Environmental Engineering and Management Journal *May 2018, Vol.17, No. 5, 1209-1216*
http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu

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GAS HOLD UP IN THE CULTIVATION OF A PETROLEUM-DEGRADING BACTERIAL CONSORTIUM

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Abstract

The hydrodynamic behavior of bubble column bioreactors (BCB) is strongly dependent on the bubbly flow regime. Therefore, the influence of superficial gas velocity (*Ug*) and Sauter mean diameter (d_{32}) on gas hold up (εg) was evaluated for the cultivation of a petroleum-degrading consortium. Hydrodynamic parameters were determined by photographic techniques. Also, the biomass cultivation was quantified by suspended solids formation (SS). Our findings indicated that the d_{32} increased at high *Ug* values (0.9-1.2 cm s^{-1}), but decreased with the presence of Tween 20 surfactant (0-0.15 mL L^{-1}) in the model medium. An enhancement in the εg was observed at high *Ug* values (1.0-1.3 cm s^{-1}). Interestingly, εg values ranging from 0.02 to 0.024 reported a high concentration level of SS (8-10 g L^{-1}) during the hexadecane degradation. Particularly, εg value of 0.024 was a convenient level to cultivate the consortium resulting in changes in bacterial population distribution, due to oxygen and hydrocarbon bioavailability. According our results, the εg is proposed as key factor related to mass transfer phenomena and agitation on the cultivation of petroleum-degrading consortium.

Key words: biodegradation, bubble column bioreactor, consortium, gas hold up, petroleum

Received: August, 2013; Revised final: August, 2014; Accepted: September, 2014; Published in final edited form: May, 2018

1. Introduction

The degree of water and soil deterioration has increased due to petroleum industry development (Bridjanian and Samimi, 2011; Preda et al., 2018). Despite benefits that petroleum industry provides, the society recognizes the serious global environmental issue due to the regular use of it (Xhelilaj and Sinanaj, 2010). Exploitation of petroleum wells, uncontrolled spills and improper disposal procedures of petroleum hydrocarbons are among the main reasons of the problem. Particularly, hydrocarbons are insoluble compounds they are widely studied due to constant persistence, environmental impact and their economic importance (Saval, 2000). The spilled petroleum is susceptible to bacterial degradation processes and several technologies of cleaning have been developed to remediate contaminated sites (Dou et al., 2016; Gong, 2012; Hasinger et al., 2012; Saraç et al., 2017). Therefore, the use of petroleum-degrading consortia has been proposed as an alternative of bioremediation. During the hydrocarbon biodegradation usually is required the cooperation of several microbial species with different metabolic capabilities, since the use of

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microbial consortia enhances the consumption rate of hydrocarbons (Ghazali et al., 2004). In the consortia, some strains possess the genotype to degrade hydrocarbons and others, the ability to produce biosurfactants (Huang et al., 2017; Saravanan et al., 2009). Interestingly, Tzintzun-Camacho et al. (2012) observed an enhanced hexadecane removal with a bacterial consortium (composed by four strains) due to both direct contact and biosurfactant-mediated uptake mechanisms. Based on these properties, the bacterial consortium could be cultured in BCB in order to increase the cultivation level.

Therefore, several operation criteria have been studied to produce or scale up the petroleum-degrading bacterial consortia for example volumetric oxygen transfer coefficient (k_La) (Medina-Moreno et al., 2005) or hydrocarbon transfer rate (Lizardi-Jiménez et al., 2012). Nevertheless, these mass transfer criteria are related to other hydrodynamic parameter, the simplest but macroscopic is the εg defined as the volume fraction of the dispersed gas phase (Veeraa et al., 2004). Particularly, εg is strongly related to air bubble diameter and it is determined by d_{32}. Large bubbles have higher rise velocity than small bubbles, therefore residence time of large bubbles decrease and cause to decrease rate of increasing εg (Moshtari et al., 2009). In addition, the εg depends on the Ug, i.e. as the Ug is increased the εg increases and at a certain Ug, coalescence of the bubbles takes place to produce the first fast-rising large bubble (Moshtari et al., 2009). Basically, the Ug and d_{32} have a strong influence on the hydrodynamic behavior in BCB. The importance of εg, related to the mass transfer phenomena and agitation is well documented in the literature (Mehrnia et al., 2005; Veeraa et al., 2004). However, there are few reports of its use in petroleum-degrading bacterial consortium cultivation processes in BCB, where oxygen and hydrocarbon bioavailability are necessary.

Therefore, the aim of this work was to evaluate the influence of Ug and d_{32} on εg, proposed as a criterion for the cultivation of a petroleum-degrading consortium, able to remediate polluted water and soil ecosystems.

2. Material and methods

2.1. Culture medium

Biodegradation assays were conducted using a mineral medium with the following composition (g L^{-1}): 6.75, $NaNO_3$ (J. T. Baker, 99. 9%); 2.15, K_2 HPO (J. T. Baker, 99.3%); 1.13, KCl (J. T. Baker, 99.9%) and 0.54, $MgSO_4 \cdot 5$ H_2O (J. T. Baker, 100.1 %). Hexadecane (HXD) was used as sole carbon source (13 g L^{-1}) and the initial pH was adjusted to 6.5, with HCl 1.0 N. Samples were analyzed in triplicate.

2.2. Bacterial consortium

Members of a bacterial consortium were isolated previously from an oil-contaminated site in Veracruz, Mexico and identified as *Xanthomonas* sp., *Acinetobacter bouvetii*, *Shewanella* sp. and *Defluvibacter lusatiensis* (Tzintzun-Camacho et al., 2012). The bacterial consortium was chosen due to enhanced degrading ability when the four strains were combined as compared to single strains. In addition, *A. bouvetti* was revealed as a biosurfactant producer, which increased the hydrocarbon degradation. All bacterial isolates were deposited in the ENCB-IPN WDCM449 culture collection and were registered with the following GenBank accession numbers: HQ424441-HQ424444.

2.3. Abiotic medium

References values of surface tension (σ) (50-65 dynes cm^{-1}) were tested according to Lizardi-Jiménez et al. (2011), by adding different Tween 20 concentrations (0-0.15 mL L^{-1}) and 13g L^{-1} of HXD. A Manual Fisher Surface Tensiometer Model 20 (Fisher Scientific International, Wisconsin, USA) was used to measure changes in σ. Viscosity (μ) was determined by using a viscometer Physica MCR Model 300 (Sttuttgart, Germany).

2.4. Bioreactors

A 10-L bubble column bioreactor (BCB) was used to determine hydrodynamic parameters. BCB cylindrical vessel was made of Pyrex glass (0.14 m diameter x1.0 m height). Airflow was sparged through the BCB with L-shaped air diffusor (stainless steel ¼ inch internal diameter; 7 orifices, 1.0 mm diameter). A second BCB (1-L) with the same specifications was used to determine changes in the population distribution within the bacterial consortium.

2.5. Analytical methods

2.5.1. Hydrodynamic parameters

Sauter mean diameter (d_{32}) and gas Hold Up (εg) were measured according to the method proposed by Ribeiro and Lage (2004), using a digital camera (Pentax Optio 50 model 5 MP, USA). A serie of photographs of the BCB (without aeration) were taken using a solid reference located in different parts of BCB at different time. Lengths were determined by means of a Software Image Analyzer Image-Pro Plus 4.1 (Media Cybernetics, USA). Due to the curvature of the BCB, a correction factor placed reference in the central and lateral bioreactor zones was used to avoid measurements inaccuracy. All determinations were conducted in triplicate and different superficial gas velocity values (Ug) were tested. In the case of d_{32}, measurements of series of bubble diameters were conducted. The εg values were determined according to the method of expanded bed (Prakash et al., 2001), which measures height difference among the unaerated liquid and aerated liquid applying (Eq. 1).

$$\varepsilon g = \frac{H_t - H_s}{H_t} \quad (1)$$

where: *Hs* is the height of unaerated liquid, and *Ht* is the height of aerated liquid.

A flexible plastic tape with a width of 1 mm was placed along the unaerated liquid level which was used to determinate *Ht-Hs*. All determinations were carried out using two different liquid phases: water and the model medium (*εg* was also determined in the biotic environment).

2.5.2. Suspended solids

Samples (10 mL) from BCB were centrifuged (J2-HS, Beckman, USA) at 4000 x g for 30 min at 4°C. Three phases were formed: HXD, aqueous and solid phase. The suspended solids (SS), including the petroleum-degrading consortium, were determined in the solid phase after heating in a low-pressure oven at 60°C for 48h (Duo Vac, Lab-line Inc. Instruments, USA). The SS fraction trapped in the HXD phase was recovered by three successive extractions, as described above. The organic phase, including residual HXD was pooled and stored at 4°C in 30 mL vials.

2.5.3. Residual hexadecane

The residual HXD was determined by gas chromatography (Varian, Star 3900 GC, California, USA) with a flame-ionization detector. An AT-1HTcolumn was used (15m x 0.25 mm x 0.10 mm, Alltech Heliflex, Illinois, USA) with helium as the carrier (30 ml min^{-1}; 40 psi). The injector and detector temperatures were constant at 290°C and 300°C, respectively. The oven was heated to 120°C (30°C min^{-1}), then the temperature was raised to 150°C (10°C min^{-1}) and 170°C (15°C min^{-1}). A 2 mL sample volume was injected and detection limit for HXD determination method was 0.003 g L^{-1}.

2.5.4. Counting bacteria

The variations of bacterial consortium growth were analyzed in a BCB (1-L) working for a period of 10 days. Bacterial strains were enumerated by plate count method using trypticase soy agar. Serial dilutions were performed using sterile saline solution, particularly; a factor dilution of 10 was used. A vortexing time of 2min was considered in each dilution to disaggregate the bacteria. The dishes were incubated at 30°C and colonies were quantified after 48 h. The counted bacteria were expressed as colony forming units (CFU).

3. Results and discussion

3.1. Hydrodynamic parameters

3.1.1. Sauter mean diameter (d_{32})

In order to estimate some hydrodynamic parameters in the BCB, the d_{32} was determined as a function of *Ug*, both in water and model medium (Eq. 2).

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (2)$$

where: n_i is the class for bubble diameters and d_i is the diameter for each particular class.

As shown in Fig. 1, d_{32} values for *Ug* ranging from 0.2 to 1.2 cm s^{-1} shown to be higher in water than d_{32} values in model medium. Variations on d_{32} could be explained by the surface tension, for example Kantarci et al. (2005) reported that the d_{32} increased with changes in the surface tension. In our study, the surface tension was higher in water than the medium model (76 dynes cm^{-1} and 50.5 dynes cm^{-1}, respectively) suggesting that the water acts as a coalescing medium. Also, the viscosity of liquid phase has been correlated with changes in the d_{32} (Li and Prakash, 1997). Similar results were observed in our work, the viscosity of the model medium was higher than water decreasing the d_{32} values.

3.1.2. Gas Hold up (εg)

3.1.2.1. Sensitivity of the photographic technique

Sensitivity of photographic technique must consider error between each *εg* measure, for instance if the mean standard error (MSE) is 0.001 (*εg* units) it would be an error to report a value of 0.031 and 0.032 as two different measures.

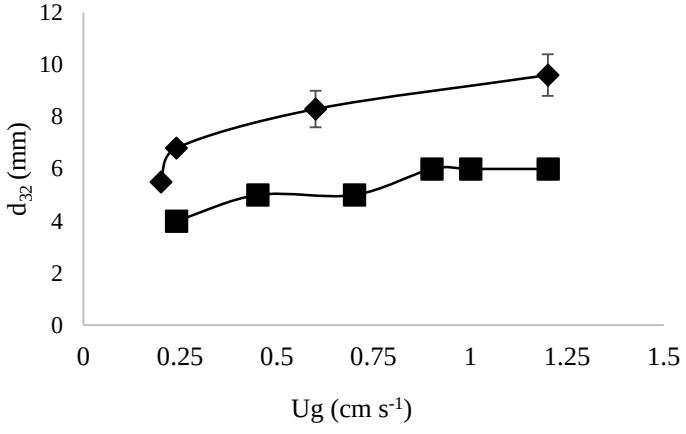


Fig. 1. Variations on d_{32} as *Ug* function in two liquid media: water (♦) and model medium (■)

In order to determinate the MSE for εg, a standard error was obtained by measuring εg for each value of tested *Ug* in the biotic medium (Table 1). The determination of εg is harder to ensure in the water and model medium, due to the sensitivity is similar in these media.

Table 1. Standard error for εg determinations at different *Ug* values

Ug (cm s^{-1})	***εg (measured)***	***Standard Error***
0.24	0.0155	0.0018
0.45	0.0211	0.0007
0.62	0.0217	0.0012
0.88	0.0236	0.0015
0.93	0.0280	0.0010
0.96	0.0319	0.0004
0.98	0.0369	0.0017
1	0.0403	0.0006
1.1	0.0417	0.0026
1.32	0.0522	0.0022
1.67	0.0588	0.0012

t-student test was applied to determine the MSE, using the NCSS 2000 software. It proposed a series of hypotheses to determine the confidence of the technique for the determination of εg. It was proposed as a null hypothesis (Ho) that is greater than 0.0017 MSE (units) and as an alternative hypothesis (Ha) the MSE is equal to or less than 0.0017 (units). Then, the set of hypotheses was: Ho: MSE > 0.0017; Ha: MSE $\leq$ 0.0017. To test the statistical hypothesis, it is defined t_0 (Eq. 3):

$$t_0 = \frac{\sqrt{n}}{S}(\overline{X} - MSE) \quad (3)$$

t_0 = -1.44, where *n* is the sample size of a population, *MSE* is the mean standard error and unknown standard deviation. $\overline{X}$ and *S* are the mean and standard deviation of the sample, respectively. Based on 10 degrees of freedom and a significance level of 10% (α= 0.1) the critical value ($t_{\alpha, n-1}$) is t 0.1, 10 = -1.37, the statistic t_0 falls in the rejection region, then rejected the null hypothesis, experimental evidence indicates that the *MSE* is less than 0.0017 (units). Then the sensitivity analysis of the proposed technique showed that to be discernible must be at least 2 x 0.0017 = 0.0034 (units of εg) away, this means that in BCB 1.5 L operating volume (and height *H*, 30 cm), the separation between two to be discernible must be at least 0.0102cm (0.0034 x 30cm).

3.1.2.2. Comparison between water and model medium

The εg value is a comprehensive approach which reflects the hydrodynamic behavior of the BCB, several factors can affect it for example superficial gas velocity (*Ug*). In this study, the εg value was measured by a photographic technique (differences of up to 0.0034 units are detected, which in the 1.5 L-column equals 0.102 mm). Variations in the εg between water and the medium model were tested at different *Ug* values (Fig. 2).

In the case of 0.7 cm s^{-1}, similar results were observed both in water and model medium. For larger *Ug* values the model medium shows higher values of εg. This can be explained by coalescence phenomena, more pronounced in the case of water, from the *Ug* value of 0.7 cm s^{-1}, whereas the model medium leading to the formation of small bubbles.

3.2. Suspended solids formation and HXD biodegradation

To determine the effect of εg variation on the cultivation of bacterial consortium (expressed as SS), kinetics of SS formation and HXD biodegradation were analyzed. The BCB was operated in batches of 14 days and the inoculum concentration was 0.4 g L^{-1} SS. As shown in Fig. 3, the maximum concentration of SS was observed at εg values ranging from 0.02 to 0.06.

These results suggest that the phenomena of mass transfer (k_La), stirring and mixing (*Re*) are very similar in this range and could be used for scaling. This implies that although higher values of εg assume greater fraction of air in the column, no limitation of oxygen was observed in the low *Ug* values (from 0.02 to 0.06).

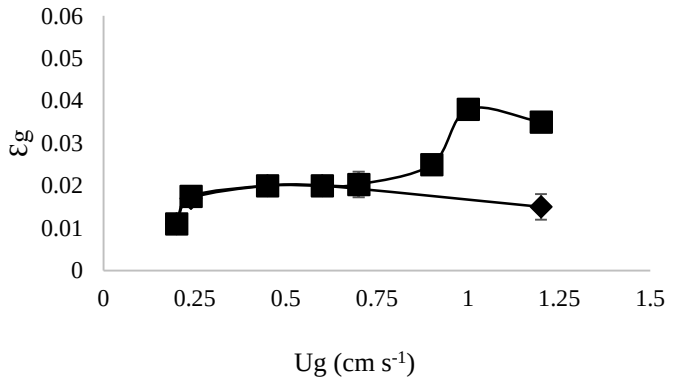


Fig. 2. Variations on εg at different *Ug* values: water (♦) and model medium (■)

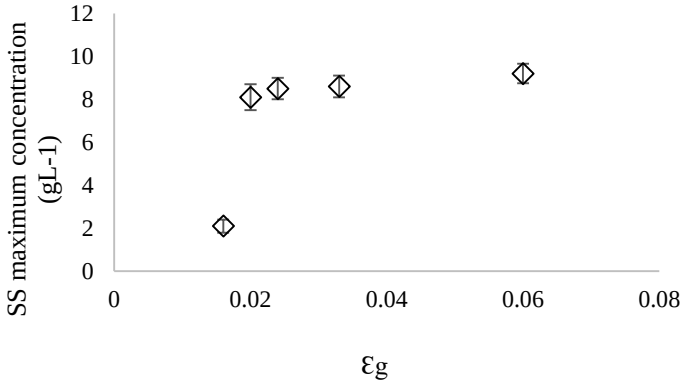


Fig. 3. Maximum concentration of SS for tested *εg* values

Profiles of SS (including petroleum-degrading bacterial consortium) were determined during over culture time, as a result the SS cultivation increased as *εg* increased (Fig. 4a). A phase of adaptation was observed for *εg* values ranging from 0.02 to 0.033. Also, the exponential growth phase was attained at day 3 and the stationary phase occurred after 7 days of culture. In contrast, for low *εg* values (0.016) the SS cultivation was below to 2 g L^{-1}; the low aeration and agitation levels in the BCB are the most probable causes of this level of cultivation. Although for high *εg* values (0.06) the stationary phase was shortened, the SS cultivation decreased after 7 days. Probably, higher levels of *εg* improve the mass transfer and agitation-mixing (generating smaller droplet sizes and HXD air bubbles), but the HXD consumption for SS cultivation could be limited.

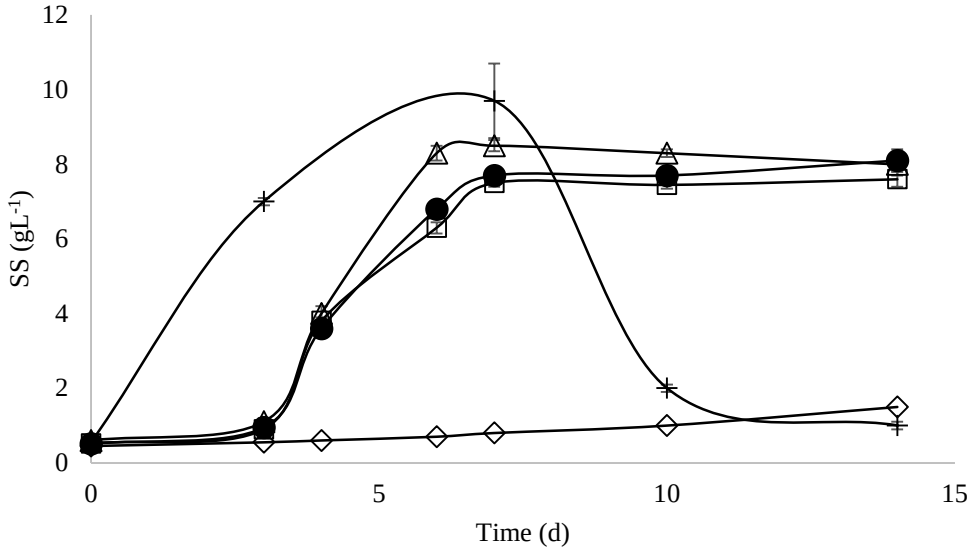
As shown in Fig. 4b, the HXD biodegradation was consistent with the SS cultivation, due to the fast depletion of HXD with high values of *εg*. Particularly, the lowest biodegradation was observed for *εg* of 0.016 and differences between 0.02 and 0.024 were not observed. Interestingly, the HXD biodegradation was completed after 14 days (*εg* values of 0.033 and 0.06). The efficiency of the bacterial consortium depends on the interaction among the species involved in the process. For example, Sugiura et al. (1997) reported 60% of biodegration of a mixture of crude petroleum by bacterial consortium, which corresponds to *εg* value of 0.016 in our findings. Additionally, experimental rates of HXD biodegradation were obtained from slopes between each of point of Fig. 4b, *i.e.* a ratio was made between variations in HXD concentration and the time between each sampling. The highest rate of biodegradation (about 4 g $L^{-1}d^{-1}$) was obtained for *εg* value of 0.033 (day 4), followed by *εg* of 0.06, about 3 g $L^{-1}d^{-1}$ (day 3). At lower *εg* the rate decreased significantly and the rates approached to zero at the end of the culture time, for all *εg* tested. According to previous reports, Díaz-Ramírez et al. (2003) found rates of 2.4 g $L^{-1}d^{-1}$ during the biodegradation of crude petroleum fractions. Differences in both studies could be explained by the type substrate and the type of agitation and aeration system tested. Díaz-Ramírez et al. (2003) used serological bottles agitated at 150 rpm. Possible mass transfer problems and limitations of oxygen and HXD could explain the results obtained (Lizardi-Jiménez et al., 2012). Regarding to hydrocarbon biodegradation in BCB, Quijano et al., (2010) obtained an overall rate of 1.2 g L^{-1} d^{-1} for a 10 day kinetic. However, in his work the hydrocarbons initial and final concentration were used to calculate the overall hydrocarbon biodegrading rate including a stationary phase reached after day 5. In our study, the SS formation rate (calculated between sampling points) reached a maximum between 3 and 4 days, for *εg* of 0.02 and 0.033 (2.7 g L^{-1}). Similar results were obtained for *εg* of 0.024 since significant differences were not observed. The SS yield (g SS per g HXD) is a fundamental variable in our process.

As a result, the highest yield was reached for *εg* of 0.33, however for *εg* values of 0.016, 0.02 and 0.024 reached similar yield values. Particularly, *εg* of 0.016 did not reach a competent SS final concentration and *εg* of 0.033 just reached a higher yield at 6 days. Conversely, *εg* value of 0.02 and 0.024 maintained high yield values for more time.

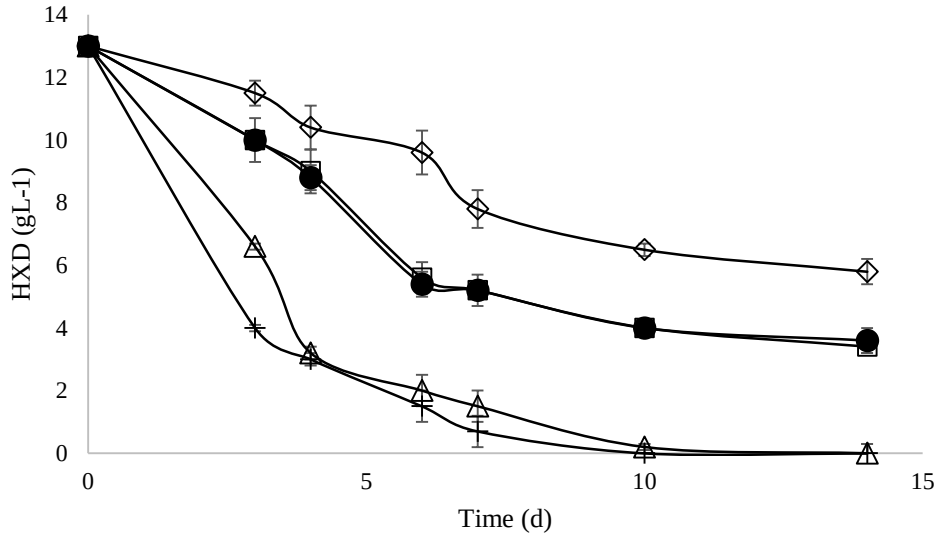
In brief, *εg* of 0.02 and 0.024 are convenient *εg* levels for petroleum-degrading consortium cultivation and with the aim of understand better the bioprocess a distribution of bacterial population was evaluated for each one: *εg* of 0.024 (Fig. 4a and Fig. 4b).

3.4. Distribution of bacterial population

Based on *εg* of 0.024, variations in the bacterial growth were determined during 10 days of culture. Conversely, the growth of *A. bouvetii* was higher than *Xanthomonas* sp. after 7 days. Interestingly, the population distribution changed at the end of culture period; *D. lusatiensis* reached a maximum value ($4.9\pm0.5x10^8$ CFU mL^{-1}). Particularly, the growth of *Shewanella* sp. was below $1x10^6$ CFU mL^{-1} during all culture period. Our findings suggest that changes in the population distribution could be explained by cooperative metabolic activities among the bacterial consortium members. According to previous studies, Tzintzun-Camacho et al., (2012) showed that HXD degrading ability of *A. bouvetii* was higher than *Xanthomonas* sp. and *D. lusatiensis*, also *A. bouvetii* was able to produce biosurfactants.



(a)



(b)

Fig. 4. Kinetics of SS formation (a) and HXD biodegradation (b) for different εg values: 0.016, (◊); 0.02, (□); 0.024, (●); 0.033, (Δ) and 0.06, (+)

In our work, both the HXD biodegradation and SS cultivation for εg values tested could be related to the HXD degrading ability of strains and oxygen bioavailability. In the case of εg 0.024, the HXD biodegradation rates increased due to predominance of cultures *Xanthomonas* sp. and *A. bouvetii* (period between 3 to 7 days). At the same time, biosurfactant capacity to pseudosolubilize and emulsify HXD was determined suggesting that biosurfactant-mediated uptake took place in the HXD removal (data not shown). Also, the growth enhancement of *D. lusatiensis* at the end of culture could be explained by biosurfactant degradation produced by *A. bouvetii*. Synergistic interactions whithin consortia during hydrocarbon biodegradation have been reported. For example, Prpich and Daugulis (2005) found synergistic benefit within a consortium consisted of two *Pseudomonas* sp., and two *Acinetobacte*r sp. during phenol biodegradation in a solid–liquid two phase partitioning bioreactor. Moreover, other studies reported that the rate of degradation of crude petroleum constituents depends on the activity and the dynamics of the different phylogenetic taxa consortium (Zrafi-Nouira et al., 2009). In our work, the population interactions among bacterial cultures were related to their degrading abilities. Also, εg value of 0.024 was a convenient level to produce smaller droplets of oxygen and increased its bioavailability resulting in the changes in population distribution. As shown in Fig. 5, similar growth profiles were observed at beginning of the culture, *Xanthomonas* sp. was predominant after 5 days.

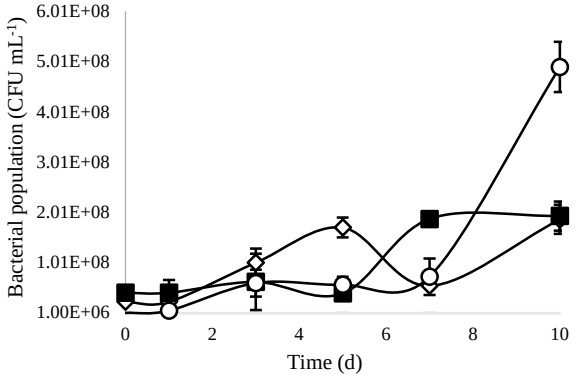


Fig. 5. Changes in the population distribution within the bacterial consortium during HXD biodegradation. Cultures: *Xanthomonas* sp., (—◊—); *A. bouvetii*, (—■—); *Shewanella* sp., (—⁎—); *D. lusatiensis*, (—○—)

5. Conclusions

The gas hold up (εg) showed an important effect on the cultivation of petroleum-degrading bacterial consortium. The highest concentration level of SS was reached for εg between 0.02 and 0.06. Particularly, εg value of 0.024 showed competent yields and was selected for distribution of bacterial population experiments. εg of 0.033 reached a high yield after six days. Interestingly, εg values of 0.02 and 0.024 maintained high yield values for more time. Also, εg value of 0.024 was a convenient level to produce the consortium and was a key factor for the changes in bacterial population distribution.

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