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ARTICLE

Stabilization of Kerosene/Water Emulsions Using Bioemulsifiers Obtained by Fermentation of Hemicellulosic Sugars with *Lactobacillus pentosus*

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The results of the present study show that *Lactobacillus pentosus* can produce extracellular bioemulsifiers by utilizing hemicellulosic sugars from grape marc as a source of carbon. The effectiveness of these bioemulsifiers (LPEM) was studied by preparing kerosene/water (K/W) emulsions in the presence and absence of these emulsifiers. Various parameters such as relative emulsion volume (EV), stabilizing capacity (ES), viscosity, and droplet size of K/W emulsions were measured. The EV values for K/W emulsions stabilized by concentrated LPEM were approximately 74.5% after 72 h of emulsion formation, with ES values of 97%. These values were higher than those obtained with dodecyl sodium sulfate as emulsifier (EV = 62.3% and ES = 87.7%). Additionally, K/W emulsions stabilized by LPEM produced polydisperse emulsions containing droplets of radius between 10 and 40 μm, which were smaller than those obtained for K/W emulsions without LPEM (droplet radius = 60 −100 μm). Moreover, the viscosity values of the K/W emulsions without and with LPEM were approximately 236 and 495 cP, respectively.

KEYWORDS: Grape marc; hemicellulosic sugars; *Lactobacillus pentosus*; emulsifiers; kerosene; polydisperse emulsion

INTRODUCTION

One criterion used to define bioemulsifiers is their ability to maintain at least 50% of the original emulsion volume 24 h after formation (*1*). Microbial emulsifiers are produced by various genera of bacteria and yeast, including lactic acid bacteria (*2*, *3*). Many of these bioemulsifiers are exopolysaccharides (EPS) that can be used in the food, pharmaceutical, and petroleum industries. For example, emulsan is an extracellular protein-associated lipopolysaccharide produced by *Acinetobacter calcoaceticus* RAG 1, having a molecular weight of approximately 1000 kDa (*4*). It does not reduce the interfacial tension appreciably but, by binding to the oil−water interface, protects the oil droplets from coalescing, thus bestowing stability to the emulsions. Emulsan could therefore be considered useful for enhanced oil recovery (*4*). Many attempts have been made to produce emulsifying agents to test their properties with kerosene and other organic compounds (*5*−*7*). In a previous study we reported that *Lactobacillus pentosus* produces cell-bound biosurfactants that not only reduce the surface tension of water but also display emulsifying properties (*8*). However, as lactic acid bacteria have the ability to produce extracellular exopolysaccharides (*9*−*11*) and as bioemulsifiers do not necessarily lower the interfacial tension, but can stabilize oil-in-water emulsions (*4*), it can be hypothesized that *L. pentosus* could produce extracellular bioemulsifiers.

The main natural mechanisms of hydrocarbon removal are photo-oxidation, evaporation, and microbial degradation. These processes may take years to stabilize and, thus, to remediate hydrocarbon-contaminated sites (*12*). Bioremediation of sites contaminated by hydrocarbons is limited by the poor availability of these hydrophobic contaminants to microorganisms, which may be improved by use of bioemulsifiers. Consequently, it is important to develop low-cost processes with high efficiency of remediation, such as processes involving the use of natural bioemulsifiers.

An emulsion consists of two immiscible liquids, with one of the liquids dispersed as small spherical droplets in the other liquid (*13*). Emulsions are classified into different types: emulsions that consist of water droplets dispersed in an oil phase are referred to as water-in-oil (W/O) emulsions, and those consisting of hydrocarbon droplets dispersed in an aqueous phase are referred to as oil-in-water (O/W) emulsions. The component that makes up the droplets is known as the "dispersed phase", and the substance that makes up the surrounding liquid is referred to as the "continuous phase" (*14*). Most surface-active compounds are produced by microorganisms. Compounds that reduce the surface tension at the air−water interface are called biosurfactants, and those that reduce the interfacial tension between immiscible liquids, or at the solid−liquid interface, are called bioemulsifiers (*15*).

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The physicochemical properties of emulsions are greatly influenced by the characteristics of the droplets that they contain; for example, the stability of an emulsion is strongly affected by the size of the droplets. When all of the droplets in an emulsion are the same size, the emulsion is referred to as "monodisperse", whereas a "polydisperse" emulsion is characterized by a wide range of particle sizes (*13*).

In the present study, we investigated the emulsifying capability of the crude extracellular bioemulsifiers produced by *L. pentosus* grown on hemicellulosic sugars obtained from grape marc. We prepared kerosene/water (K/W) emulsions in the presence and absence of these emulsifiers, and various physicochemical properties of emulsions such as droplet size, droplet concentration, emulsion viscosity, or emulsion conductivity were studied.

MATERIALS AND METHODS

Raw Material. Distilled grape marc was chosen as carbon source because it is an agro-industrial residue and it is cheaper than commercial sugars. Samples of distilled grape marc were dried at room temperature and milled to a particle size suitable for hydrolysis (approximately 1 mm).

Preparation of Hemicellulosic Hydrolysates. Prehydrolysis (acid hydrolysis using a diluted acid) of distilled grape marc was carried out in an autoclave at 130 °C, with 3% H_2SO_4 acid for 30 min and a liquid/solid ratio of 8 g/g.

Microorganism. *L. pentosus* CECT-4023T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown in MRS (de Man, Rogosa, and Sharpe) broth at 31 °C for 15 h and 150 rpm. The culture was then centrifuged, and *L. pentosus* cells were resuspended in the same volume of hydrolysate for use as inoculum.

Media Preparation and Emulsifier Production. Hemicellulosic hydrolysates from grape marc were neutralized with powdered $CaCO_3$ to a final pH of 6.0, and the precipitated $CaSO_4$ was separated from the supernatant by filtration. The clarified liquid was supplemented with 10 g/L yeast extract and 10 g/L corn steep liquor (*16*), sterilized at 100 °C for 1.25 h, to avoid the formation of inhibitory products, and used directly as fermentation medium. For comparative purposes and in order to see differences with other components present in grape marc hydrolysates, fermentations were also carried out with a mixture of commercial sugars consisting of 2.3 g/L glucose, 4.7 g/L xylose, 2.3 g/L arabinose, 1.8 g/L galactose, 1.3 g/L mannose, and 0.19 g/L fructose, which simulates the concentration of hemicellulosic sugars found in grape marc hydrolysates, also supplemented with 10 g/L of both yeast extract and corn steep liquor, and minerals (0.015 g/L $MnSO_4 \cdot H_2O$, 5.068 g/L K_2HPO_4, 0.045 g/L $NaOOCCH_3$, 16.260 g/L $CaSO_4 \cdot 2H_2O$, and 2.194 g/L $MgSO_4 \cdot 7H_2O$). The emulsifier was produced in a 2 L Biostat B batch reactor (Braun, Melsungen, Germany) with 1.5 L of working volume, by the addition of 100 mL of inoculum suspension. The fermentation was performed at 31 °C and 150 rpm for 12 h. In both cases pH was maintained at 6.5 by the addition of 5 M NaOH. At the end of the fermentation period, the culture was centrifuged and the supernatants containing the LPEM were maintained at −20 °C until analysis and emulsion experiments.

Ethanol Extraction. In some cases LPEM were precipitated from supernatants following the protocol proposed by Martínez-Checa et al. (*17*) by adding 96% ethanol to the cell-free fermentation medium at a ratio of 1:6 v/v (medium/ethanol). The precipitation was performed twice and the medium allowed to stand at 4 °C overnight. The LPEM precipitate was then resuspended in distilled water, with the same starting volume of broth to maintain the initial concentration or with a smaller volume of water, reduced by evaporation, to observe the effect of higher concentrations of LPEM on the emulsion-forming capacity.

Emulsification Studies. Emulsification was performed according to the methodology proposed by Das et al. (*18*). Kerosene was vortexed for 2 min with an equal volume (2 mL) of aqueous phase. The aqueous phase consisted of 2 mL of either nonfermented media or fermented media, always filtered through a 0.2 μm membrane to prevent interference from solids. The tubes were vortexed and left to stand for 1 h. After this time (considered as the starting time, 0 h), the relative emulsion volume (EV, %) and emulsion stability (ES, %) were calculated at 24 h intervals, up to 72 h, from eqs A.1 and A.2 proposed by Das et al. (*18*).

$$\text{EV, \%} = \frac{\text{emulsion height, mm} \times \text{cross-section area, mm}^2}{\text{total liquid volume, mm}^3} \quad \text{(A.1)}$$

$$\text{ES, \%} = \frac{\text{EV, at time, h}}{\text{EV, at 0 h}} \times 100 \quad \text{(A.2)}$$

The emulsions stabilized by LPEM were also compared with those formed by 1% (w/v) solution of the chemical surfactant sodium dodecyl sulfate (SDS) in deionized water (*18*).

In addition, the percentage of emulsified organic phase (EOP) was calculated from eq A.3

$$\text{EOP, \%} = \frac{\text{vol TOP, mm}^3 - (\text{NEOP, mm} \times \text{cross-section area, mm}^2)}{\text{vol TOP, mm}^3} \times 100 \quad \text{(A.3)}$$

where TOP is the total volume of organic phase and NEOP is the nonemulsified organic phase.

Furthermore, taking into consideration that other authors (*19*−*22*) have reported different methodologies for emulsification studies with low volumes of organic phase, 50−350 μL of kerosene was added to a test tube containing 4 mL of fermented or nonfermented medium and vortexed for 2 min. The emulsions thus formed were left to stand for 1 h, and the absorbance was measured at 500 nm, at 12 h intervals for up to 48 h. The emulsification results are presented as means of three replicates.

Droplet Size Measurements. *Microscopy*. A Nikon Eclipse E800 optic microscope equipped with a Nikon camera was used to observe the emulsion droplets. The emulsion was observed through a 10× objective lens. NIS Elements D2.30 SPI software from Nikon was used to produce photographs and to measure the radii of the droplets.

Dynamic Light Scattering. A Zetasizer Nano-ZS (Malvern Instruments, Malvern, U.K.) was used to measure the size of the droplets in dilute, nonhomogenized emulsions, achieved by mixing 4 mL of the aqueous phase (fermented media) with 50 μL of kerosene.

Droplet Concentration. The concentration of droplets in the emulsions was calculated in terms of the dispersed phase volume fraction, which is equal to the volume of emulsion droplets (V_D) divided by the total volume of emulsion (V_E), following the procedure proposed by McClements (*13*).

Emulsion Viscosity. Viscosity of emulsions was measured with a Brookfield viscometer (model LV DV-II+) at 25 °C. To perform measurements, 120 mL of emulsion was placed in a tall 150 mL beaker, at 10 rpm. The results are presented as mean values of two replicates for each sample.

Emulsion Conductivity. Conductivity of emulsions was measured to identify the continuous and disperse phases (*23*), with a Crisson GLP 32 conductivimeter.

RESULTS AND DISCUSSION

Emulsions were made with the crude cell-free broth obtained from 12 h fermentations performed with *L. pentosus* growing on supplemented grape marc hydrolysates or with commercial medium as the aqueous phase according to the methodology proposed by Das et al. (*18*). The crude cell-free broth displayed the capacity to produce metastable (kinetically stable) kerosene-in-water (K/W) emulsions; that is, the mixture remained stable for a certain period before complete separation of the phases (*13*). This indicates that the presence of extracellular bioemulsifiers produced by *L. pentosus* (LPEM) stabilized the emulsions. To rule out the presence of endogenous emulsifiers associated with the fermentation media, a series of emulsions were made with unfermented culture media as aqueous phase. The mixture emulsified to a certain degree, but the emulsions thus obtained were unstable and tended to separate into the original phases. A photograph of 24-h-old K/W emulsions stabilized with LPEM or

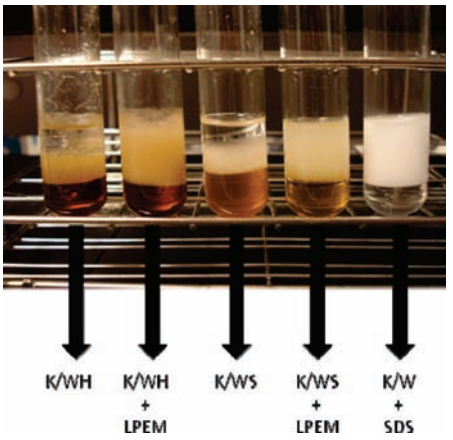

Figure 1. Photograph of 24-h-old emulsions of kerosene and aqueous solution stabilized with LPEM or SDS. K/WH, kerosene/water emulsion without LPEM, with the nonfermented grape marc hydrolysates as the aqueous phase; K/WH+LPEM, kerosene/water emulsion with LPEM, with the fermented grape marc hydrolysates as the aqueous phase; K/WS, kerosene/water emulsion without LPEM, with the nonfermented commercial fermentation medium as the aqueous phase; K/WS + LPEM, kerosene/water emulsion with LPEM, with the fermented commercial medium as the aqueous phase; K/W + SDS, kerosene/water emulsion stabilized by SDS.

SDS is shown in **Figure 1**. An emulsion layer was observed in all cases, but the almost complete absence of free kerosene was observed only in the emulsions stabilized with LPEM from fermented hydrolysates (K/WH + LPEM) or commercial medium (K/WS + LPEM). The same was observed in the case of reference emulsions stabilized with SDS (K/W + SDS).

Conductivity measurements performed on the emulsion layer confirmed the presence of K/W emulsions, with values of 328 and 148 μS/cm for the emulsions stabilized by LPEM from hydrolysate and commercial fermented media, respectively. These values indicate that the aqueous solution is the continuous phase of the emulsions, whereas kerosene droplets are trapped by the action of the bioemulsifier.

Study of Resistance to Creaming of K/W Emulsions Stabilized with LPEM. When the density of the droplets of the disperse phase is lower than that of the surrounding liquid (continuous phase), the droplets tend to move upward in a phenomenon referred to as "creaming" (*14*). Thus, emulsions formed by adding 2 mL of kerosene, as proposed by Das et al. (*18*), tended to cream rapidly, so that the emulsion layer became a cream (**Figure 1**). To assess the stability in terms of resistance to creaming, a series of low-concentration emulsions, with kerosene concentrations ranging from 50 to 350 μL, were prepared with 4 mL of aqueous phase. The absorbance of the bottom of the emulsion was measured, at 500 nm, and compared with a control consisting of the crude cell-free broth containing LPEM from hemicellulosic grape marc or commercial sugars without kerosene. It was assumed that the increase in absorbance relative to the control was because the kerosene had not creamed but had dispersed in the aqueous phase in the form of small droplets trapped by the molecules of the bioemulsifiers. The results (**Figure 2**) clearly reflect the higher resistance to creaming of the emulsions made with LPEM from hydrolysates, in comparison with those made with LPEM from commercial medium. The absorbance values were not only higher for these emulsions but also increased with increasing concentrations of kerosene, until reaching a maximum, as the ratio kerosene/bioemulsifiers was high, and thus favored creaming and, consequently, migration of kerosene from the bulk

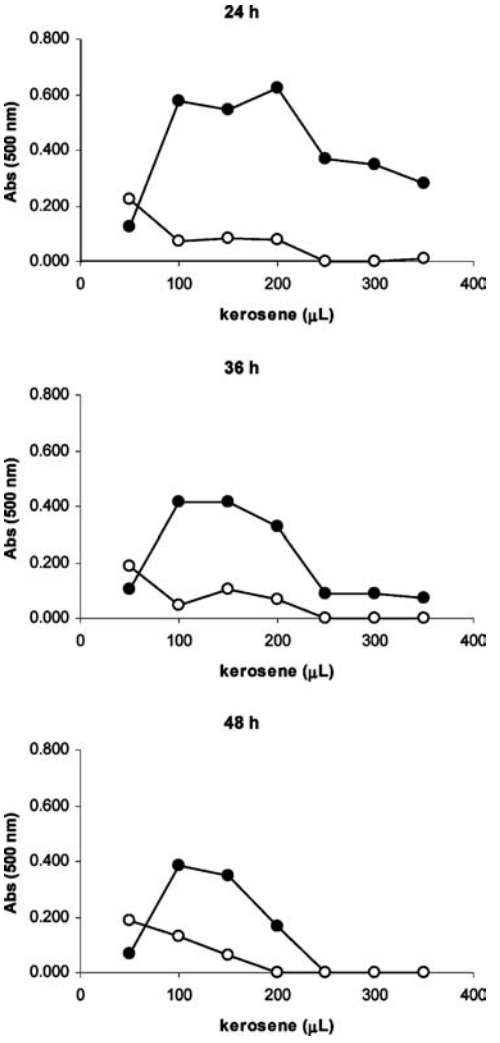

Figure 2. Absorbance (measured at 500 nm) of the bottom of the emulsions at different times after mixing 4 mL of hydrolysate (solid symbols) or commercial (open symbols) fermented media and different volumes of kerosene.

of the aqueous phase to the top. Creaming was also affected by the age of the emulsion, as a reduction in the absorbance was observed over time, before it finally stabilized at a value that depended on the volume of kerosene added: when the ratio of kerosene/bioemulsifiers was very high, kerosene droplets were poorly stabilized, and creamed, resulting in final absorbance values close to zero (**Figure 2**). However, with a low ratio of kerosene/bioemulsifiers, stabilization of the kerosene droplets is expected so that they will remain in the aqueous phase without creaming and produce higher absorbance values than those corresponding to the aqueous phase without kerosene (as occurred with the lowest volume of kerosene added).

Additionally, the size of kerosene droplets was measured by dynamic light scattering for those emulsions in which creaming was not favored (emulsions formed with 4 mL of both commercial and hydrolysate-fermented media containing LPEM and 50 μL of kerosene). Analysis was also carried out in both fermented media without addition of kerosene. **Figure 3** clearly shows the similar behavior of the commercial and hydrolysate media containing LPEM, but reflects a large difference in the media with and without kerosene and suggests the existence of some particles in the fermented hydrolysates that can be considered as bioemulsifiers. In effect, in the absence of kerosene,

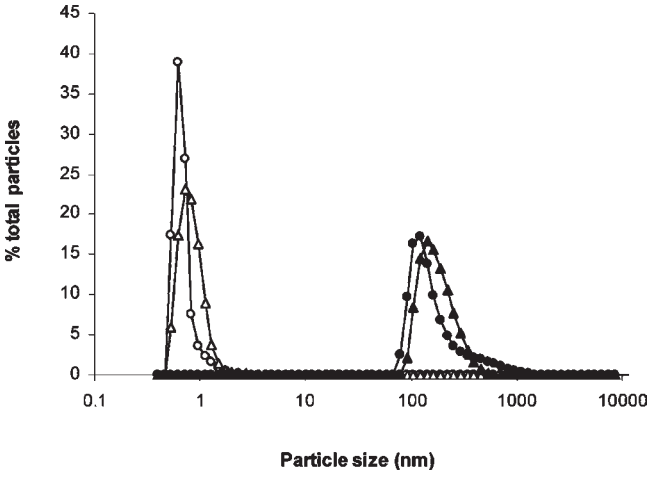

Figure 3. Particle sizes and percentage of particles (relative to the total amount of particles) for the different particle sizes measured by dynamic light scattering in the hydrolysate (circles) or commercial (triangles) fermented media without (open symbols) and with 50 μL (solid symbols) of emulsified kerosene.

small particles that may correspond to the emulsifier micelles were observed but completely disappear when the hydrocarbon is present and emulsified, and in this case larger particles that may correspond to the droplets of kerosene stabilized by the emulsifier molecules were observed.

Emulsifying and Stabilizing Capacity of LPEM. To obtain further information about the emulsifying and stabilizing capacities of LPEM, the emulsion volume and emulsion stability were determined. The emulsion-forming capacity, expressed as percentage of relative emulsion volume (EV), and stabilizing capacity, expressed as emulsion stability (ES), of kerosene/water emulsions (K/W) stabilized by extracellular emulsifiers produced by *L. pentosus* (LPEM), by fermentation of grape marc hydrolysates or commercial sugars, are shown in **Figure 4**. This preliminary study was carried out with K/W emulsions made with the crude cell-free broth as aqueous phase in the presence or absence of LPEM. The EV and ES were therefore also measured in emulsions consisting of kerosene and nonfermented media. In the absence of LPEM, these emulsions produced EV values of 46.7 and 23.7% 24 h after emulsion formation and 29.1 and 13.5% after 72 h of emulsion formation for K/W emulsions made with grape marc hydrolysates or dissolved commercial sugars as the aqueous phase. Furthermore, when EV values were obtained for K/W emulsions in the presence of LPEM, the corresponding values increased to 62.7 and 53.9% after 24 h of emulsion formation for K/W emulsions with grape marc hydrolysates or dissolved commercial sugars as the aqueous phase, which were kept after 72 h of emulsion formation (61.7 and 53.2%, respectively). The values were higher than those obtained in a previous study (*8*) in which the bioemulsifying properties of cell-bound biosurfactants produced by *L. pentosus*-induced fermentation of grape marc hydrolysates were tested and similar to the EV value of emulsions stabilized by SDS (EV = 62.3% after 72 h). The results obtained by Portilla-Rivera et al. (*8*) showed that biosurfactants from grape marc produced K/W emulsions with EV values of approximately 49.0% after 24 or 72 h of emulsion formation. Following the criterion cited by Willumsen and Karlson (*1*), only those compounds that have the ability to maintain at least 50% of the original emulsion volume 24 h after formation can be considered emulsifiers. Thus, on the basis of the previous results it is clear that *L. pentosus* has the ability to produce extracellular emulsifiers with either commercial sugars or

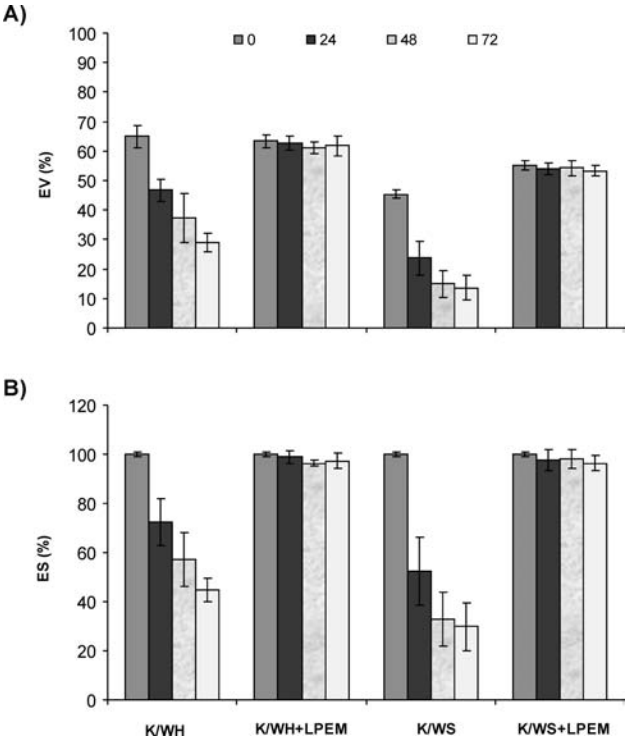

Figure 4. (**A**) Emulsion-forming capacity, expressed as percentage of relative emulsion volume (EV) and (**B**) stabilizing capacity expressed as emulsion stability (ES), respectively, of kerosene/water emulsions (K/W) stabilized by extracellular emulsifiers produced by *L. pentosus* (LPEM) during fermentation of grape marc hydrolysates or commercial sugars. K/WH, kerosene/water emulsion without LPEM, with the nonfermented grape marc hydrolysates as the aqueous phase; K/WH + LPEM, kerosene/water emulsion with LPEM, with the fermented grape marc hydrolysates as the aqueous phase; K/WS, kerosene/water emulsion without LPEM, with the nonfermented commercial fermentation medium as the aqueous phase; K/WS + LPEM, kerosene/water emulsion with LPEM, with the fermented commercial medium as the aqueous phase.

sugars from grape marc hydrolysates as the carbon source. Nevertheless, the EV value of K/W emulsion stabilized by LPEM increased, by approximately 13.7%, after 72 h of emulsion formation when LPEM were obtained using grape marc hydrolysates as carbon source rather than commercial sugars (**Figure 4A**). The ability of these surface active compounds to maintain the emulsion over time (ES) is another important parameter to consider in studying the emulsifying capacity of LPEM. The LPEM produced K/W emulsions with ES values of 97.3 and 96.4% after 72 h of emulsion formation for LPEM obtained from grape marc hydrolysates or commercial sugars and were higher than those obtained with SDS as emulsifier (ES = 87.7%), whereas emulsions without LPEM produced unstable emulsions with ES values of 44.8 and 29.9% for K/W emulsions with the crude cell-free broth from grape marc hydrolysates or dissolved commercial sugars as the aqueous phase.

The above emulsions were made with the fermentation broth as the aqueous phase in the presence or absence of LPEM, although to determine the effect of the concentration of bioemulsifiers on the emulsifying capacity of LPEM, these were precipitated with ethanol and concentrated 2- or 3-fold in distilled water, and the properties of K/W emulsions with and without LPEM were studied. The EV values obtained with concentrated or nonconcentrated LPEM are shown in **Figure 5**. When LPEM produced from grape marc hydrolysates were precipitated and redissolved in water prior to formation of the kerosene-in-water emulsion, the

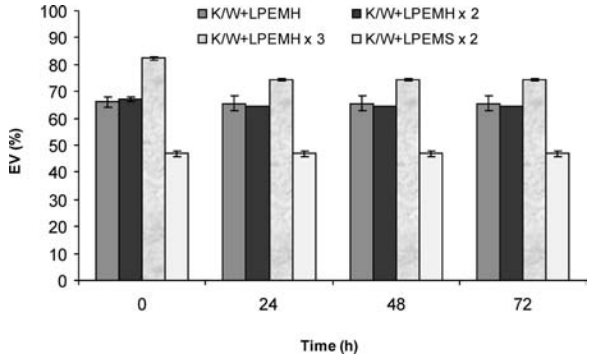

Figure 5. Emulsion-forming capacity, expressed as percentage of relative emulsion volume (EV) for K/W emulsions stabilized by extracted LPEM. K/W + LPEMH, kerosene/water emulsion stabilized by unconcentrated LPEM from grape marc hydrolysates; K/W + LPEMH × 2, kerosene/water emulsion stabilized by 2-fold concentrate of LPEM from grape marc hydrolysates; K/W + LPEMH × 3, kerosene/water emulsion stabilized by 3-fold concentrate of LPEM from grape marc hydrolysates; K/W + LPEMS × 2, kerosene/water emulsion stabilized by 2-fold concentrate of LPEM from commercial fermentation medium.

EV values of K/W emulsion increased to 65.5% after 72 h, whereas the LPEM produced from commercial sugars precipitated and concentrated 2-fold gave an EV value of 47.0%. Additionally, when the LPEM were precipitated and concentrated three times, they produced an EV value for K/W emulsion of approximately 74.5% after 72 h of K/W emulsion formation and were again clearly higher than the value proposed by Willumsen and Karlson (*1*) for a substance to be considered bioemulsifier. The ES values of K/W emulsions stabilized by extracted LPEM were approximately 97.0%, close to the value obtained with K/W emulsions stabilized by the raw LPEM and using the fermentation medium as aqueous phase.

The percentage of emulsified kerosene (EOP) in K/W emulsions is shown in **Figure 6A**, and it was found that emulsions stabilized with LPEM emulsified 87.2 and 85.9% of kerosene after 24 h of emulsion formation when LPEM obtained by fermentation of grape marc hydrolysates or commercial sugars were used. The values for K/W emulsions stabilized with LPEM maintain the percentage of emulsified kerosene after 72 h of emulsion formation, whereas K/W emulsion without LPEM produced unstable emulsions, with EOP values of approximately 45.0 and 24.1% obtained after 72 h of emulsion formation, for K/W emulsion without LPEM, and in presence of unfermented commercial sugars or grape marc hydrolysates as aqueous phase (**Figure 6A**). The EOP obtained for K/W emulsions stabilized by several different concentrations of LPEM are shown in **Figure 6B**, which shows that LPEM obtained from fermentation of grape marc hydrolysates and concentrated 3-fold were able to emulsify 100% of kerosene and that this value remained stable after 72 h of emulsion formation.

Droplet Characterization. *Droplet Concentration*. The concentration of droplets in an emulsion influences the stability of the emulsion (*13*). The droplet concentrations in the K/W emulsions stabilized by LPEM are shown in **Figure 7**; the droplet concentration was stable over time, and when the 2-fold concentrated LPEM was included, the droplet concentration in the emulsions increased to 0.8 mm^3 when LPEM obtained from fermentation of grape marc hydrolysates or commercial sugars were used as emulsifiers.

Optical Images of Droplets. The optical images of K/W creamed emulsions stabilized by LPEM obtained by fermentation of grape marc or fermentation of commercial sugars are shown in

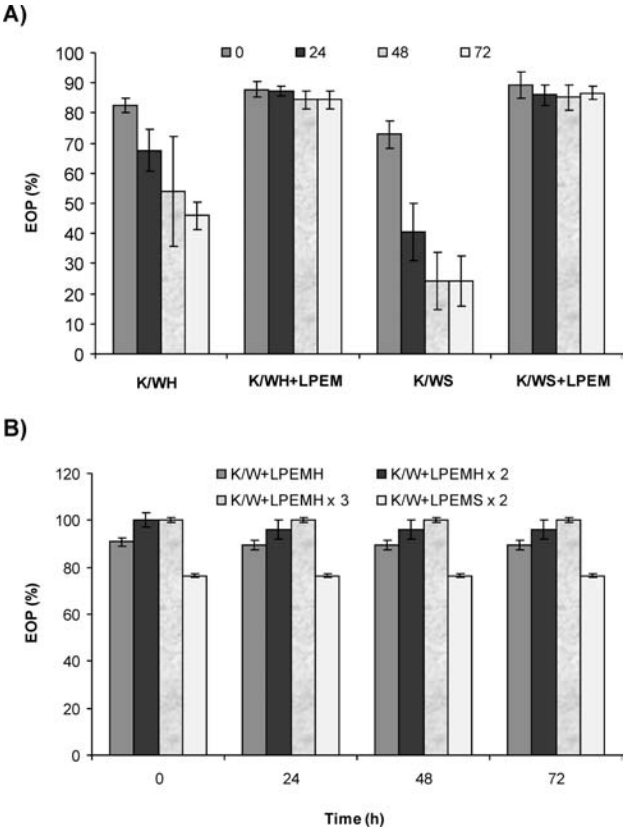

Figure 6. Percentage of emulsified kerosene (EOP) in K/W emulsions stabilized by LPEM. (**A**) Assays were carried out without extraction of LPEM. K/WH, kerosene/water emulsion without LPEM, with the nonfermented grape marc hydrolysates as the aqueous phase; K/WH + LPEM, kerosene/water emulsion with LPEM, with the fermented grape marc hydrolysates as the aqueous phase; K/WS, kerosene/water emulsion without LPEM, with the unfermented commercial medium as the aqueous phase; K/WS + LPEM, kerosene/water emulsion with LPEM, with the fermented commercial medium as the aqueous phase. (**B**) Assays were carried out with extracted LPEM. K/W + LPEMH, kerosene/water emulsion stabilized by unconcentrated LPEM from grape marc hydrolysates; K/W + LPEMH × 2, kerosene/water emulsion stabilized by 2-fold concentrate of LPEM from grape marc hydrolysates; K/W + LPEMH × 3, kerosene/water emulsion stabilized by 3-fold concentrate of LPEM from grape marc hydrolysates; K/W + LPEMS × 2, kerosene/water emulsion stabilized by 2-fold concentrate LPEM from commercial fermentation medium.

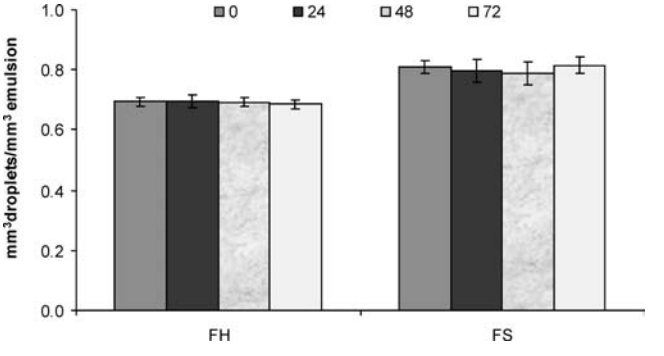

Figure 7. Droplet concentration for K/W emulsions stabilized by LPEM from grape marc hydrolysates (FH) or commercial sugars (FS).

Figure 8A,**B** and can be compared with K/W emulsions without LPEM (**Figure 8C**). It can be seen that in the K/W emulsions stabilized by LPEM (**Figure 8A**,**B**), the size of droplets in the

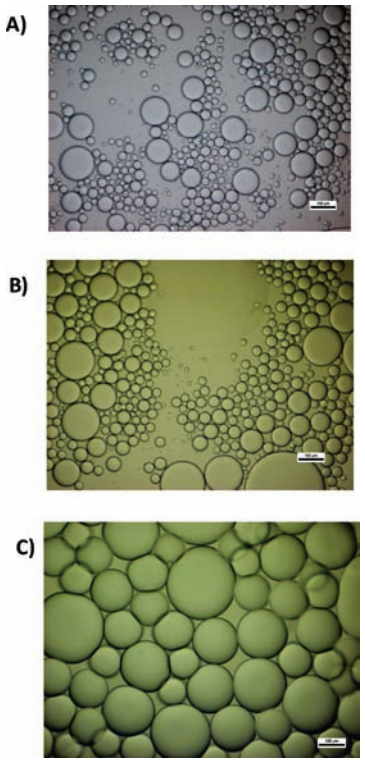

Figure 8. Optical images of K/W emulsions stabilized by LPEM obtained by fermentation of grape marc hydrolysates (**A**) or commercial sugars (**B**) and compared with K/W emulsions without LPEM (**C**).

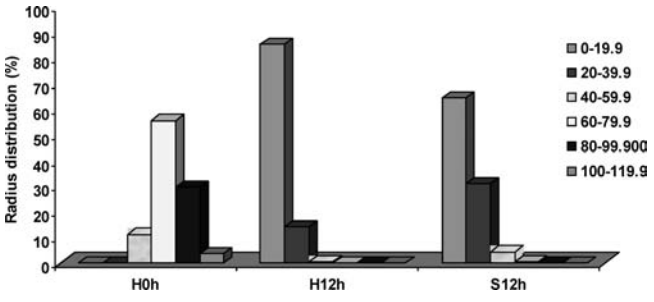

Figure 9. Radius distribution of droplets for the emulsions stabilized with LPEM from grape marc hydrolysates (H12h) or commercial sugars (S12h) and compared with emulsion without LPEM (H0h). Assays were carried out with LPEM obtained after 12 h fermentation.

emulsion decreased and thus the stability of emulsions was higher than in the control without LPEM (**Figure 8C**). In addition, panels **A** and **B** of **Figure 8** show that the K/W emulsion stabilized by LPEM is a polydisperse emulsion characterized by droplets of different sizes, whereas the droplet size distribution in the K/W emulsion without LPEM (**Figure 8C**) was more homogeneous, but with larger droplets than in the emulsions stabilized by LPEM.

Droplet Size Distribution. The distribution of droplet radius for the creamed emulsions stabilized with LPEM is shown in **Figure 9**, in which it can be observed that the K/W emulsion without LPEM comprised 55.6% of droplets of radius between 60.0 and 79.9 μm and 29.6% of droplets of radius between 80.0 and 99.9 μm respectively, whereas the K/W emulsions stabilized by LPEM obtained from fermentation of grape marc hydrolysates or commercial sugars contained 85.5 or 64.4% of droplets of radius < 19.9 μm. It is known that emulsions with smaller droplets cream more slowly and create more stable emulsions (*24*).

Viscosity. LPEM increased the viscosity of the K/W emulsion by 48.5 and 52.5% for K/W emulsions stabilized by LPEM from commercial medium or grape marc hydrolysates compared with K/W emulsions in the absence of LPEM. The viscosity values of the K/W emulsion stabilized with LPEM obtained from fermentation of grape marc hydrolysates or commercial sugars were 496 and 344 cP, respectively, whereas emulsions in the absence of LPEM gave viscosity values around 236 cP for grape marc hydrolysates and 167 cP for commercial sugars.

During the production of bioemulsifiers from grape marc using *L. pentosus*, not only are bioemulsifiers obtained but also lactic acid and biosurfactants (*8*, *25*). This fact would increase the profits of the process, because various subproducts can be obtained in a single fermentation step. On the other hand, we have to take into account that grape marc can be toxic for plants when it is spilled to the environmental without any stabilization of the organic matter (*26*). Consequently, when bioemulsifiers, lactic acid, or biosurfactants are produced from grape marc hydrolysates not only are valuable products obtained but also a residue is removed from the environmental.

On the basis of the previous assays this study concludes that *L. pentosus* produces extracellular emulsifiers by fermentation of grape marc hydrolysate and that these emulsifiers stabilize K/W emulsions with results comparable to, and in some cases even better than, those obtained with a chemical emulsifier (SDS), although further investigations are required to determine the composition of the LPEM.

LITERATURE CITED

(1) Willumsen, P. A. E.; Karlson, U. Screening of bacteria, isolated from PAD-contaminated soils, for production of biosurfactant and bioemulsifiers. *Biodegradation* **1997**, *7*, 415–423.
(2) De Vuyst, L. F.; De Vin, F.; Vaningelgem, F.; Degeest, B. Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* **2001**, *11*, 687–707.
(3) Ruas-Madiedo, P.; Reyes-Gavilán, C. G. Invited Review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *J. Dairy Sci.* **2005**, *88*, 843–856.
(4) Nerurkar, A.; Hingurao, K. S.; Suther, H. G. Bioemulsifiers from marine microorganisms. *J. Sci. Ind. Res.* **2009**, *68*, 273–277.
(5) Banat, I. M. Isolation of thermophilic biosurfactant producing *Bacillus* sp. *Biotechnol. Lett.* **1993**, *15*, 591–594.
(6) Dubey, K.; Juwarkar, A. Distillery and curd whey wastes as viable alternative sources for biosurfactant production. *World J. Microbiol. Biotechnol.* **2001**, *17*, 61–69.
(7) Das, K.; Mukherje, R. K. Comparison of lipopeptide biosurfactants production by *Bacillus subtilis* strains in submerged and solid state fermentation systems using a cheap carbon source: some industrial applications of biosurfactants. *Process Biochem.* **2007**, *42*, 1191–1199.
(8) Portilla-Rivera, O.; Torrado, A.; Domínguez, J. M.; Moldes, A. B. Stability and emulsifying capacity of biosurfactans obtained from lignocellulosic sources using *Lactobacillus pentosus*. *J. Agric. Food Chem.* **2008**, *56*, 8074–8080.
(9) Bounaix, M. S.; Gabriel, V.; Morel, S.; Robert, H.; Rabier, P.; Remaud-Siméon, M.; Gabriel, B.; Fontagné-Faucher, C. Biodiversity of exopolysaccharides produced from sucrose by sourdough lactic acid bacteria. *J. Agric. Food Chem.* **2009**, *57*, 10889–10897.
(10) Duboc, P.; Mollet, B. Applications of exopolysaccharides in the dairy industry. *Int. Dairy J.* **2001**, *11*, 759–768.
(11) Degeest, B.; Vaningelgem, F.; De Vuyst, L. Microbial physiology, fermentation kinetics and process engineering of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* **2001**, *11*, 687–708.
(12) Bognolo, G. Biosurfactants as emulsifying agents for hydrocarbons. *Colloid Surf. A: Physicochem. Eng. Aspects* **1998**, *152*, 41–52.
(13) McClements, D. J. *Food Emulsions Principles, Practices and Techniques*; CRC Press: Boca Raton, FL, 2005.

(14) McClements, D. J. Critical review of techniques and methodologies for characterization of emulsion stability. *Crit. Rev. Food Sci.* **2007**, *47*, 611–649.
(15) Batista, S. B.; Mounteer, A. H.; Amorim, F. R.; Totola, M. R. Isolation and characterization of biosurfactant/bioemulsifier-production bacteria from petroleum contaminated sites. *Bioresour. Technol.* **2006**, *97*, 868–875.
(16) Téllez-Luís, S.; Moldes, A. B.; Alonso, J. L.; Vázquez, M. Optimization of lactic acid production by *Lactobacillus delbrueckii* through response surface methodology. *J. Food Sci.* **2003**, *68*, 1454–1458.
(17) Martínez-Checa, F.; Toledo, F. L.; Vilchez, R.; Quesada, E.; Calvo, C. Yield production, chemical composition, and functional properties of emulsifier H28 synthesized by *Halomonas eurihalina* strain H-28 in media containing various hydrocarbons. *Appl. Microbiol. Biotechnol.* **2002**, *58*, 358–363.
(18) Das, M.; Das, S. K.; Mukherjee, R. K. Surface active properties of the culture filtrates of a Micrococcus species grown on *n*-alkenes and sugars. *Bioresour. Technol.* **1998**, *63*, 231–235.
(19) Vollbrecht, E.; Rau, U.; Lang, S. Microbial conversion of vegetable oils into surface-active di-, tri-, and tetrasaccharide lipids (biosurfactants) by the bacterial strain *Tsukamurella* spec. *Fett/Lipid* **1999**, *101*, 389–394.
(20) Fukuoka, T.; Morita, T.; Konishi, M.; Imura, T.; Sakai, H.; Kitamoto, D. Structural characterization and surface-active properties of a new glycolipid biosurfactant, mono-acylated mannosylerythritol lipid, produced from glucose by *Pseudozyma antarctica*. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 801–810.
(21) Kitamoto, D.; Ikegami, T.; Suzuki, G. T.; Sasaki, A.; Takeyama, Y.; Idemoto, Y.; Koura, N.; Yanagishita, H. Microbial conversion of *n*-alkanes into glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma* (*Candida antarctica*). *Biotechnol. Lett.* **2001**, *23*, 1709–1714.
(22) Mercaldi, M. P.; Dams-Kozlowska, H.; Panilaitis, B.; Joyce, A. P.; Kaplan, D. L. Discovery of the dual polysaccharide composition of emulsan and the isolation of the emulsion stabilizing component. *Biomacromolecules* **2008**, *9*, 1988–1996.
(23) Sun, W.; Sun, D.; Wei, Y.; Liu, S.; Zhang, S. Oil-in-water emulsions stabilized by hydrophobically modified hydroxyethyl cellulose: adsorption and thickening effect. *J. Colloid Interface Sci.* **2007**, *311*, 228–236.
(24) Horozov, T. S.; Binks, B. P.; Gottschalk-Gaudig, T. Effect of electrolyte in silicone oil-in-water emulsions stabilized by fumed silica particles. *Phys. Chem. Chem. Phys.* **2007**, *9*, 6398–6404.
(25) Portilla-Rivera, O.; Torrado, A.; Domínguez, S. M.; Moldes, A. B. Lactic acid and biosurfactants production from hydrolyzed distilled grape marc. *Process Biochem.* **2007**, *42* (6), 1010–1020.
(26) Moldes, A. B.; Vázquez, M.; Domínguez, J. M.; Díaz-Fierros, F.; Barral, M. T. Evaluation of mesophilic biodegraded grape marc as soil fertilizer. *Appl. Biochem. Biotechnol.* **2007**, *141*, 27–36.

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