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Biosurfactant Properties of the Extremotolerant Bacterial Strain *Acinetobacter baumannii complex* Isolated from Congenital Water

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Abstract. Formation waters associated with hydrocarbon extraction waste contain dissolved salts and metals, which make it a non-usable by-product, and its release causes a negative impact on the environment. However, for certain microorganisms, it can work as a suitable growth and metabolic development medium. In this study, three autochthonous strains were isolated from congenital water and cultivated on 1 % oil-added Bushnell-Hass Agar for further evaluation. The hydrocarbons degradation capacity was qualitatively tested in an inorganic liquid medium. The strain identified as AC362-1 showed the highest mineralization and growth in the test. Gram staining, API 20E, transmission electron microscopy, and MALDI-TOF mass spectrometry tests were performed to identify the strain; the results matched a Gram-negative bacillus of the *Acinetobacter baumannii complex* species. The bacterium demonstrated extremotolerant characteristics during growth kinetics, and its adaptation was tested under 15 % saline conditions, pH 9, and 80 ºC temperature, reaching population densities of 0.76, 0.74, and 0.44 DO_{600nm}, respectively. Additionally, it showed the capacity to synthesize biosurfactant in Kim medium, identified by a thin-layer chromatography and infrared spectroscopy as a lipopeptide type. The surface tension of the biosurfactant was measured and showed a reduction of the tension from 72.18 mN/m (water) to 53.21 mN/m. Finally, the metabolic properties of the bacterial strain facilitated the degradation of oil in a contaminated soil sample from 9000 mg/kg to 3812 mg/kg of soil, with a bacterial population of 1.56 x 10^5 CFU/g soil.
Keywords: *Acinetobacter*; microbial metabolism; biosurfactant; bioremediation.

Resumen. Las aguas de formación (congénitas) asociadas a la extracción de hidrocarburos contienen sales y metales disueltos, por lo que se consideran un subproducto no aprovechable y representa un impacto negativo en el ambiente. Sin embargo, para ciertos microorganismos, puede ser un medio propicio para su crecimiento y desarrollo metabólico. En este estudio, se evaluaron tres cepas autóctonas aisladas del agua de formación y cultivadas en agar Bushnell-Hass con 1 % de petróleo añadido. La capacidad de degradación de hidrocarburos se evaluó cualitativamente en un medio líquido inorgánico, destacando la cepa identificada como AC362-1, que mostró la mayor mineralización y crecimiento en la prueba. Para su caracterización, se realizaron pruebas de tinción de Gram, API 20E, microscopía electrónica de transmisión y espectrometría de masa MALDI-TOF. Los resultados indicaron que se trata de un bacilo Gram negativo, perteneciente a la especie *Acinetobacter baumannii complex*. La bacteria exhibió características extremófilas durante la cinética de crecimiento y su adaptación a condiciones salinas del 15 %, pH 9 y temperatura de 80 ºC, alcanzando densidades poblacionales de 0.76, 0.74 y 0.44 DO_{600nm}, respectivamente. Además, demostró

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capacidad para sintetizar biosurfactante en medio Kim, identificado mediante cromatografía de capa fina y espectroscopía infrarroja como un lipopéptido. La medición de la tensión superficial del biosurfactante mostró una reducción de la tensión de 72.18 mN/m (agua) a 53.21 mN/m. Finalmente, las propiedades metabólicas de la cepa bacteriana posibilitaron la degradación de petróleo en un suelo contaminado, disminuyendo a 9000 mg/kg a 3812 mg/kg de suelo, con una población bacteriana de 1.56 x 10^5 CFU/g de suelo.
Palabras clave: *Acinetobacter*; metabolismo microbiano; biotensioactivo; biorremediación.

Introduction

Biotechnology has made inroads in the oil sector, particularly in the development of biotransformation, biodegradation, and bioremediation processes [1]. Petroleum, a natural oleaginous liquid, is formed by 83–87 % carbon and 11–14 % hydrogen [2]. Petroleum-derived hydrocarbons can be classified into aliphatic, aromatic, and polar organic compounds, often containing sulfur compounds, oxygen, nitrogen, heavy metals, mercaptans, SO_2, H_2S, alcohols, and free or emulsified salt water [3]. Oil spills and their derivatives pose a serious global environmental problem, leading to adverse impacts on ecosystems, human health, and various activities which depend directly on natural resources, such as tourism, fishing, water sports, and aquaculture [4]. Whether an oil field is associated with an aquifer or not, it represents a potential risk in the exploitation of this resource. In Mexico, the state-owned company Petróleos Mexicanos (PEMEX) conducts crude oil desalination, resulting in wastewater effluent referred to as congenital water, containing high concentrations of inorganic salts. The disposal of this waste is challenging due to the risks of salinization in receiving bodies (water or soil) and the presence of toxic compounds [5]. Congenital water provides an ideal environment for the growth of a specific group of microorganisms that tolerate and thrive under such conditions [6]. This microbial biomass constitutes 60 % of the total biomass on the planet, existing in both marine and terrestrial subsoils that show greater genetic and metabolic diversity [7].

Extreme environments on Earth, characterized by high temperatures ranging from 55 ºC to 121 ºC or low temperatures from -22 ºC to -20 ºC, 2–5 M NaCl salinity, pH > 8 alkalinity and pH < 4 acidity, support the life of organisms adapted to survive under such conditions [8]. From a biotechnological perspective, microorganisms isolated from habitats with high salinity concentrations and extreme temperatures are of interest for both research and commercial development [9]. These microorganisms produce metabolites such as exopolymers, lipopolysaccharides, extreme enzymes, and biosurfactants. Biosurfactants are amphiphilic compounds capable of solubilizing immiscible phases or biopolymers, functioning as emulsifiers, thickeners, antioxidants, and chelators [10]. Hence, biosurfactants find wide applications in pharmaceuticals, medical, food, cosmetic, pesticide, oil and biodegradation industries [11].

Biosurfactants play a crucial role in bioremediation processes, serving as a clean and efficient technology based on the utilization of hydrocarbonaceous microorganisms, such as fungal, yeast or bacteria, with high biosurfactant production potential. These organisms employ diverse metabolic pathways that convert organic molecules into CO_2, water, and some inorganic residues [12]. Immobilization of *Pseudomonas monteilii* P26 and *Gordonia sp.* H19, is capable of producing biofilms when supported on polyurethane (PUF), leading to the degradation of 75 % of the contaminant oil at 30 ºC, as compared to immobilized cultures [13]. Similarly, the strain *Acinetobacter calcoaceticus*, isolated from oil-contaminated soil and exhibiting the potential to produce a thermostable biosurfactant for naphthalene solubilization has been documented [14]. While bioremediation processes offer an economical and environmentally friendly alternative, they encounter challenges in achieving maximum efficiency in the recovery of contaminated environments. Therefore, studies on microbial growth conditions substrate specificity, variability in *Ex situ* environments, understanding the microbiome and its biochemical properties, metabolic activity, among other factors, are suggested [15]. The objective fi of this research was to assess the biosurfactant properties of a bacterial strain isolated from congenital water and develop a biodeme dilation process for contaminated soil with hydrocarbons.

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Experimental

Materials and methods

Samples analysis and characterization

The congenital water sample was collected from oil well-362, operated by Petróleos Mexicanos (PEMEX), under collaboration agreements (E-02-G-044, E-05-R-34 and E-05-G-37). The samples were analyzed according to the procedure outlined in the Mexican Official Standard NOM-143-SEMARNAT-2003 [16]. Physicochemical parameters (alkalinity, turbidity, conductivity, total chlorides, pH) of the sample were evaluated according to Mexican standards: NMX-AA-008-SCFI-2016 [17], NMX-AA-036-SCFI-2001 [18], NMX-AA-038-SCFI-2001 [19], NMX-AA-073-SCFI-2001 [20], and NMX-AA-093-SCFI-2018 [21]. Accordingly, the crude oil sample was also collected from the same oil well-362 [22]. The physicochemical properties evaluated here were: density (ASTM D70-21) [23], API grades (ASTM D70-21, D287-22) [23], [24], Saybolt viscosity (ASTM D88-07) [25], and flash point (ASTM D92-18) [26]. Medium-fraction hydrocarbons were determined by gas chromatography with a flame ionization detector in accordance with NMX-AA-145-SCFI-2008 [27].

Isolation and selection of the *Acinetobacter baumannii complex* strain

The isolation of the bacterial strain from the congenital water sample was performed under aseptic conditions. 250 mL of HIMEDIA® (HIMEM349-500G) brand Bushnell-Hass (BH) agar culture medium was supplemented with 1 % sterile petroleum as the sole carbon source for the bacteria, then poured into Petri dishes [28]. The plates were inoculated with 0.1 mL of the sample and seeded using the extension technique, incubated at 35 ± 2 ºC for 72 h. Strain selection was performed based on viable colonies, which were identified as AC362-1, AC362-2, AC362-3. These strains were purified through cross-streaking on plates with DIBICO® (DIB-1022-A) nutrient agar culture medium and incubated at 35 ± 2 ºC for 24 h. This purification process was repeated until obtaining isolated and pure strains.

The oil mineralization produced by the bacteria was developed by means of a qualitative test, (Hydro Environment®) in a triple inorganic culture medium 17, using bioreactors at flask level with 200 mL of sterile medium. Treatments were carried out in duplicate, adding 5 mL of pure strain inoculum to each unit and 1 % crude oil as carbon source. They were kept homogenized through an aeration system for 10 days at a volumetric flow rate of 1.21 x 10^{-4} m^3/s.

Phenotypic analysis at colony level

In the oil mineralization test, the strain *Acinetobacter baumannii complex* presented a higher percentage of crude oil degradation. Hence, this strain was selected for physical and biochemical identification according to established microbiological procedures [7].

The phenotypic analysis of the strains was performed at the colony level on a plate with BH medium. Under the optical microscope characteristics such as elevation, size, border, consistency, and color -related to their morphology- were examined [7]. Likewise, the cell wall was analyzed by Gram staining test, a widely used differential stain test in bacteriology [37].

The API 20E test allows a rapid identification of Gram-negative bacilli. In this procedure, a bacterial colony was suspended in a 0.9 % saline solution, and the microtubes were inoculated with the dehydrated cultures, followed by incubation at 37 ºC for a period from 24 to 48 hours. Bacterial metabolism produced spontaneous changes in coloration and upon addition of the test reagents. The reactions that had an effect on the test were compared with the reading table of the same test. The numerical profile was obtained through the software apiwebTM REF 40011 (bioMerieux, France) and interpreted according to the procedure indicated by the manufacturer. Finally, the strain was identified by MALDI-TOF mass spectrometry, a fast and reliable analytical technique, which was performed at the ENCB Medical Bacteriology Laboratory of the National Polytechnic Institute.

Structural analysis of the bacterial cell by transmission electron microscopy (TEM)

Structural analysis of the bacterial cell was performed by the technique of transmission electron microscopy. The cell biomass was washed with 20 mL of $Na_2HPO_4 \cdot 7H_2O$ and $NaH_2PO_4 \cdot H_2O$ buffer solution at 0.2 M, were washed four times by centrifugation at 3000 rpm for 10 min. The cells were fixed with 1 mL of

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2 % glutaraldehyde ($OHC(CH_2)_3CHO$) in a sterile tube and sonicated for 5 min. From the cell concentrate, a drop was deposited on a 200 mesh Cu grid and fixed with glutaraldehyde for 5 min. Subsequently, the fixed cells were dehydrated by washing with a series of ethanol solutions of graded concentration (30 % to 100 %) for 10 min per solution. The sample was allowed to rest at room temperature and was examined by HRTEM JEOL JEM 2100 at 200 kV high resolution with LaB_6 filament, using GATAN® Digital Micrograph software, calibrated with a gold standard sample in the 1.09 mm plane with a margin of error of ± 0.1 %.

Acinetobacter baumannii complex growth kinetics

Under axenic conditions, 400 mL of culture medium was prepared in a bioreactor with nutrient broth of composition (g/L): meat extract, 1.0; yeast extract, 2.0; casein peptone, 5.0; NaCl, 5.0. The medium was inoculated with 10 mL of *Acinetobacter baumannii complex* biomass, adjusted to a pH of 7.2 and the absorbance was measured until 0.3 OD_{600nm} was reached. The bioreactor was connected to an aeration system with a volumetric flow rate of 1.21 x 10^{-4} m^3/s. For sampling, 10 mL of the biomass culture was taken, and the optical density (OD) was quantified at 600 nm in a GENESYS 20 spectrophotometer, Spectronic. The colony forming units (CFU) counting was performed by the dilutions and the plate count method over a 96 h experimental period [7]. The data were recorded for the construction of the bacterial growth curve.

Evaluation of biosurfactant production

Biosurfactant production by the bacterial strain was carried out in stages: fermentation, phase separation by centrifugation, acid precipitation and extraction. Fermentation was carried out in a bioreactor with 1.0 L of Kim medium composed of: yeast extract, 0.2; meat extract, 5.0; glucose or dextrose, 2.0; monobasic (KH_2PO_4), 0. 2; dibasic (K_2HPO_4), 0.3; $MgSO_4$, 0.1; $NaSO_4$, 0.1; $CaCl_2$: 0.1; $FeSO_4$, 0.1 and a carbon/nitrogen ratio of 2:1, specific for the biosurfactant production, adjusted to a pH of 6.3. The Kim medium was autoclaved at 121 °C, 15 min at 15 psi and 10 % bacterial biomass grown in nutrient broth with absorbance of 0.3 (OD_{600nm}) was added. The bioreactor was connected to an aeration system with a volumetric flow rate of 1.21 x 10^{-4} m^3/s, for a period of 120 h. During the bioassay, 50 mL samples were obtained at different times (0, 24, 48, 72 and 120 h), for the measurement of absorbance and CFU counting, the samples were taken in triplicate. Using the experimental data obtained, the bacterial growth curve was constructed and the maximum biosurfactant production was obtained. In order to separate the cell biomass from the liquid phase, the samples were centrifuged at 5500 rpm for 30 min, after which the CLC (cell-free broth) was obtained and preserved at 4 ºC for subsequent tests.

Biosurfactant activity produced by the *Acinetobacter baumannii complex* strain

The hemolytic test essay was performed in Petri dishes with blood-based agar supplemented with 5 % (v/v) lamb blood. Circular portions of agar were extracted with a borer, leaving a well shape of 0.8 to 1.0 cm in diameter; each well was inoculated with 0.1 mL of CLC from the samples at different times. The test control was performed in triplicate by using a sterile culture medium. The treated petri dishes were incubated at room temperature (28 ± 1 ºC). The presence of surfactant substances in the CLC had an effect on the total and/or partial hemolysis of the red blood cells contained in the agar. The result of the test was the formation of a clear zone (halo) around the inoculum which was measured by ImageJ win-32 FIJI software. The oil dispersion test was carried out in petri dishes containing 30 mL of distilled water, and 100 µL of oil was carefully added over the surface forming a thin layer and then 10 µL of CLC was added. Sterile culture broth was used to control the test. The dispersion test was performed in triplicate. The test was considered positive when a clearing zone was formed on the oil surface due to the presence of surfactant substances. Measurement of the diameter of the clearing zone was performed with a vernier, in all the samples analyzed [29].

Collapsed drop test. 0.2 mL of oil was deposited on a glass surface (slide) forming a thin film, then 40 µL of CLC coming from the samples taken at different times were added as a drop; this procedure was performed in triplicate. The test was considered positive when the drop collapsed after 1 min, due to the presence of surfactants that caused the increase in diameter, and negative if the drop remained. The size of the droplet diameter was measured with ImageJ win-32 FIJI software. Emulsion index (IE_{24}). In a test tube, 2 mL of biosurfactant and 2 mL of oil were mixed at room temperature (28 °C ± 1, 1 atm), the mixture was vortexed for 2 min. After this procedure, the emulsion was allowed to rest for 24 h h [30]. The IE_{24} index was calculated using Eq. (1):

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$$IE_{24} = \frac{Emulsion\ height}{Total\ Emulsion\ Height}\ x\ 100 \quad (1)$$

Uncultured cell-free broth was used for the control sample. The test was considered positive if the IE_{24} value ≥ was of 50 % and, negative if it was of ≤ 50 % [31].

Biosurfactant extraction

The biosurfactant was obtained by the acid precipitation method, from the cell-free broth (CLC) extracted from the samples at different times. This broth was placed in a 250 mL Erlenmeyer flask, acidified with 2 N HCl and adjusted to a pH of 2.0. The CLC was kept in agitation (2000 rpm) at 4 ºC for a period of 12 h. After this time, precipitates were observed at the bottom of the flask, then the acidified broth was placed in test tubes and was centrifuged at 6000 rpm for 30 min. The supernatant was removed from the centrifuge tube and the precipitate was stored at 4 °C for later characterization. The time to obtain the maximum biosurfactant production was estimated by calculating the approximate weight and plotted using Origin 2019 software.

Physicochemical characterization of the biosurfactant agent polar lipids identification by thin layer chromatography

The analysis was performed on a 0.25 mm thick silica gel 60 F254 plate, on which 1 µL of the sample was deposited and placed in a solvent system of chloroform/acetone/methane/acetic acid/distilled water at 6:8:2:2:2:1 v/v; the solvent solution ascended by capillarity [32]. For the disclosure of polar lipids, solutions of diphenylamine 2 g in 200 mL (glycolipids); 2 % ninhydrin (lipopeptides) and bromothymol blue 1 g in 100 mL NaOH (phospholipids) were used. The plates were asperged separately with the substances and heated at 100 °C for 5 to 10 min to reveal the bands associated with the compounds present in the sample. The observed bands were analyzed to determine the area and retardation factors (Rf), this test was reinforced by analysis of the functional groups by FTIR [33].

Organic compounds identification by Fourier Transform Infrared Spectroscopy (FTIR)

Through the infrared spectroscopy technique, the organic compounds that are part of the biosurfactant structure were identified. For the analysis, a 0.1 mL sample was used and placed in the sample holder of the Thermo Nicolet® iS a 50 spectrophotometer, in transmittance mode at a wavenumber range of 4500 to 400 cm^{-1}.

Surface tension measurement of the biosurfactant agent

The biosurfactant sample was dried at 50 ºC (vacuum for 12 h). The solid sample was dispersed in distilled water at a ratio of 100 mg/10 mL. The original dispersion was successively diluted. The surface tension of the supernatants was determined using a Ramé-Hart goniometer, Instrument Co model 250, at room temperature. The reported measurements were the average of 10 measurements in duplicate [29].

Bioassay to evaluate the *Acinetobacter baumannii complex* extremotolerant ability

The bioassay was developed based on a completely randomized one-factorial design for different treatments: temperature (20, 45, 80 °C), pH (5.0, 7.0, 9.0) and salinity (10, 15, 25 % NaCl). This test was performed in bioreactors at the in vitro level with 250 mL nutrient broth with composition: meat extract, yeast extract, peptone casein and NaCl to 1.0, 2.0, 5.0 and 5.0, respectively; NaCl (BAKER-JTB-3624-01) was supplemented, adjusted to a pH 7.2 and 10 mL of the bacteria inoculum. The bioreactors were connected to an aeration system with a volumetric flow rate of 1.21 x 10^{-4} m^3/s. Bacterial biomass was quantified by measuring absorbance every 24 h for a period of 120 h [9]. Sampling was performed in triplicate and the data generated were plotted by using Statgraphics CenturionTM software.

Bioassay to evaluate the oil degradation bacteria ability

The bioassay was carried out based on a completely randomized, one-factorial design. The treatments were established with a working mass of 3 kg oil contaminated soil with an initial concentration of 9,000 ppm TPH. Each experimental unit was performed in triplicate and inoculated with 500 mL of an *Acinetobacter*

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baumannii complex bacterial culture and 1.5 mL sterile distilled water to maintain 40 % humidity. The bioassay was monitored every 24 h for the quantification of heavy fraction hydrocarbons (HFP) by extraction and gravimetry as established in NMX-AA-134-SCFI-2006. Likewise, the CFU counting was performed by the plate count method.

Results and discussion

The physicochemical characteristics of the congenital water, obtained from the sampling results, are summarized in Table 1. High concentrations of salinity and alkalinity were observed, due to chlorides and sodium as well as alkalinity due to bicarbonates present in the water. Studies on the active well Luna-3B Tabasco, reported salinity, alkalinity, and hardness concentrations of 203000,102.4 and 26890 ppm respectively [34]. These values are above those found in this research, which is attributed to the high saturation rates of minerals corresponding to the characteristics of hydrothermal system. For this reason, it is assumed that the congenital water presents the ideal characteristics for the development of extremotolerant bacteria.

Table 1. Physicochemical parameters of the congenital water sample AC362, corresponding to Petróleos Mexicanos (PEMEX) oil well.

Parameters	Value
Alkalinity (mg/L)	300
Total chlorides (mg/L)	93.701
Electric conductivity (mS/cm)	8.51
pH (at 24 ºC)	5.5
Turbidity (NTU)	161

Crude oil can be degraded by several indigenous microorganisms, each one is capable of breaking down a specific group of molecules. It has been reported that *Acinetobacter* has the ability to degrade aliphatic and monoaromatic compounds [35]. The characterization data are presented in Table 2, whose results evidence a heavy type maya oil, according to API degrees ranging between 21.0 - 22.0. The oil was the main carbon source in the different bioassays, favoring the bacteria growth, the degradation and assimilation of the hydrocarbons, transporting them into the cell interior for the conversion to fatty acids [36].

Table 2. Characterization of a crude oil sample from well-362, property of PEMEX in Tabasco.

Test	Result	Reference
Real density (g/cm^3)	0.866	ASTM D70
API gravity (°API)	22.483 ± 0.2566	ASTM D287
Flash point (at 27 ºC)	81 ± 1	ASTM D 88
Saybolt Viscosity (s at 59 ºC)	14	ASTM D 88

The oil components were separated by using a Shimadzu model GCMS-QP2010 ultra gas chromatograph/mass spectrometer with a capillary column (5 %-phenyl)-dimethylpolysiloxane of 30 m x 0.25

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mm i.d., 0.25 m film thickness. The helium gas flow rate was 1.5 mL/min and the injection volume was 1 μL, a split ratio of 10:1, and a mass range: m/z 25–600. In Fig. 1, the chromatographic profile of the oil sample is presented, in which the aliphatic fraction of the alkanes is observed according to their molecular weights (MW). Those of lower molecular weight (C8–C16), of medium molecular weight (C17–C28), and those of high molecular weight (> C28). The degradation of n-alkanes occurs aerobically by successive oxidation of the molecules until the formation of the corresponding carboxylic acids, which are then directly incorporated in the β-oxidation; the most common oxidative pathway is the terminal oxidation: alkane (C–C), alcohol (C–OH), aldehyde (C=O), carboxylic acid (COOH) [35].

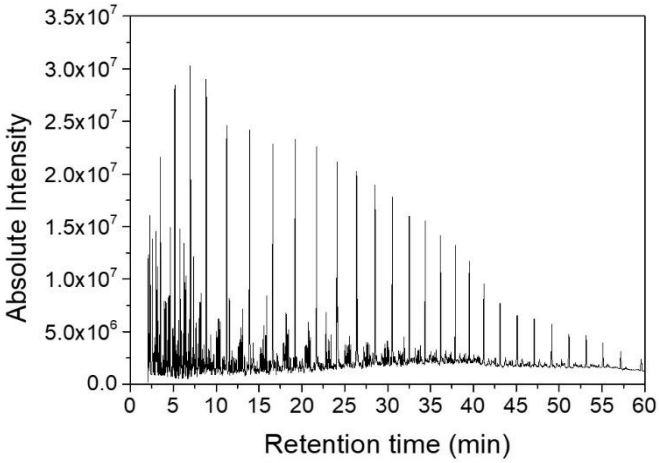

Fig. 1. Chromatographic profile of a heavy oil sample, Maya type, from well-362 (PEMEX).

Three bacterial strains identified as AC362-1, AC362-2 and AC362-3 were isolated from the solid BH medium. Strain AC362-1 (*Acinetobacter baumannii complex)* showed 80 % mineralization of the hydrocarbons present in the liquid medium (triple 17). The bacteria isolated presented a fuchsia color characteristic of the gram-negative bacteria, because they have a cell wall made up of a thin inner layer of peptidoglycan attached to a second outer plasma membrane of proteins, phospholipids and polysaccharides, which protects the bacteria. negative effects of various dyes, detergents and antibiotics, giving them greater resistance, *Acinetobacter* being a bacteria in this group [37]. The taxonomy was obtained from the Biomerieux ApiwebTM database identified strain AC362-1, belonging to the genus *Acinetobacter baumannii complex*, with an ID of 98 %. It has also been reported that strains belonging to the genera *Bacillus, Alcaligenes, Pesudomonas, Acinetobacter, Marinomonas, Kurthia* and *Azothobacter* identified as hydrocarbonoclasts have been isolated in coastal waters [38]. The results of morphological and biochemical analyses are summarized in Table 3.

Table 3. Results of biochemical tests applied to strain *Acinetobacter baumannii complex*, positive response (+), negative response (-).

Biochemical test	**Result**	**Biochemical test**	**Result**
Colonial morphology	Pointed, elevated, entire, small, smooth, matte	GEL	-
Gram Stain	Gram negative	GLU	+
ONPG	-	MAN	-
ADH	-	INO	-
LDC	-	SOR	-

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Biochemical test	Result	Biochemical test	Result
ODC	-	RHA	-
CIT	+	SAC	-
H2S	-	MEL	+
URE	-	AMY	-
TDA	-	ARA	+
IND	-	OXO	-
VP	-		

The reduction of citrate, the oxidation of glucose, arabinose, melobiose, in addition to the butanediol-type fermentation due to the presence of acetoin, are attributed to the fact that the bacteria developed the capacity to assimilate other carbon sources, which favored their growth [7]. Fig. 2 presents the image of the bacteria in a TEM equipment micrograph. The cellular structure of *Acinetobacter baumannii complex* is observed in different sections: A) The coccus united form after cell duplication. B) The preliminary analysis of the size, lengthwise (1.270 µm) and width (0.738 µm) of the cell, performed in GATAN® Digital. C) Cell division in the exponential phase and duplication of its genetic material. D) Spherical and ovoid shape of the coccus, an area which was magnified to 10 µm. The structural and morphological characteristics of the bacteria influence the mechanisms of degradation and mineralization of hydrocarbons and improve the in vitro culture conditions and, consequently, the production of biosurfactants [36,39].

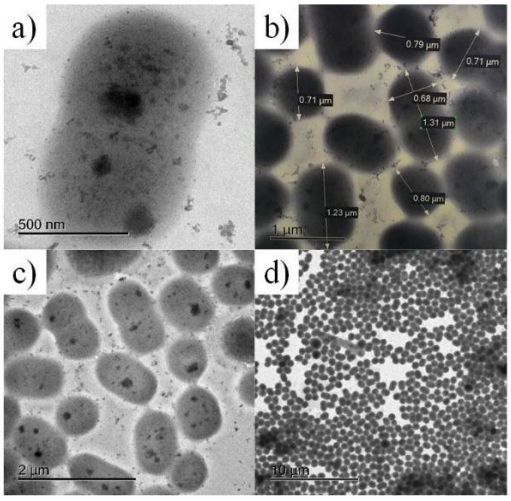

Fig. 2. Micrograph of *Acinetobacter baumannii complex*, analyzed by TEM at different scales: **(a)** duplication of coccus in nm, **(b)** length of bacteria in µm, **(c)** visualization of cell division in µm, **(d)** concentration of bacteria in a field of 10 µm.

The kinetic study of *Acinetobacter baumannii complex*, from optical density measurements at different times (t_0–t_{96}), started from a population with 0.61 OD_{600nm} and 115 x 10^6 CFU/mL. The values were adjusted to the Gompertz model, given by Eq. (2):

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$$y = D * exp\{-exp\ exp\left[\frac{\mu_{max} * e}{D} * (\lambda - t) + 1\right]\} \quad (2)$$

where y = ln (DO_t/DO_0), DO_t is the optical density at time t, DO_0 is the initial optical density at t=0. The other parameters are: D is the maximum asymptotic value equivalent to ln (DO_{max}/DO_0), μ_{max} is the maximum specific growth rate (h^{-1}) and λ is the lag phase period (h). The analysis was performed by using the SPSS version 22 package, where the estimated parameters obtained were: D = 0.479 ± 0.20; μ_{max} = 0.020 ± 0.005; λ = 0.454 ± 2.141. The model was validated with a coefficient of determination (R^2 = 0.929), which is considered an acceptable fitting range. In Fig. 3, the comparison of the observed data (black color series) of y = ln (DO_t/DO_0) and the values predicted by Gompertz (blue color series).

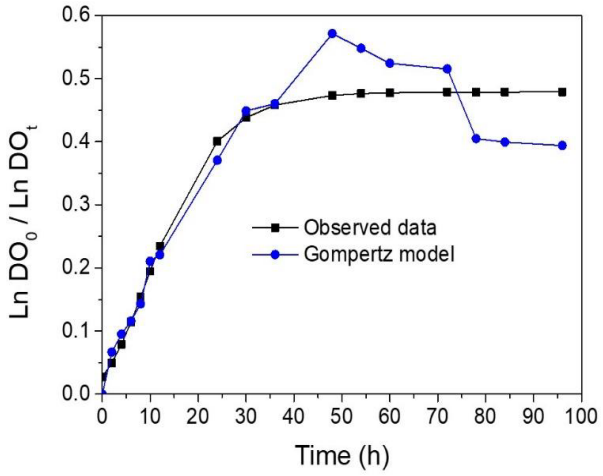

Fig. 3. *Acinetobacter baumannii complex* bacterial strain growth kinetics.

It is evident from the data analysis that the *Acinetobacter baumannii complex* strain can reach a higher metabolic rate before 96 h, under the same experimental conditions, which could favor the performance in the production of biosurfactant. On the other hand, starting from a population of 5.7 x 10^6 CFU/mL, the biosurfactant production (g/mL) and the quantification of CFU/mL were constructed. A maximum of 2.6 g/L was obtained at 96 h of experimentation (Fig. 4). Studies have reported the production of 0.52 g/L of biosurfactant isolated from the oilfield formation waters [40].

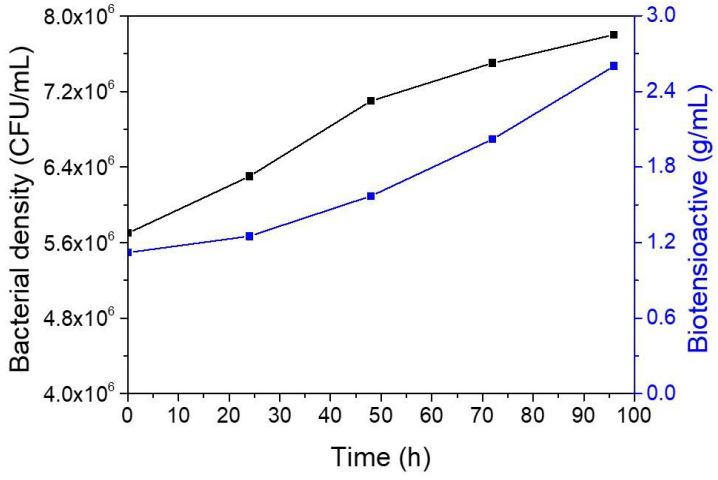

Fig. 4. Bacterial growth and biosurfactant production kinetics.

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The results of the biosurfactant activity of the *Acinetobacter baumannii complex* strain in the hemolytic test, oil dispersion, emulsification index and collapsed droplet are presented in Fig. 5.

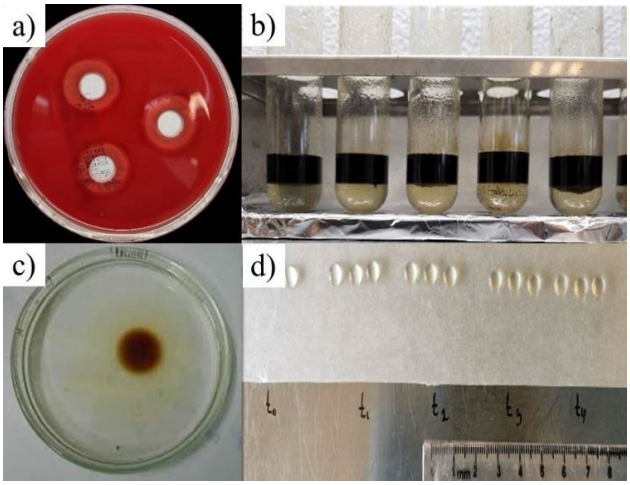

Fig. 5. Biosurfactant capacity tests in CLC. **(a)** hemolytic test, **(b)** emulsification index, **(c)** oil dispersion, **(d)** collapsed drop test.

Table 4 summarizes the average results of the tests, taking into consideration that at 96 h, when the maximum Biosurfactants production was obtained, the hemolytic tests and the collapsed drop were positive (+). The dispersion of the drop and the IE_{24} were 6.54 mm and 64.49 %, respectively. Values of 11–25 mm, 38.46 %, in droplet dispersion and IE_{24}, have been reported in the characterization of a lipopeptide produced by *Bacillus subtilis* [31]. These differences lie in the structural composition of the Biosurfactants [41]. The tests represented a useful method for the preliminary selection of the bacterium as a biosurfactant producer, but were not considered a definitive method due to its limitations. Therefore, they were validated by FTIR and surface tension analyses.

Table 4. Surfactant capacity tests in CLC.

Time (h)	**Hemolytic test**	**Collapsed drop**	**Drop dispersion (mm)**	**IE_{24} (%)**
0	-	-	0.72	48.09
24	-	+	1.14	52.17
48	+	+	3.17	55.93
72	+	+	5.62	51.62
96	+	+	6.54	64.49

In Fig. 6, it can be observed that the plate sprayed with ninhydrin presented a purple-colored marker throughout the migration, which is attributed to the amino acids present in the sample as the main component of the peptides, according to the rf value: 0.56–0.77 of the reference standard. According to the results, a lipopeptide-type biosurfactant produced by *Acinetobacter baumannii complex* bacteria was obtained. Studies have reported this same bacterium as a producer of lipopolysaccharide-type biosurfactants such as emulsan in addition to synthesizing phospholipids [30].

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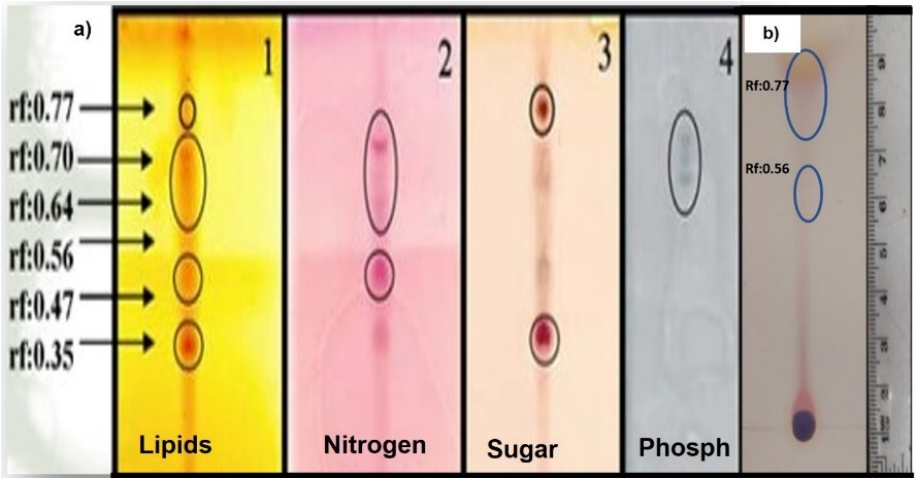

Fig. 6. Thin layer chromatography on silica plates. **(a)** Reference standard; **(b)** Sample of the BTAC362 biosurfactant.

Fig. 7 shows a FT-IR analysis of the biosurfactant in the region of 4000 to 500 cm^{-1}. A broad peak is observed at 3272 cm^{-1} related to the stretching of the O–H bonds of the hydroxyl and carboxyl functional groups. The peaks at 2928 cm^{-1} and 2872 cm^{-1} correspond to the stretching of C–H bonds (CH_2, CH_3) due to the aliphatic chains of hydrocarbons. The band at 1629 cm^{-1} is associated with the stretching of CO–N bonds, revealing the carboxyl and amide groups of the lipopeptides in the biosurfactant. The bands located at 1458 cm^{-1} and 1383 cm^{-1} represent the bending of C–H bonds, indicating the presence of carbon chains. The most intense peaks at 1118 cm^{-1} and 1070 cm^{-1} are assigned to the stretching of C–O–C bonds in the ester group [29,42].

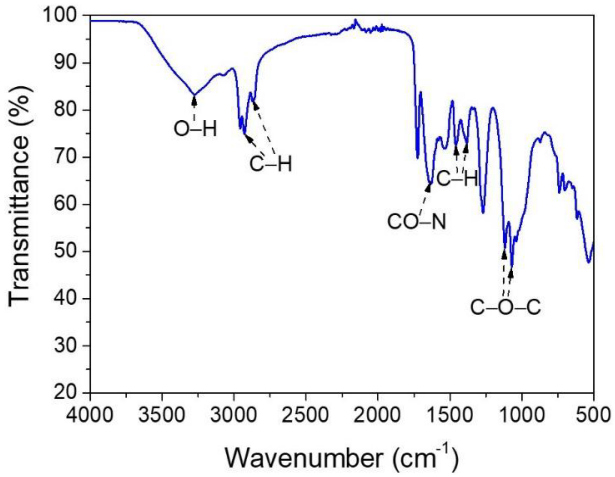

Fig. 7. FTIR spectra of the biosurfactant (BTAC362).

Fig. 8 shows the surface tension analysis, where the biosurfactant reduced the tension from 72.18 mN/m (water) to 53.21 mN/m. This favors the improvement of cell membrane permeability, which favors the biosorption of oil on the bacterial cell surface, in effect increasing the degradation rate of oil hydrocarbons [29,31].

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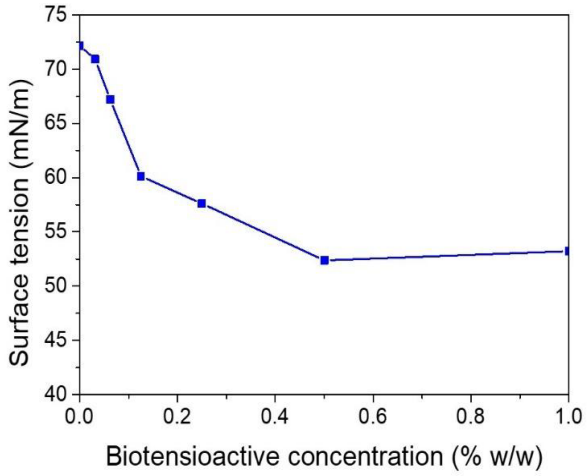

Fig. 8. BTAC362 biosurfactant surface tension.

The analysis of extremotolerant capacity was carried out based on a completely randomized single-factor design for different factors: temperature, pH and salinity conditions. Paired tests were statistically significant ($p < 0.05$). Fig. 9**(a)** this shows the absorbance curves for 3 different temperatures: 20, 45 and 80 °C. The maximum absorbance level was achieved at T= 80 °C, 0.44 at 48 hrs. **(b)** it shows 3 different pH conditions: pH 5, 7 and 9. Although the higher pH medium had a final higher absorbance value 0.74 at 120 hours, using Tukey's least significant difference (HSD) method, it was found that the pH factor did not significantly influence the bacterial growth. **(c)** it shows 3 different salinity mediums: 10 %, 15 % and 25 % NaCl. The 15 % saline concentration obtained the highest absorbance value 0.76 at 72 hrs. The salinity conditions have a significant influence on bacterial growth.

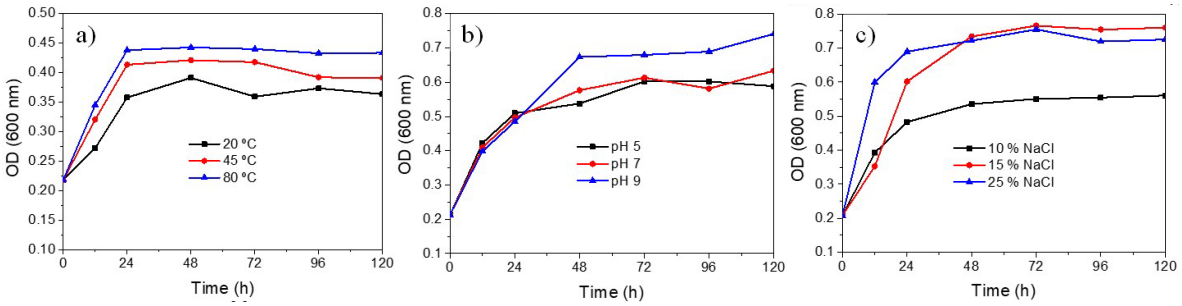

Fig. 9. Variation of optical density (600 nm) in a bioassay under extreme conditions. **(a)** Temperature conditions; **(b)** pH conditions; **(c)** Salinity conditions.

The results of the bioremediation of the contaminated soil are presented in Fig. 10, which showed a favorable behavior for the process, since it reached a degradation of 3812 mg/kg with a population of 1.56 x 10^5 CFU/g soil at time 9 (216 h) of treatment.

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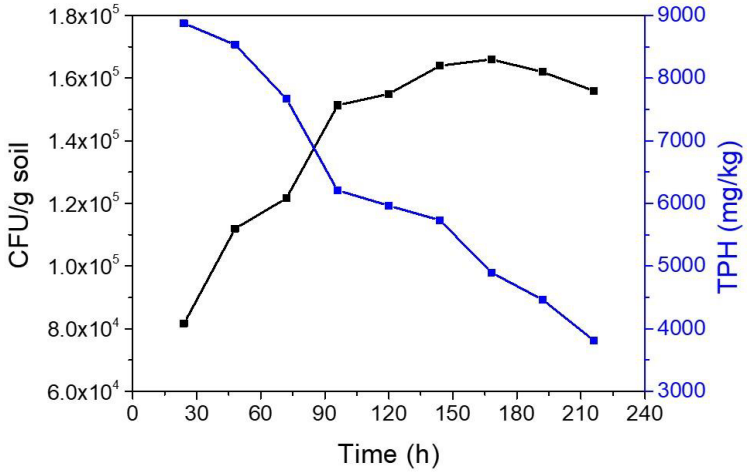

Fig. 10. Bacterial density and total hydrocarbons during soil bioremediation testing.

Conclusions

A bacterium of the genus *Acinetobacter baumannii complex* was isolated from congenital water samples under conditions of alkalinity (300 mg/L) and hardness (93,701 mg/L). The strain was characterized as a halophilic, alkalophilic and thermophilic microorganism by withstanding temperature, pH and salinity conditions of 80 ºC, 9 and 15 %, respectively.

Its extremotolerant property provides the bacteria with greater thermostability to the cytoplasmic membrane, capacity to produce hydrolytic enzymes, biofilm, which give greater protection and resistance to the cell, these metabolites are important in the production of biosurfactants. On the other hand, its Gram-negative bacillary morphology was evidenced, characterized by the formation of its double lipid membrane that protects the cell from the adverse effects of dyes, detergents and antibiotics, making it more resistant. In vitro conditions, the growth kinetics presented a maximum growth rate (0.020 ± 0.005 h^{-1}), adjusting the observed data to the Gompertz model, likewise a coefficient of determination $R^2 = 0.929$ was obtained, which is acceptable. In addition, this analysis sets the precedents for the production of biosurfactant by *Acinetobacter baumannii complex* bacteria. In this study, a yield of 2.6 g/L was obtained at 96 h of production for a lipopeptide-type biosurfactant, which reduced the surface tension from 72.18 mN/m (water) to 53.21 mN/m. The *Acinetobacter baumannii complex* strains presented a high biotechnological potential by degrading from 9000 to 3812 mg/kg of hydrocarbons in the bioremediation of soil contaminated with a Maya-type crude oil.

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