Journal of Industrial and Engineering Chemistry 55 (2017) 40–49

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

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

Vineyard pruning waste as an alternative carbon source to produce novel biosurfactants by *Lactobacillus paracasei*

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ARTICLE INFO

Article history:
Received 30 March 2017
Received in revised form 15 May 2017
Accepted 5 June 2017
Available online 15 June 2017

Keywords:
Vineyard pruning waste
Cellulosic sugars
Biosurfactant
Lactobacillus paracasei
Chemical composition

ABSTRACT

Cellulosic sugars extracted from vineyard pruning waste (VPW) were used as a low-cost carbon source for biosurfactant production by *Lactobacillus paracasei*. The results obtained showed that when glucose from VPW was used, the biosurfactant was a glycolipopeptide, whereas when it was replaced by lactose the biosurfactant produced was a glycoprotein. Additionally, it was found that the extraction process, either with phosphate-buffer or phosphate-buffer saline, influenced the biosurfactant chemical structure and emulsion capacity. Overall, these results highlight the possibility of producing biosurfactants "à la carte" with the same strain but changing the carbon source, increasing its potential in different industrial applications.

Introduction

The valorization of agricultural wastes, following the European Directive 2008/98/EC, is focused on the whole waste recycling, from generation to disposal, emphasizing their recovery and recycling. As a result, economic, environmental and social benefits increase at the same time as the industrial resource efficiency is enhanced.

VPW is the residue after pruning the vineyard trees, such as thin and thick branches, that is usually burned by the small winery producers, contributing to global warming by the emission of greenhouse gases like CO_2 during their combustion [1,2]. VPW is a renewable, abundant and attractive carbon source composed by cellulose, hemicellulose and lignin. Therefore, this lignocellulosic waste can be used as a low-cost feedstock in several biotechnological processes.

In 2012, World vineyard cultivation reached a total surface of 7487 thousands of hectares (kha), being Spain the country with the highest expansion of planted surface areas (1017 kha areas), followed by France, China and Italy with 792 kha, 706 kha and 705 kha, respectively. Regarding the Europe vineyard cultivation, Europe accounts half of the vine-growing area (about 55%), followed by Asia (24%), America (14%), Africa (5%) and Oceania (3%) [3].

Usually autohydrolysis–posthydrolysis or prehydrolysis (acid hydrolysis) treatments are employed to obtain hemicellulosic sugars (mainly glucose and xylose) from lignocellulosic residues. Several researchers evaluated the use these hydrolysates to produce biosurfactants [1,4–8], lactic acid [4,6,9–11], xylitol [12–14], antioxidants [15], as well as phenyllactic acid [16]. However, the cellulosic fraction obtained after the hydrolysis treatments remains as by-product, probably because its use involves a saccharification process using acids or enzymes. Therefore, Bustos et al. [17] proposed a delignification stage of VPW, followed by an enzymatic hydrolysis treatment, to obtain glucose solutions that can be used as carbon source in culture media (in this case to produce lactic acid by *Lactobacillus rhamnosus*). More recently, Vecino et al. [18,19] evaluated the applicability of the cellulosic fraction from VPW as an adsorbent to remove micronutrients and dyes from winery wastewater.

On the other hand, biosurfactants are promising molecules as potential alternatives to their chemical counterparts [20]. These molecules are surface-active compounds of microbial origin with well-known advantages and novel applications in the pharmaceutical, cosmetic and environmental industries [21–23]. However, their high production and recovery costs have limited their scale-up to industrial set-ups. Therefore, in order to bypass this limitation, considerable efforts have been conducted in the last years to use renewable agro-industrial substrates as cost-effective alternative substrates for their production [24,25].

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http://dx.doi.org/10.1016/j.jiec.2017.06.014
1226-086X/

Based on the above discussion, a sustainable biotechnological process using cellulosic sugars from VPW as carbon source to obtain biosurfactants from *L. paracasei* was herein developed. The effect of the carbon source and the extraction process on the biosurfactant production was studied. Furthermore, the biosurfactants were characterized regarding their critical micelle concentration, emulsification capacity and chemical composition, including elemental analysis, carbohydrates, protein and lipids contents, fatty acid profile and Fourier transform infrared spectroscopy.

Materials and methods

Strain and culture conditions

L. paracasei ssp. *paracasei* A20, isolated from a Portuguese dairy industry and previously reported as biosurfactant producer [26], was used in this study. The bacterial strain was grown in MRS (de Man, Rogosa and Sharpe) agar plates at 37 °C for 24 h. Pre-cultures were prepared by solubilizing all the cells from a plate with 5 mL of the appropriate culture medium, that were subsequently transferred to 250 mL Erlenmeyer flasks containing the same culture medium, to make a final volume of 100 mL. Subsequently, the flasks were incubated at 37 °C and 150 rpm during 24 h.

Characterization of vineyard pruning waste

VPW was obtained from a Spanish local wine industry in Galicia, located in the Northwest of Spain. The lignocellulosic residue was dried, milled to <1 mm, homogenized into a single lot to avoid differences in the composition along the work, and stored at room temperature in a dark and dry place until use. The characterization of VPW was carried out by quantitative hydrolysis in a two-stage acid treatment, following the methodology previously reported [27]. The first stage was carried out with sulfuric acid (72% w/w) at 30 °C for 1 h, whereas the second stage was carried out with sulfuric acid (3% w/w) at 121 °C for 1 h. The solid residue after hydrolysis was considered as Klason lignin. Hydrolysates were analyzed by HPLC (Agilent Technologies 1200 Series, Germany) using Rezex RHM Monosaccharide H+ (8%) column (Phenomenex, USA) maintained at a constant temperature of 65 °C (mobile phase 0.01 M H_2SO_4, flow rate 0.4 mL min^{-1}, IR and UV detection). This method allows the direct determination of sugars in the solid fraction. The composition of solid fractions solids was expressed as VPW dry weight (w/w).

a) Extraction of the cellulosic fraction

In order to remove the hemicellulosic sugars, VPW was hydrolyzed following the methodology proposed by Bustos et al. [9]: 3% H_2SO_4; 15 min at 130 °C; liquid–solid ratio 8:1 (w/w). After that, the solid fraction (consisting of lignin and cellulose) was subjected to a delignification process following the methodology proposed by Bustos et al. [17] with slight modifications: 6.5% NaOH; 60 min at 130 °C; liquid–solid ratio 10:1 (w/w). The solid residue (cellulosic fraction) resulting from this treatment was separated by filtration, washed with demineralized water and air dried for enzymatic hydrolysis.

b) Enzymatic hydrolysis of the cellulosic fraction

The cellulosic fraction obtained from the alkali delignification treatment was subjected to enzymatic hydrolysis with commercial enzyme concentrates (cellulase and β-glucosidase) provided by Novozymes (Denmark). Cellulase activity in the hydrolysates was assayed through the filter paper activity test (FPA) according to Mandels et al. [28], and was expressed as filter paper units (FPU) per mL. The β-glucosidase activity was measured according to Paquot and Thonart [29]. The operational conditions used for the enzymatic hydrolysis were as follows: 48.5 °C, pH 4.85, 48 h, liquor–solid ratio 15:1 (w/w). Regarding the cellulase activity, a cellulase–substrate ratio of 28 FPU/g of cellulose was used, supplemented with cellobiase at a cellobiase–cellulase ratio of 13 IU/FPU (during the enzymatic hydrolysis, cellobiase was added to avoid the accumulation of cellobiose in the medium, which could inhibit the cellulase activity).

Fig. 1 shows the flowchart proposed for the chemical–biotechnological processing of different fractions from VPW that were used in this study.

Biosurfactant production by L. paracasei *using glucose from VPW as carbon source*

The glucose-based medium (containing 33 g/L glucose) obtained after enzymatic hydrolysis was supplemented with 10 g/L of yeast extract (YE) and 10 g/L of corn steep liquor (CSL) as nitrogen sources; subsequently, it was sterilized at 121 °C for 15 min and used directly as culture medium. Fermentations were performed in a 2 L Applikon fermentor, with a working volume of

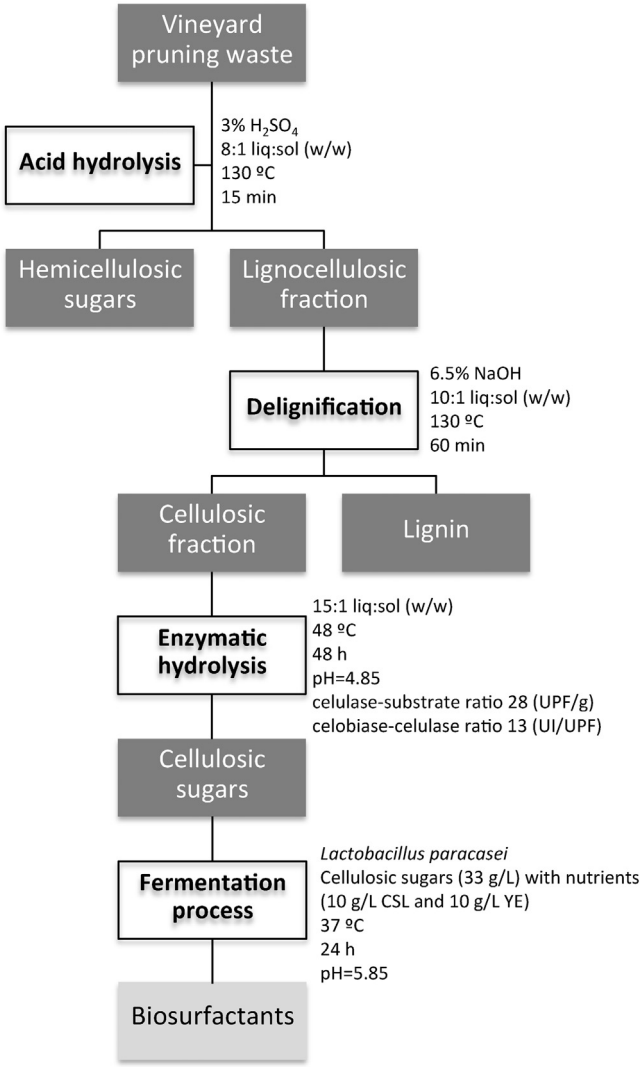


Fig. 1. Methodology used for the full valorization of vineyard pruning waste.

1.5 L, at 37 °C and 200 rpm for 24 h. The pH was adjusted to 5.85 along the fermentation by automatic addition of NaOH 4 M.

Biosurfactant production by L. paracasei *using lactose as carbon source*

For comparative purposes, a culture medium where glucose from VPW was replaced by synthetic lactose was used. The lactose-based medium contained 33 g/L of lactose, 10 g/L of YE and 10 g/L of CSL. Fermentations with this culture medium were performed using the same operational conditions described above.

Study of biosurfactant production

Biosurfactants can be excreted to the culture medium (extracellular biosurfactants) or remain attached to the cell wall (cell-bound biosurfactants). The *L. paracasei* strain used in this work has been reported to produce mainly cell-bound biosurfactants [30]. Nevertheless, the production of both types of biosurfactant was herein evaluated.

a) Extracellular biosurfactants

The surface tension (ST) of samples taken at different time points, during the fermentation, was measured and compared to the initial ST of the culture medium. As the ST of the culture medium is very close to the minimum ST value achieved in the presence of the biosurfactants produced by *L. paracasei*, the samples were diluted 4 (dilution factor (DF = 4)) and 16 (DF = 16) times in order to detect any extracellular biosurfactant production. The ST measurements were performed according to the Wilhelmy plate method using an Easy Dyne K20 Tensiometer (KRÜSS GmbH, Germany) equipped with a platinum plate.

b) Cell-bound biosurfactants

Cells were harvested by centrifugation (9000 rpm, 20 min) at the end of the *L. paracasei* fermentations, washed twice with the same volume of demineralized water and resuspended in 250 mL of phosphate-buffer saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl) or phosphate-buffer (PB: 10 mM KH_2PO_4/K_2HPO_4, without NaCl). The ratio of culture medium:buffer solution used for the extraction was 6:1. The extraction with PBS was performed at room temperature (25 °C) and 150 rpm for 2 h [7], whereas the extraction with PB was performed at 65 °C and 150 rpm for 1.5 h [31]. Afterwards, the cells were removed by centrifugation (9000 rpm, 20 min) and the remaining supernatant was filtered through a 0.2 μm pore-size filter (Whatman, GE Healthcare, UK). The solution containing the cell-bound biosurfactants was dialyzed against demineralized water at 4 °C in a Cellu-Sep© membrane (molecular weight cut-off 6000–8000 Da; Membrane Filtration Products, Inc., USA) for 48 h. Finally, the biosurfactant extracts were freeze-dried using a lyophilizer CHRIST® Alpha 1-4 LD plus (Germany).

Four different biosurfactant extracts were obtained depending on the carbon source and the methodology used for their extraction, namely biosurfactants produced in glucose-containing medium and extracted with PBS and PB (BS-Glu-PBS and BS-Glu-PB, respectively); and biosurfactants produced in lactose-containing medium and extracted with PBS and PB (BS-Lac-PBS and BS-Lac-PB, respectively).

Characterization of biosurfactants produced by L. paracasei

a) Critical micelle concentration (CMC)

CMC is the minimum concentration of biosurfactant needed to produce the maximum reduction in the surface tension of an aqueous solution, being this parameter important in terms of biosurfactant industrial effectiveness and costs. Above the CMC, the ST of a biosurfactant solution remains constant, being that surfactant tension value characteristic for each biosurfactant. On the other hand, below the CMC, the surface tension of biosurfactant solutions varies and a linear relationship between the biosurfactant concentration and the ST is observed. Therefore, the minimum biosurfactant concentration necessary to keep the ST at the minimum value corresponds to its CMC [32]. In order to calculate the CMC of the different biosurfactant extracts obtained in this work, biosurfactant solutions at different concentrations were prepared with demineralized water, and their ST were measured using the Wilhelmy plate method as previously described. The reference ST value of demineralized water was 72 mN/m.

b) Biochemical composition

The carbohydrate content of the biosurfactant extracts was determined by the phenol–sulfuric acid method [33], using D-glucose as standard. The proteins concentration was calculated by determining the N content of the extracts. The value of N was then transformed into protein content multiplying by 6.25 [34]. Elemental analysis was obtained by chromatographic analysis with thermal conductivity detection (TCD). C, N, H and S were determined in a Carlo Erba EA-1108CHNS-O element analyzer.

In addition, the functional groups of the different biosurfactant extracts were determined through Fourier transform infrared spectroscopy (FTIR). Hence, 1 mg of biosurfactant was ground with 10 mg of potassium bromide and pressed (7500 kg for 30 s) to produce translucent pellets. The FTIR analyses were carried out in a Niocolet 6700 FTIR spectrometer (Thermo Scientific). The spectral measurements were made in the transmittance mode in the range of 400–4000 cm^{-1}, with a resolution of 4 cm^{-1} and an average of 32 scans. A potassium bromide pellet was used for measuring background absorbance levels.

c) Fatty acid composition determined by GC–MS–MS

The total lipid content was estimated according to the colorimetric method [35], using cholesterol as standard. Furthermore, the fatty acids profile of the biosurfactant extracts was analyzed by GC–MS–MS (gas chromatography coupled to a mass spectrometer) on a Model Scion 451 GC (Bruker) equipped with a PTV 1019 universal capillary injector, coupled to a mass spectrometer, controlled by System Control software. Prior to analysis, the biosurfactant extracts were submitted to a derivatization process following the procedure described by Rodríguez-López et al. [36]. Afterwards, 1 μL of sample was injected using a splitless mode. The fatty acid methyl esters (FAMEs) separation was performed on a DB-WAX column (30 m long, 0.25 mm i.d., 0.25 μm film thickness) using the following oven temperature gradient: 50 °C for 2 min, then raised to 220 °C at a rate equal to 4 °C min^{-1} and then maintained for more 15 min. Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1}. The temperature of both injector inlet and the transfer line of the detector was set at 240 °C.

The mass spectra were obtained using a mass-selective detector under electron impact ionization at a voltage of 70 eV and data were acquired over an m/z range 50–400. The software used to process the peak areas was MS Data Review (version 8.1).

FAMEs were identified using a mass spectra library supplied with the GC–MS–MS system and by comparison of retention times and mass spectra of a FAME standard mix (Supelco 37 Component FAME Mix: 10 mg/mL of the FAME reference standard mix in

methylene chloride, Sigma–Aldrich) injected under the same conditions.

d) Emulsification capacity

Rosemary oil/water emulsions were formulated using the biosurfactant extracts, BS-Glu-PBS and BS-Glu-PB, as stabilizing agents. For this purpose, 2 mL of rosemary oil and 2 mL of biosurfactant solutions were vigorously mixed with a vortex for 2 min. Then, the mixture was allowed to stand for 1 h. Afterwards, the relative emulsion volume (EV, %) and the emulsion stability (ES, %) were calculated at different time intervals (2 h; 1, 2, 3, 7, 14, 21 and 28 days) according the protocol published elsewhere [37]. An emulsion with rosemary oil/demineralized water was used as negative control using the same conditions.

The macro visualizations of the emulsions were obtained 48 h after the formation of the emulsion with a regular digital camera (Sony optical SteadyShot), using an Optical Zoom 4× with a 26-mm wide-angle lens and 14.1 Megapixels. Additionally, the emulsion droplets were observed using a Leica DMI 3000B Inverted Microscope equipped with a high-sensitivity camera LEICA DFC450C. The emulsion was placed on the microscope stage and observed through a 20× objective. Moreover, the droplets were measured (at 20 ± 2 °C) and photographs were taken using the LAS 4.7 software. The size of the droplets was measured 1 month after the formation of the emulsion.

Statistical analysis

Data are reported as the mean ± SD (standard deviation) of triplicate determinations. The existence of significant differences among the results was analyzed. One-way analysis of variance (ANOVA) was used followed by the Tukey's HSD test. All statistical tests were performed at a 5% significance level using the IBM SPSS Statistics 20.0 statistical software package.

Results and discussion

Several environmentally friendly applications have been proposed for the valorization of VPW as a low-cost feedstock. This residue is composed by 34 ± 1.42% cellulose, 19 ± 1.66% hemicellulose and 27 ± 2.80% lignin. Chemical composition was similar to the VPW reported by other authors probably due to the same origin of VPW [9].

Different treatments applied to VPW in order to obtain hemicellulosic or cellulosic sugars towards the production of added value compounds can be found in the literature. However, most of these applications involve the use of the hemicellulosic fraction rather than the cellulosic sugars. In addition, biosurfactant production by *L. paracasei* using as carbon source the lignocellulosic residue VPW has not been previously reported [1,4–17].

After the hydrolysis of VPW with sulfuric acid two different fractions were obtained, namely a liquid hemicellulosic fraction composed by hemicellulosic sugars (about 18 g/L of xylose, 11 g/L glucose and 4.5 g/L of arabinose) and a solid fraction (that represents about 63% of the initial residue), that after delignification with NaOH, gave the following composition: 70.7 ± 0.25% cellulose, 1.7 ± 0.06% hemicelluloses and 25.5 ± 0.20% lignin.

The liquid fraction, rich in xylose, was discarded as pentoses are hard to ferment by Lactobacilli strains leading to low fermentation yields; whereas the solid fraction composed basically by cellulose, was subjected to enzymatic saccharification with cellulases and cellobiase. At this stage it is important to use both enzymes to avoid product inhibition of cellobiose on cellulase. After the enzymatic hydrolysis, a liquid medium composed by 33 ± 0.33 g/L of glucose was obtained, which after nutrient supplementation was used to produce biosurfactants by *L. paracasei*. At this stage the saccharification yield was about 47 ± 1.63% (see Fig. 1).

Production of biosurfactants by L. paracasei *grown on cellulosic sugars from vineyard pruning waste (VPW)*

It is well known that *Lactobacillus* strains produce cell-bound biosurfactants. However, only few studies evaluated the effect of different carbon sources on the production of biosurfactants by these bacteria [5,30]. Rodrigues et al. [38] suggested that some *Lactobacillus* strains could alter their biosurfactant production profile (from cell-bound to extracellular biosurfactants) depending on the carbon source used. Therefore, in this work, the ability of *L. paracasei* to produce extracellular biosurfactants was also evaluated. Fig. 2a shows the extracellular biosurfactant production profile of *L. paracasei* using the glucose-based medium obtained from VPW as carbon source. It can be observed that, a slight decrease in the ST of the culture medium was found at the first 8 h of fermentation. Afterwards, the ST remained almost constant until the end of the fermentation (24 h). However, that small variation in the ST of the culture medium (4 mN/m) is not enough to consider the presence of extracellular biosurfactants. Regarding the results obtained using the lactose-based medium; a similar profile was observed (Fig. 2b). The ST of the culture medium was reduced about 4 mN/m in the first 4 h, which likewise is not enough to consider that the strain produces extracellular biosurfactants.

Regarding the production of cell-bound biosurfactants by *L. paracasei* grown on glucose from VPW, Fig. 3 shows the CMC values

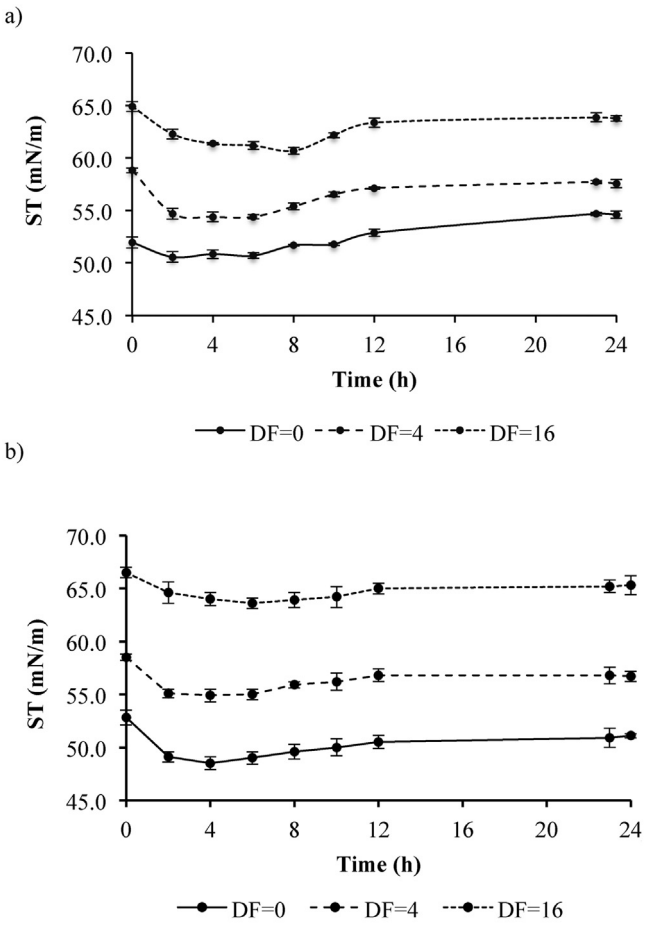


Fig. 2. Extracellular biosurfactant production by *Lactobacillus paracasei* grown in a culture medium containing cellulosic sugars from vineyard pruning waste (a), and in lactose-based medium (b).

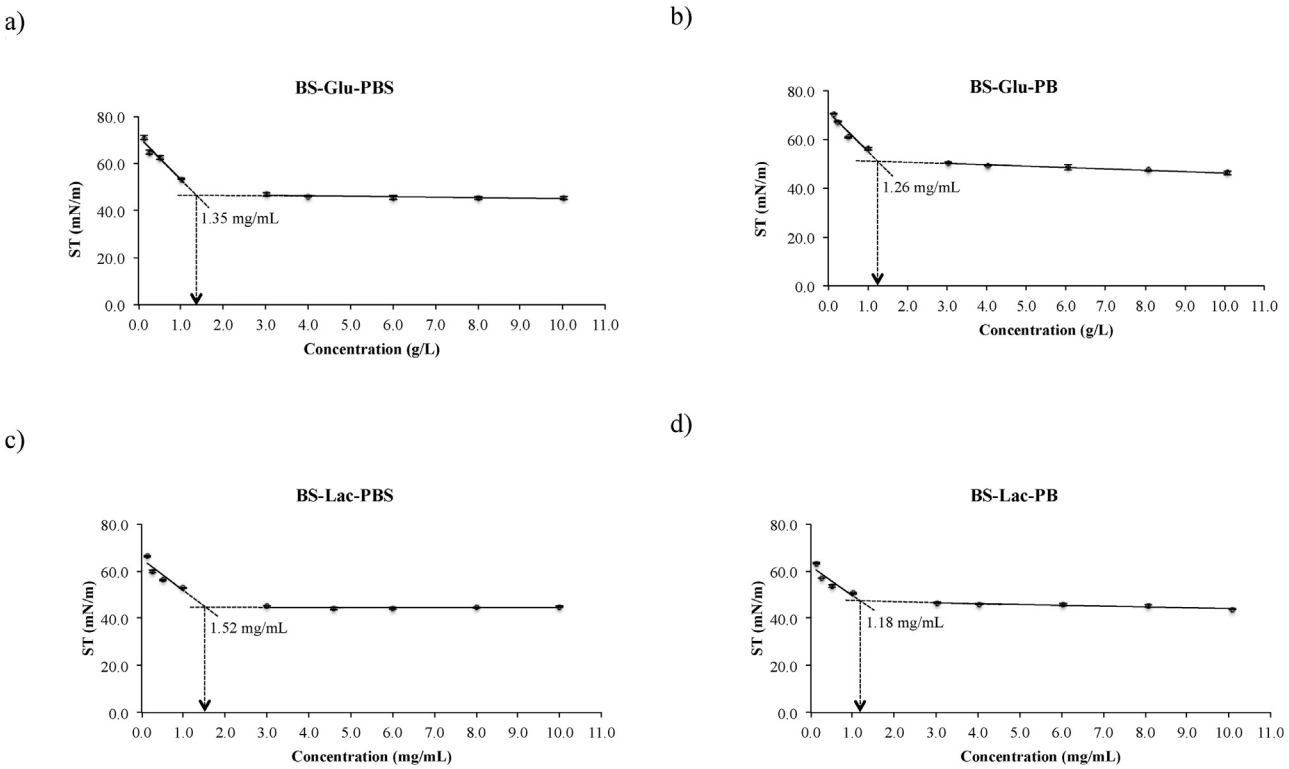


Fig. 3. Surface tension values (mN/m) versus biosurfactant concentration (mg/mL), and CMC values obtained for the four *Lactobacillus paracasei* biosurfactant extracts: (a) biosurfactant produced in glucose-based medium and extracted with phosphate-buffer saline (BS-Glu-PBS); (b) biosurfactant produced in glucose-based medium and extracted with phosphate-buffer (BS-Glu-PB); (c) biosurfactant produced in lactose-based medium and extracted with phosphate-buffer saline (BS-Lac-PBS); (d) biosurfactant produced in lactose-based medium and extracted with phosphate-buffer (BS-Lac-PB).

corresponding to the biosurfactants extracted with PBS (BS-Glu-PBS) (Fig. 3a) and PB (BS-Glu-PB) (Fig. 3b), as well as the relationship between the biosurfactants concentration and their capacity to reduce the ST of water. It was found that, independently of the buffer used to extract the cell-bound biosurfactants, the CMC obtained was similar, being 1.35 ± 0.13 mg/mL and 1.26 ± 0.11 mg/mL for BS-Glu-PBS and BS-Glu-PB, respectively. However, the capacity to reduce the ST of water was higher for the biosurfactant extracted with PBS (25.1 ± 0.49 mN/m) when compared with the biosurfactant extracted with PB (20.9 ± 0.41 mN/m). This may be due to the different composition of both biosurfactant extracts.

In a similar way, Fig. 3c and 3d show the relationship between the concentration of biosurfactants produced by *L. paracasei* grown on lactose-based medium (extracted with PBS and PB) and their capacity to reduce the ST of water. As it can be seen, the biosurfactant extract BS-Lac-PBS exhibited the highest CMC value (1.52 ± 0.12 mg/mL), whereas BS-Lac-PB resulted in the lowest CMC value (1.18 ± 0.15 mg/mL). However, the biosurfactant extracted with PBS showed the highest ST reduction (27.3 ± 0.55 mN/m), being 24.8 ± 0.61 mN/m for the biosurfactant extracted with PB.

The CMC values obtained in this work for the four biosurfactant extracts produced by *L. paracasei* using glucose from VPW or lactose as carbon sources, were lower than the CMC value obtained for the biosurfactant produced by the same strain using the MRS-Lac medium (standard MRS medium where glucose was replaced by lactose) and extracted with PBS (2.5 mg/mL) [26]. However, a higher ST reduction (30.2 mN/m) was reported in that study.

Biosurfactants with low CMC values represent an advantage from an industrial point of view due to their higher efficiency. However, that low CMC should be accompanied by low ST values. Higher CMC values than the obtained in this work have been reported for biosurfactants produced by different *Lactobacillus* strains. Gudiña et al. [39] reported a CMC of 7.5 mg/mL for the cell-bound biosurfactant produced by *Lactobacillus agilis* CCUG31450 grown in MRS medium. The biosurfactant (glycoprotein) produced by *Lactobacillus plantarum* CFR 2194 exhibited a CMC of 6 mg/mL [40]. Additionally, the CMC calculated for the glycolipid biosurfactant produced by *Lactobacillus helveticus* was 2.5 mg/mL [41]. Only the glycoprotein biosurfactant produced by *Lactobacillus acidophilus* RC14 exhibited a lower CMC value (1 mg/mL) when compared with the cell-bound biosurfactants herein produced by *L. paracasei* using cellulosic sugars from VPW as carbon source [42].

Moldes et al. [1] used hemicellulosic sugars from VPW as carbon source for biosurfactant production by *Lactobacillus pentosus*. This biosurfactant reduced the ST of water by 21 mN/m. On the other hand, Portilla-Rivera et al. [5], used *L. pentosus* to produce biosurfactants from synthetically obtained hemicellulosic sugars. These biosurfactants were found to reduce the surface tension by 14–15 mN/m. In conclusion, the biosurfactants produced by *L. paracasei* grown on cellulosic sugars from VPW exhibited a better surface activity when compared with those produced by *L. pentosus* grown on hemicellulosic sugars from VPW.

Characterization of L. paracasei *biosurfactants produced on different carbon sources*

Regarding the biochemical characterization of the biosurfactants produced by *L. paracasei*, some differences were found in the carbohydrate, protein and lipid contents depending on the carbon source and the extraction process used (Table 1).

When *L. paracasei* was grown using glucose from VPW as carbon source, the extraction with PB led to the recovery of biosurfactants with a higher carbohydrate and protein content as compared to the ones obtained when the extraction was conducted with PBS

Table 1
Characterization of biosurfactants produced by *Lactobacillus paracasei* grown in glucose-based medium obtained from vineyard pruning waste (BS-Glu-PBS and BS-Glu-PB) in comparison with the biosurfactants produced by the same strain in lactose-based medium (BS-Lac-PBS and BS-Lac-PB).

	BS-Glu-PBS	BS-Glu-PB	BS-Lac-PBS	BS-Lac-PB
CMC (mg/mL)	1.35 ± 0.13^{ab}	1.26 ± 0.11^{ab}	1.52 ± 0.12^{b}	1.18 ± 0.15^{a}
ST reduction units (mN/m)	25.1 ± 0.49^{a}	20.9 ± 0.41^{b}	27.3 ± 0.55^{c}	24.8 ± 0.61^{a}
Elemental analysis (%)				
N	3.39 ± 0.03^{a}	9.32 ± 0.50^{b}	5.82 ± 0.13^{c}	7.98 ± 0.04^{d}
C	13.83 ± 0.16^{a}	35.22 ± 2.17^{b}	30.60 ± 0.24^{c}	36.69 ± 0.19^{b}
H	1.96 ± 0.10^{a}	3.39 ± 0.17^{b}	4.74 ± 0.02^{c}	5.55 ± 0.04^{d}
S	<0.3	<0.3	<0.3	<0.3
Total protein content (%)	21.19 ± 0.18^{a}	58.22 ± 3.14^{b}	36.09 ± 0.40^{c}	49.88 ± 0.27^{d}
Total carbohydrate content (%)	5.47 ± 1.19^{a}	14.24 ± 3.81^{b}	18.6 ± 0.03^{bc}	23.3 ± 0.05^{c}
Total lipid content (%)	24.40 ± 1.15^{a}	13.66 ± 1.22^{b}	ND	ND
Major relative fatty acids (%)				
Myristic acid	8.90	6.79	No fatty acids present	
Palmitic acid	36.80	43.88		
Palmitoleic acid	2.82	4.68		
Stearic acid	35.92	26.11		
Oleic acid	7.44	1.23		
Linoleic acid	1.47	4.95		
α-Linoleic acid	–	8.85		

ND: no detected. Values correspond to the mean ± SD (n = 3) of triplicate experiments. In each column, values with different letters are significantly different ($p < 0.05$).

(Table 1). The same trend was found for the C and H percentages, being the biosurfactants extracted with PB richer in C and H than the ones extracted with PBS (Table 1). However, when *L. paracasei* was grown in lactose-based medium, the effect of the extraction process was almost negligible, resulting in similar percentages of N, C and H for both biosurfactants.

The most important differences were observed when the lipid content and the fatty acid composition were analyzed, indicating that *L. paracasei* produced different types of biosurfactants depending on the carbon source used. When *L. paracasei* was grown in lactose-based medium, the biosurfactants produced contained only carbohydrates and proteins (glycoproteins). However, when it was grown on cellulosic sugars, the biosurfactants produced were a mixture of carbohydrates, lipids and proteins (glycolipopeptides).

Table 1 shows the fatty acid profile of the biosurfactants obtained using the glucose from VPW as carbon source. A high percentage of C16 and C18 fatty acids was observed, being the most abundant palmitic acid (36.8–43.9%), followed by stearic acid (26.1–35.9%). Furthermore, differences were observed regarding the fatty acids content depending on the extraction method used. The biosurfactants extracted with PBS contained a higher percentage of oleic acid (7.4%), whereas α-linoleic acid (8.8%) was only present in those extracted with PB. Fig. 4 shows the GC–MS spectra, as well as the *m/z* spectra of the major fatty acids found in the biosurfactants produced by *L. paracasei* grown in glucose-based medium obtained from VPW.

Vecino et al. [43] reported that the biosurfactant produced by *L. pentosus* growing on hemicellulosic sugars from VPW was a glycolipopeptide, with a carbohydrate:protein:lipid ratio of 1:3:6. On the other hand, Pinto et al. [44] reported that the biosurfactant produced by the *L. paracasei* strain used in this work, grown in MRS-Lac medium (with lactose as carbon source), is a glycoprotein.

Fig. 5 shows the FTIR spectra corresponding to the biosurfactants produced by *L. paracasei* grown on glucose from VPW (Fig. 5a) and grown in the lactose-based medium (Fig. 5b), both extracted with PBS and PB.

The spectra corresponding to the biosurfactants produced from cellulosic sugars (Fig. 5a) indicated the presence of peptide groups (resulting from O—H and N—H stretching), proteins (N—H bending) and carbohydrates (C—O stretching), at wavenumbers around 3400 cm^{-1}, 1644 cm^{-1} and 1089 cm^{-1}, respectively. Comparing both extraction methods, the spectrum corresponding to the biosurfactant extracted with PB showed more intense bands than the ones observed in the biosurfactant extracted with PBS.

On the other hand, the spectra corresponding to the biosurfactants produced by *L. paracasei* grown in lactose-based medium and extracted with PBS or PB (Fig. 5b) did not show significant differences (i.e. bands exhibit the same intensity). However, when compared with the spectra corresponding to the biosurfactants produced from cellulosic sugars, the biosurfactants produced in lactose-based medium exhibited more intense bands in the wavenumbers around 1644 cm^{-1} and 1089 cm^{-1}, which is in agreement with the differences found previously in the percentages of N and C in the elemental analysis.

These results are consistent with the FTIR data reported for the biosurfactant produced by *L. pentosus* using hemicellulosic sugars from VPW as carbon source [43].

Overall, the results herein obtained suggest that the carbon source plays an important role on biosurfactant production, which is well aligned with several previous reports. For instance, Mata-Sandoval et al. [45] reported that the amount of rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* UG2 was affected by the nature of the carbon source used. The authors found that hydrophobic substrates (i.e. corn oil, lard and long-chain alcohols) led to higher rhamnolipid yields when compared with hydrophilic substrates such as glucose or succinic acid. In addition, Singh et al. [46] demonstrated that *Bacillus* sp. strain AR2 exhibits carbon source dependence. The authors reported that when the minimal salt medium was supplemented with sucrose and glycerol, the strain produced lipopeptides as a mixture of surfactin, iturin and fengycin; however, when maltose, lactose and sorbitol were used as carbon sources, only iturin was produced.

Study of the emulsifying activity of biosurfactants produced by L. paracasei *grown on cellulosic sugars*

The emulsifying activity of the biosurfactants produced by *L. paracasei* grown on glucose from VPW (BS-Glu-PBS and BS-Glu-PB) was assayed using rosemary oil as hydrophobic phase. This is the first report on the emulsifying activity of these biosurfactants.

Fig. 6 shows the macro view and the microscopic view (Fig. 6a) of oil-in-water (O/W) emulsions, as well as the relative emulsion volume (EV) and the stability of the emulsion (ES) (Fig. 6b) along

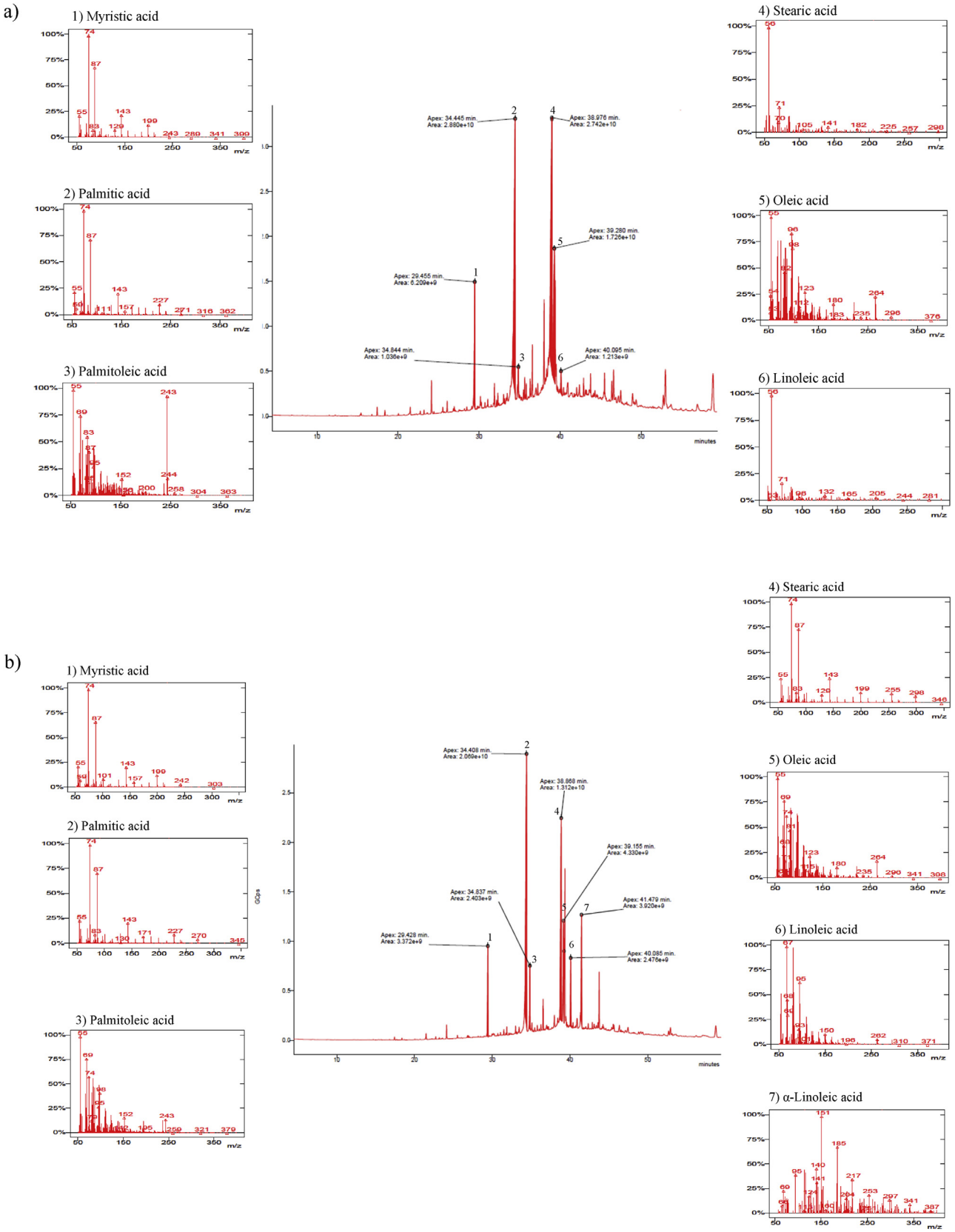


Fig. 4. GC–MS chromatograms and *m*/*z* spectra of the major fatty acids present in the *Lactobacillus paracasei* biosurfactants: a) BS-Glu-PBS; b) BS-Glu-PB.

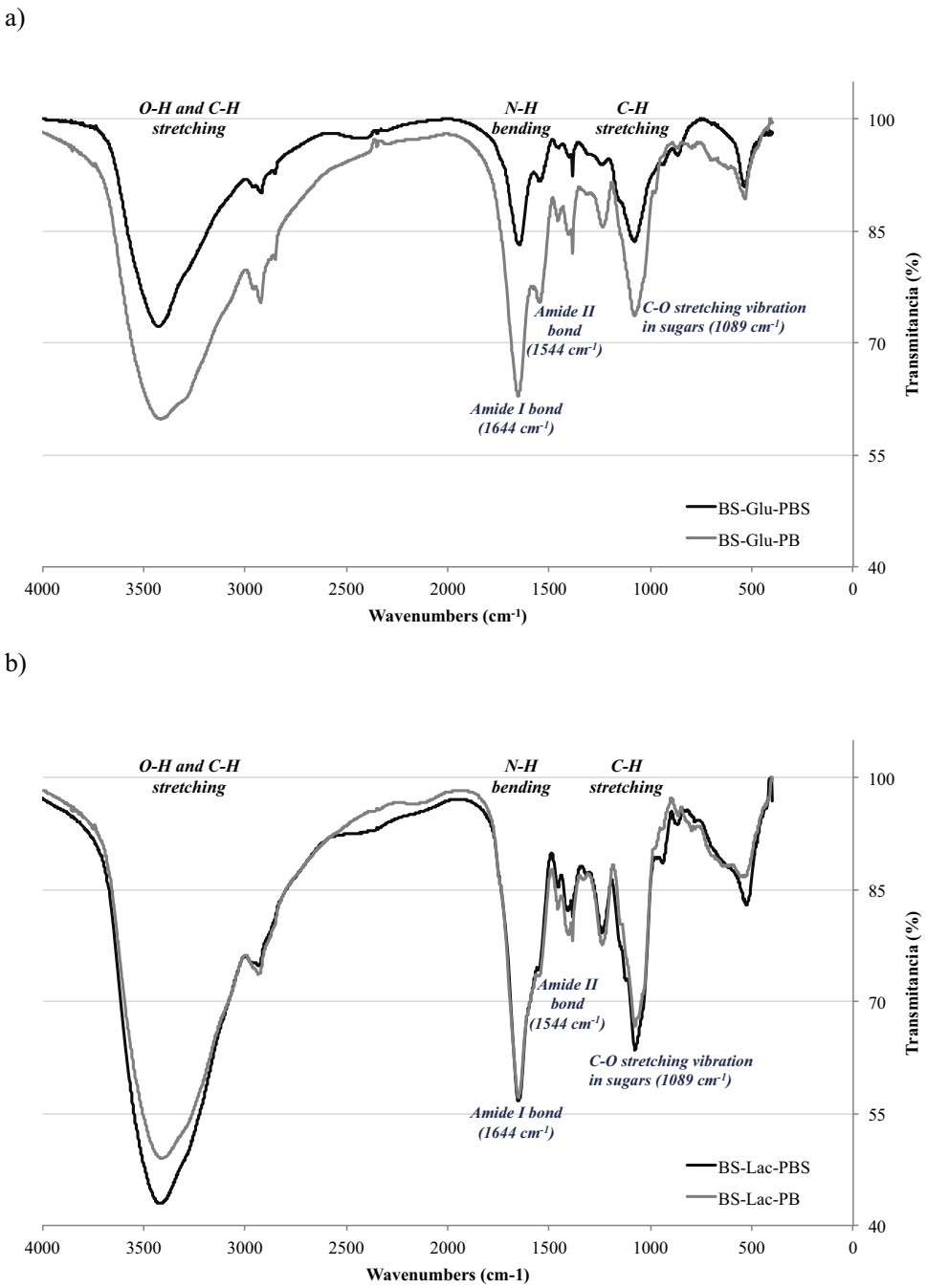
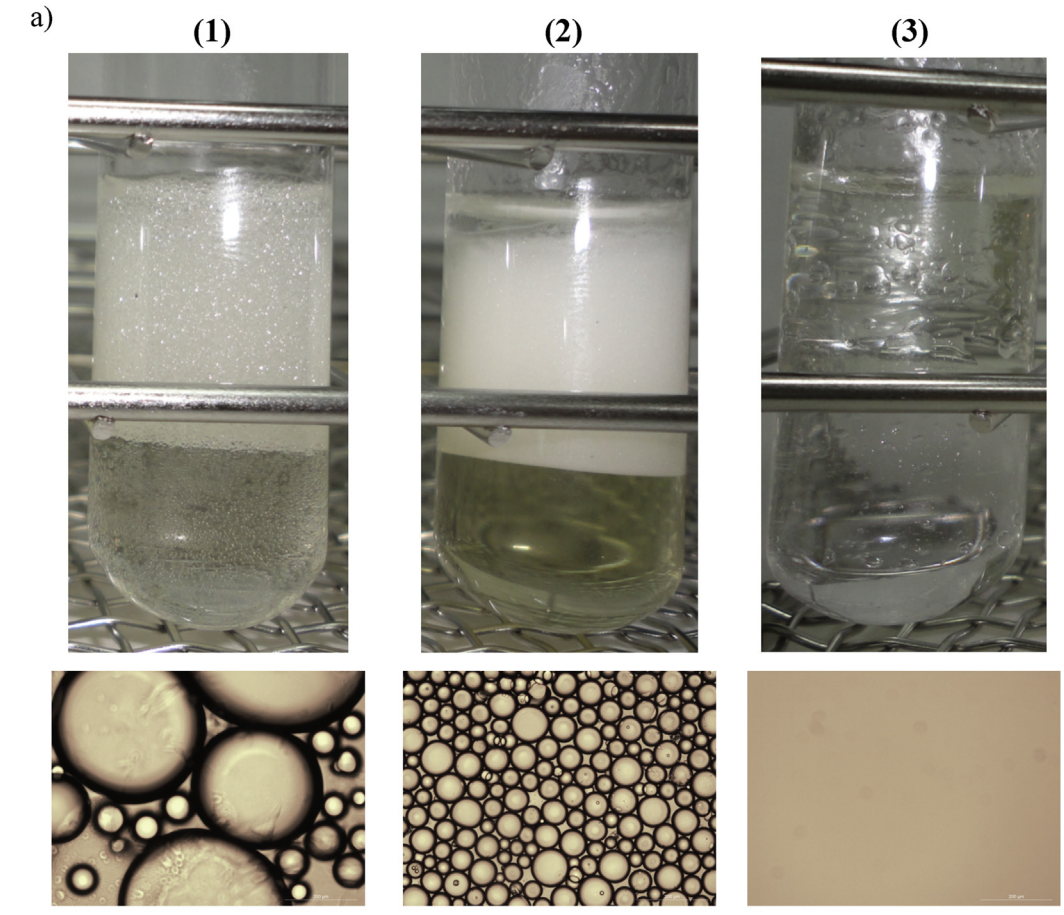


Fig. 5. FTIR spectrum of the different biosurfactants from *Lactobacillus paracasei*: (a) BS-Glu-PBS versus BS-Glu-PB; (b) BS-Lac-PBS versus BS-Lac-PB.

time (up to 28 days). As it can be seen in Fig. 6, the biosurfactants produced by *L. paracasei* resulted in similar EV percentages (69.4%–71.4%) independently of the extraction method used. However, differences were observed regarding the size of the droplets present in the emulsions stabilized with the biosurfactants obtained through the different extraction processes. The biosurfactant extracted with PB led to emulsions formed by droplets of smaller size than the ones stabilized by the biosurfactant extracted with PBS. The size of the droplets in the emulsions is an important parameter to measure their stability, as well as to define their potential applications. The results herein gathered suggest that the biosurfactant extracted with PB may be more useful for cosmetic and personal care applications, as it results in emulsions with smaller droplets, which are more suitable for these applications [23].

The results obtained regarding the emulsifying activity of the biosurfactants produced by *L. paracasei* were better when compared with those obtained using the biosurfactants produced by *L. pentosus* to stabilize rosemary oil/water emulsions. The biosurfactants were produced using hemicellulosic sugars from VPW, and the EV values obtained 24 days after the formation of the emulsion were about 55.5% [43]. The biosurfactants herein studied exhibited also a better performance when compared with those produced by *L. plantarum*, which stabilized emulsions with



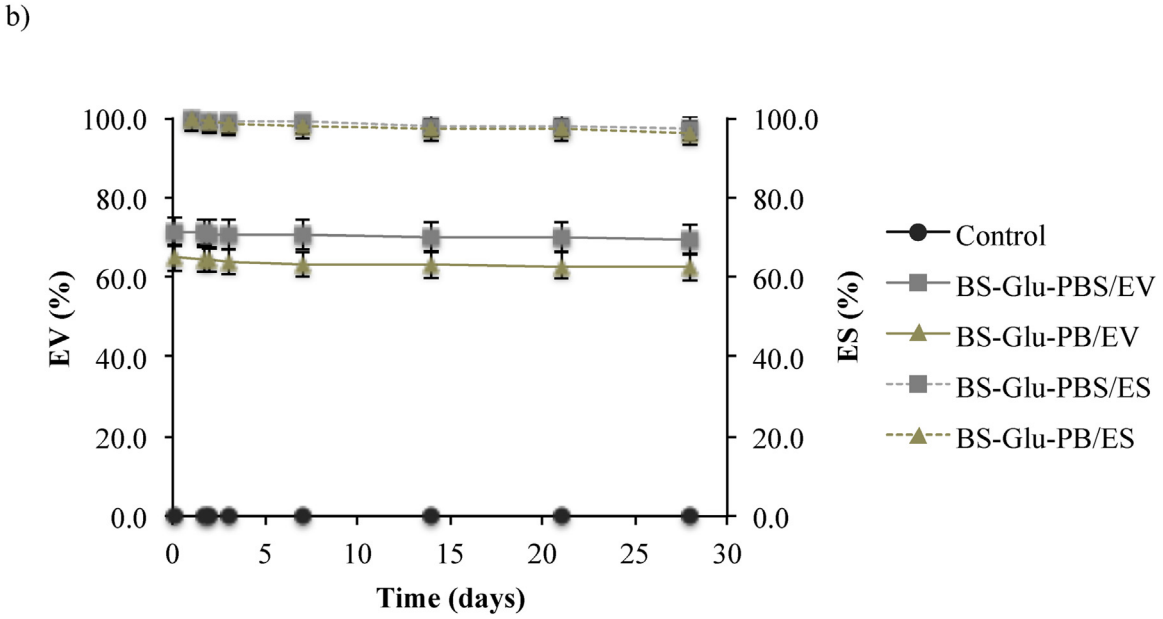


Fig. 6. Emulsification capacity of *Lactobacillus paracasei* biosurfactants (BS-Glu-PBS (1) and BS-Glu-PB (2)), in comparison with a control in the absence of biosurfactant (3): a) macro view (after 48 h), as well as microscope view (after 1 month) of the emulsions, respectively; b) EV (%) and ES (%) values 1 month after the formation of the emulsion. Pictures were taken with a 20× objective (−200 μm).

coconut and sunflower oils with EV values after 24 h of 37.9% and 19.4%, respectively [40].

Conclusions

The results obtained suggest that lignocellulosic wastes could be good alternatives as low-cost carbon sources for the fermentative production of biosurfactants using *L. paracasei*. Additionally, it can be stated that the biosurfactant composition can change according to the type of carbon source and extraction process used in their production. In this case, *L. paracasei* grown on cellulosic sugars produced a glycolipopeptide biosurfactant, whereas when it was grown on lactose-based medium, the biosurfactant produced was a glycoprotein. This finding opens the door to the production of different types of biosurfactants using the same strain, by changing the carbon source and the extraction process.

Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit, COMPETE 2020 (POCI-01-0145-FEDER-006684) and the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462), as well as X. Vecino post-doctoral grant (SFRH/BPD/101476/2014). The authors also acknowledge financial support from BioTecNorte operation (NORTE-01-0145-FEDER-000004) and the project MultiBiorefinery (POCI-01-0145-FEDER-016403) funded by the European Regional Development Fund under the scope of Norte2020—Programa Operacional Regional do Norte and the Post-doctoral grant CEB-BPD/01/2015/07 from the project UID/BIO/04469/2013, funded by FCT, to E. Gudiña. Additionally, the authors acknowledge the financial support from Spanish Ministry of Economy and Competitiveness (FEDER funds under the project CTM2015-68904) and L. Rodríguez-López acknowledges to the Spanish Ministry of Education, Culture and Sport for her pre-doctoral fellowship (FPU15/00205).

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