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Production of biosurfactants from vine-trimming shoots using the halotolerant strain *Bacillus tequilensis* ZSB10

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ABSTRACT

The strain *Bacillus tequilensis* ZSB10, isolated from Mexican brines, was able to grow and produce extracellular and cell-bound biosurfactants using nine culture broths formulated from hydrolyzates obtained from the cellulosic and hemicellulosic fractions of vine-trimming wastes. The results confirm its halotolerance since it managed to grow both in the presence and absence of salts. It also was able to consume sugars such as glucose and xylose. The process was then scaled up into a 2-L bioreactor using the mixture of hemicellulosic (50%) and cellulosic hydrolyzate (50%) supplemented with mineral salt medium as culture medium at different biomass concentrations. Crude extracellular biosurfactant yielded 1.52 g/L and lowered the surface tension to 38.6 mN/m with a critical micelle concentration of 177.14 mg/L. Furthermore, it was able to emulsify with kerosene after 24 h (E_{24} = 47%). Crude cell-bound biosurfactant only yielded 0.0783 g/L and showed lower emulsifying characteristics than extracellular biosurfactant (E_{24} = 41% with kerosene).

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1. Introduction

Biosurfactants are amphiphilic molecules produced by microorganisms that tend to reduce surface and interfacial tension of solutions and to form emulsions (Shavandi et al., 2011; Freitas et al., 2009). Bacteria, fungi and yeasts can produce biosurfactants during growth on a variety of substrates, leading to products with various biochemical and structural characteristics that can remain attached to the cell surface of the microorganism (cell-bound biosurfactants), or be expelled into the culture medium (extracellular biosurfactants) (Shavandi et al., 2011; Mukherjee et al., 2006). The surfactant-producing microorganism, culture medium (carbon source, nitrogen, phosphorus, trace elements such as Mg, Fe or Mn) and growth conditions (such as temperature, aeration or pH) determine the amount and composition of the biosurfactant (Bodour and Maier, 2003).

Biosurfactants have advantages over their synthetic counterparts since they are biodegradable, do not accumulate in the environment, have lower toxicity and, most importantly, these exhibit higher physical and chemical properties (emulsifying capacity, tolerance to pH, temperature and ionic strength) compared to those of the synthetic surfactants. However, it is necessary to find an efficient production process in order to make biosurfactants economically competitive with lower-cost chemical surfactants (Nitschke and Costa 2007; Satpute et al., 2010; Franzetti et al., 2011). Because they contain high levels of carbohydrates or lipids that favor bacterial growth and biosurfactant synthesis, agro-

Abbreviations: Abs_{600nm}, absorbance 600 nm; BS, biosurfactant; CMC, critical micelle concentration; E_{24}, emulsification index (24 h in repose); FPU, filter paper units; HMF, hydroxymethylfurfural; HPLC, high-performance liquid chromatography; MSM, mineral salt medium; MSM + GLU, mineral salt medium plus 15 g/L of glucose; MSM + XYL, mineral salt medium plus 15 g/L of xylose; MSM + HH, mineral salt medium plus hemicellulosic hydrolyzate; HH, hemicellulosic hydrolyzate; MSM + CH, mineral salt medium plus cellulosic hydrolyzate; CH, cellulosic hydrolyzate; MSM + HH + CH, mineral salt medium plus mixture of hydrolyzates (50% hemicellulosic hydrolyzate and 50% cellulosic hydrolyzate); HH + CH, mixture of hydrolyzates (50% henicellulosic hydrolyzate and 50% cellulosic hydrolyzate); ST, surface tension; ΔST, surface tension reduction.

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industrial byproducts can be low-cost, sustainable alternatives for the production of biosurfactants (Makkar and Cameotra, 2002).

The wine industry produces approximately 1.7 t/ha/year of vine-trimming shoots in Spain, and new processes have been developed to decrease the environmental impact of those residues. According to Max et al. (2010) vine-trimming shoots had the following composition: cellulose (34.0%), hemicelluloses (19.0%), lignin (27.1%), extracts (7.1%) and other minor compounds (12.7%). The valorization of vine-trimming shoots through production of a great variety of products, including biosurfactants, has been carried out in several studies (Devesa-Rey et al., 2011). Hydrolysis of these residues and further fermentation of hemicellulosic sugars by *Lactobacillus* and *Debaryomyces* strains to produce lipopeptides as biosurfactants has been carried out by several authors (Moldes et al., 2007; Bustos et al., 2007; Portilla-Rivera et al., 2008). However, low yields of cell-bound biosurfactants were obtained and additional treatments had to be done for their detachment (Vecino et al., 2015).

Bacillus strains have been shown to produce higher yield of lipopeptide as biosurfactant than those obtained with *Lactobacillus* strains using several agro-industrial wastes (Lima de Franca et al., 2015); but performance of *Bacillus* in fermentation process using the vine-trimming shoots waste as substrate is unknown.

In order to provide additional alternatives for valorization of vine-trimming shoots, the *Bacillus tequilensis* ZSB10 strain, recently isolated from brines at Zapotitlán Salinas (Puebla), Mexico, was investigated regarding its ability to use vine-trimming shoots as carbon source for the production of lipopeptides as biosurfactants. Halotolerant bacteria, such as *B. tequilensis*, have physiological properties that facilitate commercial exploitation: limited nutritional requirements, a variety of chemical energy sources, and resistance to contamination (Ramírez et al., 2004).

2. Materials and methods

2.1. Microorganism and culture conditions

B. tequilensis ZSB10 was obtained from ENCB-IPN WDCM449 culture collection (ENCB-RP-001). The strain was isolated from brines in Zapotitlán Salinas, Puebla, Mexico. It was reactivated according to the procedure reported by Saini et al. (2008), using mineral salt medium (MSM) containing (g/L): $NaNO_3$ (2.97), $MgSO_4{\cdot}7H_2O$ (0.4), KCl (1.0), $CaCl_2{\cdot}2H_2O$ (0.1), Na_2HPO_4 (24.3), NaCl (20) and yeast extract (10). The medium was adjusted to pH 7.4 and autoclaved at 121 °C for 15 min. 250 mL Erlenmeyer flasks were charged with 50 mL of culture medium and were incubated under agitation of 150 rpm in a constant temperature incubator shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) at 35 °C for 48 h.

2.2. Raw material

Samples of vine-trimming shoots were dried at room temperature, milled to a particle size less than 1 mm, homogenized in a single batch to avoid compositional differences and stored until use by sequential treatments in order to take advantage of both the cellulosic and the hemicellulosic fractions.

2.2.1. Pre-hydrolysis

The first stage consisted of a treatment with dilute acid to solubilize hemicelluloses. The raw material underwent autoclaving (Sterilclav-70 Trade Raypa, Barcelona, Spain) under the following conditions: sulfuric acid 2%, 130 °C for 15 min using a liquid/solid ratio 8 g/g, according to optimized conditions (Bustos et al., 2004). The product was filtered and the liquid phase was brought up to pH 6.0 using calcium carbonate, filtered again, and finally the liquid phase was treated with activated charcoal (10 g of hydrolyzate/1 g of activated carbon), stirred at 150 rpm, 1 h at 25 °C, and finally filtered to obtain clear liquors. The supernatant, composed mainly of xylose and glucose and smaller amounts of inhibitors such as acetic acid, furfural and HMF (hydroxymethylfurfural), was used as a culture medium.

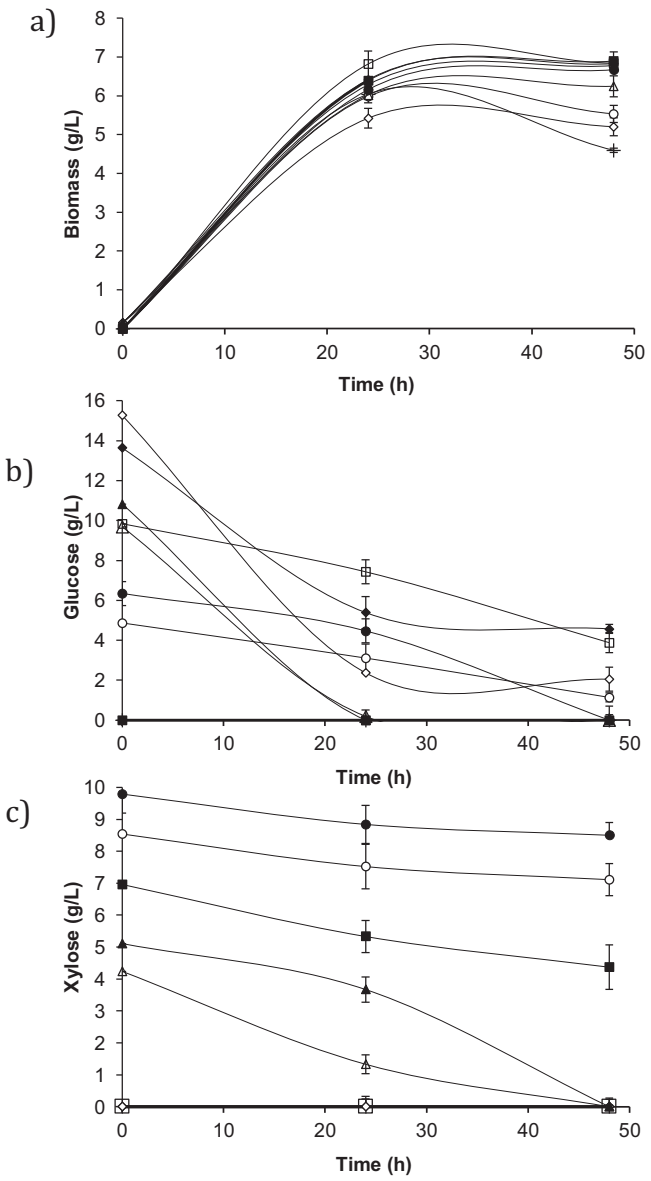

Fig. 1. Kinetics of ZSB10 strain in different culture media under the following culture conditions: 35 °C, 150 rpm during 48 h. (a) Bacterial growth; (b) glucose consumption; and (c) xylose consumption. MSM (+); MSM + GLU (□); MSM + XYL (■); MSM + HH (○); HH (●); MSM + CH (◊); CH (♦); MSM + HH + CH (△); HH + CH (▲).

2.2.2. Alkaline stage

Solids from pre-hydrolysis, containing the cellulosic fraction and lignin, were delignified to increase enzymatic digestibility. The solid was washed with distilled water to adjust to pH 6.0, oven-dried at 50 °C for 72 h, and treated in autoclave with solutions containing 8% NaOH at 130 °C during 120 min according to the conditions reported by Bustos et al. (2005). In this step, the liquor/solid ratio was fixed in 10 g/g. At the end of treatments, the solid residue containing the cellulose fraction was separated by filtration, washed with water, air dried at room temperature and used for enzymatic hydrolysis.

Table 1
Initial composition of culture broths used for the fermentation of strain ZSB10 (g/100 mL).

	MSM	MSM + GLU	MSM + XYL	MSM + HH	HH	MSM + CH	CH	MSM + HH + CH	HH + CH
$NaNO_3$ (g)	0.30	0.30	0.30	0.30		0.30		0.15	
$MgSO_4 \cdot 7H_2O$ (g)	0.04	0.04	0.04	0.04		0.04		0.02	
KCl (g)	0.10	0.10	0.10	0.10		0.10		0.05	
$CaCl_2 \cdot 2H_2O$ (g)	0.01	0.01	0.01	0.01		0.01		0.005	
Na_2HPO_4 (g)	2.43	2.43	2.43	2.43		2.43		1.22	
Yeast extract (g)	1.00	1.00	1.00	1.00		1.00		0.5	
NaCl (g)	2.00	2.00	2.00	2.00		2.00		1.00	
Glucose (g)		1.5							
Xylose (g)			1.5						
Hemicellulosic hydrolyzate (HH), (mL)				94.13	100			47.06	50
Cellulosic hydryolyzate (CH), (mL)						94.13	100	50	50
Water (mL)	94.13	92.63	92.63						

MSM: Mineral salt medium.
MSM + GLU: Mineral salt medium plus 15 g/L of glucose.
MSM + XYL: Mineral salt medium plus 15 g/L of xylose.
MSM + HH: Mineral salt medium plus hemicellulosic hydrolyzate.
HH: Hemicellulosic hydrolyzate.
MSM + CH: Mineral salt medium plus cellulosic hydrolyzate.
CH: Cellulosic hydrolyzate.
MSM + HH + CH: Mineral salt medium plus mixture of hydrolyzates (50% hemicellulosic hydrolyzate and 50% cellulosic hydrolyzate).
HH + CH: Mixture of hydrolyzates (50% hemicellulosic hydrolyzate and 50% cellulosic hydrolyzate).

2.2.3. Enzymatic hydrolysis

Two commercial enzymatic concentrates ("Celluclast" and "Novozyme 188", with cellulase and β-glucosidase acitivities, respectively), kindly provided by Novozymes, Denmark, were used to hydrolyze the cellulosic fraction in order to obtain glucose solutions. The cellulase activity of concentrates was measured by the Filter Paper Activity test according to Mandels et al. (1976) and expressed as Filter Paper Units (FPU)/mL, while the β-glucosidase activity was measured according to Paquot and Thonart, (1982) and was expressed as International Units per milliliter (IU/mL). The operational conditions used in the enzymatic hydrolysis were: temperature 48.5 °C, pH 4.85 (adjusted with citrate buffer), liquor/solid ratio 30 g/g, cellulase-substrate ratio 28 FPU/g and cellobiase/cellulase ratio 13 IU/FPU at 150 rpm (Bustos et al., 2005). The hydrolysis of the cellulosic fraction, expressed as the concentration of glucose (g/L), was monitored by HPLC for 50 h. At the end of the experiment a glucose concentration of 21.57 g/L was achieved.

2.3. Biosurfactant production by the strain ZSB10 using different culture media

Table 1 summarizes the composition of the nine culture media assayed to evaluate the production of biosurfactants using the strain ZSB10. All culture media were standardized to initial pH 7.4 using 1 N NaOH solution. Then, 50 mL was placed into 250 mL Erlenmeyer flasks. All fermentation media were sterilized in autoclave (Trade Raypa SL, Terrassa, Barcelona, Spain) at 100 °C for 60 min, and further inoculated with the strain ZSB10 in a percentage of 10% of the final volume of culture using washed cells (adjusted to 3.33 g/L), to obtain an initial concentration of 0.33 g/L. All media were incubated at 35 °C, 150 rpm and sampled at specific fermentation times (24 h and 48 h), and finally centrifuged (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) at 2755 × *g* for 15 min and 4 °C, for analysis.

2.4. Scale up of biosurfactant production in a 2-L bioreactor

One liter of culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) was prepared in order to use both the hemicellulosic and cellulosic fractions. The fermentation broth was placed in a 2-L bioreactor (Biostat B fermenter Plus, Sartorius, Germany), and inoculated with 10% of biomass (adjusted to 3.33 g/L), to give an initial biomass concentration of 0.33 g/L in the culture medium at time 0. During incubation at 35 °C and pH 7.4, the agitation speed was 150 rpm and the air flow rate was 2 L/min. Under these conditions the percentage of dissolved oxygen in medium was 0 during fermentation.

In a separate experiment to determine whether biomass concentration affects the production and activity of biosurfactant, the initial biomass concentration was increased tenfold. One liter of culture medium (MSM + HH + CH) was inoculated with 10% of biomass adjusted to 33.3 g/L, in order to start with an initial biomass concentration of 3.33 g/L in the culture medium at time 0.

2.5. Analytical methods

2.5.1. Sugar consumption analysis by HPLC

Samples (1 mL) were taken at selected fermentation times, centrifuged at 2755 × *g* for 15 min and 10 °C (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) and filter-sterilized using 0.22 μm pore-size membranes (EMD Millipore Corporation, Billerica, MA, USA). The liquid phase of the samples was employed for glucose, xylose, lactic acid, acetic acid, furfural and HMF analysis using a high-performance liquid chromatography (HPLC) system (Agilent, model 1200, Palo Alto, CA, USA) equipped with a refractive index detector and an Aminex HPX-87H ion exclusion column (Bio Rad 300 × 7.8 mm, 9 μm particles) with a guard column, eluted with 0.003 M sulfuric acid at a flow rate of 0.6 mL/min at 50 °C for 25 min.

2.5.2. Bacterial growth

Bacterial growth was monitored by measuring the turbidity of the cultures at 600 nm ($Abs_{600\,nm}$). Biomass was expressed as dry weight (g/L). In order to prepare a calibration curve, the strain ZSB10 was grown in 250 mL Erlenmeyer flasks containing 50 mL of MSM culture broth (pH 7.4) and was incubated under agitation of 150 rpm in a constant temperature incubator shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) at 35 °C for 48 h. The medium was centrifuged at 2755 × *g*, 10 °C for 15 min under sterile conditions and the precipitate was washed twice with a 4.9% NaCl solution. The absorbance of the biomass slurry was adjusted to 1.0 at a wavelength of 600 nm ($Abs_{600\,nm} = 1$) using a UV–vis Cintra 6 Spectrophotometer (GBC Scientific Equipment Ltd., Braeside, Australia). Bacterial growth was determined by triplicate measuring the absorbance of different

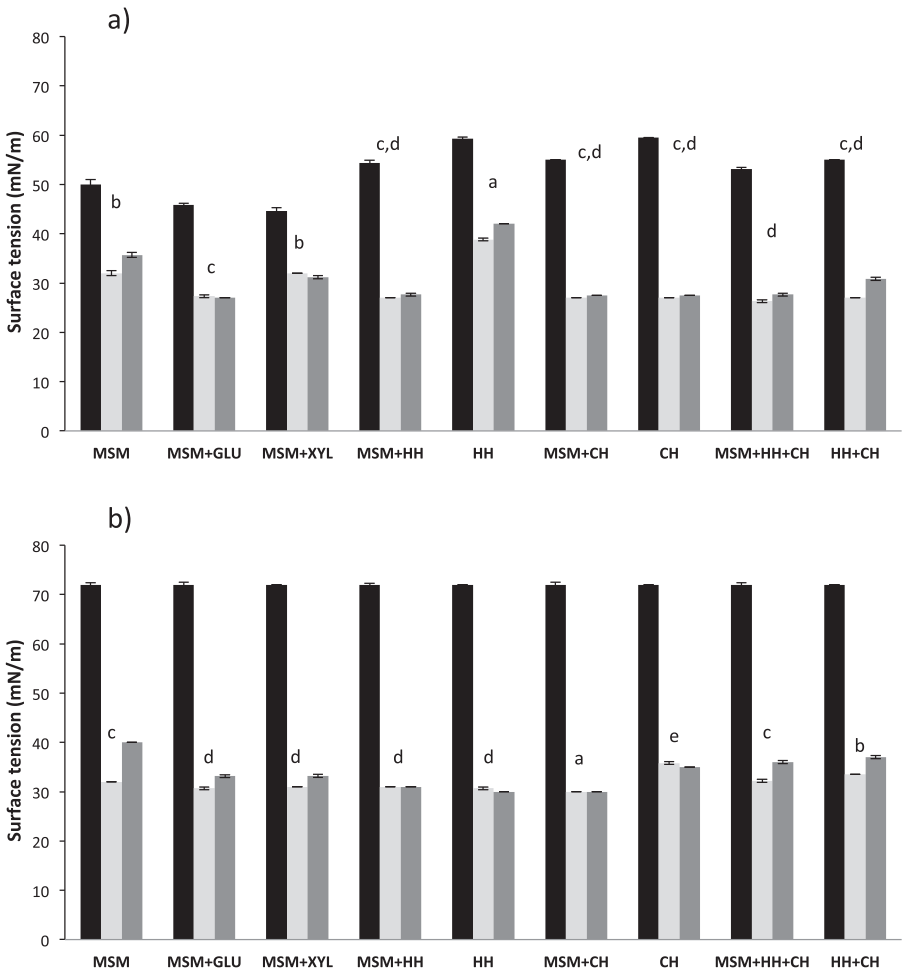

Fig. 2. Surface tension (expressed in mN/m) in (a) nine different culture media due to the production of extracellular biosurfactant, and (b) water due to cell-bound biosurfactant produced in nine different culture broths, using the ZSB10 strain. Same letters show no significant difference ($p > 0.05$). Time 0 (black); after 24 h (light grey); after 48 h (dark grey).

dilutions of the fermented culture medium at 600 nm and oven-dried at 105 °C (Celsius 2007, Memmert, Schwabach, Germany) to constant weight. The following equation was obtained: biomass dry weight (g/L) = 3.3089 (Abs_{600nm}) − 0.0578; $r^2 = 0.9958$.

2.5.3. Determination of surface tension of extracellular and cell-bound biosurfactants

Extracellular biosurfactant was obtained from the supernatant of the exhausted fermentation culture broth after centrifugation (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) at 2755 × *g* for 15 min and 4 °C. The precipitate (biomass pellet) was resuspended with distilled water in the original volume of culture broth in order to avoid dilution, washed twice with distilled water using a vortex (Classic, Velp Scientific, Italy) at 3000 rpm for 5 min, then it was centrifuged at the same conditions mentioned above to obtain cell-bound biosurfactant suspended in supernatant.

Both supernatants, extracellular and cell-bound biosurfactants, were used to determine the surface tension (ST) using a Krüss tensiometer equipped with a 1.9 cm Du Noüy wettable platinum ring at room temperature following the ring method (Kim et al., 2000). Biosurfactant activity of strain ZSB10 was determined measuring the surface tension reduction (△ST).

2.5.4. Extraction of crude biosurfactant

The extraction of crude biosurfactant was performed using extracellular and cell-bound biosurfactant supernatants of the fermentation obtained from ZSB10 strain in the culture broth MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) in a 2-L bioreactor for 52 h, starting with an initial biomass concentration of 0.33 g/L. Both supernatants were acidified to pH 2.0 with 6N HCl and left to stand at 4 °C overnight, afterwards these were centrifuged at 2755 × *g* for 15 min at 4 °C; the precipitates, extracellular and cell-bound biosurfactant were redissolved in distilled water (60 mL) and added to a separating funnel with dichloromethane (60 mL), that is, in a ratio of distilled water:dichloromethane (1:1). The organic phase was evaporated in order to obtain both crude biosurfactants as powders (Najafi et al., 2010; Shavandi et al., 2011).

2.5.5. Determination of critical micelle concentration (CMC) and emulsification index E_{24} in crude biosurfactants

Extracellular and cell-bound biosurfactants as crude powders were used to determine the CMC and emulsification index E_{24} using kerosene as hydrocarbon. The re-suspended biosurfactant powders were slightly soluble in water, in order to increase their solubility, the pH of the biosurfactant solutions should be increased to 10.5 using a solution of 3 M NaOH before CMC and E_{24} analysis. For CMC analysis, crude biosurfactant solutions at 180, 160, 140, 120, 100, 80, 60, 40, 20 and 10 ppm were prepared and the ST at each concentration was measured. Then, the ST values were plotted and the CMC was calculated by the intersection of the lines formed by the points of measurement.

The emulsification index (E_{24}) was determined in duplicate by mixing 1 mL of kerosene and 1 mL of crude biosurfactant solution

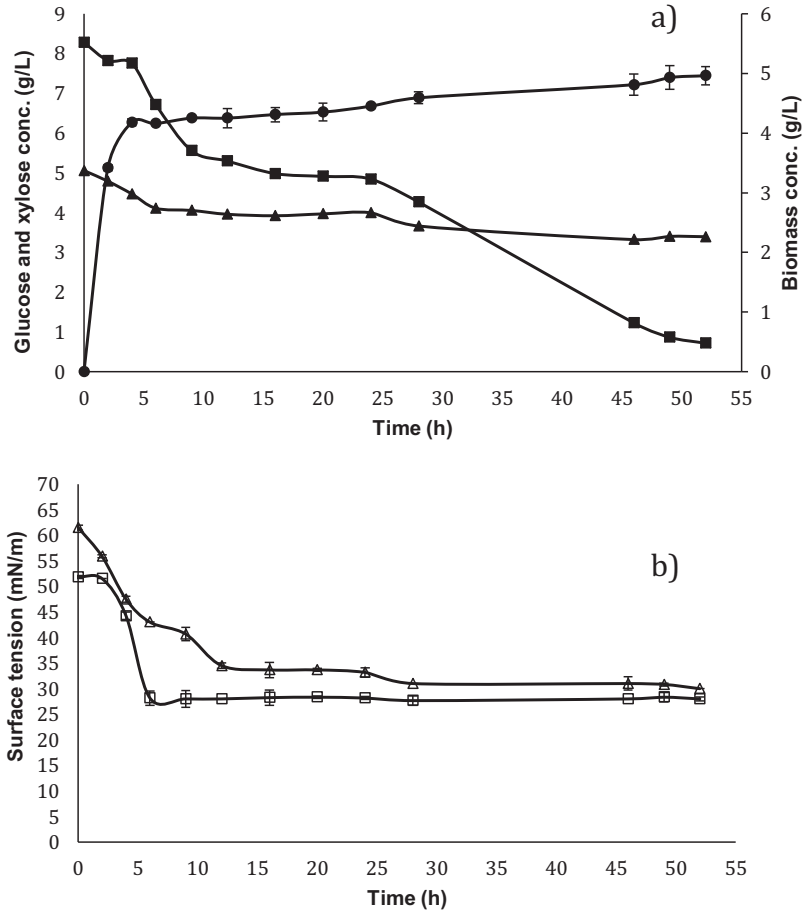

Fig. 3. Biosurfactants production by the ZSB10 strain using the culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) in a 2-L bioreactor starting with an initial biomass concentration of 0.33 g/L. Incubation conditions: 35 °C, 150 rpm, and pH 7.4. Glucose consumption (■); xylose consumption (▲); biomass (●); surface tension of extracellular biosurfactant (□); surface tension of cell-bound biosurfactant (△).

(1 mg/mL) in a 15 mL Falcon screw-capped tube (17 × 120 mm). Tubes were stirred for two minutes using a vortex to create emulsion and left to stand stable for 24 h. Then, the E_{24} was calculated by dividing the height of the emulsion layer by the height of the total mixture, and multiplied by 100 (Techaoei et al., 2011).

3. Results and discussion

3.1. Production of biosurfactants by ZSB10 strain using different growth media

Fig. 1a shows the growth of bacterial strain ZSB10. This growth appeared significant during the first 24 h (exponential growth phase), being able to grow in the 9 different culture media assayed, although the behavior was different depending on the medium composition. The strain was favored when the mineral salt medium (MSM) was added to a carbon source such as glucose or xylose, but was also able to grow well without the salts and yeast extract contained in MSM medium, meaning that could grow using only the cellulosic or hemicellulosic sugars provided by hydrolyzates from vine-trimming shoots. The results demonstrate the halotolerance of this strain, which means that it could grow either in the absence or in presence of salts. Furthermore, biosurfactants production was evaluated using the extracellular biosurfactant excreted into the culture medium and the cell-bound biosurfactants by washing the cells with distilled water. Fig. 2a shows that at time 0 (ST_0) there was a reduction of the ST regarding the value of the water (72 mN/m), which must be attributed to the components of the culture medium. A significant reduction in ST was observed on day 1 due to the concentration of excreted biosurfactant to the medium, meanwhile on day 2 a visible increase in ST was observed in most cases, which may be due to the microorganism starts consuming the metabolite. At each time surface tension reduction (△ST) was calculated as $ST_0 - ST_t$. The △ST of CH culture medium showed the highest value (△ST = 59.5–27 = 32.5 mN/m), followed by MSM + CH and HH + CH (△ST = 55–27 = 28 mN/m) and MSM + HH (△ST = 54.3–27 = 27.3 mN/m). In all cases, ST was 27 mN/m at day 1 of fermentation. The ST reduction for MSM + HH + CH was 26.8 mN/m. After one day, ST reached a minimum of 26.3 mN/m. Considering these results, it can be inferred that the strain ZSB10 can use both cellulosic and hemicellulosic hydrolyzates to produce biosurfactants. In all prior cases, the surface tension reduction was higher when MSM was used as culture broth for biosurfactant production, because it only was observed a reduction of △ST = 50–32 = 18 mN/m. In all cases, the production of extracellular biosurfactant was evidenced since a substance can be considered as biosurfactant when added to distilled water is able to lower the ST in more than 8 units (Rivera et al., 2007; Rodríguez-Pazo et al., 2013).

On the other hand, cell-bound biosurfactant was obtained by washing out of biomass with distilled water. The ST of water (72 mN/m) was considered at day 0 to evaluate the surface tension reduction. Fig. 2b shows the strong reduction in ST on biosurfac-

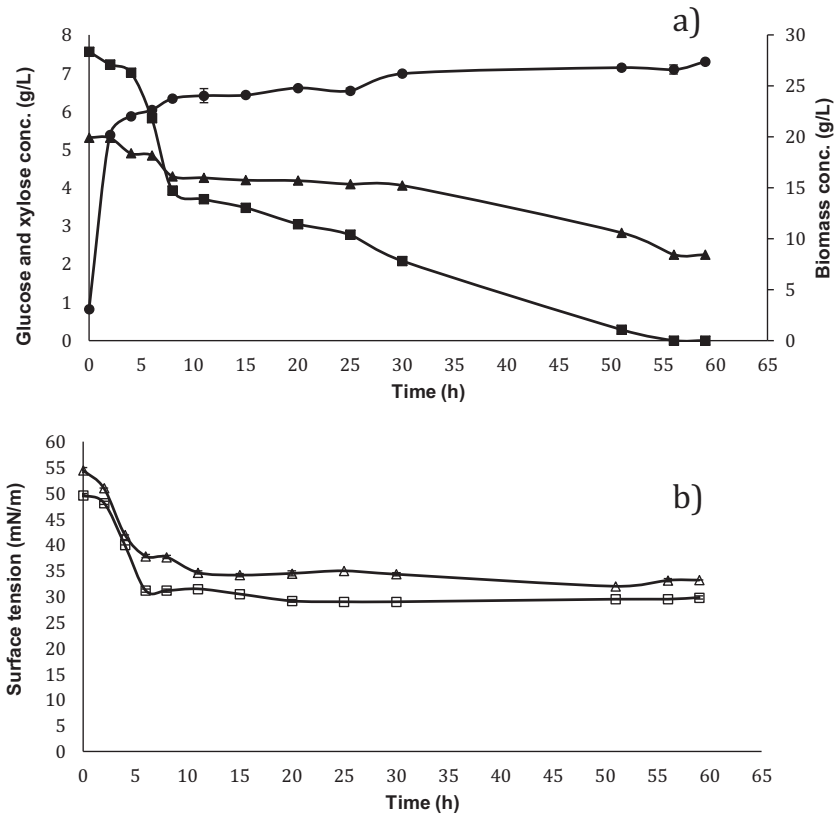

Fig. 4. Biosurfactants production by the strain ZSB10 using the culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) in a 2-L bioreactor starting with an initial biomass concentration of 3.33 g/L. Incubation conditions: 35 °C, 150 rpm, and pH 7.4. Glucose consumption (■); xylose consumption (▲); biomass (●); surface tension of extracellular biosurfactants (□); surface tension of cell-bound biosurfactants (△).

tants obtained from biomass of all culture media. Such reduction was higher at day 1 in most cases. An analysis of variance with the results of the ST of day 1 showed significant difference ($p < 0.05$), however, among some culture media there was no significant difference ($p > 0.05$).

The lowest ST value of cell-bound biosurfactants was achieved using the MSM + CH medium (ST = 30 mN/m), meanwhile the highest ST value was obtained with CH medium (ST = 35.8 mN/m). It is noticeable that the latter showed the lowest surface tension value using extracellular biosurfactant.

Comparing all these previous results measured with extracellular and cell-bound biosurfactants, it can be concluded that extracellular biosurfactants have better surfactant characteristics achieving the lowest values of ST. Rodríguez-Pazo et al. (2013), using co-cultures of *Lactobacillus plantarum* and *Lactobacillus pentosus*, reported a surface tension reduction of only 17.2 mM/m under conditions where only cell-bound surfactants were present. Similarly, Moldes et al. (2007), using *L. pentosus* grown in hemicellulosic hydrolyzates, reported a surface tension reduction of 21 mM/m.

Furthermore, the consumption of sugars by strain ZSB10 was monitored in Fig. 1b (glucose) and Fig. 1c (xylose) at times 0, 24 and 48 h. HH + CH (50% hemicellulosic and 50% cellulosic) culture medium was mainly considered in order to obtain full use of both fractions. In this case, the initials glucose and xylose concentrations were 10.83 g/L and 5.11 g/L respectively. The microorganism consumed all the glucose, remaining 3.7 g/L of xylose after 24 h, which was further consumed after 48 h. On the other hand, MSM + HH + CH culture medium presented initials glucose and xylose concentrations of 9.69 g/L and 4.24 g/L respectively. The addition of MSM to the mixture of hydrolyzates affected its behavior remaining unconsumed residual amounts of sugars (0.19 g/L of glucose and 1.3 g/L of xylose) after 24 h, although these were completely consumed at the end of fermentation (48 h) meaning that there was neither an excess nor a deficit of nutrients due to the MSM addition. In general, the consumption of glucose by the microorganism was higher than consumption of xylose. It should be noted that glucose was present in higher concentration at time 0 regarding xylose.

3.2. Scaling up biosurfactants production using the hemicellulosic and cellulosic fractions of hydrolyzates in a 2-L bioreactor

The production of biosurfactants by strain ZSB10 was scaled up using the culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates), in order to supply nutrients from MSM medium and take advantage of both hydrolyzates of vine-trimming shoots. Previous assays revealed that biosurfactants obtained from MSM + HH + CH culture medium showed good results in the surface tension reduction (Fig. 2a and b).

Fig. 3a shows the growth of the strain ZSB10 in this medium, noticing a significant increase of biomass at 2 h (3.4 g/L) and 4 h (4.2 g/L) of incubation ($p < 0.05$), but continues growing slightly, until reaching a concentration of 4.5 g/L at 24 h and 5.0 g/L at the end of fermentation (52 h). Fig. 3a also describes the consumption of sugars (glucose and xylose) from the mixture of cellulosic and hemicellulosic vine-trimming shoots hydrolyzates supplemented with MSM during 52 h. The initial sugar concentration was of 8.3 g/L glucose and 5.1 g/L xylose.

Glucose was almost depleted at the end of fermentation (91.3%), however, xylose was scarcely consumed (30.8%) after 52 h. These results performed using 1 L of culture medium in a 2-L bioreactor are worse than those attained with the same experiment performed

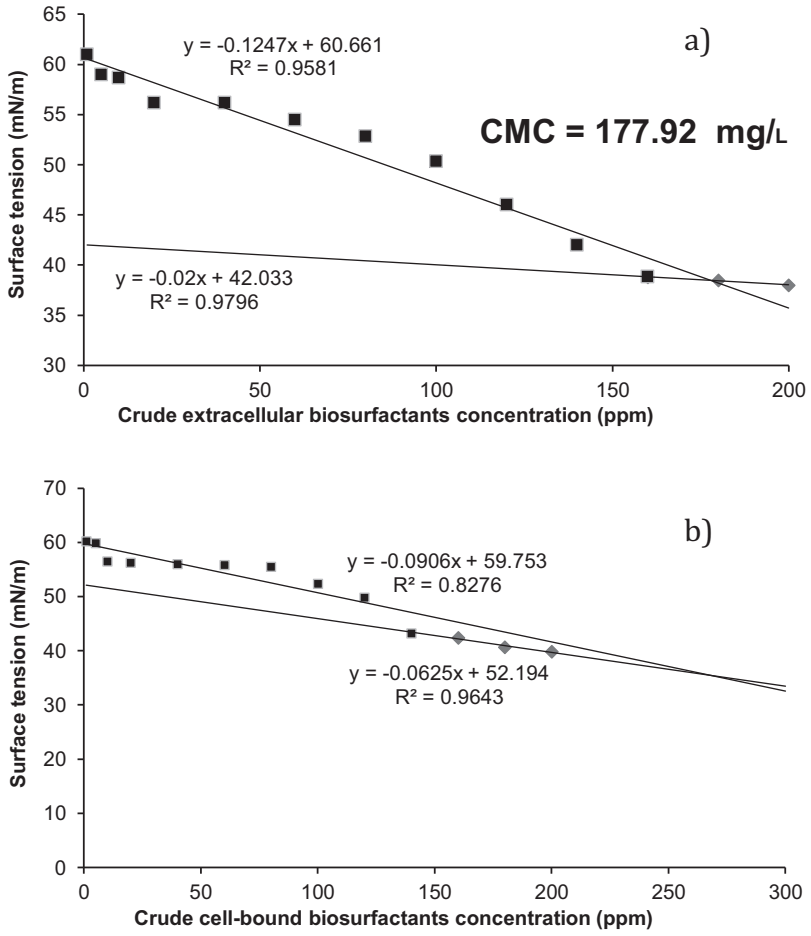

Fig. 5. Determination of critical micelle concentration (CMC) obtained from (a) crude extracellular biosurfactant and (b) crude cell-bound biosurfactant, obtained from fermentation of ZSB10 strain in MSM + HH + CH medium.

at a flask level (50 mL of medium in 250 mL Erlenmeyer flask), both inoculated with the same amount of biomass, where the substrate consumption was faster and higher. This poor performance cannot be attributed to pH, since this value only decreased slightly during fermentation from 7.4 at time 0 to 6.24 after 52 h, due to the increase of the concentration of two acids: acetic acid that rose from 0 to 0.84 g/L, but mainly to the appearance of lactic acid at 20 h of fermentation, which was augmenting its concentration until 4.0 g/L at the end of fermentation.

Regarding biosurfactants production, Fig. 3b shows the decrease in ST produced by extracellular and cell-bound biosurfactants. There was a reduction in ST at time 0 due to the components of the medium. However, as the fermentation proceeded, the decrease in ST was attributed to the biosurfactants production. It should be noted that the extracellular biosurfactant fermented medium reached the lowest ST value after 6 h, and no significant change ($p > 0.05$) turn out between 6 h (28.2 mN/m) and 52 h of fermentation (28 mN/m) indicating that it was achieved the critical micelle concentration (CMC). The CMC is defined as the concentration of surfactant required to initiate the formation of micelles. When the CMC is reached, the ST does not continue to decline even adding more surfactants (Shavandi et al., 2011). Consequently, measuring the ST of the fermented culture medium does not enable the determination of the quantity of biosurfactants produced. Expressed as surface tension reduction (△ST), extracellular biosurfactant resulted in a maximum reduction of 23.8 mN/m at 6 h, without significant differences ($p > 0.05$) between 6 and 52 h of fermentation.

Fig. 3b also shows the decrease in ST of water containing the extracted cell-bound biosurfactants. It was observed a slight decrease in ST of water at time 0 (when still there was no growth of biomass) from 72 mN/m to 61.5 mN/m due to dissolved salts in the supernatant. In this case, it was reached an important lowering in ST values during fermentation (ST = 30 mN/m, at 52 h) but these were not better than those obtained using extracellular biosurfactant.

3.3. *Scaling up biosurfactants production using the hemicellulosic and cellulosic fractions of hydrolyzates in a 2-L bioreactor increasing the concentration of the initial inoculum*

The initial concentration of inoculum was increased 10 times (3.33 g/L dry weight biomass), with the purpose to determine if there was an improvement in the production and activity of biosurfactant with a larger initial biomass concentration.

Fig. 4a shows the faster growth of ZSB10 strain in this medium, noticing a significant increase ($p < 0.05$) during the first 2 h (20.2 g/L) being slower thereafter until reaching a final concentration of 27.4 g/L at 59 h. Additionally, the consumption of sugars (glucose and xylose) from the hemicellulosic and cellulosic vine-trimming shoots hydrolyzates was also monitored, in this way in Fig. 4a can be observed that glucose was completely depleted, however, only 57.63% xylose was consumed in the same period of time.

In comparison with the previous experiment, performed with a smaller amount of biomass, it can be concluded that when the initial biomass was higher there was an increase in the utilization of glucose with a higher rate of glucose consumption. Regarding

xylose consumption, there were no big differences between experiments. In both cases the consumption of xylose occurred when the glucose was being diminished. Additionally, during fermentation occurred a small change of pH from 7.07 to 6.26 at 59 h, which can be attributed to the production of lactic and acetic acids after 11 h of fermentation, ending with concentrations of 2.1 and 1.6 g/L, respectively.

On the other hand, Fig. 4b shows the values of ST measured on the culture medium with time. Notably, the lowest ST of extracellular biosurfactant (29.2 ± 0.3 mN/m) was reached at 20 h of fermentation and no significant changes ($p > 0.05$) were observed until the end of fermentation (29.8 mN/m) meaning that CMC was reached after that time. The slight increment in ST values observed after 51 h could be due to the biosurfactants began to be consumed by the microorganism as substrate. Compared to the previous experiment, it could be observed that a faster decrease of ST and a better result (28.2 mN/m at 6 h) was obtained using a lower initial biomass concentration (0.33 g/L).

Finally, the measure of the ST in water containing the cell-bound biosurfactants showed a reduction in ST from 54.5 mN/m at time 0 to 37.8 mN/m after 6 h being almost constant from 11 h until achieving a lowest level of 32 mN/m after 51 h. ST results of cell-bound biosurfactant are slightly superiors to those achieved in the previous experiment with smaller amount of initial biomass, where the lowest ST (30 mN/m) was achieved at 52 h of fermentation. These results seem contradictory, since having more biomass should have more cell-bound biosurfactant since the metabolite is found inside or attached to the cells. It is feasible that during fermentation this cell-bound biosurfactant became extracellular due to shear in the bioreactor.

3.4. Extraction and determination of critical micelle concentration (CMC) of crude biosurfactants

The culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) fermented in a 2-L bioreactor, starting with an initial biomass concentration of 0.33 g/L, was used for crude biosurfactants extraction.

Crude extracellular biosurfactant had a yield of 1.52 g/L and lowered ST to 38.6 mN/m with a CMC = 177.92 mg/L (Fig. 5a). However, crude cell-bound biosurfactant only had a yield of 0.0783 g/L with a ST value of 39.8 mN/m as it is shown in Fig. 5b where it was not possible to determine the CMC of cell-bound biosurfactant because the experiment was carried out with a maximum concentration of 200 mg/L and this amount was not enough since in Fig. 5b did not occur the intersection of lines that is required for CMC determination.

Compared with those yields reported in literature, Rivera et al., (2007) had an intracellular biosurfactant yield of 0.0048 g/L using *L. pentosus* and grape marc, an useless agricultural residue from the wine industry, as substrate. Rodríguez et al., (2010) used wine industry waste materials such as vine-trimming shoots and distilled wine lees (vinasses) as culture media for biosurfactants production by *L. lactis,* the biosurfactant yield obtained was 0.0017 g/L and 0.0015 g/L expressed as surfactin equivalent, by two different methods. Using vine trimming shoots and a microbial co-culture of *L. plantarum* and *L. pentosus*, Rodríguez-Pazo et al., (2013) reported a maximum cell-bound biosurfactant yield of 0.0056 g/L. Consequently, the yield of extracellular crude biosurfactant produced by ZSB10 strain, using a similar culture medium, overcame these results.

Furthermore, in spite of the relatively high CMC values achieved by the strain ZSB10, the ST was decreased below 40 mN/m. The CMC value of extracellular biosurfactant resulted to be high, however, reports have been found using partially purified biosurfactants with higher CMC values than the reported in this work. For example Bodour et al., (2004) worked with a biosurfactant that diminished the ST value to 26 mN/m with a CMC value of 300 mg/L. Chen et al., (2012) used a biosurfactant produced by *B. licheniformis* TKU004 that decreased ST from 72 mN/m to 25.4 mN/m with a high CMC value of 350 mg/L.

Finally, E_{24} of crude biosurfactants indicated that both extracellular and cell-bound biosurfactants were able to emulsify kerosene, with E_{24} values of 47% and 41% respectively. Although both biosurfactants showed ability to emulsify kerosene, the emulsion of extracellular biosurfactants was more stable after 24 h in repose.

4. Conclusion

The strain *B. tequilensis* ZSB10 was able to consume hemicellulosic and cellulosic sugars obtained by sequential treatments of vine-trimming shoots to produce extracellular and cell-bound biosurfactants, in the presence or absence of salts. These results confirmed that ZSB10 strain is halotolerant. The process was scaled up from Erlenmeyer flask to 2-L bioreactor consuming effectively the mixture of hydrolyzates. Crude extracellular biosurfactants had a yield of 19.4 times the value achieved with crude cell-bound biosurfactant. Comparing the ST and CMC values between both kinds of biosurfactants, extracellular biosurfactant had better surfactant characteristics than cell-bound biosurfactant. Nevertheless, both biosurfactants showed ability to emulsify kerosene, although, the emulsion was more stable using extracellular biosurfactant after 24 h in repose.

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