Cellular Hydrophobicity is Not Determinant of Water-In-Oil Emulsification Breaking by Bacteria

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Abstract

We evaluated if the capability of bacterial isolates to break water-in-oil (W/O) emulsions depends on cell surface hydrophobicity. Among 12 bacterial isolates obtained from enrichment of solid waste compost in mineral-paraffin medium, four were shown to be efficient in breaking W/O emulsion. These strains were identified as Acinetobacter sp. and Pseudomonas mendocina. W/O emulsion-breaking activity (EBA) decreased with culture age. EBA of young cultures (22 hours of growth) was dependent on the presence of cells, and soluble deemulsifier(s) did not have significant effect at this stage. In contrast, EBA of older cultures (198 hours of growth) was attributed to the presence of non-surfactant deemulsifier compound(s) in the culture broth. Experiments with Acinetobacter sp. LBBMA LU3 revealed that cell integrity is not required for EBA. EBA was insensitive to variation of pH (3-8) or salt concentration (0-150 g l^{-1} NaCl), but increased linearly with temperature (30-60°C). Correlation between cell surface hydrophobicity and EBA was either absent, negative or positive, depending on the microbial strain or on previous growth condition for a particular strain. We conclude that hydrophobicity is not a primary factor determining EBA of bacterial cells.

Keywords: Biodeemulsifier; Deemulsification; Acinetobacter sp.; Arthrobacter nicotianae; Pseudomonas stutzeri; Sphingomonas capsulata; Xanthomonas sp.

Introduction

Stable emulsions are formed at various stages in the petroleum production chain. Emulsification is undesirable because it inhibits the subsequent separation of crude oil and production water. Regardless of the technology employed for deemulsification, physical and chemical processes are expensive and do not always provide the required basic sediment and water (BSW) values for transportation and refining (Van Hamme et al. 2003).

Several microbial species, including *Nocardia amarae* (Cairns et al. 1982), *Corynebacterium petrophilum* (Stewart et al. 1983 *apud* Van Hamme et al. 2003), *Rhodococcus auranticus* (Ramsay et al. 1983 *apud* Van Hamme et al. 2003), *Bacillus subtilis* (Janiyani et al. 1994), *Micrococcus* spp. (Das 2001), *Torulopsis bombicola* (Duvnjak and Kosaric 1987), *Alcaligenes* sp. (Huang et al. 2012), and mixed bacterial cultures (Nadarajah et al. 2002; Ward and Singh 1998) display deemulsification capability.

The ability of bacteria to break oil-in-water (O/W) or water-in-oil (W/O) emulsions is often attributed to characteristics such as cell surface hydrophobicity (CSH) and the ability to produce surfactants and deemulsifiers (Nadarajah et al. 2002).

The aim of this study was to evaluate the hypothesis that the ability to break W/O emulsions by bacterial isolates is related to CSH and to the production of biosurfactants that exhibit deemulsification activity.

Results

Microbial Isolates

Enrichment of urban solid waste previously contaminated with diesel oil in MMSM-paraffin resulted in 23 isolates. Clustering analysis of fatty acid profiles revealed 12 distinct lineages (Supporting information 1). These lineages were used in the deemulsification studies (Table 1).

Deemulsification Activity

Among the 12 bacterial strains evaluated, six showed W/O EBA higher than 50%, after 22 hours of growth in MMSM-paraffin (Table 1). These strains were considered potentially demulsifying. Among these, four strains showed capacity for deemulsification near or greater than 70%. These strains *(Acinetobacter* sp. LBBMA LU 3, *Acinetobacter* sp. LU 7a, *Pseudomonas* sp. LBBMA LU 5a, and *P. mendocina* LBBMA LU 7b) were considered efficient to break W/O emulsions. It is noteworthy that members of the genus *Acinetobacter* produce a potent emulsifier (Emulsan) that can stabilize O/W emulsion (Belsky et al. 1979).

The addition of cell suspensions from seven strains cultivated for 22 hours to W/O emulsion resulted in an elevated separation of kerosene (above 70%). The strain *Acinetobacter* sp. LBBMA LU 3 stood out from the other strains of the same genus, effecting a 98% kerosene separation after 24 hours of contact with the emulsion (Table 1).

Cultures obtained after 168 hours of incubation presented lower W/O EBA compared to the values obtained for younger cultures (22 hours, Table 1). Kerosene separation activity (KSA) was not as significantly reduced with increasing culture age as the emulsion break ratio (EBR) (Table 1). The strain *Pseudomonas* sp. LBBMA LU 14c demonstrated a different behavior: EBR and KSA increased with increasing culture age. The change of deemulsification capability with culture age was attributed to changes in cell wall components (e.g. fatty acids, Huang et al. 2012), which may interfere with deemulsification activity.

EBR and KSA of cell suspensions of cultures grown for 22 hours in MMSM were higher than of cell-free extracts (Table 1), indicating that at this stage the cells contribute with deemulsification. In contrast, the results with the cell-free extracts of older cultures (168 hours) were similar to those obtained with the respective cell suspensions for both EBR and KSA (Table 1). The result led to the conclusion that deemulsifier compounds are secreted into the extracellular medium as the cells become older. Tests for the presence of surface active compounds (biosurfactants) in the cell suspensions of the six strains that were most effective in breaking W/O emulsion, at the two growth phases (22 and 168 hours), indicated the absence of these compounds. The presence of W/O demulsifying compounds with no surface activity in cell-free extracts of old cultures contrasts with the generally accepted notion that bio-deemulsifiers are compounds with surface activity that are either secreted into the growth medium or attached to the cell surface (Huang et al. 2009, 2010; Liu et al. 2009). Therefore, it can be concluded that demulsifying compounds capable of separating kerosene contained in W/O emulsions do not necessarily have surface activity. The nature of the compounds produced by the selected bacterial strains was not investigated in this study.

Removal of cells of some strains by centrifugation (sedimentable cells) resulted in an increase in W/O emulsion break ratio (EBR) (Table 1). For some strains, this effect was observed with young cultures (*Acinetobacter* sp. LBBMA LU 1, *Pseudomonas* sp. LBBMA LU 14c); for other strains, the effect occurred with older cultures (*Acinetobacter* sp. LBBMA LU 3, *Pseudomonas* sp. LBBMA LU 5a). For *Acinetobacter* sp. LBBMA LU 4 and LBBMA LU 10, removal of sedimentable cells was beneficial either with young (22 hours of growth) and with older cultures (168 of growth). The increase in deemulsification activity upon removal of part of the cells (by using only floating cells, not separated by centrifugation) differs substantially from what was reported by Nadarajah et al. (2002b). Our results show that, in a population of cells from a bacterial culture, some cells appear to provide stability to the emulsions (sedimentable cells), while other cells (non-sedimentable or floating cells) appear to have the opposite effect.

To our knowledge, this is the first report of interactions between different cells in a population of a bacterial culture in relation to emulsion break activity.

The separation of kerosene (KSA) by non-sedimentable (floating) cells compared to those obtained with total cells suspensions was not consistent with the behavior exhibited for emulsion breaking (EBR) by the cultures after 22 hours of growth. These include, for example, *Pseudomonas* sp. LBBMA LU 5a, for which the EBR was reduced from 75% (total cells) to 64% (non-sedimentable cells), whereas the KSA increased from 85% to 92% (Table 1).

The separation of kerosene by the non-sedimentable cells suspensions after 168 hours of growth was similar to that obtained with the total cells suspensions. An exception was *Acinetobacter* sp. LBBMA LU 1, with which the KSA was reduced from 80% to 70% after the removal of the sedimentable cells (Table 1). In contrast, the complete removal of cells (cell-free extract) from this strain resulted in a KSA similar to that obtained with the total cells suspension (Table 1). These results demonstrate the existence of a negative effect of non-sedimentable (floating) cells of this strain in terms of the ability to separate the oily phase contained in W/O emulsions.

Deemulsification Stability of *Acinetobacter* sp. LBBMA LU 3

Autoclaving or freeze/thawing cell suspension of *Acinetobacter* sp. LBBMA LU 3 enhanced the W/O EBR and KSA (Table 2). There was no difference between cell suspensions that were autoclaved or subjected to freeze/thawing. The maintenance or, in this case, the increase in the deemulsification activity after autoclaving is desirable when the goal is the industrial application of bacterial suspensions for emulsion breaking, since it assures the elimination of live cells.

Effect of Ph, Salinity and Temperature on Deemulsification Activity of *Acinetobacter* Sp. LBBMA LU 3

Neither the pH of the aqueous phase (3-8) nor NaCl concentration (30-150 g L^{-1}) had significant effect ($p > 0.05$) on W/O emulsion break activity of *Acinetobacter* sp. LBBMA LU 3 or on kerosene separation (data not shown). Similar results were reported by Cairns et al. (1982). Increasing the incubation temperature of the mixture of *Acinetobacter* sp. LBBMA LU 3 and W/O emulsions resulted in a linear increase of both the EBR and the KSA (Figure 1). The result was attributed to the reduction of the viscosity of the fluids that form the emulsion with increasing temperatures, to the increase of the difference of density between the phases, and to the weakening of the interfacial film responsible for the emulsion stability, which facilitate the coalescence of the phases and, consequently, phase separation (Mohammed et al. 1994).

Relationship between Cell Surface Hydrophobicity and the Ability to Break W/O Emulsions

The change in CSH in response to maintenance in nitrogen-deficient medium was dependent on the bacterial strain and also on the previous cultivation condition (Supplement information 2). The CSH of the bacterial strains ranged between 12.6% and 62.3%. Additionally, cells of *Acinetobacter* sp. LBBMA LU 1 obtained from cultivation in TSB for 24 and 48 hours exhibited a CSH of 74%, which was the highest hydrophobicity obtained in this study.

The CSH of *Acinetobacter* sp. LBBMA LU 1 and of *S. capsulata* LBBMA 18T cultivated in TSB did not correlate ($p > 0.05$) with EBR or KSA. The CSH of *S. capsulata* LBBMA 18T cultivated in R2A correlated negatively with EBR ($p < 0.05$, $r = -0.512$) and with KSA ($p < 0.05$, $r = -0.518$). In contrast, the hydrophobicity of *Acinetobacter* sp. LBBMA LU 7a correlated positively with EBR ($p < 0.05$, $r = 0.419$) and KSA ($p < 0.01$, $r = 0.691$). Based on these results, we found no clear relationship between CSH and emulsion break capacity. The inexistence of a clear relationship between CSH and W/O EBR or KSA is supported by conflicting results from other studies. For example, Kosaric (1992) and Stewart et al. (1983) show that hydrophilic cells are more effective in promoting W/O emulsion breaking. However, Huang et al. (2012) and Wen et al. (2010) found a positive relationship between CSH and W/O breaking activity. Early studies showed that the CSH of demulsifying bacteria is an important factors determining deemulsification efficiency (Ma et al. 2006). According to Liu et al. (2004), the adsorption of demulsifying cells in the oil phase and the aggregation of dispersed droplets would be strengthened by cells with higher CSH. Huang et al. (2012) suggest that, in the deemulsification process, a higher CSH could accelerate the transference of the cells to the water-oil interface due to an improved affinity with kerosene, and the alteration of interfacial properties would contribute to emulsion breaking.

Our results contradict the findings of the abovementioned authors. Significant correlation between two variables does not necessarily mean the existence of a true relationship. In this case in particular, finding either a positive (e.g. *Acinetobacter* sp. LBBMA LU 7a), negative (e.g. *S. capsulata* LBBMA 18T cells cultivated in R2A), or no correlation (e.g. *Acinetobacter* sp. LBBMA LU 1 and of S. *capsulata* LBBMA 18T cultivated in TSB) between CSH and W/O emulsion breaking depends either on the bacterial strain that is used in the experiment, and also on the previous cultivation of a particular strain, as shown for *S. capsulata* LBBMA 18T cells cultivated in R2A or TSB. The contrasting correlation between emulsion break capacity and CSH of *S. capsulata* LBBMA 18T cultivated in TSB (no correlation) or R2A (negative correlation) shows that other cell surface characteristics, and not primarily its hydrophobicity, are essential for cell surface interaction with W/O emulsions. These differences were not caused by components secreted into the culture/nitrogen-starvation medium or by the concentration of cells, because all emulsion breaking experiments for correlation analysis were conducted with cells that were previously washed and resuspended in PBS at a concentration equivalent to an OD_{600nm} of approximately 1.2. To our knowledge, this study is the first one to report the inconsistency of the effect of cell surface hydrophobicity on W/O EBA. We conclude that hydrophobicity is not a primary characteristic involved in W/O emulsion breaking by bacterial cells.

Experimental Procedures

Municipal solid waste compost was contaminated with diesel (20,000 mg Kg-1) and incubated at room-temperature for 30 days. 5 grams were transferred to 50 ml mineral medium (MMSM; Huang et al. 2009), supplemented with 4% liquid paraffin (paraffin oil 76235 - Fluka Analytical, St. Gallen,Ch) and incubated at 200 rpm and 35°C for 48 hours. After two subcultures, the resultant mixed culture was evaluated for water in oil (W/O) deemulsification activity. Upon confirming the emulsion breaking activity (EBA), the culture was serially diluted in sterile saline and plated on R2A. Plates were incubated at 35°C for up to 4 days, and 23 colonies were selected. The isolates were identified using Sherlock® system (MIDI) with Instant Environmental TSA 1.0 library. Fatty acid methyl esters were obtained with Instant Fame Kit™, according to the recommendations of the manufacturer. The fatty acids profiles were subjected to cluster analysis by Sherlock® system to discard isolates of the same strain (same fatty acid profiles). Identification of isolates was confirmed by 16S rRNA partial sequence analysis. The sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers JX459909 to JX459919.

Isolates were cultivated in MMSM (Huang et al. 2009) containing paraffin as the sole carbon source at 200 rpm and 35°C. Emulsion break activity was evaluated after 22 and 168 hours of cultivation, using cell suspensions at optical density (OD_{600}) of 1.2. Deemulsification activity of non-sedimentable (floating) cell suspensions, obtained from supernatants after centrifugation of the cultures at 8,500 *g* for 20 minutes, and of cell-free extracts obtained after the filtration of non-sedimentable cells suspensions through 0.22 μm membranes, was also evaluated.

Emulsions were prepared as described by Das (2001), with modifications. 4.5 ml of distilled water were mixed with 4.5 ml of kerosene containing 16.7 g l^{-1} Span®80. The mixture was placed in 20 ml screw-capped glass tubes and agitated at 3,000 rpm in a digital Vortex Mixer de Luxe (Fisher Scientific) for 1 minute in the presence of 1 ml of bacterial cultures, non-sedimentable cells suspensions or their respective cell-free extracts. Controls were prepared by replacing the bacterial cultures with non-inoculated cultures or with PBS (pH 7.4). Deemulsification activity was evaluated after a 24-hour rest period at 35°C, based on the variation of the oily phase (top), of the aqueous phase (bottom) and of the emulsion (middle). Deemulsification activity was evaluated by emulsion break ratio (EBR):

EBR (%) = 1 - (Remaining emulsion volume/Original emulsion volume + Volume of added culture) x 100

Kerosene separation activity (KSA) was calculated as:

KSA (%) = (Separated kerosene volume/Added kerosene volume) x 100

All experiments in this step and in the following steps were conducted with three replicates.

Maintenance of deemulsification capability by one of the bacterial strains (Acinetobacter sp. LBBMA LU 3) was evaluated using cell suspension (22 hours of growth in MMSM, 35°C, 200 rpm) after autoclaving (121°C for 20 minutes) or freezing at -20°C for 24 hours followed by thawing at room temperature.

Effect of pH on deemulsification activity of *Acinetobacter* sp. LBBMA LU 3 was evaluated by replacing the aqueous phase of the emulsions with phosphate buffer (100 mmol l^{-1}, pH = 3 to 8). Effect of salinity was evaluated by adding NaCl to the water used for emulsion preparation at concentrations of 0 to 150 g l^{-1}, and the effect of temperature was determined by incubating the mixture of cell suspension-W/O emulsion at temperatures ranging from 30°C to 60°C.

Cell surface hidrophobicity was evaluated by the microbial adhesion to hydrocarbons (MATH) test (Sanin et al. 2003, with modifications). Cell suspensions were centrifuged at 8,500 g for 10 minutes, washed in saline and resuspended in PBS (pH 7.4) at an initial OD_{440} of 1.2. 3 ml of cell suspension were transferred to test tube and covered with 0.3 ml of hexadecane. Tubes were vortexed at 3,000 rpm for 2 minutes. The mixture was allowed to stand for 15 minutes, and the final OD_{440} of the lower phase was recorded.

MATH was calculated by:
MATH = [1 - DO_{440} (final) / DO_{440} (initial)] x 100

Two of the bacterial strains isolated in this study (*Acinetobacter* sp. LBBMA LU1 and LU7a) and *Sphingomonas capsulate* LBBMA 18T, which was previously used in studies on cell adhesion to soil particles (Borges et al. 2008), were used to study the relationship between CSH and the ability to break W/O emulsions. The strains were subjected to nitrogen starvation in nitrogen-deficient MMSM, in order to obtain cell suspensions of the same strain with different values of hydrophobicity. The isolates were cultivated (5 to 48 h) in different media (Supplement information 1), centrifuged at 8,500 g, resuspended in nitrogen-starvation mineral medium (in g L^{-1}: KH_2PO_4, 4.0; KH_2PO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract, 0.01; glucose, 20.0 and mineral solution, 1 ml l^{-1} [1 l: 1 g of $CaCl_2 \cdot 2H_2O$, 1 g of $FeSO_4 \cdot 7H_2O$ and 1.4 g of EDTA]), and incubated at 35°C and 200 rpm. CSH and EBA were measured at 24-hour intervals for up to 168 hours, after cells were centrifuged and resuspended in PBS. Data were used to Pearson correlation analysis to check for the relationship between CSH and EBA.

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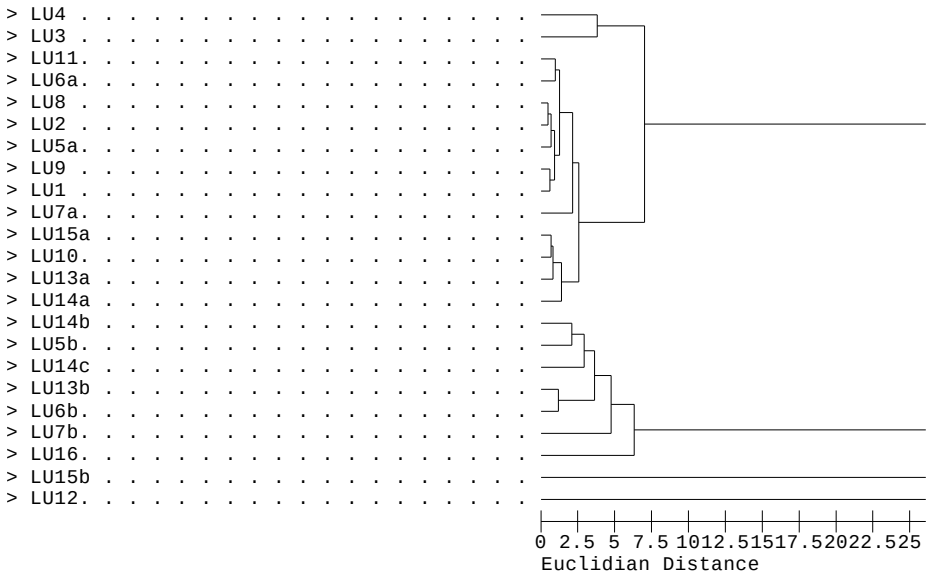
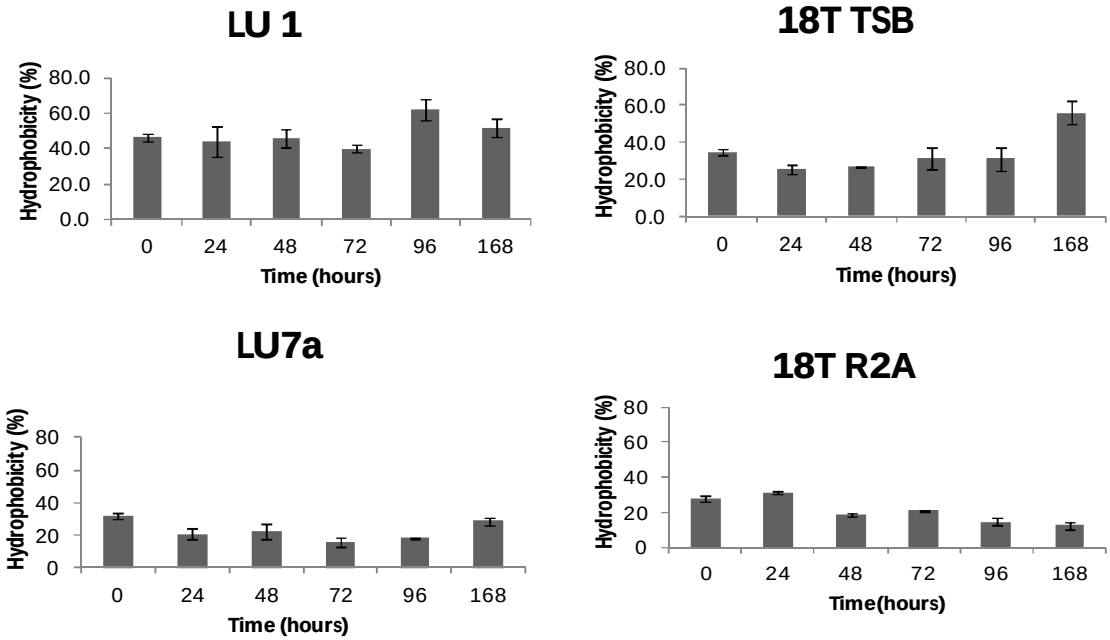
Supplement Information 1: Growth Media and Incubation Period of Isolates *Acinetobacter* Sp. LBBMA LU1 and LU7a, and *Sphingomonas Capsulata* LBBMA 18T, Before Transference to Nitrogen-Starvation Medium

Isolate	Growth medium	Incubation period (hours)
Acinetobacter sp. LBBMA LU1	TSB	6.5 24 48
Acinetobacter sp. LBBMA LU7a	TSB	5
S. capsulata LBBMA 18T	TSB R2A	5 5

Cultures were incubated at 200 rpm and 35 ^{o}C. After the indicated period, cultures were centrifuged and ressuspended in nitrogen-starvation medium.



Supplement information 2 - Cluster analysis of the fatty acid profiles of bacterial isolates obtained after the inoculation of urban solid waste that had been artificially contaminated with diesel oil in mineral medium containing paraffin as the sole carbon source. Isolates with fatty acid profiles that clustered at a Euclidean distance < 2.5 were considered to belong to the same strain. In this case, only one isolate representative of this strain was evaluated in the subsequent steps.



Supplement information 3 – Cell surface hydrophobicity (according to MATH method) of bacterial strains in response to nitrogen starvation. The cells were grown in TSB for 6.5 hours (*Acinetobacter* sp. LBBMA LU 1) or 5 hours (*Acinetobacter* sp. LBBMA LU 7a and *Sphingomonas capsulata* LBBMA 18T) at 35°C and 200 rpm. LBBMA was also cultivated in R2A for 5 hours. Sterile saline-washed cells were transferred to nitrogen-deficient medium and incubated under the same conditions. Time zero corresponds to the newly collected cells from the culture medium. Data represent the mean of three replicates. The error bars represent the standard deviation of the mean.

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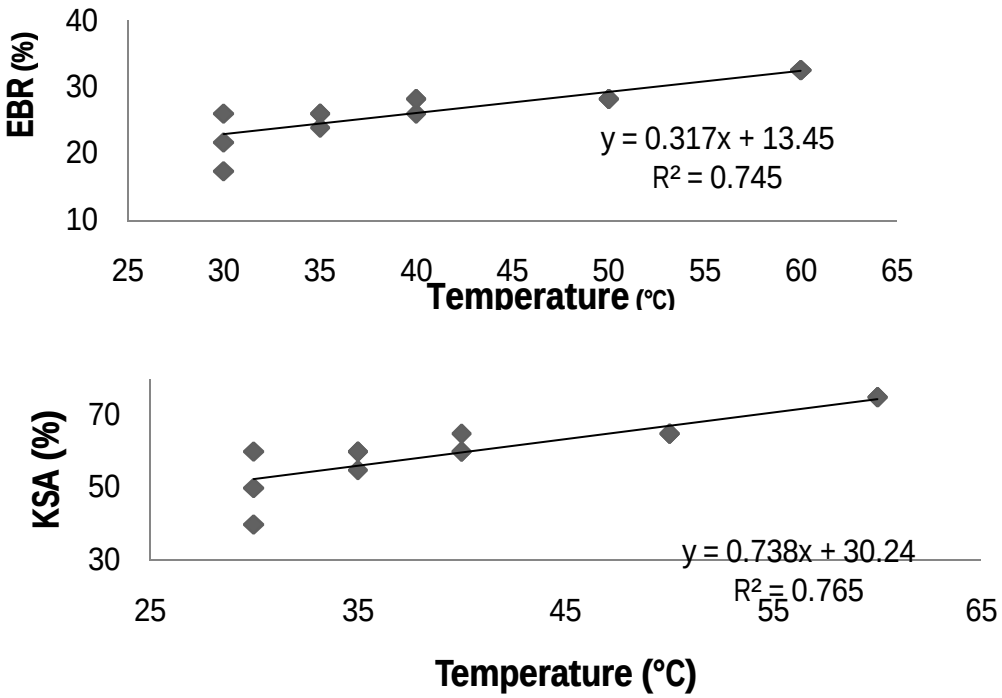

Figure 1 - Effect of temperature on the emulsion-breaking capacity (measured as the emulsion break ratio - EBR) and the kerosene separation activity (KSA) of *Acinetobacter* sp. LBBMA LU 3. The cells were obtained after 22 hours of cultivation in MMSM-paraffin (4% v:v) at 35°C and 200 rpm. The cells were washed in sterile saline solution and resuspended in PBS to an OD_{600nm} of 1.2. The cell suspension (1 ml) was mixed with 9 ml of W/O emulsion, and the mixture was incubated at rest for 24 hours at the indicated temperatures. The adjusted equations were significant at p = 0.01.

Table 1: Demulsification Activity (Water in Oil Emulsion – W/O) From Enrichment Culture of Urban Solid Waste Compost Contaminated With Diesel in Mineral Medium with Paraffin as Sole Carbon Source

Strain* LBBMA	Total Cells suspension				Non-sedimentable (floating) cells suspension				Cell-free extract			
	22 hours		168 hours		22 hours		168 hours		22 hours		168 hours	
	EBR	KSA	EBR	KSA	EBR	KSA	EBR	KSA	EBR	KSA	EBR	KSA
LU 1 (JX45991	43 (21)	75 (9)	35 (0)	80 (0)	51 (21) 83 (0) 84 (5)	82 (14)	30 (0)	70 (0)	15 (9)	17 (15)	33 (3)	85 (6)
LU 3 (JX45991	87 (2)	98 (0)	33 (0)	75 (0)	64 (6)	100 (6)	43 (21)	75 ()9	17 (9)	11 (19)	28 (3)	70 (9)
LU 4 (JX45991	64 (23)	72 (25)	44 (21)	77 (8)	44 (24) 47 (29) 75 (1)	100 (8)	64 (5)	83 (3)	8 (3)	20 (6)	36 (22)	50 (19)
LU 5a (JX45991(	75(3)	85 (0)	33 (0)	75 (0)	37 (29) 30 (0)	92 (3)	51 (18)	78 (8)	20 (11) 22	22 (39)	36 (24)	72 (29)
LU 7a (JX45991	74 (4)	87 (3)	33 (0)	75 (0)	26 (8)	75 (9)	30 (0)	70 (0)	(6)	0 (0)	28 (1)	70 (3)
LU 7b (JX45991	69 (8)	83 (11)	34 (2)	78 (4)		68 (11)	30 (0)	70 (0)	-	-	-	-
LU 10 (JX45990	44 (23)	75 (9)	33 (0)	75 (0)		85 (5)	54 (21)	82 (10)	2 (4)	6 (10)	30 (1)	76 (3)
LU 12 (JX45991	39 (24)	37 (18)	16 (6)	37 (14)		18 (24)	14 (13)	33 (29)	.	-	-	.-
LU 14c (JX4599	17 (0)	40 (0)	30 (0)	70 (0)		70 (0)	30 (0)	70 (0)	-	-	-	
LU 16 (JX45991	28 (2)	65 (5)	41 (36)	65 (33)		50 (35)	30 (12)	55 (10)	-	-	-	-
Blanc	13 (2)	30 (5)	13 (2)	30 (5)	. 13 (2)	. 30 (5)	.13 (2)	30 (5)	4 (6)	.9 (1	. 4 (6)	.9 (16)
m.s.d	44,7	30,8	ns	31,5	43,1	40,9	33,5	29,5	ns	ns	ns	42,5

The microorganisms were cultured in MMSM medium containing liquid paraffin as sole carbon source at 35 ° C and 200 rpm for 22 hours or 168 hours. 1 ml of cell suspension (OD $_{600nm}$ adjusted to about 1.2) was used in demulsification tests, together with 9 ml of water in oil emulsion. Kerosene was the continuous phase. The mixture was stirred at 3,000 rpm and kept at 35 °C for 24 hours. EBR (%) = emulsion break ratio; KSA (%) = Kerosen separation activity. Data represent the mean of three replicates. The numbers in parentheses indicate the standard deviation of the mean.

*Strains LBBMA (LU1, LU3, LU4, LU7a, LU10) = *Acinetobacter* sp.; LBBMA LU7b = *Pseudomonas mendocina*; LBBMA LU12 = *Arthrobacter nicotianae;* LBBMA (LU 5a, LU 14c and LBBMA LU16) = *Pseudomonas* sp.

** GenBank accession numbers for 16S rDNA partial sequences. Blast hits for the sequences are presented in Table S1 in the supplemental material.
m.s.d.: minimal significant difference, $p = 0.05$. (–) for cell-free extract: not-evaluated.

Table 2: Demulsification Activity (Water-In-Oil Emulsion - W/O) Of *Acinetobacter* Sp. LBBMA LU 3, in Response to Autoclaving (121°C, 20 Minutes) or Freezing (-20°C, 24 Hours) Followed by Thawing at Room Temperature

Treatment	EBR (%)	KSA (%)
Control[1]	25.4 (1.3)	58.3 (2.9)
Autoclaved suspension	31.9 (1.3)	73.3 (2.9)
Frozen/thawed suspension	31.9 (1.3)	73.3 (2.9)
Control[2]	5.1 (1.3)	12.5 (3.5)
m.s.d. 0.053	3.1	7.2

The microorganism was cultivated in MMSM containing liquid paraffin as a sole carbon source at 35°C and 200 rpm for 22 hours. Cell suspension (1 ml, OD_{600nm} adjusted to approximately 1.2) was used in 9 ml of emulsion. The mixture was agitated at 3,000 rpm and kept at 35°C for 24 hours. EBR (%) = emulsion break ratio. KSA (%) = kerosene separation activity of the emulsion. The data represent the mean of three replicates. The numbers in parentheses indicate the standard deviation of the mean. [1] - cell suspension without autoclaving or freezing/thawing. [2] - MMSM culture medium used in place of the cell suspension. 3 - minimal significant difference, p = 0.05.