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Demulsifying properties of extracellular products and cells of *Pseudomonas aeruginosa* MSJ isolated from petroleum-contaminated soil

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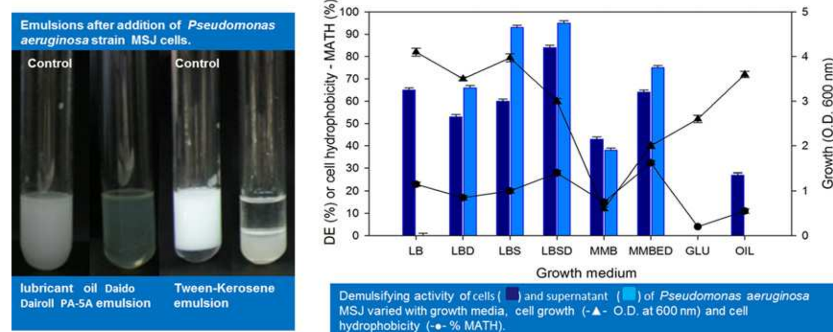
HIGHLIGHTS

- ▶ Cells and supernatant of *Pseudomonas aeruginosa* cultures showed emulsifying activity.
- ▶ Demulsifying activity was relatively stable in different physicochemical conditions.
- ▶ The extracellular biodemulsifier is composed of fatty acids and carbohydrates.
- ▶ The demulsifier was active against industrial emulsions with oil Daido Dairoll PA-5A.
- ▶ Demulsification varied according to growth media, culture age and cell hydrophobicity.

GRAPHICAL ABSTRACT



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ABSTRACT

A strain of *Pseudomonas aeruginosa* isolated from a site contaminated with refined oil products exhibited demulsification capabilities against Tween 80–Span 80 stabilized oil-in-water (O/W), Tween 80-stabilized water-in-oil (W/O) model emulsions (kerosene–water), and an industrial emulsion (Daido Dairoll PA-5A). GC–MS analysis confirmed the presence of fatty acids and carbohydrates in the extracellular biodemulsifier. The demulsifying activity of cells and culture supernatants was favored by growth in media containing 1% diesel oil. There was a correlation between culture age, de-emulsification and cellular hydrophobicity, and highest activities were observed for cells and supernatants from 96-h cultures. Activity increased with addition of up to 60 mg cells or 300 μL supernatant to emulsions. The activity was relatively stable at 20–40 °C and to freezing, but was reduced by 69% by washing the cells with chloroform–methanol-water. This demulsifier has potential for application in biotreatment of emulsified oily wastewaters to promote recovery and/or degradation of oil.

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1. Introduction

An emulsion is a system of two immiscible liquids in which one liquid (dispersed phase) is suspended in the form of small droplets in a second liquid (continuous phase). Depending on the kind of liquid forming the continuous phase, emulsions are classified as either water-in-oil (W/O) or oil-in-water (O/W) (Larson et al., 1994).

Stable emulsions are generated in diverse enterprises, such as the steel, aluminum, food, textile, leather, petrochemical and metal finishing industries (Becker, 1997). For instance, the metal working industry often uses oil–water emulsion as coolants and lubricants

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to increase the useful life of tools. In these sectors, it is often necessary to separate water from oil for further oil processing and/or refinement or to treat oily effluents. A combination of physical and chemical methods involving centrifugation, heat, electrical treatment or chemical demulsification has been used to promote breakage of emulsions (Becker, 1997; Ha and Yang, 1999; Li and Wang, 1999).

The demulsifiers used by the oil industry are made up of one or more surfactants derived from alcohols, fatty acids, fatty amines, glycols and alkylphenols dispersed in a solvent system (Stalss et al., 1991). Demulsifiers containing mainly nonylphenol alkoxylates and related compounds have a toxic effect on organisms in the marine environment (Peigne, 1993) and conventional chemical demulsifiers typically do not meet desirable biodegradation levels. Consequently, it is likely that the vast majority of conventional demulsifiers will be prohibited from offshore use in the near future. Therefore, the need exists for oily effluent demulsifiers with an improved environmental profile, including high biodegradability and low toxicity.

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Microorganisms are believed to modify emulsion properties by using hydrophobic cell surfaces or the dual hydrophobic/hydrophilic nature of biosurfactants to displace or alter emulsifiers present at the oil–water interface (Das, 2001). Bacteria belonging to the genera *Nocardia*, *Corynebacterium*, *Rhodococcus*, *Mycobacterium*, *Bacillus*, *Aeromonas*, *Alcaligenes* and *Micrococcus* have been examined in demulsification studies with satisfactory results (Cairns et al., 1982; Kosaric et al., 1987; Janiyani et al., 1994; Das, 2001; Ma et al., 2006b; Singh et al., 2007; Huang et al., 2009; Liu et al., 2011). Microbial demulsifiers have low toxicity, biodegradability and high efficiency under extreme conditions (Liu et al., 2011). In addition, if hydrocarbons of the emulsion can support microbial growth, growth and demulsification can occur simultaneously (Kosaric et al., 1987). The ability of a demulsifier to adsorb to a water–oil interface is crucial to its demulsifying activity. This process can be affected by cell surface hydrophobicity and hydrophobic–hydrophilic balance of the cell wall, when the demulsifying activity is linked to the cell surface, and by electrochemical properties of the extracellular demulsifiers (Kosaric et al., 1987; Ma et al., 2006a; Singh et al., 2007).

In the present study, strain MSJ, isolated from petroleum-contaminated soil was evaluated for its demulsifying activity in W/O or O/W and industrial emulsions when cultivated in media with different carbon sources. In addition, the physiological characteristics of the strain, its identification based on 16S rRNA gene sequencing and phenotypic tests, and factors that influence its demulsifying activity are described.

2. Methods

2.1. Microorganism identification and cultivation medium

Strain MSJ was isolated from soil contaminated with refined oil products in Brazil. Enrichment culture was performed aerobically in flasks with 100 mL mineral medium (MMB) containing (g/L) K_2HPO_4, 1.5; KH_2PO_4, 0.5; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; NH_4NO_3, 3.0; $FeSO_4 \cdot 7H_2O$, 0.002; $CaCl_2 \cdot 2H_2O$, 0.002; yeast extract, 0.002; and 1 mL/L trace mineral solution, pH 7.0, containing (mg/L) $MnCl \cdot 4H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot 2H_2O$, 2.6 and $Na_2MoO_4 \cdot 2H_2O$, 6.0. After addition of diesel oil (1% v/v) and 10 g of soil, the flasks were incubated at 25 °C on an orbital rotary shaker at 200 rpm for 96 h. Colony isolation was carried out on Luria Bertani (LB) plates incubated at 25 °C for 120 h. Individual colonies on plates were evaluated based on demulsification activity using the kerosene model emulsion (ME) (Nadarajah et al., 2002).

Strain MSJ, with the highest demulsification activity, was identified according to morphological and biochemical characteristics using the Vitek automated system with the GNI card (bioMérieux Vitek Inc. – Hazelwood, MO, USA), API 20NE (bioMérrieux), and 16S rRNA gene sequencing.

For genomic DNA extraction, cells grown for 24–48 h in LB broth were removed by centrifugation at 13,000*g* for 5 min. The pellet was washed with TE buffer (0.01 M Tris–HCl, pH 8.0, and 0.001 M EDTA) and suspended in 500 μL of the same buffer and transferred to a 1.5-mL conical tube for further cell disruption for at least 20 s using approximately 60 mg acid-washed glass beads (Sigma, 150–212 μm) in combination with strong vortexing. The suspension was purified by adding equal volumes of phenol–chloroform–isoamylalcohol (25:24:1), followed by isopropanol precipitation at −20 °C for 12 h. Subsequently, the pellet was washed with 70% ethanol and dissolved in 20 μL Milli-Q water or TE buffer. DNA and protein concentrations were determined using a NanodropTM 1000 instrument at 260 and 280 nm. The 16S rDNA region was amplified by PCR using the primers 8F (5′-GGATCCAGACTTTGATYMTGGCTCAG-3′) and 907R (5′-CCGTCAA TTCMTTTGAGTTT-3′) (Lane, 1991). PCR was performed in a final volume of 50 μL containing 200 ng of the extracted DNA, 0.02 mM of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 5 μL PCR buffer (10×, Fermentas, Barcelona, Spain), 0.3 μM of each primer and 2.5 U Taq-DNA polymerase (Fermentas, Barcelona, Spain). The amplification was carried out with initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 1 min., 55.3 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 8 min. Amplifications were performed in a GeneAmp PCR System 9700-Thermocycler from PE Applied Biosystems. PCR products were analyzed together with a molecular weight ladder (1 kb DNA ladder, Promega, Madison, USA) by electrophoresis on a 1% agarose gel containing ethidium bromide in Tris–acetate–EDTA buffer (TAE: 40 mM Tris–acetate, 1 mM EDTA). The amplified DNA was concentrated, cleaned (Kit Wizard Plus SV Minipreps DNA Purification; Promega, USA), and sequenced using the BigDye® Terminator kit and ABI-3730XL automated sequencer (Applied Biosystems). The strain was identified by searching databases using the BLAST sequence analysis tool (http://.ncbi.nlm.nih.gov/BLAST/) and RDP (http://www.rdp.cme.msu.e161du).

2.2. Demulsification assays

Strain MSJ was grown in 100 mL of MMB with 1% (v/v) diesel oil (Petrobras) in an orbital rotary shaker at 200 rpm, 28 °C for 144 h. Growth was monitored by measuring optical density (OD) at 600 nm in a spectrophotometer (Shimadzu UV120). After cultivation, bacterial cells were separated from the medium by centrifugation at 13,000*g* for 15 min, and the cells and supernatants was preserved at 4 °C to determine the demulsification activities using a Tween–kerosene model emulsion (ME) and an industrial emulsion (IE).

The ME (W/O) was prepared by mixing 0.2% Tween 80 (Sigma) and kerosene at 1:2 (v/v) at 24,000 rpm for 2 min in a Turrax-type agitator (Marconi). As necessary, other emulsions were prepared

with toluene and olive oil instead of kerosene. To obtain Span–Tween–Kerosene ME (O/W), a stock solution of kerosene was prepared by mixing 0.08 g Span-80 (Sigma) with 100 mL kerosene on a stir plate, and a stock solution of Tween-80–water was prepared by dissolving 0.1 g Tween-80 (Merck) in 100 mL de-ionized water. Subsequently, 0.9 mL Tween-80–water and 2.1 mL Span-80–kerosene solutions were mixed at 24,000 rpm for 2 min. The emulsion type was verified by the Oil Red *O*-test (Kim et al., 1998).

IEs were prepared by mixing 0.5% lubricant oil (Daido Dairoll PA-5A) in distilled water for 2 min in a Turrax-type agitator Turrax (Marconi). The emulsions were kept in stoppered glass bottles for no longer than 1 h. The oil was purchased from a steel company that uses it as metalworking oil in machining and thus generates wastewater containing high concentrations of emulsified oil.

In the demulsification test, 40 mg wet cells suspended in distilled water in a total volume of 50 μL and 300 μL culture supernatant or 300 μL of medium (control) were added into a 14.8 × 100 mm tube containing 2 mL of emulsion and sealed with silicone rubber. The test tube was agitated in a vortex (Phoenix) for 30 s at 13,800 rpm to achieve complete mixing. The tube was kept undisturbed in an upright position in a water bath at 35 °C. The changes in the volume of the oil phase (top), water phase (bottom), and emulsion phase (in-between) were recorded at certain time intervals for up to 7 d.

Demulsification of MEs was based on the volume of separated emulsion compared to the original volume (EV) and expressed as the demulsification percentage – %DE = [(initial volume (2 mL) − final emulsion volume at interphase (mL))/initial volume (mL) × 100]. All experiments were carried out in duplicate and variations in reproducibility between experiments were less than 10%. Using the %DE values for different time points, the half-life of the emulsion was calculated as $(\log_e 2)/k$ from the plot of $\log_e(EV)$ vs. time, where k is the slope of the respective straight line. If the emulsions collapsed before the last noted time, and $\log_e(0)$ may have become undefined, the point corresponding to the time of collapse, i.e., 0%EV, was not considered in the calculation.

In the demulsification assays using the IE, glass tubes (12 × 75 mm) were used, and the %DE was expressed as the reduction in the emulsion turbidity evaluated by absorbance at 600 nm (Micronal B542) before and after incubation at 24-h intervals for up to 7 d.

The effect of cell amount and supernatant volume on demulsification was studied with MEs in a manner similar to that described above, except that different quantities of cells (20, 40, 60, 80, 100, and 120 mg) suspended in distilled water in a total volume of 50 μL and different supernatant volumes (50, 100, 200, 300, 400, and 500 μL) were used. The control for the first assay consisted of the addition of 50 μL sterile distilled water; 500 μL sterile medium was used as a control for the second test. The emulsions were kept at room temperature for up to 7 d and demulsifying activity was evaluated at intervals of 24 h.

To evaluate the effect of culture medium on demulsification, strain MSJ was cultivated in Erlenmeyer flasks containing MMB plus 1% diesel oil (MMBD), 1% glucose (GLU), 1% olive oil (OIL), or 1% yeast extract and diesel (MMBED); LB, LB plus 1% diesel (LBD), LB without NaCl (LBS) and LB without NaCl plus 1% diesel (LBSD). Flasks were incubated under constant orbital agitation at 200 rpm and 28 °C for up to 144 h. After cultivation, bacterial growth was determined by measuring OD at 600 nm; cells (40 mg) and supernatant (300 μL) and 300 μL growth medium without added cells (control) were added to the ME, and demulsification activity was assessed.

Microbial surface hydrophobicity was assessed by the microbial adhesion to the hydrocarbon (MATH) method described by Rosemberg (1991) with modifications. The cells were washed twice and resuspended in 100 mM sodium phosphate buffer, pH 7, to an initial OD of 0.7 at 600 nm. Hexadecane (0.5 mL) and cell suspensions (2.0 mL) were vortexed in a test tube for 2 min and left undisturbed for 30 min. The bottom aqueous phase was carefully removed with a Pasteur pipette, and the OD 600 nm was measured. Adherence was expressed as the percent decrease in absorbance of the lower aqueous phase following mixing compared with the OD 600 nm of the cell suspension prior to mixing.

2.3. Effect of culture age

Strain MSJ was inoculated to an OD of 0.05 at 600 nm in a fermenter (New Brunswick) containing 3 L LBSD medium and incubated for 144 h at 28 °C under agitation at 200 rpm. One-hundred-milliliter samples were taken at 24-h intervals to assess cell growth (OD at 600 nm), medium pH, cell surface hydrophobicity (MATH) and %DE of cells and supernatant samples.

2.4. Effect of pH and temperature on demulsification

The aqueous phase of model emulsions was prepared by adding 0.2% Tween in buffer solutions with pH values ranging from 4 to 8. Citrate buffer (100 mM) was used for pH 4 and 5 and 100 mM sodium phosphate buffer for pH 6, 7, and 8.

To evaluate the effect of temperature, Tween–kerosene (W/O) and Span–Tween–kerosene (O/W) model emulsions were mixed with 40 mg cells, 300 μL supernatant or 300 μL medium without growth (control) and incubated at 20, 30, 40, 50 and 60 °C. Demulsifying activity was evaluated at intervals of 24 h for up to 7 d.

2.5. Effect of physical and chemical treatments

Cells and supernatant were submitted to moist heat under pressure (121 °C for 20 min), freezing at −20 °C for 24 h followed by thawing, freezing in liquid nitrogen followed by thawing and freeze-drying, and demulsification activity assays were performed using ME (W/O) mixed with 40 mg treated cells or 300 mL treated supernatant. Controls consisted of tubes with untreated cells or supernatant samples.

To evaluate the effect of chemical treatments on demulsification, 40 mg cells collected by centrifugation were treated separately with kerosene or a mixture of chloroform–methanol–water (CMW) (5:10:4) as described by Das (2001) with modifications. Cells were added to 10 volumes of solvent and kept at room temperature for approximately 30 min with intermittent agitation. After centrifugation at 13,000*g* for 15 min, solvents were discarded, and cells were washed twice with sterile saline (0.85% NaCl). Cells were further tested for demulsification as described in Section 2.2 using cells that were not washed with solvent as a control.

2.6. Extraction and partial characterization of the chemical composition of the demulsifier

The isolate was grown for 72 h in LBSD under constant orbital agitation at 200 rpm and 28 °C. After centrifugation of the culture at 10,000 g for 15 min, demulsifying agent in the supernatant was precipitated by acidification to pH 2 with 6 M HCl at 4 °C overnight. The precipitate was recovered by centrifugation at 10,000 g for 1 h and dissolved in deionized water. Two volumes of chloroform:methanol (2:1, v/v) were added and the mixture was shaken for 30 min. The organic phase was removed and evaporated to dryness. Finally, the extract was dissolved in deionized water to 100 mg/mL and the fatty acid and monosaccharide content was determined by GC–MS.

To determine the fatty acid composition of the lipid moiety of the demulsifier, approximately 0.5 mg of extract was placed in an airtight vial with a screw cap, dissolved in 1 mL of methanol P.A.

(Merck), and stirred until homogeneity was reached. Approximately, 50 µL of trifluoroacetic acid (Sigma) was added to this mixture. The resulting sample was placed in 2.5-mL microtubes and incubated at 120 °C on a hot plate for 4 h. The solvent was completely evaporated using a nitrogen stream. Fatty acid methyl esters (FAMEs) were extracted three times with *n*-hexane and analyzed using a GC–MS (SHIMADZU, model QP 5050 A equipped with a PTE 5 capillary column (30 m × 0.25 mm ID, 0.25 µm film). The column temperature was initially set at 120 °C, rising linearly to 300 °C at a rate of 5 °C/min, and remaining at 300 °C for 1 min. Helium was used as carrier gas at 27.8 ml/min. The split ratio total pressure was 1:20 and 100 kPa, respectively. Injector port and detector interface were maintained at 250 °C. Mass ranges were collected at 40–400 atomic mass units range and scanned 5 times/s. Data were analyzed by the AMDIS program using the NIST v. 2.0 library.

To perform carbohydrate composition analysis, demulsifier (1 mg) was hydrolyzed in a sealed tube with 150 µL of 2 M trifluoroacetic acid (CF3COOH) at 120 °C for 4 h. After evaporation, the residue was washed twice with methanol, reduced with 1 M aqueous sodium borohydride (NaBH4, 100 µL) and acetylated with a mixture of potassium acetate (100 µg) and acetic anhydride (100 µL) at 100 °C for 2 h. The excess reagent was removed by evaporation, and the sample was washed several times with ethanol. The alditol acetates were extracted with ethyl acetate and water (1:1, v:v) and analyzed using a GC–MS (SHIMADZU, model QP 5050 A) equipped with a PTE-5-Supelco (30 m × 0.25 mm ID, 0.25 µm film) column. The column temperature was programmed to increase from 100 °C (1 min) to 200 °C at a rate of 4 °C/min, followed by a 20 °C/min increase to 300 °C, and maintenance at this temperature for 5 min. Helium was used as carrier gas at 8.5 mL/min. The split ratio was 1:10 and total pressure was 40 kPa. Injector port and detector interface were maintained at 250 °C. Mass ranges were collected at 40–400 atomic mass units range and scanned at 5 times/s. Data were compared using a series of alditol acetates as internal standards. The following sugar standards were purchased from Supelco, USA: glucose, mannose, galactose, rhamnose, fucose, ribose, arabinose, and xylose.

2.7. Statistical analyses

The experimental design consisted of randomized blocks with three replicates and treatments arranged in full factorial design. All statistical calculations were made using Sigma-Stat 3.5 statistical software (Jandel Scientific, San Rafael, CA, USA). Analyses of variance (ANOVA) and Duncan means comparison tests with a significance level of 0.05 were applied. For the ANOVA, the occurrence of interaction between culture fraction, treatment or incubation time, and conduction of folding were evaluated. Pearson's correlation coefficient was used to evaluate the correlation between the variables analyzed, and values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Strain identification

Strain MSJ stained as a Gram-negative rod, did not ferment sugars, was catalase- and oxidase-positive, grew at room temperature or 37 °C and produced a yellow-green fluorescent pigment that was highly water-soluble. BLAST and RDP searches of the 16S rDNA sequence obtained from strain MSJ indicated a close relationship with species of the *Pseudomonas* genus and a 99% similarity with the corresponding sequence from *P. aeruginosa* K2 (FJ972528.1). The sequence obtained for MSJ was deposited in Genbank with the number of accession JQ901102.

3.2. Effect of the composition of growth media on demulsifying activity

The medium significantly affected growth and demulsification activity, and there was a significant difference between the activities of the cells and the supernatant ($p < 0.05$) (Fig. 1). From an initial inoculum with an OD 600 nm of 0.05, the poorest growth was observed in MMB medium (OD 600 nm = 0.5), and LB medium favored the increase in biomass most (OD 600 nm = 4). The best demulsifying activity was observed in cells and supernatants from cultures grown in LBSD medium, reaching values of 84% and 95%, respectively, followed by cells and supernatants of LBS cultures. These data show the NaCl effect on the demulsifying activity. In LB medium, adding diesel oil had a positive effect on demulsifying activity of the supernatant but resulted in decreased activity of the cells (82–53%). Salt concentration can affect biosurfactant production (Yakimov et al., 1995). Emulsions are stabilized by steric stability and electrostatic mechanisms and electrolytes such as NaCl can affect this stability by influencing surface and interfacial behaviors of surface-active molecules (Raza et al., 2010).

Addition of 1% yeast extract to the MMBED medium increased the demulsifying activity from 43% to 64% in cells and from 38% to 75% in the supernatant. The cells and supernatants of MMB plus glucose cultures did not have demulsifying activity, and the addition of olive oil to this medium only promoted a low demulsifying activity (27%) of cells. Although the tested media supported *P. aeruginosa* MSJ growth, the highest rates of demulsification were observed after growth on hydrophobic substrates derived from petroleum, suggesting an effect of the carbon source on promoting demulsifying activity.

This effect has been described in other studies. Nadarajah et al. (2002) used a mixed bacterial culture and observed that growth on crude oil, motor oil (10W30) or rapeseed oil promoted higher demulsifying activity in a kerosene–water model emulsion, with values above 80%. In contrast, growth on hydrophilic substrates, such as glucose, starch and sucrose, resulted in lower rates of demulsification, with values below 80%. The authors suggested that the high production of components associated with cells or biosurfactants responsible for demulsifying activity was induced

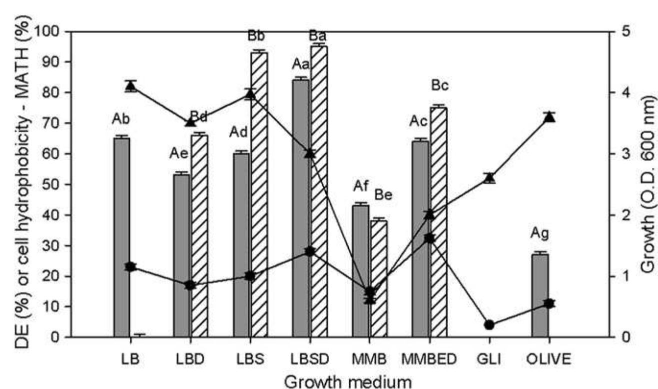

Fig. 1. Demulsifying activity of Tween–kerosene model emulsion (ME) by cells (▓) and supernatant (▨) of *Pseudomonas aeruginosa* MSJ grown in the following media: Luria Bertani (LB); LB plus 1% diesel oil (LBD); LB without NaCl (LBS); LBS plus 1% diesel oil (LBSD); bacteria mineral plus 1% diesel oil (MMBD); MMB plus 1% yeast extract and 1% diesel oil (MMBED); MMB plus 1% glucose (GLU) and 1% olive oil (OIL); (-N-) OD at 600 nm; (-d-) % MATH. Emulsions were maintained at room temperature for up to 7 d; no changes were observed in emulsion controls. Averages followed by the same uppercase or lowercase letters do not differ by Tukey test at 5% probability. Capital letters are related to the fractions of culture, cells (A) and supernatant (B), and lowercase letters are related to the treatments.

by the hydrocarbon constituents of petroleum. Cairns et al. (1982) also observed a positive effect of hydrophobic substrates on demulsifying activity of *Nocardia*. Cells grown in mineral medium containing hydrophilic substrates (1% yeast extract and 1% dextrose) had lower demulsification rates than those grown in medium containing a hydrophobic substrate (4% hexadecane). Positive effects of growth in hydrophilic substrates have also been described. For example, Janiyani et al. (1994) observed a high demulsification rate of an O/W emulsion using pure cultures of *Bacillus subtilis* grown on a medium containing glucose.

The microbial adhesion to hydrocarbon (MATH) values changed with the cultivation medium. The highest values were observed in MMBED and LBSD media (Fig. 1). A positive correlation between MATH and demulsification activity was also observed ($r = 0.91$and $p = 0.035$), suggesting that cell surface hydrophobicity during demulsification by this strain is correlated with its emulsion-breaking performance. This result is in accord with others showing that higher cell surface hydrophobicity and a lower negative charge of the demulsifying strain contribute to a higher dispersion rate into the water–oil interface, which improved demulsifying efficiency (Araujo et al., 2010; Liu et al., 2011).

3.3. Demulsification of W/O and O/W emulsions

Samples of cells and supernatant of cultures of the *P. aeruginosa* MSJ grown for 7 d in LBSD medium were evaluated for their ability to break W/O (Tween–kerosene ME, Tween–toluene ME, Tween–olive oil ME) and O/W (Span–Tween–kerosene ME and Daido Dairoll PA-5A IE) emulsions (Table 1). There was a significant difference between the activities of cells and supernatant and between the activities of different emulsions ($p < 0.05$). In general, higher rates of demulsification were observed for the cell fraction, which was able to break all the different emulsions. Tubes with W/O and Span–Tween–kerosene emulsions and cells or supernatants exhibited a clear separation of phases: a kerosene phase at the top, and interface and an aqueous phase at the bottom. The volume of the median phase was measured, and the percent demulsification was calculated. A different result was observed for the IE. At the top of the tube, a film of oil formed, and in the remaining emulsion, a continuous turbid phase was observed.

For emulsions prepared with a hydrophilic (Tween 80) or a hydrophobic (Span 80) surfactant, a greater effect of cells and supernatant of MSJ cultures on emulsion stability was observed. The emulsion began to destabilize at the moment cells or supernatant were added, and complete demulsification was observed after 72 h. For the IE, a low demulsification was observed, with values corresponding to 15% and 27% for cells and supernatant, respectively.

Differences in the composition and viscosity of emulsions could explain the variation in the percentage of demulsification. Generally, emulsions containing large quantities of water are easier to break than those containing small quantities (Mouraille et al., 1998). This variation was also observed with respect to the $t_{1/2}$ of the emulsions (Table 1). The ME treated with cells or supernatant had a $t_{1/2}$ of 107.2 and 78.8 h, respectively. A lower $t_{1/2}$ was observed for the emulsion of Span–Tween–kerosene, with values of 11.1 h (addition of cells) and 13.38 h (addition of culture supernatant). However, the highest $t_{1/2}$ values were achieved by IE-treated with cells (701.4 h) or supernatant (318.2 h). *P. aeruginosa* MSJ had demulsifying activity against different types of W/O and O/W emulsions. This profile is not observed in all organisms with demulsifying activity. Often, microorganisms can destabilize emulsions containing only one type of organic phase. According to Huang et al. (2009), the selection of microorganisms using emulsions prepared with crude oil resulted in demulsifiers with specific activities for emulsions prepared with the same oil.

However, when using an emulsion prepared with kerosene, the microorganisms had the potential to demulsify emulsions containing different oils with less specificity. In addition, the ME with a kerosene phase was appropriate for the selection of bacteria with high activity against different W/O and O/W emulsions. These results suggest that the demulsifying agent from *P. aeruginosa* MSJ has great potential for use in the treatment of industrial emulsified effluents, such as those generated during the recovery and processing of oil and machining operations or by the steel industry, which uses Daido Dairoll PA-5A oil-based emulsions. The MSJ strain could also be of interest to the food industry because it had high demulsification activity against emulsions with olive oil as organic phase (Table 1).

3.4. Effect of culture age

An increase in demulsification was observed with an increase in culture age (Fig. 2). Maximum values were obtained for cells and supernatants of 96-h-old cultures, with activities of 87% and 95%, respectively. This period corresponded to a higher biomass of the strain (OD 600 nm = 1.54) (Table 2). During this period, the pH of the culture remained near 5.6. Nadarajah et al. (2002) also observed an increase in demulsification rate with an increase in culture age. Maximum demulsification was observed for 48-h-old cultures. Kosaric et al. (1987), using pure cultures of *Nocardia amarae*, observed that cells obtained in the initial stages of growth were able to produce agents for demulsifying W/O emulsions, whereas the model O/W emulsion was broken efficiently by cells in the late stages of growth.

Bacterial growth was monitored during the culture period, and an increase in OD was observed for up to 96 h. After this period, OD values decreased, remaining close to 1 after 96 h. This decrease could be explained by cell lysis due to the toxicity of diesel oil, or because the cells became more hydrophobic and were present on the surface of the medium with the diesel. A direct correlation between increasing culture age and cell hydrophobicity over a 96-h period was observed ($r = 0.936$ and $p = 0.052$) (Table 2). In 24-h-old cells, the rate of demulsification was 60% and cell hydrophobicity was 19%. The highest demulsifying activity and cell hydrophobicity was observed in 96-h-old cells: 87% and 38%, respectively.

Table 1
Values of $t_{1/2}$ of water-in-oil and oil-in-water emulsions added of cells and supernatant of *Pseudomonas aeruginosa* MSJ.

Emulsions		Emulsion type	%DE[ab]		$t_{1/2}$[a] (d/h)	
Aqueous phase surfactant	Organic phase		Cells	Supernatant	Cells	Supernatant
Tween 0.2%	Kerosene	W/O	90.3 ± 2.4 Ab	95.3 ± 3.6 Bb	2.12 (50.8)	1.6 (38)
Tween 0.2%	Toluene	W/O	83.3 ± 2.2 Ac	0	2.74 (65.7)	1.6 (38.3)
Tween 0.2%	Olive oil	W/O	78 ± 2 Ad	85 ± 3.3 Bc	3.19 (76.5)	2.5 (60)
Span 0.08% and Tween 0.01%	Kerosene	O/W	96.7 ± 6.4 Aa	99.7 ± 2.2 Ba	0.46 (11.1)	0.54 (13.38)
Industrial emulsion		O/W	15 ± 2.3 Ae	27 ± 0.7 Bd	29.22 (701.4)	13.3 (318.2)

[a] Standard deviations were lower than 3% of media value.
[b] % demulsifying activity. Averages followed by the same uppercase or lowercase letters do not differ by Tuckey test at 5% probability. Capital letters are related to the fractions of culture, cells (A) and supernatant (B), and lowercase letters are related to the treatments.

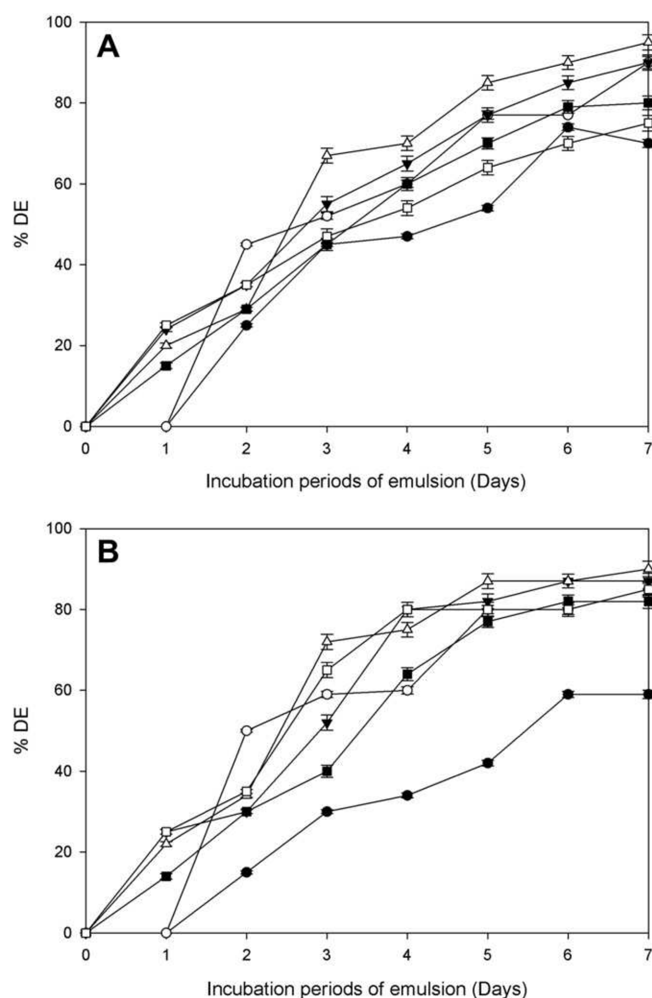

Fig. 2. Effect age culture age of *Pseudomonas aeruginosa* MSJ on the demulsifying activity of Tween–kerosene model emulsion (ME) by culture fractions. % Demulsification by cells (A) and supernatant (B) in different incubation periods: 24 h (-d -), 46 h (-s -), 72 h (-. -), 96 h (-△-), 120 h (-j -), and 144 h (-h -). Forty milligram cells or 300 μL supernatant or 300 μL media without growth (control) were utilized in the demulsification assays. Emulsions were maintained at room temperature for up to 7 d; no changes were observed in emulsion controls.

Table 2
Biomass, cell hydrophobicity, $t_{1/2}$ of model emulsion and demulsifying activity during growth of *Pseudomonas aeruginosa* MSJ in Luria Bertani medium without NaCl plus 1% diesel (LBSD).

Age of culture (h)	Cell concentration (OD at 600 nm)	MATH[a] (%)	$t_{1/2}$[b] (d/h)	
			Cells	Supernatant
0	0.05	–	–	–
24	0.89	19 ± 0.25	4.49 (107.2)	3.28 (78.8)
48	1.00	23 ± 0.10	2 (48.5)	2.06 (49.5)
72	1.24	37 ± 0.06	1.95 (47)	1.73 (41.5)
96	1.54	38 ± 0.10	1.67 (40)	1.75 (42)
120	1.00	35 ± 0.31	2.08 (49.2)	2.64 (63.4)
144	1.00	43 ± 0.40	2.35 (56.4)	3.57 (85.8)

[a] Cell hydrophobicity express as % cells adhered to hexadecane (MATH). A significative linear correlation was observed between the increase of age of culture and cell hydrophobicity (r = 0.986 and p = 0.0027).
[b] $t_{1/2}$ of model emulsions after addition of 40 mg cells or 300 μL supernatant. The addition of 300 μL sterile growth medium as control does not alter the half-life of emulsion during a 144-h incubation. Standard deviations were lower than 3% of media value.

It has been reported that growth of microorganisms on hydrophobic substrates may cause an increase in hydrophobicity of their cell surfaces. Cell hydrophobicity may facilitate cell adherence to oil drops of emulsions, promoting their coalescence (Park et al., 2000). Huang et al. (2009) observed that a change in the cell surface of different species of the genus *Alcaligenes* from hydrophilic to hydrophobic promoted the breaking of both W/O and O/W emulsions. Demulsification of the ME by cells and supernatants of cultures of different ages had 1st-order kinetics (Fig. 2). From these data, the $t_{1/2}$ of each emulsion was calculated (Table 2).

The half-life of emulsions treated with cells or supernatant decreased with culture age, reaching a minimum value in 96-h-old cultures. However, the coefficients of linear correlation between culture age and $t_{1/2}$ values of emulsions treated with cells (r = −0.835, p = 0.165) and supernatants (r = −0.867, p = 0.133) were not significant. Cells and supernatants of cultures older than 96 h probably resulted in an increase in half-life because values corresponding to 56.2 and 85.8 for emulsions treated with cells and supernatants from a 144-h-old culture, respectively, were observed.

3.5. Effect of cell and supernatant amount on demulsifying activity

The demulsification kinetics using different concentrations of MSJ cells and supernatant were evaluated (Table 3). Demulsifying activity increased with the addition of up to 300 μL supernatant, with an activity of 95%. The addition of aliquots greater than 300 μL did not result in an increase in the demulsification rate. There was a trend of increased demulsification activity with an increasing amount of cells used in the assay; the highest activity was obtained in tests with 60 mg cells (95%). Amounts higher than 60 mg resulted in a decrease in activity. However, activities in the assays using 20, 40, 80 and 100 mg cells were not significantly different (p < 0.05). The increase from 20 to 60 mg of cells reduced the $t_{1/2}$ of the emulsion from 53.2 to 38 h (Table 3), but values above 60 mg increased the $t_{1/2}$ values considerably, to 61 h for 120 mg of cells. In contrast, emulsion coalescence increased with increasing supernatant volume from 50 to 300 μL, with $t_{1/2}$ values of 83 and 36 h, respectively. However, values higher than 300 μL did not result in a decrease or increase in $t_{1/2}$ (r = −0.810, p = 0.049). The decrease in activity with higher quantities of microbial cells could be due to the saturation of the emulsion by the high cell number or the formation of a protective layer around the immiscible phases that inhibit rather than promote the coalescence of oil droplets (Kosaric et al., 1987). Das (2001) used cells of *Micrococcus* sp. and observed that the stability of the O/W emulsion was more sensitive to the concentration of added cells. Increasing the cellular concentration from 2 to 4 mg/mL significantly reduced the half-life of emulsion from 4.6 to 1 h. However, the demulsification of the W/O emulsion was not directly proportional to the concentration of cells. A positive correlation between the percent demulsification and the concentration of cells was also observed by Park et al. (2000) and Nadarajah et al. (2002).

3.6. Effect of temperature on demulsifying activity

Demulsification rates varied significantly with culture fractions and incubation temperatures (p < 0.005). For the Tween–kerosene ME (W/O), the values increased with increasing incubation temperatures up to 40 °C, varying from 70.7% (assays with cells) and 80% (assays with supernatant) at 20 °C to 90.7% and 95.3%, respectively, at 40 °C. The increase in incubation temperature promoted a decrease in the $t_{1/2}$ of emulsions treated with cells or supernatants (Table 4). In both assays, a reduction in the $t_{1/2}$ of approximately 50% was observed in emulsions incubated at 40 °C.

For the Span–Tween–kerosene ME (O/W), higher DE rates (85%) were observed for emulsions treated with cells and incubated at 30 and 40 °C (Table 4) than those observed at 20, 50 and 60 °C. The activity of the supernatant was more stable than that of cells at

Table 3
Effect of cells and supernatant quantities of *Pseudomonas aeruginosa* MSJ cultures on the $t_{1/2}$ of model emulsion.

Supernatant volumes (μL)	%DE[a]	$t_{1/2}$[b] (d/h)	Cell quantities (mg)	%DE	$t_{1/2}$[b] (d/h)
50	73 ± 0.7c	3.46 (83)	20	88 ± 0.7 b	2.22 (53.2)
100	90 ± 1.2b	2.12 (50.8)	40	90 ± 0.6 b	2.03 (48.7)
200	92 ± 2b	2.05 (49)	60	95 ± 0.2 a	1.6 (38)
300	95 ± 1.3a	1.5 (36)	80	90 ± 0.2 b	2.05 (49.1)
400	95 ± 0.5a	1.5 (36)	100	90 ± 0.2 b	2.12 (50.8)
500	95 ± 0.7a	1.5 (36)	120	85 ± 0.3 c	2.55 (61.1)

[a] % Demulsifying activity. Assays were conducted at room temperature for up to 7 d of incubation. The letters on the columns represent the degree of similarity between treatments. Averages followed by same letters do not differ by Tuckey test at 5% probability.
[b] $t_{1/2}$ of Tween–kerosene model emulsion after addition of 50–500 μL supernatant or 20–120 mg cells. The addition of 500 μL sterile growth medium as a control does not alter the half-life of emulsion during 144 h incubation. Standard deviations were lower than 1% of media value.

Table 4
Effect of incubation temperature on the demulsifying activity and $t_{1/2}$ of Tween–kerosene and Span–Tween–kerosene model emulsions.

Temperature (°C)	%DE[a]		$t_{1/2}$[b] (d/h)	
	Cells	Supernatant	Cells	Supernatant
Tween–kerosene ME (W/O)				
20	70.7 ± 0.9Ac	80 ± 0.4Bc	4.22 (101)	3.03 (73)
30	85.3 ± 0.4Ab	90 ± 0.6Bb	2.57 (61.8)	1.96 (47)
40	90.7 ± 0.9Aa	95.3 ± 0.4Ba	2.12 (50.8)	1.6 (38)
Span–Tween–kerosene ME (O/W)				
20	80 ± 0.1Ab	25 ± 0.1Bd	0.86 (20.7)	4.82 (115.6)
30	95 ± 0.2Aa	60.3 ± 0.4Bc	0.46 (11.1)	1.5 (36)
40	95 ± 0.1Aa	70 ± 0.5Bb	0.46 (11.1)	1.15 (27.6)
50	75 ± 0.2Ac	75.3 ± 0.4Ba	1 (24)	0.94 (22.7)
60	59.3 ± 0.7Ad	75 ± 0.2Ba	1.5 (36)	1 (24)

[a] % Demulsifying activity. Averages followed by the same uppercase or lowercase letters do not differ by Tuckey test at 5% probability. Capital letters are related to the fractions of culture, cells (A) and supernatant (B), and lowercase letters are related to the treatments.
[b] $t_{1/2}$ of model emulsions after the addition of 40 mg cells or 300 μL supernatant. The addition of 300 μL sterile growth medium as a control does not alter the half-life of emulsion during a 48-h incubation. Standard deviations were lower than 1% of media value.

temperatures above 40 °C; and the $t_{1/2}$ at 50 and 60 °C were statistically equal, with values of approximately 75%. It has been suggested that increases in incubation temperature reduce the viscosity of the oil phase, thereby increasing the difference in density between the phases and stabilizing the weakening interfacial film of emulsions (Mohammed et al., 1994). These changes result in an elevation in the collision rates of oil droplets, increasing coalescence and demulsification rates. Kosaric and Duvnjak (1987) observed that demulsification of the W/O emulsion for activated sludge samples was faster at 85 °C, requiring approximately five times less sludge to break the emulsion when compared to room temperature. Nadarajah et al. (2002) also observed a positive effect of increased temperature on the rate of demulsification of a mixed bacterial culture; the highest rates were obtained at 50 °C. In general, the half-life of emulsions treated with cells was lower than that of emulsions treated with supernatant. The $t_{1/2}$ of emulsions treated with supernatant decreased with an increase in incubation temperature up to 50 °C, and the values ranged from 115.6 h at 20 °C to 22.7 h at 50 and 60 °C. Emulsions treated with cells had a $t_{1/2}$ of 20.7 and 11.1 h when incubated at 20 and 40 °C, respectively.

The stability of the demulsifying ability of *P. aeruginosa* MSJ at different temperatures is favorable to its industrial application, with the possibility of maintaining the desired demulsifying characteristics during transport and storage. Furthermore, the process of demulsification can be optimized with temperature elevation, since an increase in temperature led to a gradual destabilization of the crude oil/water interfacial films (Becker, 1997).

3.7. Effect of pH of the aqueous phase of the emulsion

Demulsification rates were significantly different for emulsions of different pHs treated with cells and supernatant of *P. aeruginosa* MSJ (Table 5). The demulsifying activity of ells (positive correlation, $r = 0.955$, $p = 0.0113$) and supernatant (positive correlation, $r = 0.917$, $p = 0.0282$) increased with an increase in pH of the aqueous phase. The highest demulsifying activity was observed when cells and supernatant were added to emulsions of pH 8, with values of 88% and 85%, respectively. There was a significant negative correlation between pH and the half-life of emulsions treated with cells ($r = -0.948$, $p = 0.0142$) or supernatants ($r = -0.623$, $p = 0.262$). Lower $t_{1/2}$ values were observed for emulsions of pH 8.0, regardless of whether they were treated with cells or supernatant.

Table 5
Effect of aqueous phase pH on the demulsifying activity and $t_{1/2}$ of model emulsion.

pH	%DE[a]		$t_{1/2}$[b] (d/h)	
	Cells	Supernatant	Cells	Supernatant
4	22 ± 1Af	17 ± 1.5Be	18.13 (435.18)	24.27 (582.6)
5	35.3 ± 1.5Ae	55.3 ± 2Bd	11.4 (276.6)	6.7 (161)
6	40.7 ± 2Ad	55.3 ± 1.1Bd	7.6 (183)	6.7 (161)
7	83.3 ± 2.1Ac	78 ± 0.9Bc	3.6 (87)	2.8 (66)
8	88 ± 2.2Ab	86 ± 0.7Bb	4 (96)	2.4 (56)
Control	90 ± 1.5Aa	95.3 ± 0.6Ba	2.11 (50.6)	1.6 (38)

[a] % Demulsifying activity. Averages followed by the same uppercase or lowercase letters do not differ by Tuckey test at 5% probability. Capital letters are related to the fractions of culture, cells (A) and supernatant (B), and lowercase to the treatments.
[b] $t_{1/2}$ Tween–kerosene ME after addition of 40 mg cells or 300 μL supernatant. The addition of 300 μL of sterile growth medium as control not altered the age half of emulsion during 144 h incubation. Standard deviations were lower than 2% of media value.

The effects of pH on demulsifying activity are quite varied. Cairns et al. (1982) observed that the demulsifying activity of *Nocardia amarae* was minimally affected by pH ranging from 3 to

Table 6
Effect of physical and chemical treatments of the culture of *Pseudomonas aeruginosa* MSJ on demulsification of model emulsions.

	%DE[a]		$t_{1/2}$[b] (d/h)	
	Cells	Supernatant	Cells	Supernatant
Physical treatments				
Control	90 ± 1.5Aa	93.5 ± 0.6Ba	2.12 (50.8)	1.59 (38)
Freezing	80.3 ± 1Ab	85.7 ± 1.2Bb	3.03 (72.72)	2.57 (61.68)
Nitrogen liquid	80 ± 1.5Ab	75.3 ± 2Bb	3 (72)	3.55 (85.2)
Freeze drying	18 ± 0.2Ad	16 ± 0.27Bd	21 (504)	26 (624)
steam under pressure	31 ± 0.43Ac	20 ± 0.38Bc	10.5 (252)	16 (384)
Chemical treatments				
Control	90 ± 1.6a	–	2.12 (50.8)	–
Kerosene	85 ± 1.7b	–	2.22 (53.2)	–
CMW	28 ± 0.9c	–	14 (336)	–

[a] % Demulsifying activity. Averages followed by the same uppercase or lowercase letters do not differ by Tuckey test at 5% probability. Capital letters are related to the fractions of culture, cells (A) and supernatant (B), and lowercase letters are related to the treatments.
[b] $t_{1/2}$ Water–kerosene model emulsion after addition of 40 mg cells or 300 μL supernatant. The addition of fractions of culture without treatment as control not altered the half-life of emulsion during 144 h incubation. Standard deviations were lower than 1% of media value.

10. Ly et al. (2008) used an O/W emulsion stabilized by milk proteins at pHs ranging from 3 to 7.5 and observed destabilization by bacterial strains only at pH 3. The effect of pH on demulsification suggests that the demulsification mechanism promoted by the cells or culture supernatants of MSJ was sensitive to the degree of ionization of any ionizable group on the bacterial surface or in compounds present in the culture supernatant.

3.8. Effect of physical and chemical treatments

Freezing and rapid freezing in liquid nitrogen affected the demulsifying activity to a lesser extent than lyophilization or exposure to steam under pressure (Table 6). Lyophilized cells and autoclaved cells promoted only 18% and 31% demulsification, respectively, after 144 h of incubation. These results are in partial agreement with those of Nadarajah et al. (2002), who observed that freezing and thawing did not affect demulsification activity, but that a lyophilized culture provided only 56% demulsification, and autoclaving completely destroyed the demulsification capability of the culture.

CMW-treated cells promoted a significant reduction in demulsification, from 90% to 28% ($p < 0.05$) (Table 6), possibly due to the removal of active component(s) associated with the cell surface. For cells treated with kerosene, the demulsification activity was 85%. A significant effect of solvent treatment on emulsion half-life was observed. The $t_{1/2}$ values suggested that washing cells with solvents has an influence on demulsification properties. Emulsion decay became slower after the addition of kerosene and CMW-treated cells. The $t_{1/2}$ of emulsions treated with unwashed cells was 50.8 h, in contrast to 53.2 h for kerosene-treated cells and 336 h for CMW-treated cells. In general, microorganisms grown in the presence of aliphatic hydrocarbons accumulate considerable quantities of lipid material in their cell wall (Rapp et al., 1979). Solvents such as *n*-pentane can remove neutral lipids (MacDonald et al., 1981; Kosaric et al., 1987), *n*-hexane is able to remove some glycolipids (Syldatk and Wagner, 1987), and CMW and kerosene remove total extractable lipids (Kosaric et al., 1987; Syldatk and Wagner, 1987). The reduction in demulsifying activity of cells after washing with solubilizing lipid solvents is an indication that the agent in the cellular structure may be composed of lipids. More studies to characterize the demulsifier are necessary to understand the mechanisms governing its activity.

3.9. Partial characterization of the chemical composition of the demulsifier

Previous studies have revealed that the active ingredient of tensoactives produced by *P. aeruginosa* is rhamnolipid (Abalos et al., 2004; Wei et al., 2005; Raza et al., 2010). The demulsifier produced by strain MSJ also appears to be of this type since it contains fatty acids of type methyl 2 hydroxydodecanoate (27.1%), methyl 3 hydroxydecanoate (11.9%), dodecanoic acid (16.5%), methyl 3 hydroxytetradecanoate (25.6%), hexadecanoic acid (19%), methyl ester and 9-dodecenoic acid (14.6%). More than 28 different congeners of rhamnolipids are produced by *P. aeruginosa* strains growing on different carbon sources (Déziel et al., 1999), most of them are di-rhamnolipids. These rhamnolipid molecules contain one or two b-hydroxy fatty acids of various chain length (C_8–C_{22}) esters linked to a monorhamnose (R) or di-rhamnose (RR) moiety (Perfumo et al., 2006). The main problem with GC analysis is that the relationship between the 3-hydroxyfatty acids in the dilipid portion of the RLs is lost, along with the relationship between the dilipids and their substitution with one (or two) rhamnose moieties. In adition, mannose, one of the main components in the polysaccharide encoded by the *psl* locus (Ma et al., 2006a,b), was detected in the supernatant of the culture. These biomolecules will be further investigated using techniques such as thin-layer chromatography (TLC) and ^{1}H and ^{13}C NMR spectroscopy.

4. Conclusion

The addition of cells and supernatants of cultures of *P. aeruginosa* MSJ promoted breakage of W/O and O/W emulsions consisting of different organic phases and emulsifying agents and an industrial emulsion. This activity was relatively stable in different physicochemical conditions. GC–MS analysis confirmed the presence of fatty acids and carbohydrates in the extracellular biodemulsifier. More studies will have to be conducted for confirmation of the use of oil waste as substrate for fermentation of demulsifier and the further direct use of culture or cell-free supernatant for the oil recovery and/or biotreatment of emulsified oily effluents on pilot and field scales.

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References

Abalos, A., Viñas, M., Sabaté, J., Manresa, M.A., Solanas, A.M., 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 15, 249–260.

Araujo, E.A., Andrade, N.J., Silva, L.H.M., Carvalho, A.F., Silva, C.A.D., Ramos, A.M., 2010. Control of microbial adhesion as a strategy for food and bioprocess technology. Food Bioprocess Technol. 3, 321–332.

Becker, J.R., 1997. Crude oil Waxes Emulsions and Asphaltenes. PennWell, Oklahoma, pp. 5–29, ISBN 0-87814-737-3.

Cairns, L., Cooper, D.G., Zajic, J.E., Wood, J.M., Kosaric, N., 1982. Characterization of *Nocardia amarae* as potent biological coalescing agent of water-in-oil emulsions. Appl. Environ. Microbiol. 43, 362–366.

Das, M., 2001. Characterization of de-emulsification capabilities of *Micrococcus* species. Bioresour. Technol. 79, 15–22.

Déziel, E., Lépine, F., Dennie, D., Boismenu, D., Mamer, O.A., Villemur, R., 1999. Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. Biochim. Biophys. Acta 1440, 244–252.

Ha, J., Yang, S., 1999. Break up of a multiple emulsion dropin a uniform electric field. J. Colloid Interface Sci. 213, 92–100.

Huang, X.F., Liu, J., Lu, L.J., Wen, Y., Xu, J.C., Yang, D.H., Zhou, Q., 2009. Evaluation of screening methods for demulsify bacteria and characterization of lipopeptide bio-emulsifer produced by *Alcaligenes* sp. Bioresour. Technol. 100, 1358–1365.

Janiyani, K.L., Purohit, H.J., Shanker, R., Khanna, P., 1994. De-emulsification of oil-in-water emulsions by *Bacillus subtilis*. World J. Microbiol. Biotechnol. 10, 452–456.

Kim, K.L., Lee, J.J., Kim, D.W., Lee, J.C., 1998. De-emulsification of petroleum emulsion using *Nocardia amarae*. J. Biotechnol. Bioeng. 13, 209–213.

Kosaric, N., Duvnjak, Z., 1987. De-emulsification of water-in-oil emulsion with sludges. Water Pollut. Res. J. Can. 22, 437–443.

Kosaric, N., Cairns, W.L., Gray, N.C.C., 1987. Microbial deemulsifiers pp. 247–320. In: Kosaric, N., Cairns, W.L., Gray, N.C.C. (Eds.), Biosurfactants and Biotechnology. Marcel Dekker, New York, NY.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. Wiley and Sons Ltd., Chichester, United Kingdom, pp. 115–175.

Larson, K., Raghuraman, B., Wiencek, J., 1994. Electrical and chemical de-emulsification techniques for microemulsion liquid membranes. J. Membr. Sci. 91, 231–248.

Li, X., Wang, J., 1999. Effects of mixed anionic–cationic surfactants and alcohol on solubilization of water-in-oil microemulsions. J. Disp. Sci. Technol. 20, 993–1007.

Liu, J., Lu, L.J., Huang, X.F., Shang, J.J., Li, M.X., Xu, J.C., Deng., H.P., 2011. Relationship between surface physicochemical properties and its demulsifying ability of an alkaliphilic strain of *Alcaligenes* sp. S-XJ-1. Process Biochem. 46, 1456–1461.

Ly, M.H., Aguedo, M., Le, M.L., Cayot, P., Teixeira, J.A., Le, T.M., Belin, J., Waché, Y., 2008. Interactions between bacterial surfaces and milk proteins, impact on food emulsions stability. Food Hydrocolloids 22, 742–751.

Ma, L., Jackson, K.D., Landry, R.M., Parsek, M.R., Wozniak, D.J., 2006a. Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. J. Bacteriol. 188, 8213–8221.

Ma, T., Liang, F.L., Xi, Y.W., Liu, R.L., 2006b. Performance of demulsification by *Rhodococcus* sp. PR21. Environ. Sci. 27, 1191–1196.

MacDonald, C.R., Cooper, D.G., Zajic, J.E., 1981. Surface active lipids from *Nocardia reythropolis* grow on hydrocarbons. Appl. Environ. Microbiol. 41, 117–123.

Mohammed, R.A., Baily, A.I., Luckham, P.F., Taylor, S.E., 1994. Dewatering of crude oil emulsions 3. Emulsion resolution by chemical means. Colloids Surf. A: Physicochem. Eng. Asp. 83, 261–271.

Mouraille, O., Skodvin, T., Sjoblom, J., Peytavy, J.L., 1998. Stability of water–crude oil emulsion: role played by the stage of solvatation of asphaltenes and by waxes. J. Disp. Sci. Technol. 19, 339–367.

Nadarajah, N., Singh, A., Ward, O., 2002. Evaluation of mixed bacterial culture for de-emulsification of water-in-petroleum oil emulsions. World J. Microbiol. Biotechnol. 18, 435–440.

Park, S.H., Lee, J.H., Ko, S.H., Lee, H.K., 2000. Demulsification of oil-in-water emulsions by aerial spores of a *Streptomyces* sp. Biotechnol. Lett. 22, 1389–1395.

Peigne, G., 1993. Know-how on the use of demulsifiers to enhance oil recovery operations. In: Proceedings of a Workshop on formation and breaking of water-in-oil emulsions. MSRC Technical Report Series 93–018. API, Washington, DC.

Perfumo, A., Banat, I.M., Canganella, F., Marchant, R., 2006. Rhamnolipid production by a novel thermotolerant hydrocarbon-degrading *Pseudomonas aeruginosa* AP02-1. J. Appl. Microbiol. Biotechnol. 72, 132–138.

Rapp, P., Bock, H., Wray, V., Wagner, F., 1979. Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on *n*-alkanes. J. Gen. Microbiol. 115, 491–503.

Raza, Z.A., Khalid, Z.M., Khan, M.S., Banat, I.M., Rehman, A., Naeem, A., Saddique, M.T., 2010. Surface properties and sub-surface aggregate assimilation of rhamnolipid surfactants in different aqueous systems. Biotechnol. Lett. 32, 811–816.

Rosemberg, M., 1991. Basic and applied aspects of microbial adhesion at the hydrocarbon: water interface. Crit. Rev. Microbiol. 18, 159–173.

Singh, A., Hamne, J.D.V., Ward, O.P., 2007. Surfactants in microbiology and biotechnology: Part 2. Applications aspects: a review. Biotechonol. Adv. 25, 99–121.

Stalss, F., Bohm, R., Kupfer, R., 1991. Improved demulsifier chemistry: a novel approach in the dehydration of crude oil. SPE Prod. Eng. 6, 334–338.

Syldatk, C., Wagner, F., 1987. Production of biosurfactants. In: Kosaric, N., Cairns, F., Gray, N.C.C. (Eds.), Biosurfactants and Biotechnology. Marcel Dekker, New York, pp. 89–120.

Wei, Y.H., Chou, C.L., Chang, J.S., 2005. Rhamnolipid production by indigenous Pseudomonas aeruginosa J4 originating from petrochemical wastewater. Biochem. Eng. J. 27, 146–154.

Yakimov, M.M., Timmis, K.N., Wray, V., Fredrickson, H.L., 1995. Characterization of a new lipopeptide surfactant produced by Thermotolerant and Halotolerant Subsurface *Bacillus licheniformis* BAS50. Appl. Environ. Microbiol. 61, 1706–1713.