Carbohydrate Polymers 107 (2014) 1–6

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Characterisation of a new exopolysaccharide obtained from of fermented kefir grains in soymilk

Priscilla S. Botelho[a], Maria I.S. Maciel[a,*], Luciano A. Bueno[a], Maria de Fátima F. Marques[b], Djalma N. Marques[b], Tania M. Sarmento Silva[a]

[a] Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros, S/N, Dois Irmãos, Recife CEP: 52171-900, Brazil
[b] BioLogicus-Ind. e Com. de Produtos Naturais S.A., Av. Prof. Luiz Freire, 700, Cidade Universitária, Recife CEP: 50740-540, Brazil

ARTICLE INFO

*Article history:*
Received 1 November 2013
Received in revised form 6 February 2014
Accepted 11 February 2014
Available online 20 February 2014

*Keywords:*
Microbial polysaccharides
Thermal analysis
Structural characterisation

ABSTRACT

Microbial exopolysaccharides (EPSs) have been widely studied in recent decades due to similarity to gums used in the food industry. Exopolysaccharides can be used in food processing as a thickener and/or stabiliser. This study aimed to investigate the physicochemical properties, thermal behaviour and structural composition of the lyophilised EPS obtained from the fermentation of kefir grains in soymilk. The EPS in concentration 18 mg/mL exhibited water activity of 0.204 and pH = 6.20 at 25 °C, reducing sugars content of 22.10% (v/v) and protein content of 2% (v/v). The thermogravimetric curve obtained was similar to those reported in the literature for other EPSs. The degradation temperature was 351.84 °C and showed that the EPS in this study had a high thermal stability. Characteristic polysaccharide bands were observed in the infrared spectrum. The analysis by liquid chromatography coupled to electrospray ionisation mass spectrometry (LC–ESI-MS) showed that the EPS is only composed of glucose.



## 1. Introduction

Exopolysaccharides (EPSs), also called extracellular polysaccharides, are defined as carbohydrate polymers that can be produced by widely different micro-organisms. These biopolymers are associated with the cell surfaces in the form of capsules or are secreted into the extracellular medium in the form of mucus. Microalgae and bacteria produce large amounts of EPSs, while yeasts and fungi produce small amounts. Some micro-organisms produce EPSs in amounts satisfactory for use on an industrial scale, including curdlan, xanthan and dextran gums (Silva et al., 2006; Sutherland, 1990).

Exopolysaccharides are water soluble gums that have long linear or branched chains consisting of monosaccharides, mainly glucose, galactose, mannose and glucuronic acid or derivatives of these sugars such as rhamnose, in different proportions (Welman and Maddox, 2003). These biopolymers are used as emulsifiers, stabilisers, gelling agents, binders, lubricants, coagulants and thickeners, and they are also used in the production of suspensions and films. These applications rely on the chemical structure of the biopolymers. The food industry has widely used these biopolymers, especially to alter the texture, viscosity and elasticity of food (Duboc and Mollet, 2001; Rottava et al., 2009).

The EPS obtained from the fermentation of kefir grains in soymilk has been minimally studied. In a previous study, it was observed that this EPS from the fermentation of soymilk is composed of the monomers glucose and galactose in the ratio of 1.00:0.43. This composition was compared to the monosaccharide composition of the polysaccharide obtained from kefir in cow's milk, which has a ratio of 1.00:1.14 (glucose:galactose) (Liu, Chen, and Lin, 2002). Using the above information as a starting point, this study aimed to verify some of the physicochemical properties of the EPS extracted from fermented kefir grains in soymilk and structurally characterise this EPS for possible further applications in the food and cosmetic industries.

## 2. Methods

### 2.1. Materials

The glucose, anthrone reagent and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Saint Louis, MO, USA).

### 2.2. Obtaining the exopolysaccharide (EPS)

The EPS was obtained from the commercial kefir product called Kefir BioLogicus® Ind. e Com. de Produtos Naturais S.A., which is located in the city of Recife, State of Pernambuco, Brazil. The extraction was performed according to the procedure used by Yokoi,

* Corresponding author. Tel.: +55 81 33206536.
*E-mail addresses:* m.inesdcd@gmail.com, m.ines@dcd.ufrpe.br (M.I.S. Maciel).

http://dx.doi.org/10.1016/j.carbpol.2014.02.036
0144-8617/

Watanabe, Fujii, Toba, and Adachi (1990), with some modifications. The obtained solution was precipitated with cold ethanol and centrifuged in a refrigerated CIENTEC CT-6000R 115 mm centrifuge (10,000 × g, 20 min, 20 °C) to remove most other compounds present in the solution and obtain only the EPS. The obtained EPS was then subjected to lyophilisation. The lyophilised EPS was macerated and stored under refrigeration at 5 °C for further analysis.

### 2.3. Physicochemical analysis

The concentrations of the reducing sugars were determined by the method of Antrona (Yemm and Folkes,1954), using glucose as a standard. The protein content was verified by the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard. The water activity ($A_w$) of the lyophilised EPS was determined using a Water Activity AquaLab 4TEV meter. The pH of the solution containing the EPS at a concentration of 10% (w/v) was read directly by a Tecnal pH meter, using buffers with pH values of 4 and 7 as references.

### 2.4. Infrared (IR) spectroscopic analysis

The IR spectra were obtained by a 640-IR spectrometer (Varian), using the total internal reflection or attenuated total reflectance (ATR) technique. The spectra were recorded over the wavenumber interval of 600–4000 $cm^{-1}$, using 32 scans to achieve the best signal noise ratio.

### 2.5. Thermogravimetric analysis (TGA)

To study the thermal properties of the obtained EPS, we used a SHIMADZU TGA system under atmospheric pressure. A sample of 5.5 mg of EPS was placed in a Pt crucible. The system was programmed with a heating rate of 10 °C/min under a $N_2$ atmosphere, a flow rate of 50 mL/min and a sweep range from 25 to 600 °C.

### 2.6. Liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS)

The monosaccharide composition of the EPS was analysed by LC–ESI-MS using an Esquire 3000 Plus chromatograph (Bruker Daltonics) with a capillarity of 4000 V, a gas flow of 5 L/min, a temperature of 320 °C and a pressure of 12 psi. The EPS was hydrolysed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h and derivatised with *p*-aminobenzoic acid ethyl ester (ABEE) (Gomis, Tamayo, and Alonso, 2001; Kwon and Kim, 1993) before being subjected to LC–ESI-MS.

## 3. Results and discussion

### 3.1. Physicochemical analysis

The pH of the EPS solution was slightly acidic but near neutral (pH = 6.20 ± 0.05). The pH of kefir reported in the literature is between 4.2 and 4.6. This lower pH value can be influenced by the other constituents present, such as $CO_2$, acids, lactose, ethanol and the amounts and types of protein and the fat content (Dadkhah, Pourahmad, Assadi, and Moghimi, 2011; Irigoyen, Arana, Casteilla, Torre, and Ibanez, 2005; Kesenkaş et al., 2011). The difference between the pH values of the EPS solution and the kefir is most likely related to the extraction process. The extraction process removes compounds that decrease the pH, such as $CO_2$ and lactic acid, which are present in the fermented beverage.

The EPS showed an $A_w$ value of 0.204 ± 0.001 at 25 °C, which places it in zone A according to Fennema, Damodaran, and Parkin (2010). In this zone, food supplies have good shelf life stabilities and are therefore resistant to deterioration over time.

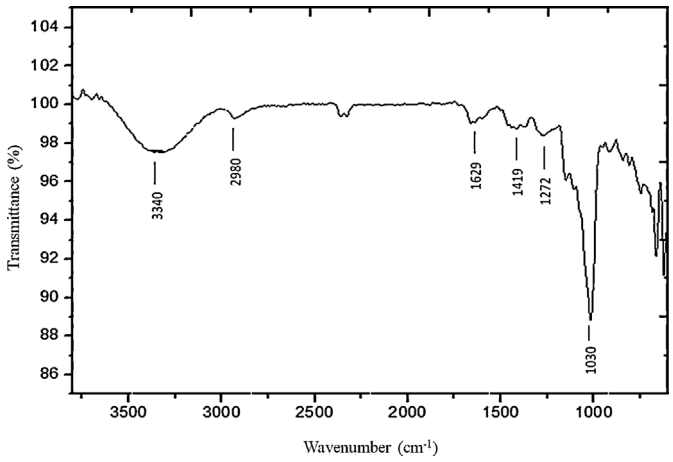


**Fig. 1.** Infrared absorption spectrum of EPS.

The protein analysis using the method described by Bradford (1976) showed that the EPS exhibited a low protein content of approximately 2% (v/v) of the total sample. Values near those found in this investigation (2.3% protein) were found by Liu et al. (2002) in the EPS obtained by the fermentation of kefir grains in soymilk after precipitation with ethanol and by Piermaria, La Canal, and Abraham (2008). Using the same method of analysis, Piermaria et al. (2008) also identified a low protein content for a solution of EPS obtained from the fermentation of kefir grains in bovine milk. The concentration of the reducing sugars or total soluble sugars (ATS) of the studied EPS was 221.03 mg/L, or 22.10% (v/v) of the sample. This result can be attributed to the small amount of terminal reducing sugars present in the EPS solution. This low reactivity can also be explained by the partial hydrolysis of the sugars using the proposed method of analysis, which is insufficient to break all of the glycosidic linkages present in the EPS.

### 3.2. IR spectroscopic analysis

In the IR spectrum shown in Fig. 1, a band was found at 3440 $cm^{-1}$ that corresponded to the hydroxyl groups (Bramhachari et al., 2007; Howe, Ishida, & Clark, 2002; Wang, Ahmed, Feng, Li, & Song, 2008; Wang et al., 2010). The band at 2980 $cm^{-1}$ can be attributed to methyl and methylene groups (Bramhachari et al., 2007; Wang et al., 2008, 2010). The bands in the range between 1660 and 1220 $cm^{-1}$ approximately, are asymmetric and symmetric characteristics stretching of links C—O (Haxaire, Maréchal, Milas, and Rinaudo, 2003; Wang et al., 2008, 2010). The region of 1000–1200 $cm^{-1}$ has showed an intense band which is characteristic of stretches C—O—C and C—O of alcohol groups in carbohydrates. The band at 1030 $cm^{-1}$ is characteristic of polysaccharide compounds (Haxaire et al., 2003; Wang et al., 2008, 2010). These bands indicate that the substance is a polysaccharide, according to the findings of Cho, Amy, Pellegrino, and Yoon (1998) and Nataraj, Schomacker, Kraume, Mishra, and Drews (2008).

### 3.3. Thermogravimetric analysis (TGA)

The literature reports that certain common substances, both volatile and non-volatile, are formed by the thermal degradation of different types of carbohydrates. According to Fagerson (1969), during the initial temperature increase, the primary events that appear in the thermograms are gelatinisation and swelling. Increasing the temperature further causes the following three events to occur: dehydration, pyrolysis and the reorganisation of linkages.

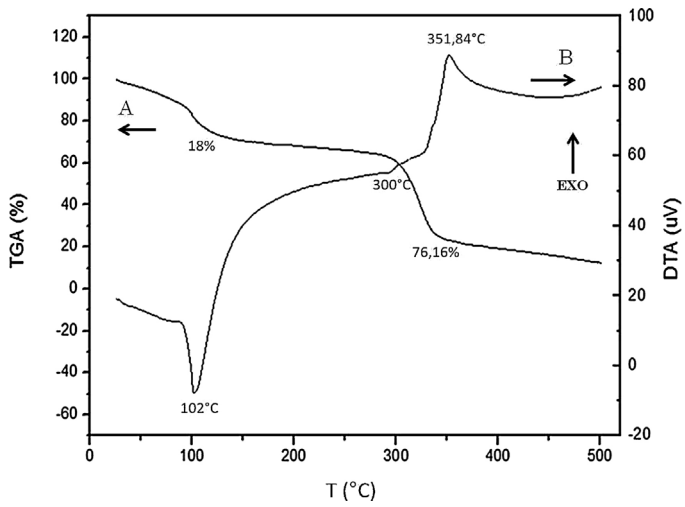


**Fig. 2.** TGA (A) and DTA (B) curves of EPS.

During these last three events, volatile and non-volatile products are generated by the breakdown of the connections in the polysaccharides and the formation of new bonds.

In the TGA EPS curve (Fig. 2), three events can be observed during the time band during which the temperature was increased. The first event occurred between 26 and 102 °C, with the maximum mass loss (approximately 18%) occurring at 101.65 °C. This first mass loss event may be associated with the loss of moisture (Marinho-Soriano and Bourret, 2005; Parikh and Madamwar, 2006; Wang et al., 2010).

From 101.65 °C upward, EPS began to release energy, and at a temperature of approximately 300 °C the second event occurred. This second event shows a release of energy that is represented by an exothermic peak with a maximum at 351.84 °C. At this point, a large mass loss (76.16% of the initial mass) can be observed. Then, the mass loss gradually decreased to 430 °C but continued to 500 °C, and at temperatures above 500 °C, degradation proceeded until 13% of the original mass remained. These results can be compared to the degradation temperature ($T_d$) and curve (TGA) observed by Parikh and Madamwar (2006) for the EPSs produced by the cyanobacteria *Oscillatoria* sp. and *Nostoc* sp. and for guar gum. The major mass loss events occurred over the temperature ranges of 350–490 °C (*Oscillatoria* sp.), 340–530 °C (*Nostoc* sp.) and 340–550 °C (guar gum). Compared to the results presented for ZW3 EPS (Ahmed, Wang, Anjum, Ahmad, and Khan, 2013) and EPS KF5 (Wang et al., 2010) it can be observed that the temperature of degradation ($T_d$) of the EPS in this study was slightly higher than the $T_d$ of the EPSs analysed by other authors. The EPS cited Zw3 and KF5, showed approximated temperature of degradation, with maximum at 299.62 °C and 278.53 °C, respectively, while there was a shift to $T_d$ of the EPS studied in this work, with maximum at 351.84 °C. However, the TGA profiles of the curves were the same. This difference between the $T_d$ values can be explained by the different structural compositions of the EPSs. The thermal characteristics of polysaccharides, such as the absorption and emission energies, are accompanied by physical changes, including the structural deformation or melting of the crystalline polysaccharide (Wang et al., 2008; Wang et al., 2010).

Due to the thermal properties of the EPS studied in this work, it can be said that it possesses good thermal stability for potential applications in industries where processing temperatures reach approximately 150 °C (Ahmed et al., 2013). The similarities between the presented EPS and the previously explored EPSs may be related to the monosaccharide compositions.

### 3.4. LC–ESI-MS analysis

LC–ESI-MS was carried out to determine the monomeric sugars that composed the EPS, and the results showed the presence of oligosaccharides composed of glucose molecules. The sequence of the sugars present in the composition of the EPS was determined according to their retention times. The chromatogram obtained after the hydrolysis of the EPS is shown in Fig. 3.

It can be observed that hydrolysis provided the greatest amount of glucose fragments (peak 4, Fig. 3). The next largest amount was observed for maltose fragments (two glucose molecules, peak 3, Fig. 3), and lower concentrations of other fragments occurred, with three or four glucose molecules.

The sequences observed for the carbohydrates in the LC–ESI-MS analysis are consistent with the observed retention times. The more polar compound represented by the first peak had a retention time that was shorter than that of the peak for compound 4, which was formed by a single derivative linked to glucose. The compound corresponding to peak 4 interacted more strongly with the nonpolar C-18 column than the compound of peak 1.

Peak 1 (retention time = 8.8 min) corresponds to four glucose molecules linked to benzocaine (Fig. 4).

The molecular ion is represented by $[M+1]^+$ or $[M+Na]^+$, the other fragments obtained are represented by letters in the sequence (A) to (H) and the cleavage sites are shown by arrows. The same type of representation is used to indicate structural cleavages in the other spectra. The suggested fragmentation pattern was determined by consulting the work of Stahl, Rocke, and Bayer (2002).

In the spectrum shown in Fig. 4, it can be observed that the molecular ion is related to four glucose molecules linked to benzocaine ($m/z$ = 814). Some fragmentations are represented by

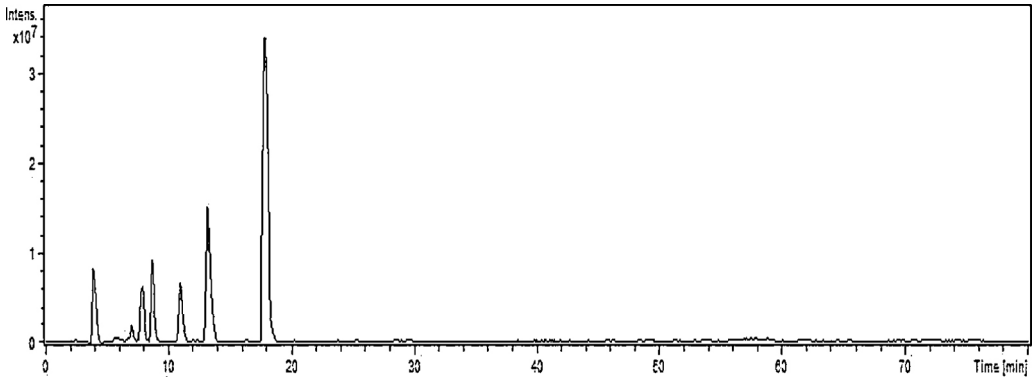


**Fig. 3.** Chromatogram of the derivatised EPS hydrolysates analysed by LC–ESI-MS.

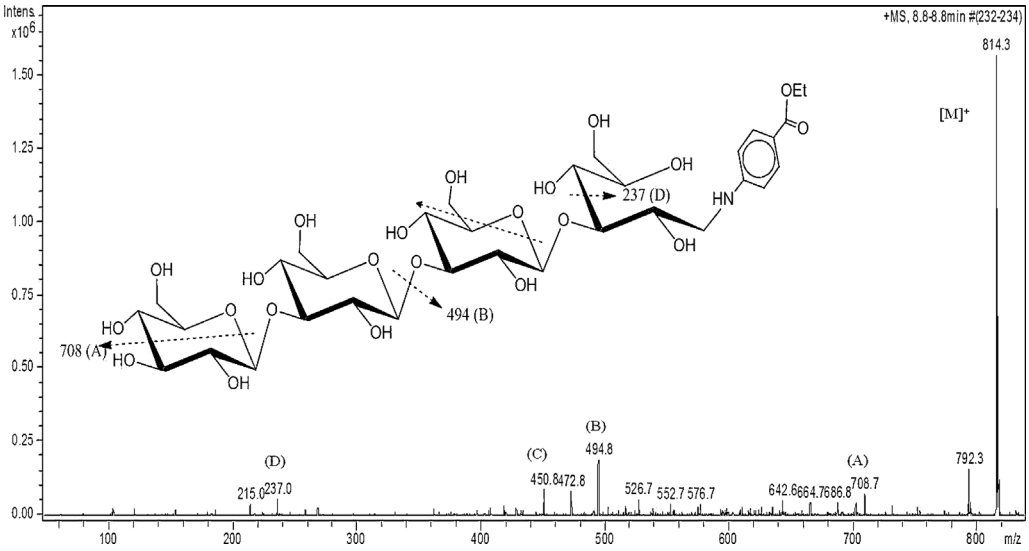


**Fig. 4.** Mass spectrum using electrospray ionisation for peak 4 present in the chromatogram of EPS, which eluted at 8.8 min.

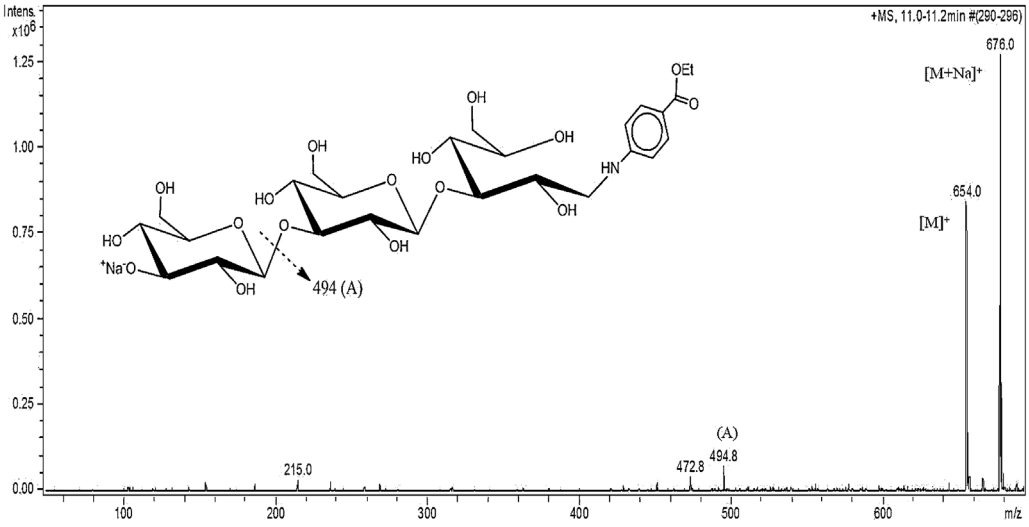


**Fig. 5.** Mass spectrum using electrospray ionisation for the peak present at 11.0 min in the chromatogram of EPS.

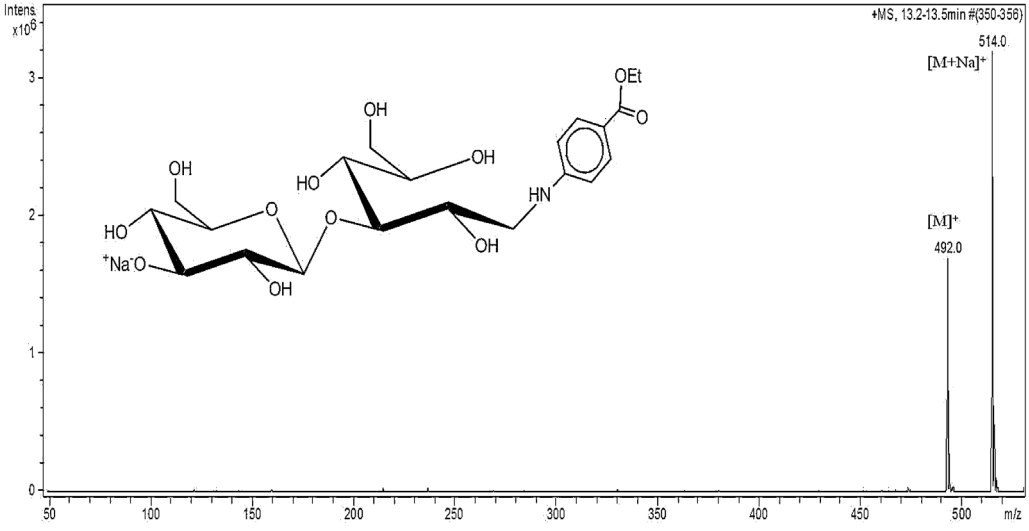


**Fig. 6.** Mass spectrum using electrospray ionisation for the peak present at 13.2 min in the chromatogram of EPS.

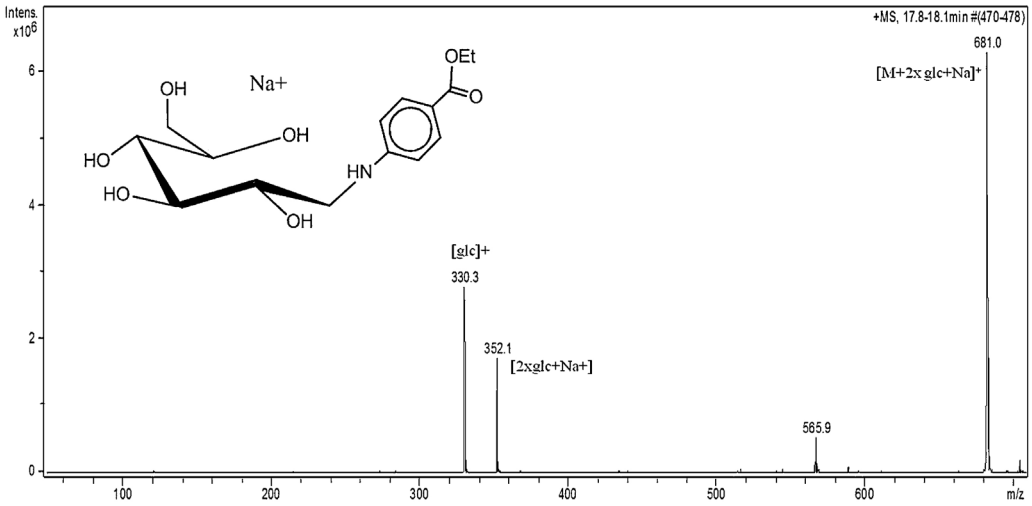


**Fig. 7.** Mass spectrum using electrospray ionisation for the peak present at 17.8 min in the chromatogram of EPS.

(A) to (D). In the first fragmentation (A), the intra-glycoside ring is cleaved, resulting in an ion with a $m/z$ of 708.

The second ion, represented by (B), results from the cleavage of the glycoside bond and leaves two glucose molecules linked to benzocaine. The peak at an $m/z$ value of 450 is represented by (C). In the third fragmentation represented by (D), a glycoside cleavage within the ring can also be observed producing an ion with an $m/z$ value of 237.

The peak at 11.0 min (peak 2, Fig. 3) shows the molecular ion bound to sodium $[M+Na]^+$, which has an $m/z$ value of 676, corresponding to three glucose molecules and the $Na^+$. The peak with an $m/z$ value of 494, represented by (A), indicates the cleavage of a glycosidic bond, forming a molecule with two glucoses linked to benzocaine (Fig. 5).

Therefore, for peak 5 (13.2 min), the observed value of $[M+Na]^+$ refers to two glucose molecules linked to a sodium ion with an $m/z$ value of 514. Once the molecular ion with an $m/z$ value of 492 was identified, the mass of the molecule without the sodium ion could be determined (Fig. 6).

The identity of the latter signal obtained using LC–ESI-MS, which was already labelled as glucose by the chromatographic analysis in this work and already cited works (HPLC-UV and HPLC-IR), was confirmed by the molecular ion in the mass spectrum obtained that is depicted in Fig. 7.

The molecular ion at an $m/z$ value of 681 $[M+2xglc+Na]^+$ represents a dimer formed by two glucose molecules bound to a sodium ion. The formation of dimers can occur due to a high concentration of glucose. This is the major peak observed in the chromatogram. The second peak, identified with $m/z$ 352, refers to a glucose molecule attached to benzocaine with the sodium ion. In this case, there was separation of the $[2xglc+Na]^+$ dimer. The third peak at an $m/z$ value of 330 refers to the glucose after losing the sodium ion.

It can be observed by LC–ESI-MS that the EPS molecule is a biopolymer composed of glucose molecules and that, after undergoing hydrolysis, the EPS fragments exist in the forms of mono-, bi-, tri- and tetrasaccharides.

## 4. Conclusion

In this work, a new EPS was obtained from soymilk fermented with kefir grains. The molecular structure of this EPS is composed of glucose units, which can be detected by LC–ESI-MS analysis. The physicochemical characteristics of this EPS showed similarity with other known polysaccharides, and an industrial use is visualised.

## Acknowledgments

This work was supported by the National Scientific and Technological Development (CNPq) and Science and Technology Foundation of Pernambuco State (FACEPE).

## References

Ahmed, Z., Wang, Y., Anjum, N., Ahmad, A., & Khan, S. T. (2013). Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir – Part II. *Food Hydrocolloids*, *30*, 343–350.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Bramhachari, P. V., Kavi, K. P. B., Ramadevi, R., Kumar, R., Rao, B. R., & Dubey, S. K. (2007). Isolation and characterization of mucous exopolysaccharide (EPS) produced by *Vibrio furnissii* strain VB0S3. *Journal of Microbiology and Biotechnology*, *17*, 14–51.

Cho, J., Amy, G., Pellegrino, J., & Yoon, Y. (1998). Characterization of clean and natural organic matter (NOM) fouled NF and UF membranes, and foulants characterization. *Desalination*, *118*, 101–108.

Dadkhah, S., Pourahmad, R., Assadi, M. M., & Moghimi, A. (2011). Kefir production from soymilk. *Annals of Biological Research*, *2*(6), 293–299.

Duboc, P., & Mollet, B. (2001). Applications of exopolysaccharides. *International Dairy Journal*, *11*, 759–768.

Fagerson, I. S. (1969). Thermal degradation of carbohydrates: A review. *Journal of Agricultural and Food Chemistry*, *17*(4), 747–750.

Fennema, O. R., Damodaran, S., & Parkin, K. L. (2010). *Química de Alimentos de Fennema*. Porto Alegre: Artmed.

Gomis, D. B., Tamayo, D. M., & Alonso, J. M. (2001). Determination of monosaccharides in cider by reversed-phase liquid chromatography. *Analytica Chimica Acta*, *436*, 173–180.

Haxaire, K., Maréchal, Y., Milas, M., & Rinaudo, M. (2003). Hydration of polysaccharide hyaluronan observed by IR spectrometry. I. Preliminary experiments and band assignments. *Biopolymers (Biospectroscopy)*, *72*, 10–20.

Howe, K. J., Ishida, K., & Clark, M. M. (2002). Use of ATR/FT-IR spectrometry to study fouling of microfiltration membranes by natural waters. *Desalination*, *147*, 251–255.

Irigoyen, A., Arana, I., Casteilla, M., Torre, P., & Ibanez, F. C. (2005). Microbiological, physicochemical and sensory characteristics of kefir during storage. *Food Chemistry*, *90*, 613–620.

Kesenkaş, H., Dđnkçđ, N., Seçkđn, K., Kinik, O., Gönç, S., Ergönül, P. G., et al. (2011). Physicochemical, microbiological and sensory characteristics of soymilk kefir. *African Journal of Microbiology Research*, *5*(22), 3737–3746.

Kwon, H., & Kim, J. (1993). Determination of monosaccharides in glycoproteins by reverse-phase high-performance liquid chromatography. *Analytical Biochemistry*, *215*, 243–252.

Liu, J. R., Chen, M. J., & Lin, C. W. (2002). Characterization of polysaccharide and volatile compounds produced by kefir grains grown in soymilk. *Journal of Food Science*, *67*(1), 104–108.

Marinho-Soriano, E., & Bourret, E. (2005). Polysaccharides from the red seaweed *Gracilaria dura* (Gracilariales, Rhodophyta). *Bioresource Technology*, *96*, 379–382.

Nataraj, S., Schomacker, R., Kraume, M., Mishra, M. I., & Drews, A. (2008). Analyses of polysaccharide fouling mechanisms during crossflow membrane filtration. *Journal of the Membrane Science*, *308*, 152–161.

Parikh, A., & Madamwar, D. (2006). Partial characterization of extracellular polysaccharides from cyanobacteria. *Bioresource Technology*, *97*, 1822–1827.

Piermaria, J. A., La Canal, M. L., & Abraham, A. G. (2008). Gelling properties of kefiran, a food-grade polysaccharide obtained from kefir grain. *Food Hydrocolloids*, *22*, 1520–1527.

Rottava, I., Batesini, G., Silva, M. F., Lerin, L., De Oliveira, D., Padilha, F. F., et al. (2009). Xanthan gum production and rheological behavior using different strains of *Xanthomonas* sp. *Carbohydrate Polymers*, *77*, 65–71.

Silva, M. L. C., Martinez, P. F., Izeli, N. L., Silva, I. R., Vasconcelos, A. F. D., Cardoso, M. S., et al. (2006). Caracterização Química de Glucanas Fúngicas e Suas Aplicações Biotecnológicas. *Quimica Nova*, *29*(1), 85–92.

Stahl, M., Rocke, A. V., & Bayer, E. (2002). Mass spectrometry of oligosaccharides. Carbohydrate analysis by modern chromatography and electrophoresis. *Journal of Chromatography Library*, *66*(27), 961–1042.

Sutherland, I. W. (1990). *Biotechnology of microbial polysaccharides. Cambridge studies. Biotechnology* (Vol. 9) Cambridge, UK: Cambridge University Press.

Wang, Y., Ahmed, Z., Feng, W., Li, C., & Song, S. (2008). Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir. *International Journal of Biological Macromolecules*, *43*, 283–288.

Wang, Y., Li, C., Liu, P., Ahmed, Z., Xiao, P., & Bai, X. (2010). Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet kefir. *Carbohydrate Polymers*, *82*, 895–903.

Welman, A. D., & Maddox, I. S. (2003). Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. *Trends in Biotechnology*, *21*(6), 269–274.

Yemm, E. W., & Folkes, B. F. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal*, *57*(3).

Yokoi, H., Watanabe, T., Fujii, Y., Toba, T., & Adachi, S. (1990). Isolation and characterization of polysaccharide-producing bacteria from kefir grains. *Journal of Dairy Science*, *73*, 1684–1689.