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NOVEL EXOPOLYSACCHARIDE PRODUCED BY *Acinetobacter bouvetii* UAM25: PRODUCTION, CHARACTERIZATION AND PAHs BIOEMULSIFYING CAPABILITY

NUEVO EXOPOLISACARIDO PRODUCIDO POR *Acinetobacter bouvetii* UAM25: PRODUCCIÓN, CARACTERIZACIÓN Y CAPACIDAD BIOEMULSIFICANTE DE HPAs

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Abstract
Environmental pollution that polycyclic aromatic hydrocarbons (PAHs) cause, results in ecological damage. Bioremediation tackles this issue by using living organisms and metabolites such as bioemulsifiers (BEs) which can pseudo-solubilise hydrocarbons making them more accessible for biodegradation. The aims of this work were (i) to produce in an airlift bioreactor a novel BE through the UAM25 strain, (ii) to evaluate the BE emulsifying activity and stability against commercial emulsifiers using three PAHs, and (iii) to characterize the biomolecule after purification. The UAM25 strain, produced a high molecular weight (1,010 kDa) macromolecule when grown in an airlift bioreactor with n-hexadecane as its sole carbon and energy source. Under these conditions, the yield was 150 mg·L^{-1} of pure BE with 160 emulsifying activity units. The BE emulsified n-hexadecane and β-methylnaphthalene, as well as blends of pyrene and phenanthrene in a non-specific mode. This novel BE displays an exopolysaccharide (repeated rhamnose and galactose units) structure different from previously reported within the same bacterial genus. We observed remarkable PAH-emulsifying capabilities in the BE, compared to commercial emulsifiers such as Tween 80 and Triton X-100. Our work suggests potential biotechnological applications to enhance the bioremediation of soil, sediments, and water.
Keywords: *Acinetobacter bouvetii*, bioemulsifier, polycyclic aromatic hydrocarbons, exopolysaccharide, airlift bioreactor.

Resumen
Los hidrocarburos aromáticos policíclicos (HPAs) causan contaminación ambiental provocando daño ecológico. La biorremediación resuelve este problema utilizando organismos vivos y metabolitos tales como los bioemulsificantes (BEs) los cuales pueden pseudo-solubilizar hidrocarburos facilitando su biodegradación. Los objetivos de este trabajo fueron: (i) producir en un biorreactor airlift un nuevo BE utilizando la cepa UAM25; (ii) evaluar la actividad del BE contra emulsificantes comerciales utilizando tres HPAs; y (iii) caracterizar la biomolécula después de su purificación. La cepa UAM25 produjo un BE macromolecular (1,010 kDa) cuando se inoculó en un biorreactor airlift, con n-hexadecano como única fuente de carbono y energía. Bajo estas condiciones, el rendimiento fue de 150 mg·L^{-1} de BE puro con 160 unidades de actividad emulsificante. El BE fue capaz de emulsificar n-hexadecano y β-metilnaftaleno, así como mezclas de pireno y fenantreno de manera inespecífica. Este nuevo BE muestra una estructura de exopolisacárido (unidades repetidas de ramnosa y galactosa) diferente de las previamente reportadas para este mismo género bacteriano. Se observó una alta capacidad del BE para emulsificar HPAs, al compararlo con emulsificantes comerciales, tales como Tween 80 y Triton X-100. Las propiedades de este BE, demuestran su potencial para mejorar técnicas de biorremediación de suelos, sedimentos y agua.
Palabras clave: *Acinetobacter bouvetii*, bioemulsificante, hidrocarburos policíclicos aromáticos, exopolisacárido, reactor airlift.

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1 Introduction

Pollution of the environment by hydrocarbons derived from petroleum-industry activities results in damage to various ecological systems. The bioremediation approach aims to tackle this issue by using living organisms such as bacteria to reduce or eliminate environmental hazards resulting from the accumulation of toxic chemical wastes (Cisneros-de La Cueva *et al*., 2016, 2014; Souza *et al*., 2014). In this regard, hydrocarbons low solubility result in a mass transfer limitation which directly affects microbial growth (Jiménez-González *et al*., 2015). However, some microorganisms can produce one of the most extensively studied secondary metabolites, bioemulsifiers (BEs), which are metabolites that affect surfaces and can pseudo-solubilise pollutants such as polycyclic aromatic hydrocarbons (PAHs), making them more accessible for biodegradation (Martínez Trujillo *et al*., 2015). BEs are polymeric amphipathic molecules of high molecular weight that can form and stabilize emulsions, since they bind tightly to aqueous, dispersed hydrocarbons and oils, preventing them from merging (Ron and Rosenberg, 2001). The resulting emulsifying activity (EA) is related to the chemical composition and structure of the BE (Uzoigwe *et al*., 2015). PAHs such as pyrene, naphthalene, and phenanthrene, are persistent organic compounds with two or more fused aromatic rings. In the environment, they have high carcinogenic and mutagenic potential, which makes their elimination a critical need. Interest in BEs has increased because of their different functional properties, such as wetting, foaming, oil solubilizing, enhancing PAH biodegradation (which benefits the rheological characteristics), high biodegradability, and low toxicity compared to chemically synthesized emulsifiers (Monteiro *et al*., 2010). On the other hand, effective BE production in bioreactors is a challenge due to the shear stress that rotating impellers impose and outcome to be detrimental to cells growth depending on the level of intensity. However, pneumatic airlift bioreactors (ALBs) improves the mixing performance and reduces the mechanical forces on cells, additionally, it generates well-defined fluid flow patterns, high gas-liquid mass-transfer rates, low operating costs, with less dead zones and cell clumping in the bioreactor (Guo *et al*., 2015; Villegas *et al*., 2016).

The *Acinetobacter* spp. are widely studied macromolecular BE producers, this genus, depending on the species, could produce BEs with differences in molecular structure and emulsifying mechanisms. For example, the emulsifying capacity of the BE that *Acinetobacter* sp. RAG-1 forms is mainly due to its polysaccharide and lipidic components, whereas the emulsifying capacity of the BE from *A. radioresistens* results from the presence of protein in the macromolecule (Ron and Rosenberg, 2001; Toren *et al*., 2001). In this regard, *Acinetobacter bouvetii* UAM25, isolated from the rhizosphere of *Cyperus laxus* Lam in a highly polluted swamp, showed BE production capacity, but these capabilities had not been described so far (Díaz-Ramírez *et al*., 2008; Tzintzun-Camacho *et al*., 2012).

The aims of this work were: (i) to produce, in an airlift bioreactor, a BE with a high EA by the strain UAM25; (ii) to evaluate the BE emulsifying activity and stability against commercial emulsifiers using three PAHs; and (iii) to characterize the biomolecule after purification. The insight into the relationship between the BE's emulsification capabilities and chemical structure may enable us to shade light on emulsifying hydrocarbons to design new and better hydrocarbon-emulsifying and degradation methods for bioremediation.

2 Materials and methods

2.1 Microorganisms and chemicals

Microbiological media and salts (J.T. Baker, Mexico) were used. Tween 80 and Triton X-100 (HYCEL, México) were also used to compare the BE's performance as a hydrocarbon emulsifier. Solvents were 99 % pure reagents purchased from Sigma Aldrich, USA. The *Acinetobacter bouvetii* UAM25 strain (Tzintzun-Camacho *et al*., 2012) (GenBank accession number HQ424441.1) is available at the WDCM449 culture collection from The National Polytechnic Institute (Mexico) with the number ENCB-MG-076.

2.2 Bacterial growth and residual HXD

The Bacterial growth and kinetic studies were done in ALBs. Culture medium consisted of mineral medium with the following composition: ($g \cdot L^{-1}$); 3.87 $NaNO_3$; 1.08 K_2HPO_4; 0.56 KCl; and 0.55 $MgSO_4 \cdot 7H_2O$

with 7.6 g·L^{-1} of n-hexadecane (HXD) as the sole carbon source. The initial pH was adjusted to 6.5 with 1.0 N HCl. Kinetic studies were modelled with the re-parameterized sigmoidal Gompertz model (Zwietering *et al*., 1990) calculated from colony forming units (CFU·mL^{-1}). Culture growth was determined by viable counts on Petri dishes containing TSA incubated at 30 °C. Colonies were quantified after 24 h. Cells and residual HXD were separated from the broth in the ALB by centrifugation at 10 000 x *g* for 20 min at 4 °C (Eppendorf; Centrifuge 5810 R; Germany), then the supernatant was analysed for total carbohydrates by the sulfuric acid phenol method (DuBois *et al*., 1956) and for proteins by Bradford, (1976) method. Residual HXD was separated from the cell-free broth by liquid-liquid extraction with a hexane-acetone (1:1, v/v) mixture. Funnels were left 30 min at room temperature until the mixture reached complete phase separation. The recovered HXD was quantified by gas chromatography in a Varian Star 3900 GC (USA) equipped with an ionization detector and an AT-1HT column (15 m x 0.25 mm x 0.10 μm, Alltech Heliflex, USA) with helium as carrier (30 mL·min^{-1}; 40 psi). The oven was heated to 120 °C (30 °C·min^{-1}), then increased to 150 °C (10 °C·min^{-1}) and finally increased to 170 °C (15 °C·min^{-1}). Injected volume sample was 2 μL.

2.3 BE production and purification

The BE was produced in glass ALB vessel equipped with stainless steel concentric draft tube. The dimensions and geometric relationships were previously reported (Lizardi-Jiménez and Gutiérrez-Rojas 2011; Lizardi-Jiménez *et al*., 2014). Mineral medium (1 L) with carbon (HXD) to nitrogen ratio of 10.5 was inoculated with 1×10^6 CFU·mL^{-1} of *Acinetobacter bouvetii* UAM25. To purify the BE, aliquots (100 mL) were taken from the ALB after 48 h. Cells were separated by two sequential centrifugation steps: (i) 8 000 x *g* for 30 min at 4 °C; the supernatant was collected and (ii) centrifuged again at 10 000 x *g* for 20 min at 4 °C, then the resulting supernatant was filtered through cellulose acetate membranes (0.45 μm). The supernatant was diafiltrated and concentrated by ultrafiltration with a stirred cell system using an Omega polyethersulfone 30 kDa cut-off ultrafiltration membrane (PALL, USA). The air injected into the ultrafiltration system passed through a polytetrafluoroethylene membrane (20 μm) at 20 psi. Diafiltration was conducted in a non-continuous regimen, such that every 100 mL concentrated 50 times, was then diluted to the original volume until the conductivity of the filtered solution was less than 0.01 mS·cm^{-1}, using distilled water's conductivity as reference.

2.4 Emulsifying activity

To evaluate the EA of the purified and non-pure BEs in the cell-free mineral medium, a modification of the standard assay by Rosenberg *et al*., (1979) was used. Samples (0.4 mL) were introduced into 10 mL glass tubes containing 2.6 mL of buffer mix (20 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.0), then 40 μL of a 1:1 (v/v) mixture of HXD and β-methylnaphthalene were added. The tubes were vortexed at room temperature for 2 min and allowed to standing for 10 min before measuring optical density (OD) (Deng *et al*., 2010), determined in a Varian Cary 50 UV-Vis spectrophotometer (Agilent, USA). One unit of EA was defined as the capacity of 1 mL of supernatant to raise the OD measured at 600 nm by 0.1 units.

2.5 Emulsification of PAHs, stability assays, and zeta potential

EA was assayed with blends of HXD: pyrene (PYR) at 1% (w/v), HXD: β-methylnaphthalene 1:1 (v/v), and HXD mixed with PYR and phenanthrene (PHE), both at 1%. PAHs assayed were mixed with HXD in proportions that mimicked common environmental contaminants, such as diesel fuels (Sjögren *et al*., 1995). Control emulsion was formed using individual HXD. PAHs within the emulsion were detected at 276 nm (PAHs maximum peak), the emulsion OD was measured at 600 nm (standard assay). Synthetic surfactants suitable for oil in water PAHs emulsification, Tween 80 and Triton X-100, with respective hydrophilic lipophilic balances (HLB) of 13.4 and 15, were used as synthetic commercial references (Ghosh and Mukherji, 2016). The assayed concentrations were 150 mg·L^{-1} for both the BE and the reference samples. Cell-free medium (1 mL) was diluted to a 1:10 ratio, deionized water was used for zeta potential determinations, in the 2-10 pH range, using a Zetasizer Nano ZS ZEM3500 (Malvern Instruments, UK) (Sotelo-Boyas *et al*., 2015).

2.6 BE characterization by NMR analyses

The ^{1}H, ^{13}C and bi-dimensional (COSY and NOE) NMR spectra were recorded in a unity spectrometer

(Varian, USA) at 400 or 100 MHz using deuterated water and DMSO-d6 as solvents. The BE purified samples were previously hydrolysed by an acidic method, mixing concentrated BE with a 2.0 N HCl solution in a 1:1 ratio (v/v), later the mixture was heated in a water bath for 15 min. The hydrolysate was then lyophilized to a translucent soluble film prior to analyses. Signals were assigned according to the bacterial carbohydrate structure database (Egorova and Toukach, 2014).

2.7 *Protein analysis*

Proteins in the cell-free media were precipitated by the addition of different ratios of water saturated with ammonium sulphate at 4 °C. After 12 h at 4 °C the tubes were centrifuged (8 000 x *g* for 30 min) to obtain a pellet that was later dialyzed against water with a cellulose regenerated dialysis membrane (10 kDa). Samples were analysed by the Laemmli, (1970) method. The EA of purified proteins was also tested.

2.8 *Size exclusion chromatography (SEC)*

Molecular weight distributions (number-average molecular weight, M_n, and polydispersity index, PDI) of the dried non-hydrolysed BE concentrate samples (5 mg) were acquired in an Agilent 1260 series liquid chromatograph equipped with a refractive index detector (RID) and two PL aquagel-OH (Waters, USA) columns in series placed in a thermostat at 25 °C and calibrated with polyethylene glycol (PEG) standards (Agilent, USA). Deionised water (MilliQ-UV, Merck) was used as the mobile phase at 0.8 mL·min^{-1} flow. Samples were dissolved in the mobile phase and filtered (0.45 μm) prior to chromatographer injection. Results were analysed with Cirrus GPC/SEC software (Agilent, USA).

2.9 *Statistical analysis*

One-way ANOVA with Tukey's post hoc tests were performed to make all pair-wise comparisons (α = 0.05) with SPSS v.15.0 statistical software (IBM, USA).

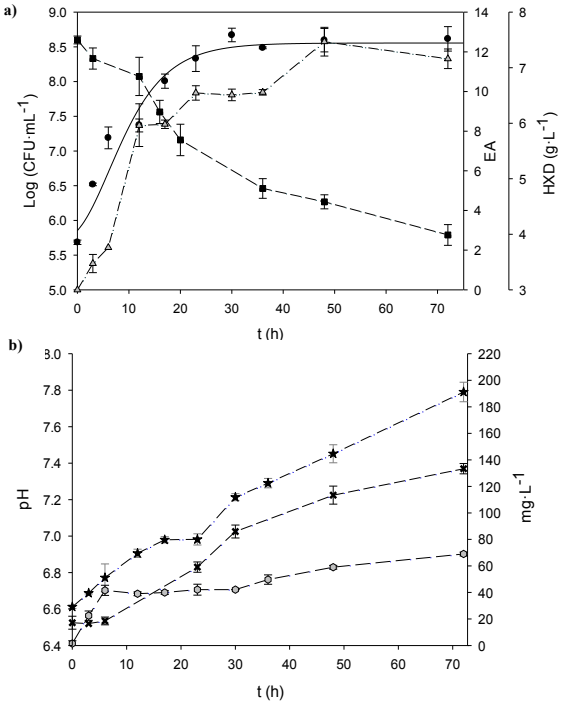

Fig. 1. a) Cell growth (– ●–), emulsifying activity (EA) (-·-▲-·-), and residual HXD (- -■- -). b) Carbohydrates (-·-★-·-), protein (- - ● - -), and pH kinetics (- - × - -); each value is the mean of three replicates ± standard deviations (error bars).

3 Results

3.1 *Cell-growth, EA and residual HXD kinetics in ALB*

To determine the growth phase in which the BE was produced with the highest EA in the shortest time, growth kinetics were analysed. Figure 1a shows cell-growth, residual HXD, and BE production kinetics. BE attachment was observed on ALB surfaces. Growth pattern was fully explained by the re-parameterized sigmoidal Gompertz model with R^2 = 0.99: no *lag* phase was detected, the maximum specific growth rate (μ_{max}) was 0.395 ± 0.073 h^{-1} with a maximum growth of 8.6 ± 0.17 Log CFU·mL^{-1}, and no significant differences were observed after 20 h of growth (α = 0.05). A maximum EA value (12.51 ± 0.72 units) after 48 h was observed. The EA rose significantly after 15 h of culture and remained without significant changes. At the end of

the kinetic growth phase, 47 ± 2.4% of the HXD was consumed, however, 24 ± 3.7% was removed after 20 h (exponential phase). The remaining 23% of HXD required 52 h time lapse (stationary growth phase) to be consumed. EA rose significantly five hours before the end of the exponential phase, reaching a maximum in the stationary phase while the HXD uptake rate remained low.

3.2 Protein, carbohydrates, and pH kinetics in ALB

A preliminary characterization of the BE was performed through an analysis of the protein, carbohydrate, and pH kinetic variables in ALB. Carbohydrate concentrations increased significantly after 20 h with a value of 144.61 ± 6.77 $mg{\cdot}L^{-1}$ at 48 h, which correlates to the BE yield production and EA with pH increase up to 7 (Fig 1b). The purified protein content from the culture broth showed low EAs (less than 0.5 units) with yields of less than 1% of the total purified BE. Additionally, the SDS-PAGE of these purified proteins (data not shown) revealed blurry bands with molecular weights below 21 kDa.

3.3 PAHs emulsification and zeta potential

Figure 2a shows that *A. bouvetii* UAM25 BE emulsified different aromatic compounds with no significant differences among the three PAHs tested (α = 0.05). The presence of PAHs within the emulsion were evidenced (Fig 2, b and c) at a 276 nm wavelength. Tween 80 and Triton X-100 were unable to emulsify PAHs other than β-methylnaphthalene, in a low extent compared to the BE (Fig 2b). Emulsification of the HXD component by BE folded 4.5 and 3.3 times the EA of Triton X-100 when PYR and PHE were in the mix, respectively.

The pH and zeta potential were correlated to get insight on physicochemical BE properties. Figure 3a shows that the BE was able to form a stable emulsion that remained after 96 h. The pH change had no significant effect on stability from pH 4 to 8, but at pH 3, a decrease in stability, zeta potential electronegativity (Fig 3b) and EA response was observed. At pH 9, the EA response was slightly higher and the stability was not significantly different to that at pH levels of 4, 5, and 7 (α = 0.05).

3.4 SEC and NMR analyses

The BE yield was 150 ± 1.12 $mg{\cdot}L^{-1}$ after 48 h of culture. The purified BE exhibited an EA of 160 $U{\cdot}mg^{-1}$ (24 000 U per ALB). The molecular weight distribution analysis by SEC revealed a high molecular weight molecule by only one narrow peak in the chromatogram with a weight of 1,010 kDa and a PDI of 1.38 against PEG standards. Resonance analyses showed a low intensity signal at (δ) 8 ppm in the ^{1}H NMR spectra that might indicate the presence of unsaturation to a low extent (Fig 4a). It is noteworthy that the singlets at 2.76, 2.89, and 3.05 ppm might be assigned to chlorinated methyl impurities due to the hydrolytic treatment and therefore are not related to the molecular structure of the BE. The signals at low field (5-5.5 ppm) within this massive broad signal could be assigned to the anomeric protons of the saccharide region according to Jain *et al.*, (2013). In agreement with the database, the pattern of signals at (δ) (ppm) of 5.18, 3.78, 4.04, 4.18, 4.11, and 3.74 correlates to α-D-galactose (DGalp) in a chain of six sugars. Similarly, the signals (δ ppm) at 5.09, 4.10, 4.00, 3.58, 3.95, and 1.33 were assigned to an α-L-rhamnose (LRhap) side chain of the polysaccharide backbone. Interestingly, the shifts at (δ ppm) 4.49, 3.61, 3.80, 4.33, and 4.27 corresponded to a β-D-galactose (DGalpA) with an uronic acid unit. The signal at 2.1 ppm suggested methyl groups of O-acetyl moieties, which might indicate the presence of acetylated residues. More importantly, the signal at δ (ppm) of 1.26 (-$(CH_2)_n$-), 1.44-1.46 (-CH_2-$(CH_2)_n$-), and 2.00-2.30 (-CH_2-CH_2-$(CH_2)_n$-) evidenced the presence of an aliphatic region that might be attributed to alkyl side chains (Dhasayan *et al.*, 2014) in a branched structure. Additionally, 2D-NMR studies might evidence the presence of cross-linking between chains, as Figure 5 shows. The analysis of the spectrum revealed that the H1 and H2 hydrogens of the carbohydrate component are attached to the aliphatic component (Fig 5, Int A, B, and C). The sample solubilized in DMSO-d6 also showed shifts in the 7.00-7.50 ppm range, corresponding to the H2 shift of galactose, which interacted with anomeric protons (Fig 5, Int D and Int C). Interpreting the ^{13}C NMR gives further evidence (as the spectrum of Fig 4b shows) where signals at δ (ppm) of 96-100, 54-56, 71-78, and 65 correlated to the carbons C1, C2, C3-C4, and C6 of galactose. The signal at 17 ppm relates to a C6 rhamnose unit; both sugars were also identified in the ^{1}H NMR spectra. More importantly, the ^{13}C NMR spectra displayed intense signals between 168-172 ppm assigned to carbonyl groups. This intense signal in the ^{13}C NMR spectrum and the absence of aldehyde protons in the ^{1}H NMR suggest the presence of either acetyl or ester moieties forming ester-type linkages between chains.

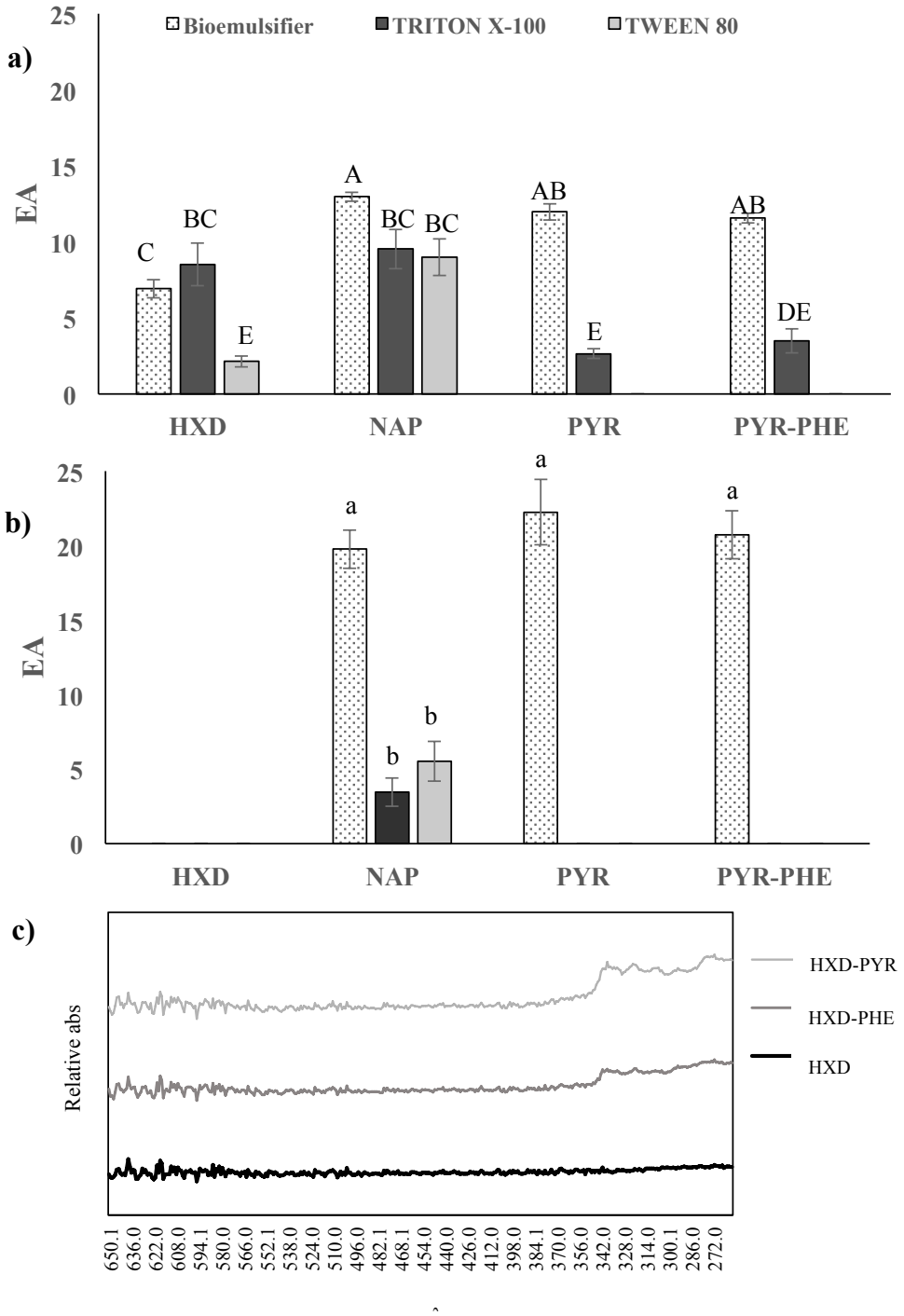

Fig. 2. EA of the BE mixed with sole HXD, HXD: β-methylnaphthalene (NAP), HXD: pyrene (PYR), and HXD: pyrene: phenanthrene (PYR-PHE). Measurements were done at a) 600 nm; and b) 276 nm (each value is the mean of six replicates ± standard deviations (error bars), pair-wise comparisons by Tukey's post hoc, different letters (capital or lowercase) designate significant differences ($\alpha = 0.05$)); and c) Spectrophotometric scan of hydrocarbon blends.

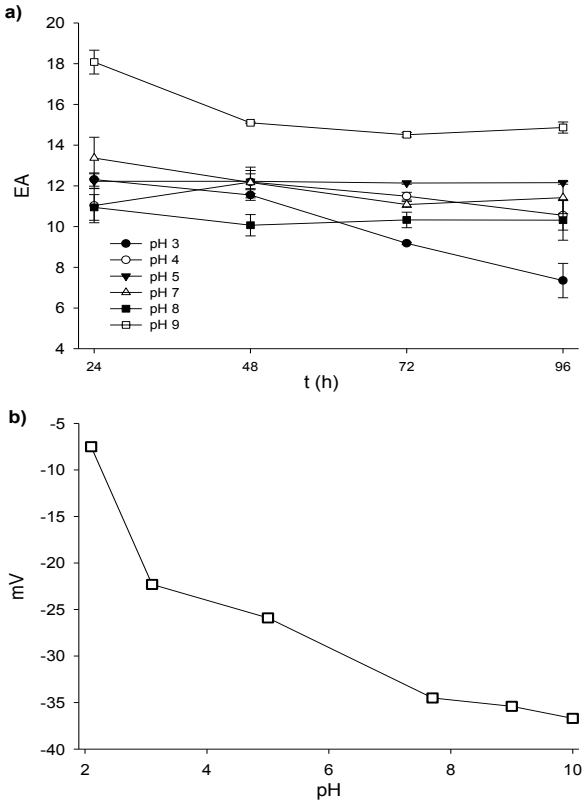

Fig. 3. a) Kinetic emulsion stability of BE; and b) zeta potential at different pH values.

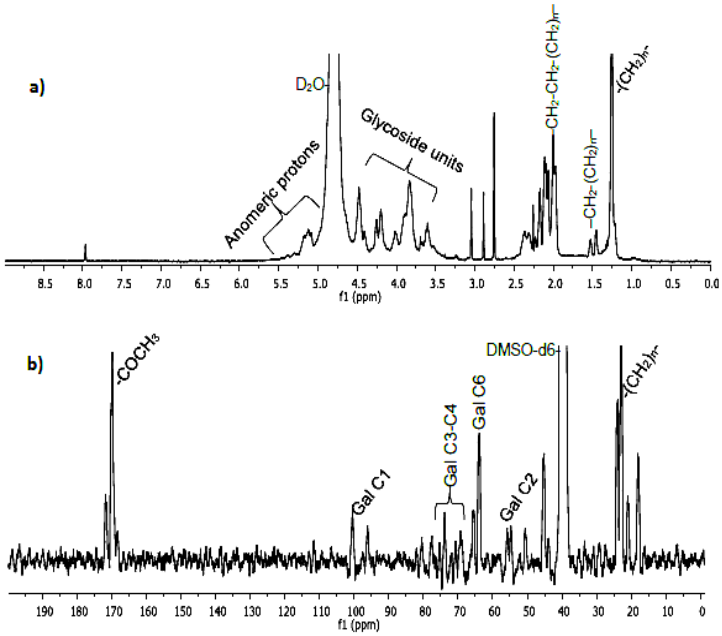

Fig. 4. a) ^{1}H NMR; and b) ^{13}C NMR spectra of the purified BE.

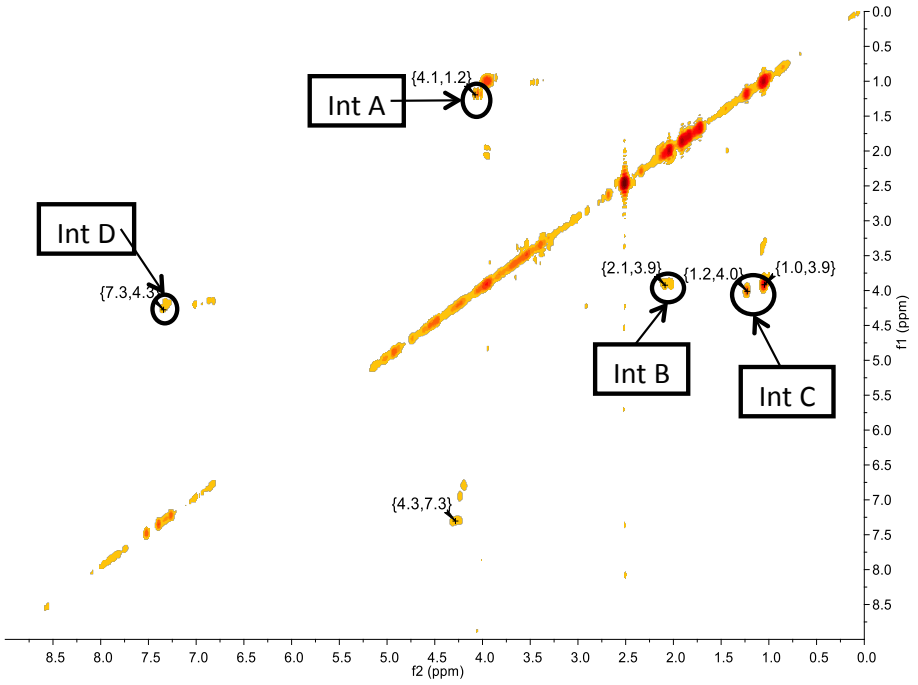

Fig. 5. COSY NMR spectrum of purified BE; the interactions (Int) are represented.

4 Discussion

4.1 EA kinetics and BE production in ALB

Figure 1a shows the low emulsifying capacity in the medium during the initial hours of culture. Despite this, the culture could produce BE in association with the cell wall as a biofilm before its detachment upon favourable conditions (Goldman *et al*., 1982), in which case the detected EA was low in the mineral medium at this initial stage. The presence of the BE on the cell wall also enhanced the cell hydrophobicity, and consequently its initial contact and carbon source consumption (Baldi *et al*., 1999). Likewise, Mehdi and Giti, (2008) observed increased affinity towards HXD and various hydrocarbons for Gram-negative microorganisms as the culture reached the early stationary phase, with biofilm production. In our study, correlation between cell growth and EA was observed, similar results were reported for biosurfactant production by *Halomonas* sp. MB-30 (Dhasayan *et al*., 2014). Thereby, we can ascribe the EA activity as an indirect measure of BE production. Additionally, after a lapse of 48 h, its EA maintained and showed no significant changes (α = 0.05), which correlates with growth behaviour and strengthens BE production association with cell growth. Therefore, this time was adequate to stop the culture and purify the BE with the highest EA. Experimental evidence indicates that the BE might be released at the late exponential phase and throughout the stationary phase when the bacteria and BE are forced to detach from the ALB wall surfaces. BE production and the EA were therefore associated with growth.

The *Acinetobacter* genus microorganisms are reported to grow with HXD as organic carbon source, along with a consequent rise of pH, during the culture time (Chen *et al*., 2012). Our experimental (Fig 1b) data agree with those earlier reports, which show that the strain also raised the pH from 6.5 to 7.4. It had a higher rate of protein production before five hours had passed and a pH level was lower than 6.8. The protein rate production seemed to lower after five hours, when proteins involved in the biodegradation of HXD, fatty acid metabolism, and oxidative stress defence reached proper concentration, which occurs because alkane metabolism includes its terminal oxidation and the β-oxidation of fatty acids (Jung *et al*., 2011). Culture media changes were observed by the end of the growth kinetics due to the presence of a gel-like substance which adhered to the ALB walls. Importantly, the recovery of the soluble fraction of *A. bouvetii* BE in the culture broth is favoured by an airlift-mixing condition

that allows UAM25 to colonize the reactor surfaces with low shear strength.

In the present work, the protein concentration was low during culture, which contrasts with commercially produced Alasan by A. radioresistens in a similar culture time, in which protein is reported to play the role of emulsifier. On the other hand, the carbohydrate production (Fig 1b) was superior to other yields reported for BEs of the polysaccharide type. Su *et al*., (2009) reported a total amount of emulsan production of 60 $mg{\cdot}L^{-1}$ after 120 h of cell growth; our BE yield was similar to this sugar concentration for the equivalent culture time.

4.2 PAHs emulsification

When emulsifying capabilities were studied in systems that mimic water contamination, no significant differences (α = 0.05) on PAHs emulsification were observed (Fig 2). Hence, emulsification occurred in a non-specific mode (emulsification independent of PAH type), avoiding coalescing the dispersed droplets and improving the stability due to a steric hindrance effect similar to other exopolysaccharide (EPS) BEs reported (Jain *et al*., 2012). The hydrocarbons non-specificity of *A. bouvetii* UAM25 BE suggests that it is a good candidate for application in hydrocarbon remediation and oil recovery (Beltrani *et al*., 2015). In order to compare BE differences such as specificity and emulsification activity, regarding commercial emulsifiers, Tween 80 and Triton X-100 emulsifiers were assayed. Specificity for β-methylnaphthalene organic mixture was observed, in contrast for HXD and the rest of the PAHs, when commercial emulsifiers were used. No EA was observed at 276 nm when HXD was tested individually (Fig 2b), as no aromatics were present. Notwithstanding this, EA increased when aromatics were present in the mixture. Rosenberg *et al*., (1979) reported that the RAG-1 strain poorly emulsified pure aliphatic and aromatic hydrocarbons, but binary blends containing aliphatic and aromatic hydrocarbons raised the EA. In our case, the presence of β-methylnaphthalene, PYR, or PHE favoured the EA response for BE but not for Tween 80 and Triton X-100. Tween 80 could not emulsify HXD when PYR and/or PHE were in the blend. Triton X-100 showed HXD emulsification to a lower extent than the emulsification that BEs produced, which could have been due to Triton X-100 HXD pseudo-solubilization capabilities (Zhong *et al*., 2016). Although Cheng *et al*., (2016) reported an enhancement of HXD removal by Triton X-100, its degradation is difficult when compared to EPS BE because of its chemical composition, which includes an aromatic ring and polymeric ethylene oxide structure. Beltrani *et al*., (2015) also reported specificity for certain hydrocarbons by Tween 80 and Triton X-100 when compared them to EPS BE, Pdb-Z produced by *Pedobacter* sp. MCC-Z. It is noteworthy that emulsification could be related to solubilisation, and therefore also related to hydrocarbon degradation. For example, Mahanty *et al*., (2006) described a linear increment of aqueous solubility of PYR and anthracene alongside BE production and degradation.

4.3 BE stability and zeta potential

BE was stable at broad range of pH conditions (Fig 3a), although slight decrease in emulsion stability at acidic conditions was observed, which could be due to the protonation of the EPS BE carboxylic groups that results in coalescence between positively charged droplets (Dhasayan *et al*., 2014). Concomitantly, the pH effects were reflected by the zeta potential of the BE (*i.e.*, they affect the dispersion characteristics of the emulsion as a colloid (Fig 3b)). Stability might be ascribed to the absence of protein and the inherent precipitation. It is worth noting that the viscous nature of the EPS BE gel probably provides stability even at pH 9. In this regard, Grinberg *et al*., (1995) reported an Ukrainian Acinetobacter species that produced viscous BE when pH was lowered.

At pH 9, electrostatic repulsion (that higher EA values reflect) was favoured due to the increased electronegativity in the zeta potential. Wicek and Chibowski (2002) found more stability (one-week age) when the zeta potential of dispersed n-tetradecane droplets with β-casein has increased electronegativity (-60.3 mV) at pH 11. In our work, two remarkable aspects emerged: (i) emulsion stability at a wide range of pH; and (ii) non-specific emulsification manner, both mainly due to steric effects that correspond to physicochemical exopolysaccharide characteristics.

4.4 Chemical characterization

The purified BE showed an EA (160 $U{\cdot}mg^{-1}$), which two-fold that of the non-pure BE. Therefore, the presence of impurities in the mineral medium (low molecular weight proteins and salts) probably had a negative effect on the emulsion stability and EA through its effect on the superficial charges that promote the coalescence of dispersed droplets. The

low concentration of proteins found, and the lack of correlation with EA, ruled out any relation to protein chemical composition, unlike the BE Alasan reported by Toren *et al*., (2001), in which the main EA capacity is associated with a protein of 45 kDa. With the remarkable difference due to the gel-type consistency, our BE was similar to other polysaccharide-based BEs, such as Emulsan, Ethapolan (Pirog *et al*., 2009), and Biodispersan, moreover the carbohydrate concentration, which was correlated to the BE yield production and EA, supported the BE carbohydrate nature. To confirm the polysaccharide structure, NMR analyses were performed. Signals corresponding to β-D-galactose (DGalpA) with a uronic acid unit. In a previous study by Vinogradov *et al*., (2010), the presence of uronic acid was identified at the end of the main polysaccharide chain. The presence of uronic acid is related to BEs ability to emulsify and detoxify hydrocarbons (Uzoigwe *et al*., 2015). This moiety could enhance the hydrocarbon-emulsifying capability over the tested commercial emulsifiers. Aliphatic chain component associated with the carbohydrate moieties was identified (Fig 5) which explains the amphipathic BE nature. The BE was therefore identified as a high-weight molecule with a polysaccharide chemical structure composed mainly of repeated units of rhamnose and galactose linked to an aliphatic side chain.

We have discussed for the first time the EPS characteristics and capabilities of the BE produced by *A. bouvetii* UAM25.

Conclusions

The BE is a high-molecular-weight molecule and was produced with the highest emulsifying activity. The BE production was associated with the microbial growth pattern in the ALB. The BE exhibits exopolysaccharides associated with aliphatic compounds and was capable of emulsifying several polycyclic aromatic hydrocarbons, actually was 4.5 times more effective at emulsifying the hydrocarbons than commercial emulsifiers when PYR was in the blend. This is the first study dealing with the BE of *A. bouvetii* UAM25 chemical and its functional characterization. Our work suggests potential biotechnological applications of this novel BE to enhance the bioremediation of soil, sediments, and water.

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