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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Efficiency in hydrocarbon degradation and biosurfactant production by *Joostella* sp. A8 when grown in pure culture and consortia☆

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ARTICLE INFO

Article history:
Received 6 May 2017
Revised 11 August 2017
Accepted 14 August 2017
Available online 18 August 2017

Keywords:
Joostella
Biosurfactants
Co-cultures
Hydrocarbon degradation
Biodegradation efficiency

ABSTRACT

Joostella strains are emerging candidates for biosurfactant production. Here such ability was analyzed for *Joostella* strain A8 in comparison with *Alcanivorax* strain A53 and *Pseudomonas* strain A6, all previously isolated from hydrocarbon enrichment cultures made of polychaete homogenates. In pure cultures *Joostella* sp. A8 showed the highest stable emulsion percentage (78.33%), hydrophobicity rate (62.67%), and an optimal surface tension reduction during growth in mineral medium supplemented with diesel oil (reduction of about 12 mN/m), thus proving to be highly competitive with *Alcanivorax* and *Pseudomonas* strains. During growth in pure culture different level of biodegradation were detected for *Alcanivorax* strain A53 (52.7%), *Pseudomonas* strain A6 (38.2%) and *Joostella* strain A8 (26.8%). When growing in consortia, isolates achieved similar abundance values, with the best efficiency that was observed for the *Joostella-Pseudomonas* co-culture. Gas-chromatographic analysis revealed an increase in the biodegradation efficiency in co-cultures (about 90%), suggesting that the contemporary action of different bacterial species could improve the process. Results were useful to compare the efficiencies of well-known biosurfactant producers (*i.e. Pseudomonas* and *Alcanivorax* representatives) with a still unknown biosurfactant producer, *i.e. Joostella*, and to confirm them as optimal biosurfactant-producing candidates.

Introduction

The genus *Joostella* (family *Flavobacteriaceae*) was firstly described by Quan et al. (2008) who isolated *J. marina* strain En5^T gen. nov., sp. nov., from coastal seawater (Quan et al., 2008; Hameed et al., 2014). A summary classification and a set of features for *J. marina*, in addition to the description of the complete genomic sequencing and annotation, was then presented by

☆ Dedication: Carmen Rizzo would like to affectionately dedicate this paper to Luigi Michaud for his special and unforgettable support, and to her mentor Angelina Lo Giudice, for all teachings and her constant presence.

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http://dx.doi.org/10.1016/j.jes.2017.08.007

Stackebrandt et al. (2013). A second species, *J. atrarenae*, was described by Kim et al. (2011), but not yet appeared on a validation list. To date, information on *Joostella* spp. remain scarce and fragmentary. Only recently, members in the genus *Joostella* have been reported as biosurfactant (BS) producers by Rizzo et al. (2013) who isolated *Joostella* strain A8 from crude oil enrichment cultures, which were set up with homogenates of the polychaete *Megalomma claparedei* (Gravier, 1906). Further in depth investigation were carried out with the attempt to enhance BS production by *Joostella* strain A8 by varying culture conditions, including the carbon source (Rizzo et al., 2014), and link the influence of heavy metals on BS activity (Rizzo et al., 2015). Overall, gained data on *Joostella* strain A8 (Rizzo et al., 2013, 2014, 2015) were encouraging about its strong potentiality as BS producers and its possible application in microbial-mediated bioremediation process. Surface active agents, such as biosurfactants, facilitate the cellular uptake of insoluble substrates, by reducing the surface and interfacial tension, so increasing the solubility and emulsification of them, and resulted able to overcome the toxicity of synthetic compounds (Edwards et al., 2003). As it is well known, a limited range of petroleum substrates are metabolized by individual microorganisms, while mixed populations could exploit a variety of enzymatic abilities. This improve the biodegradation efficiency of complex hydrocarbon substrates, thanks to the complementary action of more than a single species with substrate specificity (Patil et al., 2012; Varjani and Upasani, 2013; Thomas et al., 2014). According to this, bacterial consortia should be regulated by a wide range of metabolic mechanisms for the enhancement of oil components transformation (Antoniou et al., 2015; Thomas et al., 2014). To date, bacterial consortia have been used to investigate microbial degradation efficiency, which is generally higher if compared to mono-cultures (Kadali et al., 2012), probably thanks to synergistic interactions among members of the association (Sampath et al., 2012). In contrast, the majority of studies on BS-mediated biodegradation were carried out with the use of mono-culture, and in rare occasions, mixed cultures (or co-cultures) were used (Ławniczak et al., 2013).

The improvement of microbial hydrocarbon degradation and BS production represents a promising approach in the control and remediation of this kind of pollution, so that various hydrocarbon-degrading bacteria have been isolated during last decades. The present study had a dual aim. Firstly, BS production and hydrocarbon degradation by *Joostella* sp. A8 were compared with those by isolates of the same origin (Rizzo et al., 2013) belonging to well-known genera in this field, i.e. *Alcanivorax* strain A53 and *Pseudomonas* strain A6. The genus *Alcanivorax* is an alkane degrader and producer of an efficient glucose–lipid surfactant (Fernàndez-Martìnez et al., 2003). The genus *Pseudomonas* is the best known rhamnolipid producer, able to use different substrates such as fructose, glycerol, mannitol, glucose, n-paraffin and vegetable oils (Desai and Banat, 1997), and most promising candidate for BS-production on large scale. Secondly, BS production and hydrocarbon degradation capacity was evaluated in co-cultures of *Joostella* strain A8 grown together with *Alcanivorax* strain A53 or *Pseudomonas* strain A6 in order to establish if they might have reciprocal advantage in substrate degradation thanks to the involvement of different metabolic abilities.

1. Material and methods

1.1. Bacterial strains

BS-producing bacterial strains used in this study were previously isolated from crude oil enrichment cultures which were set up with homogenates of the Polychaete annelids *Megalomma claparedei* (Gravier, 1906) (*i.e. Joostella* strain A8, **J**, Accession number **JX298555**; *Pseudomonas* strain A6, **P;** Accession number **JX298544**) and *Branchiomma luctuosum* (*i.e. Alcanivorax* strain A53, **A,** Accession number **JX298541**) from the brackish Lake Faro, Messina, Italy (Rizzo et al., 2013). Main features of the three BS-producing isolates are summarized in Table 1. Isolates were grown in both pure cultures and co-cultures (consortia) to monitor cell abundances, hydrocarbon degradation and BS production over time, as described in the following sections.

1.2. Culture set-up

1.2.1. Pure cultures

Experiments were carried out in 500 mL Erlenmeyer flasks containing 150 mL of the mineral salt medium Bushnell Haas Broth (BH) supplemented with NaCl (3%, W/V) and diesel oil (DO; 2%, V/V). Culture broth was inoculated with 10% (V/V) of an overnight pre-culture ($OD_{580} \approx 0.6$), and incubated at 25°C in a shaker (160 r/min) for 480 hr. Biodegradation assays were carried out under three parallel culture sets, as follows: Set I, Bacterial cells plus BH plus DO was used for chemical analyses; Set II, Bacterial cells plus BH plus DO was used for monitoring BS production and bacterial abundance; Set III, Bacterial cells plus BH plus DO plus sodium dodecyl sulfate (SDS) was used to investigate the effect of a synthetic surfactant on hydrocarbon degradation. Uninoculated control experiments were simultaneously carried out, with 150 mL of BH supplemented with DO (2%, V/V) to monitor abiotic losses of the substrate.

1.2.2. Co-cultures

Equal volumes of bacterial cultures ($OD_{580} \approx 0.700$) (for a total inoculum of 10%) were combined to inoculate 150 mL of BH *plus* DO (2%, V/V). Consortia were incubated at 25°C under shaking (160 r/min) for 480 hr. In detail, *Joostella* strain A8 was grown together with *Pseudomonas* strain A6 (consortium J-P) or *Alcanivorax* strain A53 (consortium J-A). Uninoculated control experiments were simultaneously carried out, with 150 mL of BH broth supplemented with DO (2%, V/V) to monitor abiotic losses of the substrate.

1.3. Estimation of bacterial abundances

1.3.1. Estimation of bacterial abundances in pure cultures

Aliquots from the pure cultures were collected at regular intervals (48 hr) to monitor bacterial abundance by optical density measurement at 580 nm (OD_{580}) using a spectrophotometer (UV-mini-1240, Shimadzu, Japan). Additionally, sub-samples were collected at 0, 240 and 480 hr of incubation (T_0, T_{240} and T_{480}, respectively) and fixed with formaldehyde (final concentration 2%, V/V) for the subsequent total counts using epifluorescence microscope and flow cytometry, as follows. For microscope

Table 1 – Main results obtained for biosurfactant production by *Joostella* strain A8, *Pseudomonas* strain A6 and *Alcanivorax* strain A53.

Medium [a]	Temperature (°C)	E_{24} [b] (%)	ST [c] (mN/m)	C-TAB [d]	BA [e]	TLC [f]	Reference
Joostella strain A8							
MB	28	62.5	5.3	–	–	+	Rizzo et al. (2013)
ONR7a-Tet	28	57	8.9	–	–	nd	Rizzo et al. (2013)
BH-Tet	15	72.5	35.6	–	–	nd	Rizzo et al. (2014)
BH-Tet	25	62	16.6	–	–	nd	Rizzo et al. (2014)
Pseudomonas strain A6							
MB	28	62.9	6.3	–	–	+	Rizzo et al. (2013)
ONR7a-Tet	28	–	7.1	–	–	nd	Rizzo et al. (2013)
BH-Tet	15	8.5	18.9	–	–	nd	Rizzo et al. (2014)
BH-Tet	25	57.5	32	–	–	nd	Rizzo et al. (2014)
Alcanivorax strain A53							
MB	28	51.4	18.2	+	+	+	Rizzo et al. (2013)
ONR7a-Tet	28	59	1.7	–	–	nd	Rizzo et al. (2013)
BH-Tet	15	61	19.5	–	–	nd	Rizzo et al. (2014)
BH-Tet	25	61	20.7	–	–	nd	Rizzo et al. (2014)

[a] MB, Marine Broth; ONR7a-Tet, ONR7a supplemented with tetradecane (2%, V/V); BH-Tet, Bushnell Haas broth supplemented with tetradecane (2%, V/V).
[b] E_{24}: maximum value of stable emulsion.
[c] ST^{48}: surface tension reduction after 48-hr incubation.
[d] C-TAB assay.
[e] Blood Agar assay.
[f] Yellow spots on thin layer chromatography plates.

observations, samples were filtered on black polycarbonate membranes (Isopore; pore size, 0.22 μm; diameter 25 mm) and stained with 4,6-diamidino-2-phenylindole (DAPI; final concentration 5 μg/mL). The number of microorganisms was determined by counting stained cells by epifluorescence microscopy on 20 randomly selected fields (Porter and Feig, 1980). Flow cytometry analysis was carried out on a Beckman Coulter flow cytometer (Cytomics FC 500 MPL) with five fluorescent channels equipped with two lasers emitting at 488 nm (argon laser) and 635 nm (red emitting diode). For the cell staining, samples (0.5 mL) were mixed with SYBR Green-I (Molecular Probes) diluted 1:10,000 (V/V) final concentration from a stock solution as supplied by the manufacturer, and incubated in the dark for 20 min at room temperature. Samples were then run in the cytometer at low-medium speeds (15.8–40.6 μL/min) and data were acquired in log mode and visualized in plots of side scatter light (SSC), which is considered to be related to the size, *versus* green fluorescence (FL1) channels, with the threshold made in the latter. Additionally, by plotting green (FL1) *versus* red (FL4) fluorescence channel, we were able to discriminate heterotrophic bacteria from cytometric noise that frequently may appear close to the former and interferes its determination. When necessary, samples were diluted with filtered sheath fluid to hold the rate of particle passage below 1000 events/sec to avoid coincidences and thus allowing obtaining more accurate results. Fluorescent beads of 1 μm of diameter were used as size markers. All parameters were collected as logarithmic signals to generate a four-decade channel scale. This cytometer procedure allowed separation of two main subpopulations of bacteria that distinguished depending of their green fluorescence intensity, being this proportional to their DNA content. We refer to these as high DNA (HDNA) and low DNA (LDNA) bacteria. Considering that the green fluorescence can be related to the apparent nucleic acid content per cell, we assumed that HDNA subpopulation were metabolically more active and constituted the dynamic fraction of the bacterial assemblage (Gasol et al., 1999; Lebaron et al., 2001). Cell concentrations of these bacterial populations were obtained considering the processed volume gravimetrically. The active cell index (ACI) was calculated by dividing the number of HDNA bacterial cells in each sample by the total abundance, and expressed as a percent (Jellett et al., 1996).

1.3.2. Estimation of bacterial abundances in consortia

Bacterial abundances in consortia were monitored at intervals of 48 hr by both DAPI-staining (as described above for pure cultures) and fluorescent *in situ* hybridization (FISH) (Glöckner et al., 1999), to distinguish *Joostella* strain A8 (CF group of the *Bacteroidetes*) from *Alcanivorax* strain A53 and *Pseudomonas* strain A6 (both belonging to the *Gammaproteobacteria*). Briefly, cells were concentrated from samples (5–10 mL) on white polycarbonate filters (diameter, 25 mm; pore size, 0.22 μm) and subsequently fixed for 30 min at room temperature by overlaying filters with a freshly prepared paraformaldehyde (final concentration 4%) phosphate buffer saline (PBS; 130 mmol/L NaCl, 10 mmol/L $NaHPO_4$, and 10 mmol/L NaH_2PO_4, pH 7.4) solution (3 mL). The fixative was removed by applying vacuum, and filters were washed twice with 3 mL of PBS and, finally, with distilled water. Filters were labeled, air dried, and stored in a sterile Petri dish at −20°C until processing. Filter sections were hybridized with the CY3-labeled oligonucleotide probes: EUB338I (probe sequence GCTGCCTCCCGTAGGAGT; target group most but not all Bacteria), EUB338II (probe sequence GCAGCCACCCGT AGGTGT; target group *Planctomycetes*), EUB338III (probe sequence GCTGCCACCCGTAGGTGT; target group *Verrucomicrobiales*) (Egli et al., 2003); and CF319a (probe sequence TGGTCCGTGTCTCAG TAC; target group CFB group of *Bacteroidetes*) and GAM42a

(probe sequence GCCTTCCCACATCGTTT; target group *Gammaproteobacteria*) (Manz et al., 1992) (MWG-Biotech, Germany). Probes EUB338I, EUB338II, and EUB338III were equimolarly mixed together to obtain the EUB-mix, and a negative control probe, non-EUB (5′-ACTCCTACGGGAGGC AGC-3′), was also used for nonspecific probe binding. Results with this negative control probe, which accounts for autofluorescence of cells and nonspecific probe binding, were subtracted from the percentages detected with probes for the bacterial groups. Filters were mounted on glass slides with Citifluor AF1 (Citifluor Ltd., Canterbury, United Kingdom) and enumerated by epifluorescence microscope (Axioplan, Zeiss) equipped with specific filter sets for CY3. For each sample and probe, minimum 20 fields and 200 cells were enumerated. Additionally, flow cytometry analysis was performed at T_0, T_{240} and T_{480} as described above for pure cultures.

1.4. Biosurfactant production

Bacteria growing in both pure cultures and consortia were screened for BS production at regular intervals (48 hr), as follows. When necessary (see below), cultures were preliminary centrifuged at 4700 r/min for 20 min at 4°C, and only the obtained supernatants were used. For each test, uninoculated medium was treated exactly as the cultures and then used as negative control.

1.4.1. Emulsification assay

A 500 μL amount of cell culture broth was added to an equal volume of kerosene (Petroleum ether, Panreac) and vigorously vortexed for 1 min. The mixture was allowed to stand for 2 min. After 1 min of stabilization, the emulsification ability was measured and calculated by dividing the height of the emulsion layer by the total height of the mixture, and multiplying by 100 (Christova et al., 2004).

1.4.2. E_{24} index detection

In case of positive result to the emulsification test, the stability of produced emulsions was tested, and expressed by the index of emulsification E_{24}, in agreement with Amiriyan et al. (2004). A 2-mL portion of the sample from each culture was added to 2 mL of kerosene as test-oil and the mixture was shaken at high speed for 2 min. After 24 hr, the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture, and multiplying by 100 (Rizzo et al., 2014).

1.4.3. Surface tension measurement

The surface tension of the cell free supernatant was determined with a digital tensiometer K10T (Krüss, Hamburg, Germany) by using Wilhelmy Plate method (Rosenberg et al., 1980), which measures the weight of the liquid drawn by a plate when a plate is lifted from or through the surface of liquid. The weight of the liquid is proportional to the surface tension of the liquid.

1.4.4. Bacterial adhesion to hydrocarbons (BATH assay)

Cell hydrophobicity was assessed photometrically by measuring bacterial adherence to hydrocarbons (BATH) as described by Rosenberg et al. (1980). The method is based on the degree of adherence of cells to liquid hydrocarbons. After centrifugation (see above), cells were washed twice and resuspended in phosphate urea magnesium sulfate buffer (PUM buffer; 22.2 g $K_2HPO_4{\cdot}3\ H_2O$,7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4{\cdot}7\ H_2O$ and distilled water to 1000 mL; pH 7.1), to an initial OD_{550} of the cell suspension of 0.5–0.6. Cell suspension (1.2 mL) was transferred to a clean, round-bottom, test tubes and vortexed with hexadecane (0.2 mL) at high speed for 2 min and equilibrated for 1 hr. The OD_{550} of the bottom aqueous phase was then measured. Hydrophobicity was expressed as the percentage of cell adherence to hydrocarbon, calculated as follows: 100 × (1-OD of the aqueous phase/OD of the initial cell suspension).

1.4.5. Penetration assay

The cavities of a 96 well microplate were filled with a hydrophobic paste consisting of oil and silica gel, and covered with oil. After centrifugation (see above), 90 μL of supernatant was colored by adding 10 μL of a highly concentrated staining solution of red phenol in each well and pipetting up and down the whole solution. Then, the colored solution was pipetted smoothly on the paste. BS-containing supernatant changed from clear red to cloudy white within the first 15 min, whereas BS-free supernatant turned cloudy but stayed red (Walter et al., 2010).

1.5. Biodegradation efficiency

Residual hydrocarbons were extracted at T_0 and T_{480} by liquid–liquid extraction, as previously reported (Michaud et al., 2007). After incubation, biodegradation activity was stopped by acidifying cultures with HCl 10 mol/L to achieve pH 2. Bacterial cells were removed by centrifugation at 5000 r/min at 4°C for 20 min. Hydrocarbons were extracted three times with CH_2Cl_2 (10%, V/V) in separatory funnels, which were vigorously shaken for 3 min during each extraction. The three organic phases were pooled and the solvent evaporated to dryness under reduced pressure in a rotary evaporator (Heidolph).

The biodegradation efficiency (BE) of bacterial isolates grown in pure cultures and consortia was assessed by gas-chromatography mass-spectrometry, as follows. Squalene (1 mL, V/V) was added before hydrocarbon extraction and used as internal standard for gas-chromatographic analysis. Extracts were dissolved in CH_2Cl_2 by using mass spectrometry detector (GC2010-MS Shimadzu, equipped with a quadrupole) equipped with a column fused silica capillary Supelco SPB-5MS (5% diphenyl-95% dimethyl polysiloxane; 30 mm × 0.25 mm; 0.25 μm film stationary phase). Helium was used as carrier gas at flow velocity in column of 0.8 mL/min. The oven was programmed as follows: initial temperature 50°C (5 min), then increased to final temperature 280°C (10 min) at rate of 10°C/min. Injector and interface temperatures were both maintained at 250°C. Further parameters were: ion source temperature maintained at 200°C split injection with a split ratio 1:10; electronic ionization (EI) at 70 eV; acquisition in full scan from 40 to 500 Da. The degradation of DO as a whole was expressed as the percentage of DO degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples (external standard technique). The BE, based on the decrease in the total concentration of hydrocarbons, was evaluated by using the following

expression: BE (%) = 100 – (A_s * 100 / A_{ac}), where A_s is the total area of peaks in each sample; A_{ac} is the total area of peaks in the appropriate abiotic control; BE (%) is the efficiency of biodegradation (Michaud et al., 2004).

1.6. Statistical analyses

Analyses of variance (ANOVA) and the Tukey tests with a significance level of 0.05 were applied using the MiniTab software (version 16.0). Pearson's correlation coefficient was used to evaluate the correlation between the analyzed variables, and values of $p < 0.05$ were considered statistically significant.

2. Results

2.1. Bacterial pure cultures

2.1.1. Estimation of bacterial abundances and BS production

All bacterial strains were able to grow in the presence of DO as the sole carbon and energy source, with the exception of cultures containing SDS (set III). Even if a considerable reduction of surface tension was recorded, a strong bacterial growth inhibition was shown by SDS which resulted very toxic even at low concentration (0.1%, V/V).

Figure 1 shows bacterial growth in terms of OD_{580} and total counts for each strain. The maximum cell growth was generally achieved between T_{192} and T_{288}, with a decline phase that occurred after T_{336}. Total bacterial abundances obtained by DAPI staining and flow cytometry were in the same order of magnitude, ranging between 2.7×10^7 and 21.85×10^7 cells/mL, and between 1.27×10^7 and 18.4×10^7 cells/mL, respectively (Table 2). However, bacterial abundances determined by flow cytometry always resulted underestimated compared to the obtained by DAPI staining (Table 2), in percentages that ranged from 16.1% to 68.4% (mean = 35.7%). This underrating was a consequence of the occurrence of cell aggregates, which are not reflected in the cytometer analysis but considered in the microscopic counts. This underestimation was roughly the same for three bacterial strains although somewhat higher in the case of *Joostella* strain A8. Despite such issues, time-course bacterial abundances followed the same pattern that microscopic counterparts (Table 2), being in the three strains higher cell numbers achieved at time T_{240} compared to T_0 and T_{480}.

Based on the flow cytometry analysis, bacteria were divided into two main groups, considering only the disaggregated cells as the total. The first corresponded to the LDNA fraction, which had a low green fluorescence (FL1) signal and was located in the lower part of the flow cytometer plot, and the second to the HDNA fraction, corresponding to a high green fluorescence (FL1) signal and located in the upper part of the plot (Fig. 2). The ratio of HDNA bacteria to total bacterial abundance (HDNA plus LDNA), expressed as the active cell index (ACI), ranged broadly from 0.2% to 38.9%, with an average for three strains of 15.9% (Table 2). In the case of *Pseudomonas* strain A6, and particularly in *Alcanivorax* strain A53, the ACI values were higher at the initial phases and decayed notably at times T_{240} and T_{480}. On the other hand, *Joostella* strain A8 maintained higher ACI during time course incubations, being even higher than the initials at time T_{480}.

All strains showed emulsification activity and stable emulsion production, starting from T_{96} (*Joostella* strain A8 and *Pseudomonas* strain A6) and T_{144} (*Alcanivorax* strain A53). *Joostella* strain A8

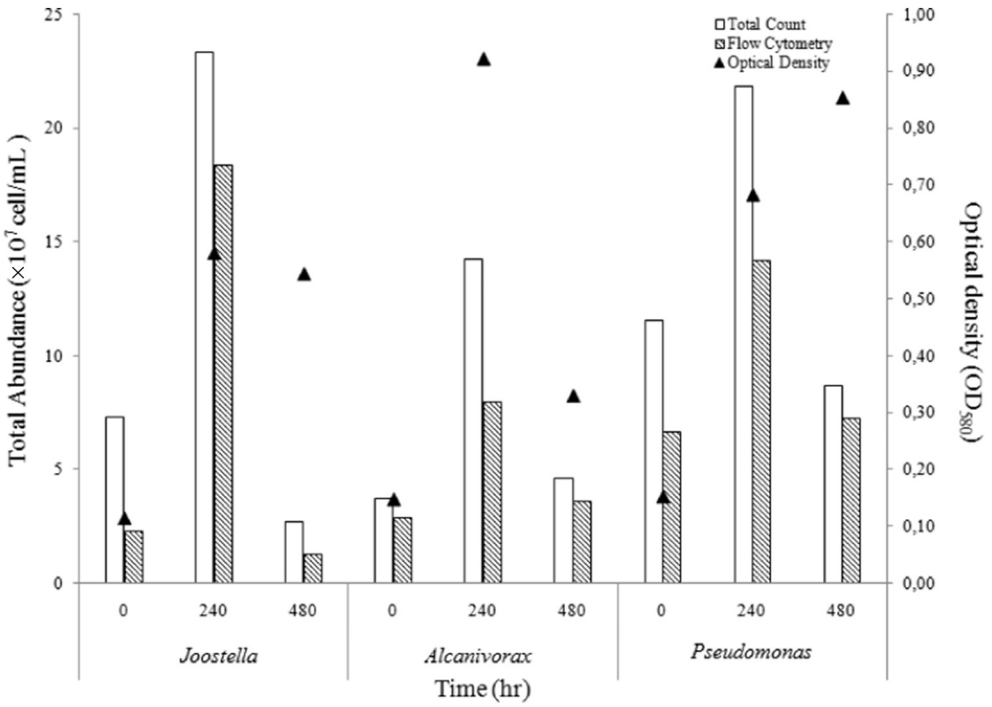

Fig. 1 – Bacterial abundances by *Joostella* strain A8, *Pseudomonas* strain A6 and *Alcanivorax* strain A53 during incubation in pure cultures at 25°C in BH supplemented with DO. Optical density (OD_{580}) values (lines) and total cell counts (histograms; cell $mL^{-1} \times 10^7$).

Table 2 – Bacterial counts obtained from pure cultures by DAPI staining and flow cytometry, expressed as $\times 10^7$ cell/mL.

Type of bacteria	*Alcanivorax* strain A53			*Pseudomonas* strain A6			*Joostella* strain A8		
Time	T_0	T_{240}	T_{480}	T_0	T_{240}	T_{480}	T_0	T_{240}	T_{480}
DAPI staining									
Total	3.7	14.25	4.6	11.55	21.85	8.65	7.3	23.3	2.7
Flow cytometry									
Total	2.92	8.09	3.61	6.65	14.2	7.26	2.31	18.4	1.27
ACI (=%HDNA)	32.8	0.2	0.4	38.9	2.5	6.4	17.8	10.2	33.9
% Underestimation *	21.08	43.23	21.52	42.42	35.01	16.07	68.36	21.03	52.96

* Underestimation with respect DAPI counts.

produced stable emulsion with values that ranged between 21.67% and 78.33%, by following an exponential trend corresponding to bacterial growth pattern, as showed by optical density values. The same profile was showed by *Pseudomonas* strain A6 and *Alcanivorax* strain A53, with E_{24} values ranged from 15.67% to 71.67% and from 8.33% to 66%, respectively. All the emulsions created remained stable for more than one month. The strains followed a similar kinetics, by starting firstly to show emulsification activity, and then stable emulsion production, after achieving the exponential phase or near to it. A strong positive correlation between stable emulsion production and optical density values was observed only for *Alcanivorax* strain A53 ($r = 0.71$; $p = 0.01$). By considering the E_{24} values, the pattern followed by the three strains were, from the higher to the lower stable emulsion percentage, in the order *Joostella* > *Pseudomonas* > *Alcanivorax* (Fig. 3a). However, emulsifying activities by the three strains during all the incubation period were not significantly different ($p > 0.05$). Bacterial strains showed different ability to reduce surface tension, starting from about 45 mN/m and achieving final surface tension values of 33.43 for *Joostella* strain A8, 27.27 mN/m for *Pseudomonas* strain A6 and 25.20 mN/m for *Alcanivorax* strain A53. Statistical analysis showed that surface tension measurements reported for *Alcanivorax* strain A53 were significant different from those obtained by *Joostella* strain A8 and *Pseudomonas* strain A6.

Figure 3b shows surface tension trends in relation to the hydrophobicity values of each strain during incubation. *Joostella* strainA8 had a maximum cell adhesion with diesel oil (62.67%), followed by *Alcanivorax* strain A53 and *Pseudomonas* strain A6. *Joostella* strain A8 began to show affinity towards hydrophobic substrates after T_{48}, and maintained it until the end of incubation with a minimum value of 20.67% after T_{288}. *Alcanivorax* strain A53 showed the maximum value of hydrophobicity (51.67%) after T_{240} and maintained it until T_{336}, after which it entered into the decline phase. Finally, *Pseudomonas* strain A6 showed hydrophobicity values ranged between 7 and 18.33%, starting from T_{144} and ending at T_{432}. The maximum value of hydrophobicity corresponded to the maximum value of optical density. In the case of hydrophobicity index, there was a significant difference between *Joostella* strain A8 percentages and values obtained for the other strains ($p < 0.05$). Moreover, both for *Joostella* strain A8 and *Alcanivorax* strain A53 a strong positive correlation was highlighted between stable emulsion production and hydrophobicity values (respectively $r = 0.81$ and $p = 0.002$; $r = 0.93$ and $p = 0.001$).

All strains showed a positive reaction to the penetration assay.

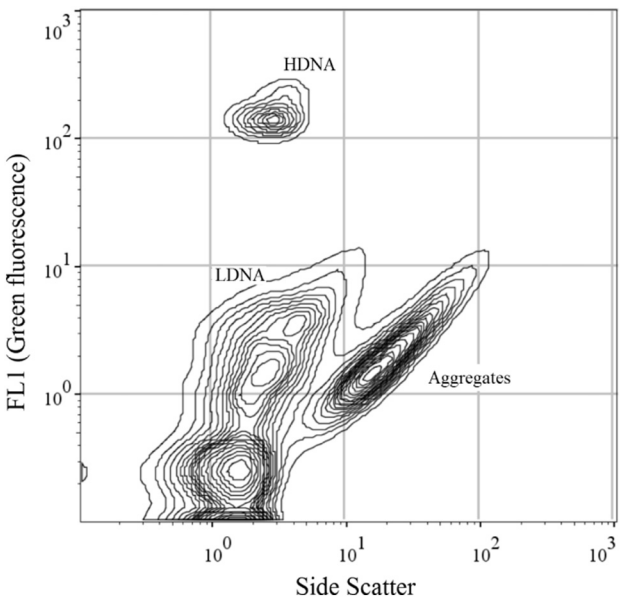

Fig. 2 – LDNA and HDNA of *Alcanivorax* strain A53 in pure culture after 0, 240 and 480 hr of incubation.

2.2. Bacterial consortia

2.2.1. Estimation of bacterial abundances and BS production

All mixed bacterial consortia were able to use DO as the sole carbon source for growth, and the strains were able to grow together in the same culture medium without evident inhibitory activity one towards each other. In detail, Fig. 4a and d highlight that in both consortia strains reached similar values of bacterial abundance (as it was determined by FISH), following a similar temporal profile. In the J-P consortium (Fig. 4a), bacterial abundances of individual strain showed a fluctuating trend: in a first phase, the higher abundance value was achieved after 96 hr of incubation, with 11×10^7 cells/mL for *Pseudomonas* strain A6, and 7.6×10^7 cells/mL for *Joostella* strain A8. Results obtained by flow cytometry were not used, because of the difficult interpretation caused by the occurrence of too many aggregates. For this reason bacterial abundances from flow cytometry for bacterial consortia are not reported.

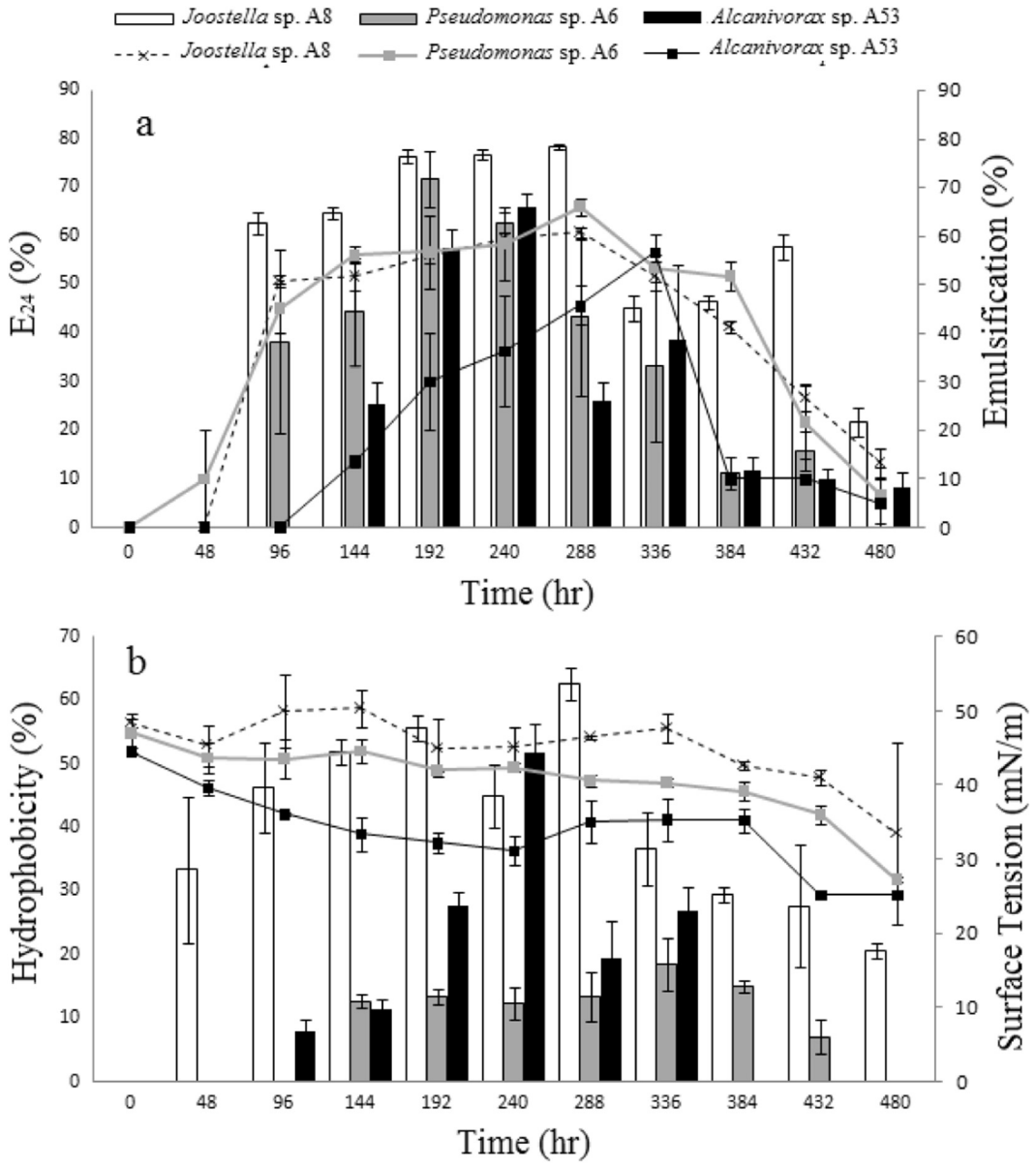

Fig. 3 – Biosurfactant production by *Joostella* strain A8, *Pseudomonas* strain A6 and *Alcanivorax* strain A53 during incubation in pure cultures at 25°C in BH supplemented with DO. a) **E_{24} index (histograms) and emulsification activity (lines);** b) **Hydrophobicity percentages (histograms) and surface tension (lines).**

The emulsification activity and stable emulsion production started from T_{48}, in correspondence of the initial increasing phase of *Pseudomonas* strain A6 (see Fig. 4a and b), and had a linear profile only until the achieving of exponential phase, with a maximum stable emulsion percentage (68%) after 240 hr. Then, both values showed an inconstant trend, until the end of incubation.

Joostella strain A8 started a decreasing phase after 336 hr of incubation, until a minimum of 2.75×10^7 cells/mL, while *Pseudomonas* strain A6 continued to show fluctuating values, and achieved also a minimum after 336 hr of incubation (4.9×10^7 cells/mL). When *Pseudomonas* strain A6 started the last decreasing phase, *Joostella* strain A8 increased again with 9×10^7 cells/mL after 432 hr.

Figure 4c shows the hydrophobicity percentages and the surface tension measurements obtained for the J-P consortium during the entire incubation period. Hydrophobicity followed an exponential trend as well as emulsification activity, and increased until T_{192} with a maximum percentage of 51%, and then started to decrease and assume a constant value (≈4%) until the end of incubation. The surface tension reduction ability showed a starting value of 57.3 mN/m and a final value of 34.4 mN/m, with a total reduction of 22.9 units. The maximum cell adhesion in the presence of DO (51% achieved after 192 hr) corresponded to the starting time point for the surface tension reduction.

In the J-A consortium (Fig. 4d), both strains showed a more linear trend in terms of bacterial abundance, and achieved

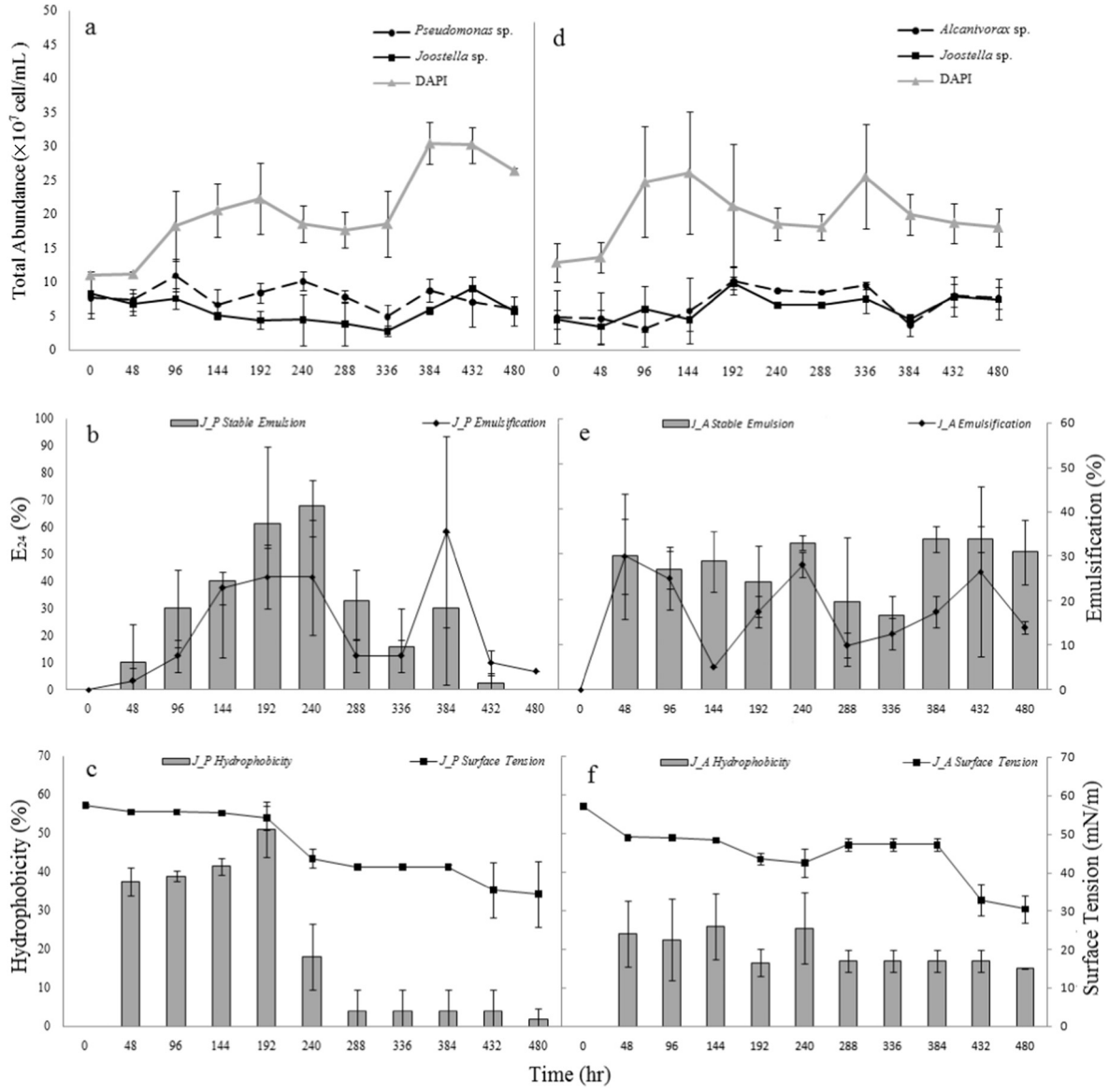

Fig. 4 – Bacterial abundances and biosurfactant production in the *Joostella-Pseudomonas* (J-P, graphs on the left) and *Joostella-Alcanivorax* (J-A, graphs on the right) consortia at 25°C in BH supplemented with DO. (a) Total bacterial abundances and *in situ* abundance of *Joostella* strain A8 and *Pseudomonas* strain A6 as determined by DAPI and FISH in the J-P consortium; (b) E_{24} index (histograms) and emulsification activity (lines) measured in the J-P consortium; (c) hydrophobicity percentages (histograms) and surface tension (lines) measured in the J-P consortium; (d) total bacterial abundances and *in situ* abundance of *Joostella* strain A8 and *Alcanivorax* strain A53as determined by DAPI and FISH in the J-A consortium; (e) E_{24} index (histograms) and emulsification activity (lines) measured in the J-A consortium; (f) hydrophobicity percentages (histograms) and surface tension (lines) measured in the J-A consortium.

together the maximum value of exponential phase after 144 hr of incubation (*Alcanivorax* strain A53, 18.1 × 10^7 cell/mL; *Joostella* strain A8, 20.6 × 10^7 cell/mL).

The fluctuating aspect of emulsifying activity and stable emulsion production showed by the J-P consortium was also observed in the J-A consortium. It appeared to be stronger and extended to the entire incubation period. In this case, stable emulsion production covered the entire period of incubation starting from T_{48}, and the highest emulsification index of 56.5% was obtained after 384 and 432 hr of incubation (Fig. 4e).

After 192 hr of incubation, both strains started the decreasing phase until a minimum of abundance after 384 hr of incubation, with 3.9 × 10^7 and 4.75 × 10^7 cells/mL for *Alcanivorax* strain A53 and *Joostella* strain A8, respectively. Figure 4f shows surface tension trends in relation to the hydrophobicity values in the J-A consortium. The maximum cell adhesion percentage in the presence of DO (26%) was achieved after 144 hr of incubation, and assumed overall values ranging from 15% and 26% during the entire incubation period. Surface tension was reduced of 26.8 mN/m.

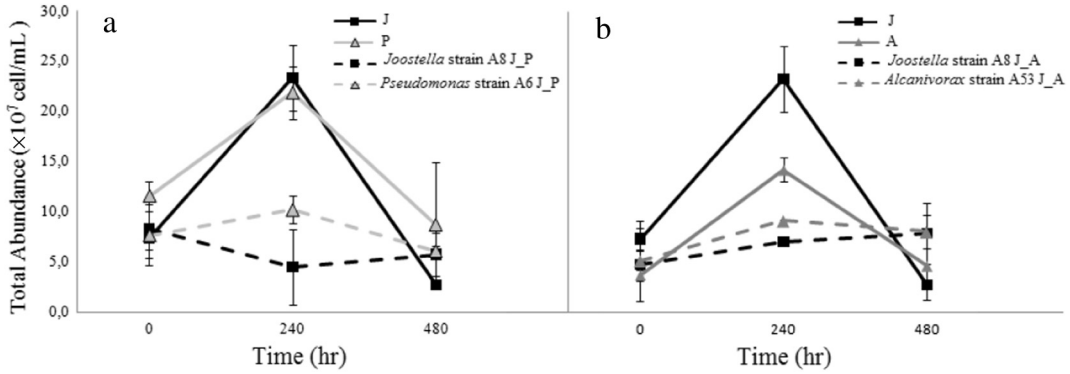

Fig. 5 – Total bacterial counts in pure cultures and consortia. (a) *Joostella* strain A8 and *Pseudomonas* strain A6 in pure culture (J and P, respectively) and consortium (J in J-P; P in J-P); (b) *Joostella* strain A8 and *Alcanivorax* strain A53 in pure cultures (J and A, respectively) and consortium (J in J-A, A in J-A). J-A, *Joostella* plus *Alcanivorax* consortium; J-P, *Joostella* plus *Pseudomonas* consortium.

Figure 5 reports on the correlation between total counts obtained when isolates grew in pure cultures and consortia at T_0, T_{240} and T_{480}. Figure 5a highlights that, even if *Joostella* strain A8 and *Pseudomonas* strain A6 followed the same pattern in pure culture, in the co-culture *Joostella* decreased when *Pseudomonas* increased. On the other hand, *Joostella* strain A8 and *Alcanivorax* strain A53 maintained the same pattern in enrichments and co-culture (Fig. 5b). Results obtained by each screening test in both consortia were statistically equal ($p > 0.05$).

2.2.2. Biodegradation efficiency

Strains showed similar BE on diesel oil when growing in pure cultures (Fig. 6a). The highest value of BE was obtained for *Alcanivorax* strain A53 with a mean percentage of 52.7%, followed by *Pseudomonas* strain A6 (38.2%) and *Joostella* strain A8 (26.8%). The biodegradation efficiency in bacterial consortia reached values of 99.4% and 99.2% in the *Joostella - Alcanivorax* and *Joostella - Pseudomonas* consortium, respectively (Fig. 6b). No statistical differences in BE percentages were highlighted among pure culture and consortia. Conversely, a significant difference among BE values was recorded ($p = 0.02$) in both pure culture and consortia.

3. Discussion

The discovery of new tensioactive agents, or new bacterial genera able to produce them, is an important goal for the application of bioremediation treatment, which destroys pollutant hydrocarbons occurring in a certain environment and does not allow the contaminants to accumulate (Banat et al., 2000). According to this, in consideration of the potentialities previously showed by *Joostella* strains (Rizzo et al., 2013, 2014, 2015), we decided to investigate more in depth the abilities of most promising isolates. This study was aimed at evaluating such individual abilities in comparison with two other BS-producing bacterial strains, *i.e. Alcanivorax* strain A53 and *Pseudomonas* strain A6, possessing distinct features in hydrocarbon degradation and/or BS production, as well as when growing in consortia. *Alcanivorax* spp. exhibit growth profiles that are highly restricted to certain aliphatic and aromatic hydrocarbons (Schneiker et al., 2006), and significantly contribute to the degradation of oils in natural environments, producing a large fraction of the microbial biomass after a spill event. On the other hand, *Pseudomonas* spp. are well known BS producers in the presence of a wide range of possible carbon sources (Chen et al., 2007).

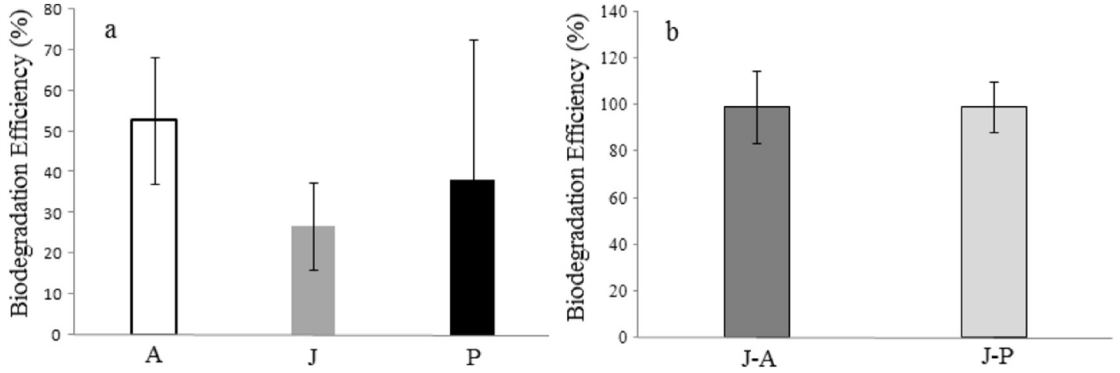

Fig. 6 – Biodegradation efficiency of bacterial strains grown in (a) pure cultures and (b) consortia. A, *Alcanivorax* strain A53; J, *Joostella* strain A8; P, *Pseudomonas* strain A6; J-A, *Joostella* plus *Alcanivorax* consortium; J-P, *Joostella* plus *Pseudomonas* consortium.

Despite changes in microbial surface hydrophobicity is one of the strategy used in BS adsorption described in literature (Chrzanowski et al., 2009), only a limited number of experiments have highlighted a correlation between cell hydrophobicity, biodegradation efficiency and BS production (Franzetti et al., 2009; Obuekwe et al., 2007). Contrastingly, some reports established that the changes in cellular properties may not be necessarily associated to biodegradation ability (Chakraborty et al., 2010). In this work, diesel oil was used as a carbon source, as it is a complex hydrocarbon combination deriving from the distillation of crude oil having carbon numbers approximately from C9 to C20, such as paraffin, olefins, naphtha and aromatic compounds. This oil mixture represents an excellent substrate in the study of hydrocarbon biodegradation due to its composition (Bicca et al., 1999). The results allowed observing a relation between hydrophobicity changes and interface activities, such as surface tension reduction and emulsifying activity. The highest affinity to DO, as it was determined by the BATH assay in pure cultures, was shown by *Joostella* strain A8, even if the reduction of surface tension operated by such strain (11.57 mN/m) was lower than those observed for both *Alcanivorax* strain A53 and *Pseudomonas* strain A6 (19.8 and 17.7 mN/m, respectively).

Two different screening tests were performed for the emulsifying activity to distinguish the possible bacterial production of emulsions from the ability to produce stable emulsions. The production of emulsion with stability over time in addition to the emulsifying activity alone could give an idea of the BS strength (Satpute et al., 2010) or its concentration (Walter et al., 2010). Despite the different hydrophobicity rates, all strains showed emulsifying activity values higher than 50%. This is in agreement with other authors, who reported how both hydrophilic and hydrophobic bacteria were able to produce surfactants, also when grown on glucose or hexadecane (Bouchez-Naitali et al., 1999). *Joostella* strain A8 was the best stable emulsion producer as it produced stable emulsion during the entire incubation time, starting after only 96 hr with a E_{24} index of 62.67%, and a maximum value that was reached during the exponential phase (78.33% at T_{288}). *Pseudomonas* strain A6 produced higher E_{24} values than *Alcanivorax* strain A53, whereas higher hydrocarbon affinity was highlighted for this latter. This difference is not necessary to be attributed to the BS potentiality, but probably to the higher cellular biomass produced by *Pseudomonas* strain A6.

In summary, the parallel trend in hydrophobicity and stable emulsion percentages observed especially for *Joostella* strain A8 seem to confirm that cell surface properties are important factors affecting hydrocarburic substrate biodegradation, and could be linked to BS production. Our results showed that bacteria in monoculture interact with hydrocarbons by emulsification rather than interfacial action on the surface tension, as supported by Amiriyan et al. (2004), who recorded different results depending on the type of hydrocarbon used for the E_{24} detection. Moreover, here a correlation between emulsifying activity and hydrophobicity was found, but not between hydrophobicity and surface tension reduction. This was probably due to a different possible mechanisms operated by our bacterial strains, which could produce molecules that act with a reduction of the interfacial tension between immiscible liquids or at the solid–liquid interface (low-molecular-mass molecules) and molecules that usually exhibit emulsifying capacity but without a necessary surface tension reduction (high-molecular-mass molecules) (Batista et al., 2006).

The flow cytometry analysis furnished more details about the growth dynamics of each strain in relation to the hydrocarbon degradation. Pure cultures were characterized by an underestimation in cell counts if compared with counts obtained by DAPI staining. This result was probably dependent on the formation of bacterial aggregates on hydrocarbon droplets, thus resulting too large in size to be properly detected by the cytometer. However, this underrating was lower in *Alcanivorax* strain A53 and *Pseudomonas* strain A6 compared to *Joostella* strain A8 after 480 hr of incubation. In the case of *Alcanivorax* strain A53, this was probably due to the more efficient and rapid degradation of DO by such strain (up to 53%) which caused a decrease in the cell aggregate number, thus allowing to a more precise bacterial counts. The degradation ability of *Alcanivorax* strain was an expected result, due to its well-known specialization in the use of aliphatic and aromatic substrates (Yakimov et al., 1998). The biodegradation efficiencies showed by *Joostella* strain A8 and *Pseudomonas* strain A6 when grown in pure cultures were comparable, differing only of about 10%.

After investigating individual potentialities of the bacterial strains used in this study, *Joostella* strain A8 was grown in co-cultures with *Pseudomonas* strain A6 or *Alcanivorax* strain A53 in order to establish if they might have reciprocal advantage in substrate degradation thanks to the involvement of different metabolic abilities. Overall, *Joostella* strain A8 seemed to produce more efficiently BS when grown in mono-culture than in co-cultures in terms of emulsification activity. Conversely, interfacial activity by mean of surface tension reduction was higher in consortia (22.9 and 26.8 mN/m for J-P and J-A consortium, respectively) than in *Joostella* mono-culture (15 mN/m). With regard to hydrocarbon affinity, only the hydrophobicity percentages obtained from the J-P consortium were comparable with those obtained from *Joostella* pure culture.

Hydrocarbon-degrading bacterial consortia exist in nature and are well settled in polluted areas, where they assume a strong competitiveness towards autochthonous bacterial communities. In the present study, different results were obtained for the two consortia. Owsianiak et al. (2009) suggested that in microbial consortia the surfactant-mediated biodegradation effectiveness is dependent on the specification of microorganisms and not on the type of surfactant, as confirmed by results here reported. In fact, we observed that the initial hydrophobicity percentages were different between strains in pure culture and comparable to the emulsion trend for each of them. By observing the same parameters in co-cultures, the trends appear to oscillate, probably proving the different approach of the single strain in the consortia. For this reason, in agreement with Owsianiak et al. (2009), the cell surface hydrophobicity is not an optimal descriptor of biodegrading potential for mixed cultures, but could be a useful tool in support of other parameters observation, for studying the bacterial activity and for comparing it in single and mixed cultures.

Joostella represents a sort of *new entry* in this debate, and the consortium acquires a great importance because allow to

understand if this genus may be an optimal potential BS producer also in cooperation with bacteria belonging to other genera. Our results allow to suppose that the metabolic patterns and capacities of *Joostella* and *Alcanivorax* are probably not compatible, and had as resultant response in a loss of efficiency in BS production by the single strain when grown in co-culture. These findings support a change in the bacterial approach of degradation, as suggested by a surface tension reduction near to 30 units in mixed culture, and so a possible increase of interfacial activity of isolates when inoculated together with other strains.

As it was reported by Antoniou et al. (2015), bacteria could also act mainly as hydrocarbon degraders rather than BS producers in mixed cultures. This was confirmed by results obtained for the estimation of biodegradation efficiency that resulted higher (near 99%) in both consortia and comparable between J-P and J-A. Co-culturing did not seem to produce a strong inhibitory interaction between strains. The lower values of bacterial counts for individual strain in co-cultures than in pure cultures, as it was determined by FISH, could be explained with a reduction in the availability of space and carbon source. Finally, the strong occurrence of aggregate in the case of consortia culture made impossible the use of results, but suggests a probable stronger attack of bacteria towards the hydrocarburic substrate.

4. Conclusions

Joostella strain A8 was confirmed as a promising candidate for possible application in the bioremediation of hydrocarbon contaminated sites, both in pure and mixed cultures. It showed excellent performances in all the screening tests, sometimes showing a better efficiency respect to the other two tested strains. Lower values of emulsification indices that were measured in the consortium experiment rather than in pure cultures were counterbalanced by a more efficient biodegradation capability. This led to suppose that in the course of the experiment, a positive and beneficial interrelationship between strains occurred, suggesting that a sort of "functional complementation" between strains might exist. The benefits deriving from cooperative strategies may be very complex, and could include the removal of toxic metabolites with potential inhibitory action, or specie-specific attack of different hydrocarbon moieties. The strong competitiveness of *Joostella* strain A8 in pure culture encouraged new further insights for the optimization of BS-production on industrial scale, and for testing any possible additive uses of its BS. Additionally, further insights are envisaged to characterize qualitatively and quantitatively the BSs produced by strains in pure culture and co-culture.

Acknowledgments

The authors wish to thank Rossana Rando (Department of Environmental Science, Safety, Territory, of Food and Health, University of Messina) for residual hydrocarbon analysis.

This research was supported by the Doctoral School in "Scienze Ambientali: Ambiente Marino e Risorse" of the University of Messina, Italy. Carlos Rochera is a recipient of a VALid postdoctoral contract of Generalitat Valenciana APOSTD/2015/088.

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