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Production and characterization of a thermostable EPS produced by a new strain of Lactobacillus fermentum in medium containing sugarcane molasses

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ABSTRACT
The aim of this study was to identify a new bacterial strain, isolated as industrial alcoholic fermentation contaminant, and characterize chemically the EPS produced. The EPS yield was 44.5g/L in a molasses-sucrose medium under static fermentation conditions. Results of HPLC indicated that the main EPS sugars were glucose and galactose. FTIR and UV-vis spectroscopic analysis confirmed the presence of carboxyl and hydroxyl groups and absence of both proteins and nucleic acids. The structural characterization of EPS (^{1}H NMR and ^{13}C NMR) showed the form of α-glucopyranose rings linked by bonds, such as α-(1→6)-linked glucose, α-(1→2)-linked galactose, α-(1→3)-linked glucose and terminal branch α-(galactose). The chemical structure of the EPS was proposed on basis of these results. High thermal stability was observed by thermogravimetric analysis performed in a dynamic process suggesting the EPS potential application in the chemical, cosmetic, food and pharmaceutical industries. Molecular identification based on 16S rDNA sequencing showed that the strain was *Lactobacillus fermentum*, classified as GRAS. Sequence data were deposited with GenBank as accession number KY703871. The results indicated that the strain is a promising thermostable EPS producer that can promote a sustainable and economical fermentation process using sugarcane molasses as an alternative carbon source.

Keywords **-** Exopolysaccharide; *Lactobacillus fermentum*; sugarcane molasses; thermostability.

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I. INTRODUCTION

Microbial exopolysaccharides (EPSs) are polymers formed from sugars and their derivatives, presenting with large structural variety (e.g., size and branches) and chemical composition (e.g., homo - or heteropolysaccharide). The biotechnological functions and applications of EPSs are defined according to their structural properties [1, 2]. For example, their physicochemical properties render them useful as gelling agents, thickeners, and stabilizers in the food industry, used to improve the texture and viscosity of foodstuff [3]. A number of therapeutic effects have also been associated with EPSs, such as antitumour [4,5], antioxidant [6], immunomodulatory [7] and antimicrobial activities [8]. This biotechnological aptitude has promoted studies aimed at isolating bacterial strains to produce new EPSs, exploiting agro-industrial waste, reducing costs, and increasing productivity [9, 2].

Some strains of lactic acid bacteria, such as the genus *Lactobacillus*, produce EPSs used in the food industry. *Lactobacillus fermentum* is part of many food products and is considered as GRAS "Generally Recognized As Safe" organism (GRAS) by the US Food and Drug Administration (FDA) and is on the European Food Safety Authority's (EFSA 2011) list of biological agents with a "Qualified Presumption of Safety" (QPS) [10,11].

Species from genus *Lactobacillus* have also been identified as prevalent bacterial contaminants in ethanol fermentation. These bacteria are well adapted to stressful conditions, such as high ethanol concentration, low pH, low oxygen, and nutrient competition [12]. Consequently, the biosynthesis

and structural diversity of EPSs are influenced by ecological and environmental conditions, reflected in the survival of these bacteria [13].

L. fermentum is a beneficial microorganism found in many environments, foods, and in the human body [14, 15, 16, 17]. It has been described as able to protect the host from various diseases caused by pathogenic bacteria [18], inflammation [19] and oxidative stress [20], as well as having potential probiotic properties [21, 22]. The species is resistant to bile acids and salts, survives well in the gastrointestinal tract, and has the ability to produce antibacterial [21] and biosurfactant agents [23].

L. fermentum is recognized as a producer of EPSs with structural and chemical diversity [24, 25, 26, 27]. Depending on the substrate, the EPS produced by this bacterium may be composed of repeating units of glucose and galactose in the molar ratios of 5:2 [25], 4:3 [27] and 2.6:1 [24]. As well, EPSs contain units of rhamnose, raffinose, and maltose [26]. The research reported has shown the biotechnological importance of studying the production of EPSs using low-cost substrates.

In recent years, the industrial application of bacterial production of polysaccharides is increasing and with this the attempt to find new products.

For all the reasons cited above, the aim of this work was the isolation and chemical characterization of the EPS produced by a new strain of bacterium isolated as a contaminant from an ethanol fermentation industrial plant, as well as identification of the producer specie.

II. MATERIAL AND METHODS

2.1 Microorganism isolation, molecular identification and culture growth conditions

Several samples of industrial fermentation for ethanol production were collected from a sugar and alcohol plant in the state of Pernambuco-Brazil in order to study contaminants. In these samples, one bacterium attracted attention for its special characteristics. This bacterium, denominated X1, was growing in a Molasses-Sucrose-Agar medium, forming large, light yellow colonies with cartilaginous appearance. It was quite resistant to contact with the inoculation loop and looked like hard rubber, extremely suggestive of an uncommon polysaccharide. When the strain was cultivated in a fluid sugarcane molasses-based medium, a large amount of a gel with high viscosity was produced.

Isolation of the strain was proposed using a Molasses-Sucrose-Agar medium (called MSA medium), containing(g/L): molasses, 50 g; sucrose, 75 g; K_2HPO_4,1 g; $(NH_4)_2SO_4$, 1 g; yeast extract, 1.5 g; peptone, 1.5 g; and agar, 20 g; pH 6.5; and kept at 35 °C for 48 h of incubation.

Microscopic observations and biochemical tests were performed to identify the bacterial genus of the strain [28]. After this, a 16S rDNA gene sequence analysis of X1 strain was carried out to identify the bacterium specie.

Total genomic DNA was extracted from the isolated bacterium [29] for molecular identification by 16S ribosomal DNA (16S rDNA) sequence analysis, using the universal primers fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC)[30]. The specific nucleotide sequence was compared against sequences in the GenBank public database (http://www.ncbi.nlm.nih.gov/BLAST/).

In order to find a suitable carbon source for the bacterial growth and EPS production, a qualitative screening using glucose, sucrose, fructose, glucose-fructose mix was carried out. These qualitative tests to verify the use of carbon sources to the EPS production were developed in Petri dishes in triplicate with the base culture medium (1.0 g/L K_2HPO_4, 1.0 g/L $(NH_4)_2SO_4$, 1.5 g/L yeast extract, 1.5 g/L peptone, and 20 g/L agar) with each dish containing a different carbon source: glucose (100 g/L), fructose (100 g/L), sucrose (100 g/L), or glucose-fructose (50 g/L each). This test enabled observation of which sugars provided good strain growth and production of the polysaccharide. That is, to see if growth and production were taking place only in sucrose, glucose, or fructose; or growth and production took place in a mixture of glucose and fructose. This simple experiment provided preliminary information not only about the most appropriate sugar for the EPS synthesis, but also orientation for which alternative sources of wastes to test. After 24 h of incubation at 35 °C in different carbon sources, the growth and morphology of the colonies were evaluated. The superior carbon source was selected for use in the further studies.

2.2 Production, recovery and purification of EPS

The inoculum of cells was produced using a MS (molasses-sucrose) medium (composition g/L): 50 molasses, 75 sucrose, 1 K_2HPO_4, 1 $(NH_4)_2SO_4$, 1.5 yeast extract and 1.5 peptone) and cultured at 35 °C for 24 h.

L. fermentum is considered facultative anaerobic bacteria and consequently, fermentation studies were carried out to verify the influence of stirring on the growth and yield of EPS from L. fermentum X1 strain in a MS medium.

The EPS production was carried out in MS medium, in batch cultures in cylindrical borosilicate flasks with wide mouth, capacity 500 mL with 300 mL of working volume. The scale-up factor of 10 (i.e. inoculum of 10 % v/v) was adopted. To test the performance of growth and production under static and shaking conditions, flasks were incubated aerobically in a shaking orbital incubator set at 210 rpm (New Brunswick Scientific C25KC) and in a

nonshaking incubator, in triplicate at 35 °C for 72 h. Samples were taken from the flasks every 24 h to measure pH, cell biomass (by dry weight cells), sugar (glucose, fructose and sucrose by HPLC) and EPS concentrations (by dry weight).

The biopolymer was isolated and purified according to the procedure described in a previous paper [5] with modifications, as follows: the fermentation broth was filtrated through cellulose membranes (0.22 μm), and the EPS-containing supernatant was precipitated by ethanol at a low temperature (4 °C), at the molar ratio of 1:3. The mixture was centrifuged at 13600×g for 10 min, and then the EPS-containing precipitate was washed twice with cold ethanol, followed by three washes with acetone. The sample was dried under vacuum for 12 h and then stored at –4 °C. The EPS concentration (g/L) was estimated by dry weight.

2.3 EPS characterization

Sugars (i.e., glucose, fructose, and sucrose) in the supernatant cell-free culture were analysed using high-performance liquid chromatography (HPLC) coupled with a refractive index detector and a degasser (1100 series; Agilent Technologies, Santa Clara, CA). The column used was Aminex HPX-87HX (Bio-Rad Laboratories, Hercules, CA), measuring 300×7.8 mm; the mobile phase consisted of 5 mmol/L sulfuric acid, at a flow rate of 0.6 mL/min; and the temperature was set at 60 °C. The mobile phase was prepared with ultrapure water and then filtered through a 0.22 μm microporous membrane. Glucose, galactose, fructose, sucrose, rhamnose were used as standard monosaccharides.
All samples and standards were first diluted in the mobile phase solution and then filtered through a 0.22 μm membrane, after which 5 μL was injected into the column. Samples of EPS (2 mg) were treated with 250 μL of 2 mol/L trifluoroacetic acid for 5 h in a 100 °C water bath. Then, the solutions were concentrated using a rotary evaporator [31].

Ultraviolet–visible absorption spectra were obtained from a 3.6 mg/mL aqueous solution of EPS, using an Agilent 8453 UV-Vis spectrophotometer, at a wavelength range between 200 and 800 nm. An amount of 2 mg of EPS was mixed with 100 mg of potassium bromide and the mixture was pressed to form pellets suitable for measurements by Fourier transform infrared (FT-IR) spectroscopy. The spectrum was obtained using a Varian 640-IR FTIR spectrometer, at a wavelength ranging from 4000 to 400 cm^{-1}, with a spectral resolution of 4 cm^{-1}. The EPS structure was determined using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. A Varian Unity Plus 300 MHz spectrometer was employed at a frequency of 300 MHz for ^{1}H and 75 MHz for ^{13}C.

Thermogravimetric analysis and a differential scanning calorimetry of the EPSs were carried out using the thermal analysers NETZSCH STA 449 F3 Jupiter and Shimadzu DSC 60, respectively. The analytical conditions were 10 mg of EPS, platinum crucible (in the case of TGA) and aluminium pans (in the case of DSC), temperature from 30 to 500 °C, and under argon flow rate of 50 mL/min and heating rate of 5 °C/min. In order to determine changes in the physical properties of the EPS, a Fisatom 430 melting point apparatus (230 V, 60 Hz, and 50 W) was used to determine the melting point or thermal decomposition of the sample.

III. RESULTS AND DISCUSSION

3.1 Bacterium identification and EPS production

The X1 strain was isolated in a pure culture and cultivated in Molasses-Sucrose-Agar medium, and stored at 4 °C.

Microscopic observations and biochemical tests showed that the isolated bacterium belonged to the genus *Lactobacillus* [28].

A 16S rDNA gene sequence analysis of the X1 strain was carried out to identify the bacterium. When its gene sequence (1500 base pairs, PCR amplified) was compared with sequence data deposited in the GenBank database, 99% of similarity was observed with several strains of *L. fermentum*, such as *L. fermentum* strain LBBY024 (GenBankAccession Number: HM101305.1). As result, this strain was determined as *L. fermentum* and the sequence data was deposited in the GenBank as Accession Number KY703871.

After the molecular identification, the strain X1 was deposited in the "Microorganism Culture Collection of the Antibiotic Department of Federal University of Pernambuco", official Collection registered in the WFCC – World Federation for Culture Collections, with the number 114. The deposited strain is registered in the "UFPEDA Collection" as *L. fermentum* UFPEDA 1059B.

Lactobacilli belong to the Firmicutes phylum, a large heterogeneous group of Gram-positive prokaryotes considered facultative anaerobic bacteria with fermentative metabolism [28].

In spite of being found in different environmental niches (from water, soil and sewage to the commensal microbiota of plants and humans), they are considered fastidious from the point of view of nutritional requirements [32].

They are able to produce unique EPSs with particular properties. Nowadays, bacterial EPSs are seen as alternatives for polysaccharides produced by plants, seaweeds or by chemical synthesis for use in many sectors, such as pharmacy, foods, medicine, biotechnology and cosmetics. EPSs from lactic acid bacteria (LAB), which generally have a GRAS

status, play an important role in food industry producing fermented dairy products (yoghurt, cheese and milk-based products and more) [33]. Moreover, recent studies have demonstrated a wide variety of biological effects exhibited by lactobacilli-produced exopolysaccharides, such as: prebiotic potential, antitumour and immunomodulatory activity, antioxidant properties, anti-atherosclerotic effects, heavy metal and mutagen binding capacities [32].

A drawback for the EPS-LAB industrial use is in the small amount produced by the majority of strains. There are studies comparing lactobacilli EPSs production that report these amounts: 0.599g/L by *L. plantarum* KX041, 1.5 g/L by *L. plantarum*, 0.2 g/L from *L. paracasei* HCT, 0.26 g/L from *L. plantarum* YML009, 0.4 g/L from *L. rhamnosus* [33]. Many others authors have also reported yields for bacterial EPSs from approximately 80 mg/L to 2.5 g/L of culture media. This makes its use difficult in large scale production for commercial proposes [32].

In the qualitative screening using different carbon sources (glucose, sucrose, fructose, glucose-fructose mix), we observed growth in all carbon sources, but EPS was only produced in media with sucrose. In a sucrose medium the colonies grow large, lumpy and yellowish, covered by the polysaccharide. In the other media with glucose, fructose, or a glucose-fructose mix, the colonies were very small spots, like small points, without EPS. Therefore, sucrose was selected as the carbon source for the further studies.

It is also well known that *L. fermentum* has the ability to grow in different carbon sources (such glucose, lactose), however sucrose appears to be an inducer for the production of EPS [34]) for lactic acid bacteria [34]. In fact, the medium composition (carbon sources, nitrogen) and the culture conditions (such as temperature, pH, time) play crucial roles in EPS yield and its properties [35, 36]. Although the effect of carbon sources on the EPS composition is not clear, it is obvious that the preference for certain sugars that lead to maximum polysaccharide yield is strain dependent [31].

The MRS culture medium is widely used for the production of EPS by lactic acid bacteria (LAB), however, it contains components (yeast extract, protease peptone) that may interfere in the quantification, composition and structural analysis of EPS [37]. Besides, these components are relatively expensive for large scale production, whereas sugarcane molasses is considered a nutritionally rich, cheap substrate and is an abundant by-product of the sugar and ethanol production industry in Brazil.

The selection criteria in the fermentation process, microorganism efficiency, and low-cost substrates are indispensable to making any biotechnological process feasible [36]. Thus, a significant effort has been devoted to studies promoting the development of cost-effective and efficient EPS yield processes, including investigation of the potential use of agro-industrial waste (e.g., molasses and sugarcane syrup) as cheaper fermentation substrates [9].

The results showed that the biomass and the EPS concentration per litre of MS-medium were higher in the static culture (biomass: 7.37 g/L; EPS yield: 44.5 g/L) than in the stirred fermentation culture (biomass: 2.37 g/L; EPS yield: 8.0 g/L). Other authors found no influence of stirring on the EPS yield from *L. fermentum* grown in a chemically defined medium [35]. The maximum productivity (Qp) and maximum product yield from substrate ($Y_{P/S}$) were observed after 24 h, both for static (Qp 1.497 g·L-1·h-1; $Y_{P/S}$ 0.417 g/g) and stirred fermentations (Qp 0.283 g·L-1·h-1; $Y_{P/S}$ 0.128 g/g). With regard to the product yield from biomass ($Y_{P/X}$), the maximum values were 11.279 g/g for the static fermentation after 48h and 4.295 g/g for the stirred fermentation after 24 h (Table 1).

Table 1. Biomass concentration (X) and EPS concentration in grams per litre of fermented medium; productivity (Qp), yield of EPS from sucrose substrate ($Y_{P/S}$), and yield of EPS from biomass ($Y_{P/X}$), with L. fermentum, during 72 h, under stirred (210 rpm) and static conditions.

Time	*X g/L*		*EPS g/L*		Q_p (g·L⁻¹·h⁻¹)		$Y_{P/S}$ (g/g)		$Y_{P/X}$(g/g)	
	Static	Stirred	Static	Stirred	Static	Stirred	Static	Stirred	Static	Stirred
24 h					1.497	0.283	0.417	0.128	7.840	4.295
48 h					0.793	0.129	0.354	0.096	11.279	3.235
72 h	7.37	2.37	44.5	8.0	0.562	0.108	0.369	0.115	5.487	3.284

Aerobic growth may promote oxidative stress and cellular damage in lactic acid bacteria due to the production of reactive oxygen species (ROS; hydrogen peroxide, H_2O_2; superoxide anion, O_2–; hydroxyl radical, OH•). Accumulation of ROS may reduce the survival and fitness metabolic of LAB and may induce the production of undesirable compounds in fermented foods [38].

The yield of hetero EPS in Lactobacillus species is quite diverse. For example, L. fermentum TDS030603 showed a yield of crude EPS 586.6 mg/L and 151.2 mg/L of purified EPS in a MRS medium (30°C, 72h) [25]. Crude EPS production by three strains of L. fermentum isolated from Burkina Faso fermented milk ranged, 322 to 713mg/mL in a MRS medium at 35°C [39], although these results were obtained under optimized culture conditions. These results can be considered low from an industrial point of view.

L. fermentum Lf2 is a strain which was isolated as a contaminant culture in cheese manufacture and cited as producing high levels (approximately 1g/L) of crude EPS when grown in

semi-defined broth [40]. Although many of these results were obtained under optimized culture conditions, however, we can consider these low yields when compared to those obtained in our research with *L. fermentum* X1. The best results found in the literature, refer to yields of EPSs at 4.6 g/L [41] and 28.85 g/L [26].

It seems that the supplementation of the fermentation medium plays a very important role in the production of EPS by *L. fermentum*. A number of studies concerned with fermentation optimisation have demonstrated that substrate composition has a major influence on EPS production. Additionally, other growth conditions, such as pH, temperature, salt concentrations and bacterial microencapsulation, can all be manipulated to increase EPS production. Future experiments need to be carried out to explore these fermentation parameters.

In summary, comparing our results with those in the literature review, we can say that the *L. fermentum* X1 strain presents great advantages, with EPS concentrations around 44.5 g/L (without optimisation of the process), using a fermentation medium with molasses that is considered cheaper as raw material, being a waste from the biotechnological industry. Carbon sources represent the major cost of the bioprocess. It is well known that agro-industrial by-products can be used as an alternative to reduce fermentation costs. Molasses is attractive economically and contributes to economic viability on an industrial scale.

3.2 EPS characterization

The thermal behaviour of an EPS plays a fundamental role in its industrial exploitation [8]. Thermogravimetric analysis of the EPS, from *L. fermentum* X1, was carried out to determine its thermal characteristics of weight loss and confronted with DSC events themselves (Fig. 1).

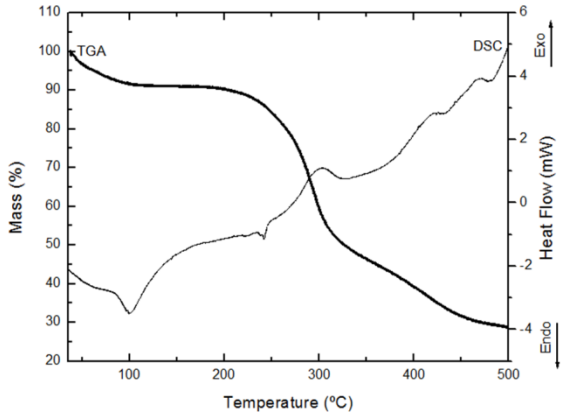

Fig.1. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of the EPS from *L. fermentum* X1.

The first step in EPS degradation occurred between 38.9 °C and 99 °C, with a weight loss of 8.43%, which is associated with loss of moisture, suggesting that the EPS was not entirely anhydrous. Approximately 90% of the EPS weight remained stable up to 200 °C, suggesting that the polymer should not be submitted to temperatures higher than this, i.e. physical integrity not affected. Subsequently, the EPS mass decreased gradually until a final temperature of 500 °C, leaving a residue of 28.8% of its initial mass. The melting point of the EPS from *L. fermentum* was at 225 °C (showed by melting point apparatus), and confirmed by TGA and DSC.

Figure 1 demonstrates that the polymer showed two stages of mass loss: a first stage between 40 and 100 °C, due to the loss of the moisture content and a second stage with a degradation temperature (Td) of greater than 240 °C. The weight of the polymer was reduced dramatically at about 300°C after which it decreased gradually. The results were obtained by TGA in an inert atmosphere. The data showed that at the evaluated temperatures for the thermal behaviour of the polymer remained stable, with mass-loss activity only the mass-loss in the first reaction, due to the vaporization of the water.

The EPS from *L. fermentum* X1 was chemically analysed using HPLC. The major constituents were two residues of sugar; namely, glucose and galactose at a ratio of 1.15:1, at the retention time of 9.202 and 9.858 min, respectively. No other monosaccharide was detected among the analysed standards. Glucose was the most abundant monosaccharide, accounting for 53 mol% of the carbohydrate content of EPS, whereas galactose comprised about 47 mol%.

Glucose and galactose residues in microbial EPSs are quite common, although they may be found in varying amounts [42, 27]. The EPS analysed from *L. fermentum* F6 produced in a milk-based medium supplemented with 2% of glucose, galactose, fructose, and lactose, and found the characteristic HPLC peaks of galactose and glucose at a molar ratio of 4:3. EPS produced by *L. fermentum* MR3 composed predominantly of galactose (99.2%) and glucose (0.8%) was described by [39]. Similar to this study, EPS with a higher glucose : galactose ratio were described [43], with a ratio of 2:1, and with molar ratio ranging from 2.6 to 2.8 depending on the culture medium used [31].

The ultraviolet-visible spectrum of the EPS, obtained from *L. fermentum*, showed no absorption at 260 and 280 nm, indicating the absence of nucleic acids (DNA residues) and proteins, respectively, in the polymer sample. Because no absorption bands were found in the sample, its molar absorption coefficient could not be determined. In addition, no chromophore groups were found, nor the occurrence of any possible conjugated unsaturated chain.

The FTIR (Fourier Transform InfraRed) spectrum of the EPS (Fig. 2) showed several absorption peaks between 4000 and 400 cm^{-1}.

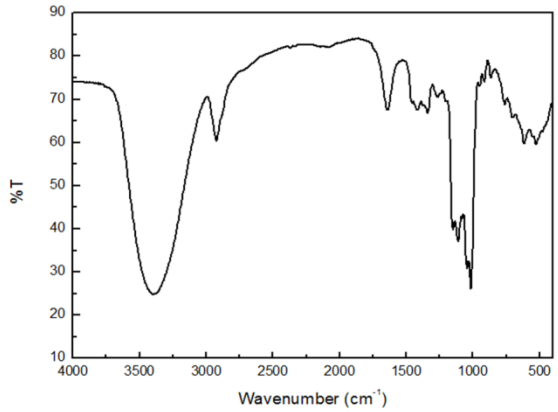

Fig**. 2.** FTIR characteristics peaks of the EPS produced by *L. fermentum* X1 (EPS in KBr tablet).

The presence of high frequency levels of vibration in the hydroxyl group (-OH) was identified by the broad absorption peak at around 3000–3750 cm^{-1}, confirming the nature of the polysaccharide polymer. The absorption peak at 2923 cm^{-1} was due to C-H axial stretching. The absorption band at 1643 cm^{-1} probably represents adsorbed water molecules in the polymer chain; the band at around 1416 cm^{-1} refers to C-C stretching; the band at 1338 cm^{-1} was due to C-O angular deformation and the band at 1010 cm^{-1} was attributed to C-O-C axial deformation, which is consistent with the flexibility properties around the α-(1→ 6) bond of EPS [44].
Researchers in a previous study produced and characterized four different lactic acid bacteria: *Lactobacillus suebicus*, *Leuconostoc mesenteroides*, and two other strains used for production of β-glucans, *Pediococcus parvulus* and a recombinant *Lactococcus lactis*. Among these, *L. lactis* showed bands similar to those found in the polysaccharide produced by our strain. β-glucans produced by *L. lactis* showed bands similar to those found in the polysaccharide from *L. fermentum* in the work at: 3393, 2915, 1639, 1423, 1377, 1079, 1033, and 894 cm^{-1}. The absorption band at around 890 cm^{-1}, not found in the polysaccharide studied, is attributed to β-anomers [45].

Others authors have analysed the infrared spectrum of a purified EPS from *Lactobacillus kefiranofaciens* ZW3, showing its functional properties. The strong absorption band at 1067 cm-1indicated that the substance was a polysaccharide [46].

^{1}H NMR is mostly used to characterize the configuration of the glycosidic bond in polyssacharides [47].

The ^{1}H NMR spectrum of the EPS from L. fermentum(Fig. 3) showed a signal at δ4.96 ppm, corresponding to the protons of the first carbon monosaccharide unit (C1), suggesting a (1→6) bond at the glycosidic residue in the main chain. Additionally, there were three signals between δ4.0 and δ3.5 ppm, suggesting protons in the sugar ring, which is typically found in polysaccharides.

According to the results of the ^{1}H NMR reported [31], the signals at δ4.978 and δ5.314 ppm can be attributed to a glucose α-anomeric configuration. This was similar to the EPS in our study, showing the same characteristic of ^{1}H NMR signals with a similar peak. This leads to the conclusion that all of the monomeric units are connected by alpha bonds.

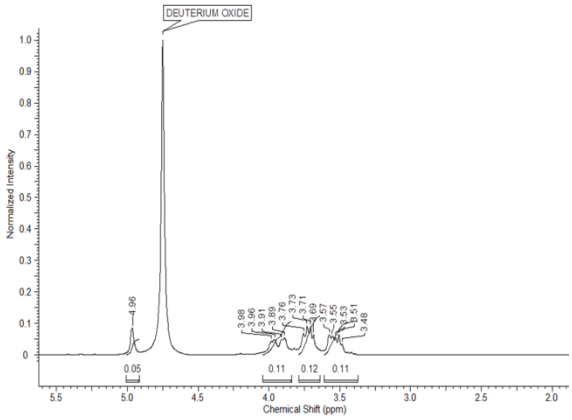

Fig. 3.^{1}H NMR spectrum of the EPS from *L. fermentum* X1 (Solvent D_2O, 300MHz).

The ^{13}C NMR spectrum of EPS (Fig. 4) showed chemical shift of an anomeric carbon at δ 97.66 ppm, indicating that the EPS had an all-glycosidic bond in an α-anomeric configuration, which was also attributed to the α-(1→6) bond. In addition, seven signals were included, corresponding to regions of carbon rings (δ 50–85 ppm), which was the same linking pattern (1→6) found in the EPS from lactic acid bacteria. The same linking pattern (1→6) was found in the EPS produced by *Leuconostoc lactis* KC117496 isolated from idli batter [48]. In addition, the signal at around δ 65.52 ppm may indicate a change in the C-6 bond of glucose, suggesting that O-6 was substituted [42].

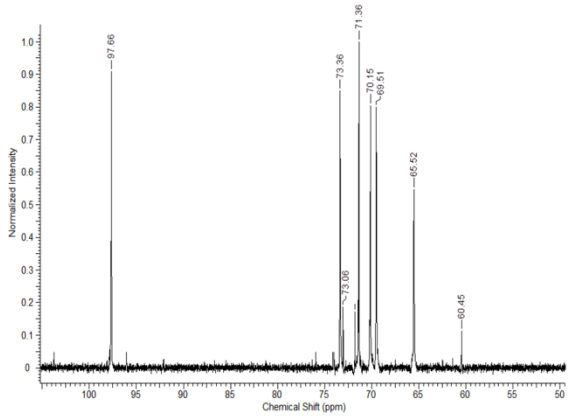

Fig. 4.^{13}C NMR spectrum of the EPS from *L. fermentum* X1 (Solvent D_2O, 75 MHz).

The results from spectroscopy and chromatography results lead to the conclusion that the main EPS components were glucose and galactose, in the form of α-glucopyranose rings linked by bonds, such as α-(1→6)-linked glucose, α-(1→2)-linked galactose, α-(1→3)-linked glucose and terminal branch α-(galactose), as confirmed by the signals at δ 97.66, δ 73.36, and δ 73.06 ppm, respectively. All of these data corroborate the structural formula proposed of the EPS, showed in (Fig. 5).

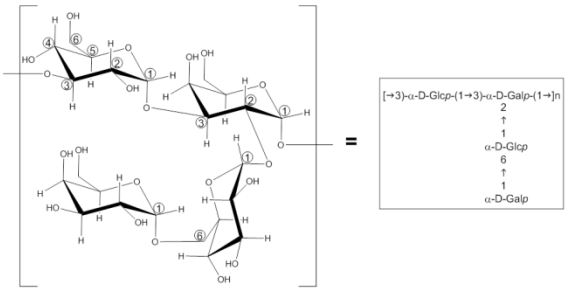

Fig. 5. Structural formula proposed of the EPS produced by *L. fermentum* X1.

IV. CONCLUSION

The EPS produced in this study and identified as a LAB from the specie *L. fermentum* deposited in the "UFPEDA Collection" as *L. fermentum* UFPEDA 1059B can be considered as having a GRAS status, which means, theoretically, it can be used as a probiotic or in industries such as food, medicine, biotechnology and cosmetics. EPSs are non-toxic natural products that can be utilised in modern medicine, thus meriting, future investigations to explore the efficacy of this EPSs of *L. fermentum* X1 for a wide variety of biological activities.

It is pointed, that a major obstacle in the utilisation of EPSs in commercial products is the relatively low levels produced by the majority of bacteria. This is not a problem for the use of *L. fermentum* X1, considering that its yield of EPS is superior to many other strains cited in the literature. It is important to remember that yield of EPS with X1 was obtained in the fermentation without an optimization process, in a medium with molasses. This waste from the biotechnological industry represents an inexpensive alternative source of nutrient.

The results of thermogravimetry indicated a thermostable polymer, providing opportunities for future research.

Taking all the results together, we can say that X1 is a promising and economically viable producer of EPS. These first studies represent an important point for an EPS future commercial application, besides other characteristics or biological activities that it may present.

The isolated EPS was characterized as a heteropolysaccharide consisting of glucose and galactose (in a molar ratio of 1.15:1. In the literature review, we did not find any reports of EPSs with an equal chemical structure suggesting that it is a novel LAB EPS. Moreover, to the best of our knowledge, the production and chemical characterization, with the structural formula proposed, of an EPS of *L. fermentum* produced in sugarcane molasses, have not been carried out before.

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