CONCLUSION

Due to wide applications of EPS, it becomes important to determine different strains of LAB that can produce EPS with high yield and technological properties. This study was aimed to test *Lb. brevis* E25 for it's EPS production potential under varied incubation conditions and structural characterization of EPS produced. The LAB strain, initial pH of the medium, incubation temperature and time were shown to affect the EPS yield. Results indicated that the optimal incubation conditions based on all responses were found to be 35 °C, 18 hours and initial pH of 6.5 which resulted in an average production of EPS ranged from 10 to 35 gL^{-1}. Decreasing in EPS yield with the longer incubation times might be attributed to EPS production is growth-associated being thus primary metabolites. Their molecular weight was bigger than 10^5 Da. EPS samples are characterized by moderate thermostability making them promising candidates for further food applications. Further studies should be done to better evaluate the optimal conditions obtained from this study i.e. in some further cultivations.

4.1. Acknowledgements

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