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Biosurfactants Production During Diesel Biodegranation by Mixed Microbial Consortia Selected From Polluted Spolls

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ABSTRACT: This work studies the biosurfactants production which enables the diesel biodegradation by using mixed microbial consortia from polluted sites. It was carried out by culturing three microbial consortia (named as XA, XB and XC) obtained from polluted soils, and enriched in diesel as sole carbon source.Batch experiments were done to study the effects of three variables (temperature,hydrocarbon concentration and the origin of the consortia) on the diesel biodegradation and the surface tension evolution. The three enriched consortia contained similar bacterial genera and degraded diesel with similar efficiencies (approximately 90%). Thermal inhibition was observed at 35 ºC. The evolution of surface tension was similar in all experiments: an initial fast reduction followed by an increase once the diesel had been consumed. All three consortia were found to be efficient biosurfactants producers. Consortia XB and XC had similar low biosurfactant yields (1.3 and 1.8 g g^{-1}, respectively) and lower critical micelle concentration values (0.42 and 0.45 g L^{-1}, respectively), while XA generated a greater quantity of biosurfactants (6.9 gg^{-1}). It was noted that the maximum diesel biodegradation rate increased versus the biosurfactants yields. Despite some differences between the consortia due to their different origins, especially concerning biosurfactants production, the diesel-enrichment process resulted in adapted consortia with similar efficiencies for diesel biodegradation.

Key words: Surface tension, Hydrocarbon, Diesel-enrichment process, Thermal inhibition, Critical micelle concentration

INTRODUCTION

The contamination of soil and groundwater with petroleum hydrocarbons is unfortunately a common phenomenon and causes serious environmental problems. Bioremediation is considered to be one of the best approaches for restoring hydrocarbon-contaminated soils and wastewater because the technology is cost-effective and environmentally benign (Menezes Bento *et al*., 2005).

The success of a bioremediation technology is also dependent upon a microbial ability to access the complex hydrocarbon mixtures (Margesin&Schinner, 2001), which are compounds with low water solubility and thus not readily available to microorganisms. Because of this, bacterial consortia display a wide array of metabolic mechanisms for coping with the breakdown of oil components including the production of surface-active agents and emulsifiers (Willumsen&Karlson, 1997). These agents are small surfactant molecules with a hydrophilic portion and a hydrophobic one, and this complex structure provides them with amphipathic properties enhancing the bacterial growth and the bioremediation rate.

Surfactants can be synthetic, but in many cases it has been shown that biosurfactants are more effective than chemical surfactants in increasing bioavailability of hydrophobic compounds (Wong *et al*., 2004). Additionally, biosurfactants are less toxic and generally have higher biodegradability, better environmental compatibility, higher substrate selectivity and lower critical-micelle concentrations than the synthetic ones (Bordoloi&Konwar, 2009). Due to their low purification yields, they have not to date been commercially competitive with synthetic surfactants. However, recently several studies about isolation and characterization of biosurfactants and its application in bioremediation have been carried out (Bordoloi&Konwar, 2009; Nayak*et al*., 2009; Vasileva-Tonkova *et al*., 2011), showing that these substances can be easily "manufactured" and used in the improvement of bioremediation technologies.The biosurfactants production is related to the utilisation

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of available hydrophobic substrates by the producing microbes from their natural habitat, presumably by increasing the surface area and increasing their apparent solubility (Ron & Rosenberg, 2002; Maier, 2003; Das & Mukherjee, 2005).

The aim of the present work was to study thebiosurfactant generation that enables the treatment of wastewater contaminated with diesel hydrocarbons. This objective was carried out using three different microbial consortia, obtained from three hydrocarbon-contaminated soils, and adapted to the degradation of diesel. During batch diesel-biodegradation studies, the evolution of the surface tension (ST) was linked with the production of biosurfactants and the effects of three variables (temperature, hydrocarbon concentration, and the origin of the consortium) were tested.The work does not focus on the chemical identification of the biosurfactants obtained, but how the different variables could affect the production of them.

MATERIALS & METHODS

Three diesel contaminated soils (denoted as SA, SB and SC) were collected from different sites around a petroleum refinery in central Spain. The highest level of TPH (total petroleum hydrocarbons) was measured,according to UNE-EN 14039:2005,in SB (770.4 mg/kg) and the other two soils, SA and SC, had 16.6 and 25 mg/kg, respectively. The pH values were 8.01, 8.46 and 8.26 and the electrical conductivity values were 342µS/cm, 366µS/cm and 497µS/cm, for SA, SB and SC, respectively. The soil A was a sandy clay soil consisting of 40 % (w/w) clay, 15 % (w/w) silt, and 45 % (w/w) sand and the soils B and C were a sandy clay loam soilconsisting of 22.7 % (w/w) clay, 26.9 % (w/w) silt, and 50.4 % (w/w) sand. Different consortia of microorganisms contained in the soils (denoted XA, XB and XC) were isolated, maintained and enriched over several weeks, as described below.

Five grams of each soil sample were added to sterile flasks containing 50 mL of BH (Bushnell-Haass) basal-enrichment medium broth (BD, Franklin Lakes, NJ USA, ref. 257820), and the bottles were placed in an Ecotron incubator-shaker overnight at 50 rpm and 26ºC. The supernatants were inoculated at a ratio of 1% (v/v) into three new flask bottles containing sterile BH broth with 1 % (v/v) diesel as the sole carbon source. These consortia were maintained by weekly subcultivation and enrichment under aerobic conditions in the Ecotron incubator-shaker.

Serial dilutions (1/10) of the adapted consortia were grown in Luria Bertani agar plates with 25 g/L of glucose at 26 ºC. To characterise the isolates, their oxidase reactions, catalase reactions and morphology were determined, and a Gram stain test was conducted. Bacteria were identified with physiological test kits, according to Analytical Profile Index micromethods API 20 NE, API 20E and API Staph (BioMérieux, Lyon, France). The hydrocarbons used in this work were those contained in a conventional petroleum-derived diesel fuel from a petrol station in Ciudad Real, Spain. The density of the diesel was 832 g/L (EN-ISO 3675, 1998). Chain length of the *n*-alkanes was also identified using a diesel standard pattern (Absolute Standards, Inc. Hamden, Connecticut, USA) by comparing the retention times. The results indicated the presence of n-alkanes with chain lengths ranging from 10 to 26 carbon atoms and other typical compounds such as pristane and phytane. A total of 21 batch biodegradation experiments were carried out to determine the diesel-biodegradation abilities of the different consortia (Table 1).

Table 1. Batch biodegradation experiments

C_0 (% v/v)	Consortium	T (ºC)		
		25	30	35
0.5	Abiotic	x	x	x
	XA	x	x	--
	XB	x	x	--
	XC	x	x	--
1	Abiotic	x	x	x
	XA	x	x	x
	XB	x	x	x
	XC	x	x	x
3	Abiotic	x	x	x
	XA	x	x	--
	XB	x	x	--
	XC	x	x	--

Symbols: C0, initial diesel concentration; XA, consortium A; XB, consortium B; XC, consortium C; x, tested; --, not tested

The experiments were performed in 1-L Erlenmeyer flasks with rubber stoppers in an orbital shaker bath at 130 rpm. The dissolved oxygen concentration in the flasks was measured with an YSI 5000 oxygen probe and calibrated versus the shaking rate to ensure aerobic conditions.

Each flask contained 500 mL of BH broth and wereinoculated with 1% (v/v) of a microbial consortium. The influences of temperature (25, 30 and 35 ºC), and initial diesel concentration (C_0 = 0.5, 1 and 3 % v/v) were studied with the three different consortia. Aliquots of 10 mL were sampled in duplicate over 8 days to measure the evolution of diesel biodegradation, biomass growth and ST. Uninoculated control experiments were simultaneously carried out, with 500 mL of BH broth and each diesel concentration, to monitor abiotic losses of the substrate.

Microbial growth was determined by measuring the optical density (OD) of the culture medium at 600 nm, as described by Sadouk *et al.* (2008), using a Shimadzu UV-1700 spectrophotometer. A calibration curve ($r^2 = 0.976$) was prepared relating OD and total volatile solids concentrations, measured according to ASTM E 1755-01 (1991), in order to calculate biomass concentration (g/L).

Diesel was extracted from the 10 mL aliquots, using 2 mL of *n*-hexane, then analysed by gas chromatography to determinate the concentration ofTPH. The equipment used was a Thermo-Fischer Trace GC Ultra gas chromatograph equipped with a flame-ionization detector, where the hydrocarbons of the samples were separated in a micro Ultra Fast capillary column (5 m × 0.1 mm id × 0.4 µm). Qualitative analysis was performed using an *n*-alkane reference-calibration mixture (Absolute Standards Inc - Hamden, CT, USA) and calibration curves were prepared from serial dilutions for quantitative analysis.

STmeasurements were made on a Du Nouy tensiometer (Lauda TD2) calibrated with air and water to a reading of 71±1 mN/m (ASTM D971 – 99a, 2004). The values of ST were averaged from three replicate measurements with a 0.5 % deviation error.

Three additional experiments were carried out to determine the quantitative relationship between ST and the biosurfactants generation. Each experiment used one type of consortium (XA, XB or XC) and the remaining operating conditions, which were selected according to the best results obtained in the previous experiments. The experiments were made in 1-L Erlenmeyer flasks in an orbital shaker bath at 130 rpm and 25 ºC. Each flask contained 500 mL of BH broth with 1 % (v/v) diesel hydrocarbons and 1 % (v/v) inoculum. The three batch experiments were studied over 8 days to monitor the evolution of the ST and the concentration of biosurfactants. The concentration of biosurfactants was obtained by acid precipitation from the supernatant as described by Porsunthorntawee *et al.* (2008).

All samples for ST and biosurfactant recovery were performed in triplicate, and the results reported as the average of these three values.The removal of volatile biosurfactants was not quantified, but was likely to be small considering the generally high molecular weight of these compounds (Cassidy *et al.*, 2002).

RESULTS & DISCUSSION

Enriched cultures grew rapidly on diesel as the sole carbon source and became turbid after 24 h of incubation. After 3 months of successive transfers, several pure bacterial strains were isolated from each consortium; only eight were identified in each of them according to the Analytical Profile Index shown in Table 2. Microbes in the 3 consortia that are members of the same bacterial genera were identified, including *Staphylococcus lentus*, *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens*. The isolation of these bacteria is not surprising due to their frequency in soils; moreover, most of them have been shown to degrade hydrocarbons in previous studies (Chaineau *et al.*, 1999; Barathi&Vasudevan, 2001; Rahman*et al.*, 2002; Medina-Moreno *et al.*, 2005; Ueno *et al.*, 2007; Owsianiak *et al.*, 2009; Lafortune *et al.*, 2009) and some are well known to be biosurfactant producers (Banat, 1995).

Fig. 1 presents bacterial growth, the diesel degradation and the ST evolution over time.This chart applies only to the XA consortium with a 0.5 % (v/v) initial diesel concentration at a temperature of 30 ºC, but serves to illustrate the general behaviour of the three consortia under all the different conditions (except experiments at 35ºC). Allmixed bacterial consortia used diesel as the sole carbonsourcefor growth. Therefore, as the amount of biomass increased, the concentration of diesel was reduced, noting that the largest reduction corresponded to the exponential stage of bacterial growth.

As shown in Fig. 1, there was a rapid reduction in the ST (around 40 mN/m) at the beginning, which was related to the biosurfactants production to improve diesel accessibility and biodegradation (Desai& Banat, 1997). After approximately 30 h, during the exponential stage of bacterial growth, the ST increased considerably, to approximately 70 mN/m (the value of measure air and pure water), and then remained constant at that value during the stationary stage. It was assumed that, at the end of the exponential stage, the consortium metabolized the diesel and then also the biosurfactants produced, therefore increasing the surface tension. After this stage, the carbon source was completely consumed, so there was no further generation or elimination of biosurfactants and the ST remained constant. Lu *et al.* (2003) reported that the surface tensions of fermentation broths were related to the growth of bacteria. They also found a rapid reduction of surface tensions during exponential periods indicating that the production of biosurfactants might be active. Thereafter, the ST remained constant, suggesting that biosurfactants could be further metabolised by the bacteria.

Fig. 2 shows that the evolution of the diesel degradation was connected to the ST evolution in all experiments according to the previously explained behaviour (performed with the XA consortium). The

Table 2. Biochemical and growth characteristics of isolated bacterial cultures

Isolate type	Colour	Convex	Opaque	Margin (E=Entire; I=Irregular)	Cell morphology (R=Rod; C=Coccus)	Gram coloration	Oxidase	Catalase	Identification API	Genus	Species
XA1	Yellow	+	-	I	R	-	w	w	20 NE	NI	
XA2	Greyish	-	+	E	R	-	+	+	20 NE	*Rhizobium*	*radiobacter*
XA3	White	+/-	-	E	R	-	+	+	20 NE	*Aeromonas*	*sobria*
XA4	Orange	+/-	+/-	E	C	+	-	+	Staph	*Staphylococcus*	*lentus*
XA5	Cream	+	+	I	R	+	-	+	20 NE	NI	
XA6	White	+	-	I	C	-	-	-	20 NE	NI	
XA7	Cream	+	+	I	R	-	+	+	20 NE	*Ochrobactrum*	*anthropi*
XA9	White	-	+	E	R	-	+	+	20 NE	*Achromobacter*	*xylosoxidans*
XB1	Yellow	-	-	E	R	-	w	+	20 NE	*Burkholderia*	*cepacia*
XB2	White	+	+/-	E	R	-	+	+	20 NE	*Pseudomonas*	*fluorescens*
XB4	Yellow	+	-	E	R	-	-	+	20 E	*Stenotrophomonas*	*maltophilia*
XB5	Cream	+	+	E	R	-	+	+	20 NE	*Achromobacter*	*denitrificans*
XB6	Orange	+/-	+/-	E	C	+	-	+	Staph	*Staphylococcus*	*lentus*
XB7	Cream	+	+	I	R	-	+	+	20 NE	*Ochrobactrum*	*anthropi*
XB8	White	+	+	E	R	-	+	w	20 NE	*Sphingomonas*	*paucimobilis*
XB9	White	-	+	E	C	-	-	+	20 NE	NI	
XC1	Yellow	+	-	E	R	-	-	+	20 E	*Stenotrophomonas*	*maltophilia*
XC2	Cream	+	-	E	R	-	+	w	20 NE	*Sphingobacterium*	*multivorum*
XC3	Yellowish	-	-	E	R	-	+	w	20 NE	*Sphingobacterium*	spp
XC4	Yelllow	-	-	E	C	+	-	w	Staph	*Staphylococcus*	*sciuri*
XC5	Orange	+/-	+/-	E	C	+	-	+	Staph	*Staphylococcus*	*lentus*
XC6	White	-	+	E	C	-	-	+	20 NE	NI	
XC7	White	-	+	I	C	+	-	+	Staph	*Micrococcus*	spp
XC8	White	+	+	E	R	-	+	+	20 NE	*Pseudomonas*	*fluorescens*

Symbols: +, positive; -, negative; +/-, variable; w, weak; NI, No identified

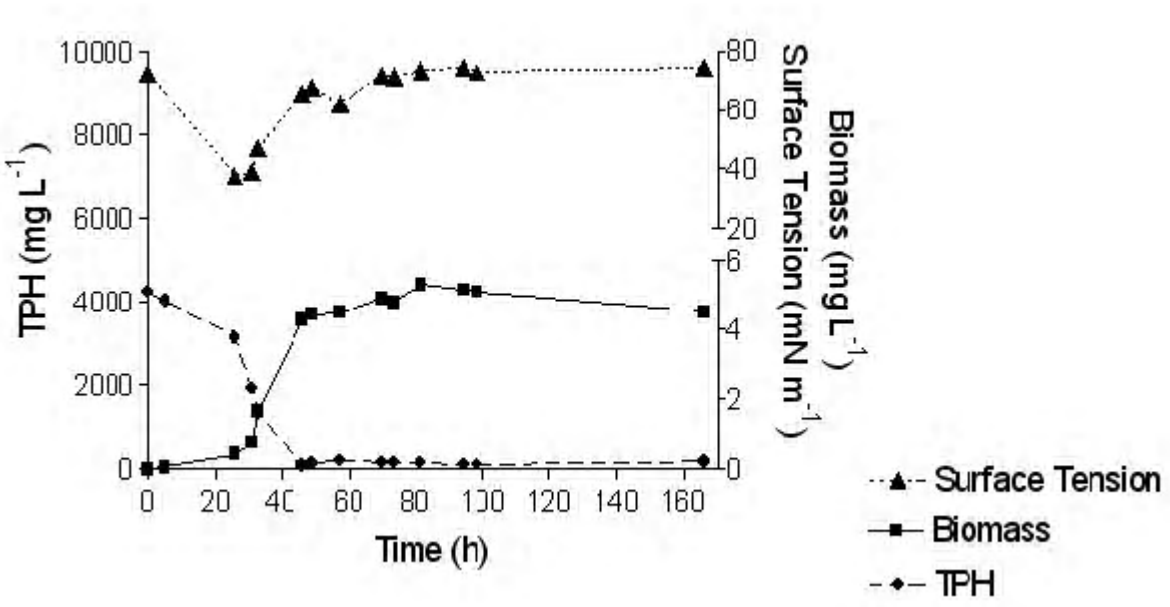

Fig. 1. Diesel-oil biodegradation, biomass production and surface-tension evolution for XA consortium at 30 °C at an initial diesel concentration 0.5% (v/v). Lines indicate the trends only

evolutions of the other two consortia XB and XC were similar, so Fig. 2 is presented here as an example. Fig. 2 shows again the rapid reduction of the initial substrate; over 90 % of TPH was degraded at 40 h in all the experiments, except from the one at 35 ºC. As previously discussed, a rapid reduction in the ST was observed during the diesel consumption, which was assumed to be due to the generation of biosurfactants that would increase the diesel accessibility. Subsequently, an increase in the ST was observed after the diesel had been consumed, and it was further assumed that the biosurfactants had been also degraded.

To summarise the results of the biodegradation process, Table 3 shows the dieselbiodegradation efficiency and the time required to degrade 90 % of the initial diesel concentration. As shown in this table, the three consortia had similar behaviours, degrading high percentages of the initial diesel concentrations (higher than 91 %, except for one experiment). It was noted that the three consortia behaved more or less similar efficiencies and provided similar results, and no important differences were observed among the three consortia. This indicated that, although they came from different contaminated soils, the enrichment process with diesel resulted in mixed consortia with very similar efficiencies. This is confirmed having found some bacteria of the same species in the three consortia.

Despite the diesel removal efficiencies were approximately the same, it should be noted that there was a slight trend in relation to the effects of diesel concentration and temperature: an increase of them provided a slight increase in the removal rate. However, the existence of a thermal inhibition at 35 ºC should be highlighted; this led to a low value of the diesel removal efficiency and therefore there was no exponential growth stage of the consortia at this temperature.

The ST reduction rates and percentages among all the experiments were analysedalso in Table 3. A slightly variation in the ST reduction rate with an increase in temperature was observed (except at35 ºC), likely related to the improvement in microbial growth. However, it seemed to be that initial diesel concentration did not cause a clear trend.

The relationship between the ST reduction and thebiosurfactants productionfor each consortium can be observed in Fig. 3. The figure plots the ST data versus the biosurfactants concentration data, drawing also two lines for each consortium, to indicate the trend and to obtain the CMC (critical micelle concentration) as their intersection. Overall, a rapid reduction of the ST was observed as the biosurfactants concentration increased to the CMC, after which the ST remained constant. By definition, the CMC is the surfactant concentration at which an abrupt change in the rate of the ST reduction with increasing the surfactant concentration is observed, i.e., an inflection point in

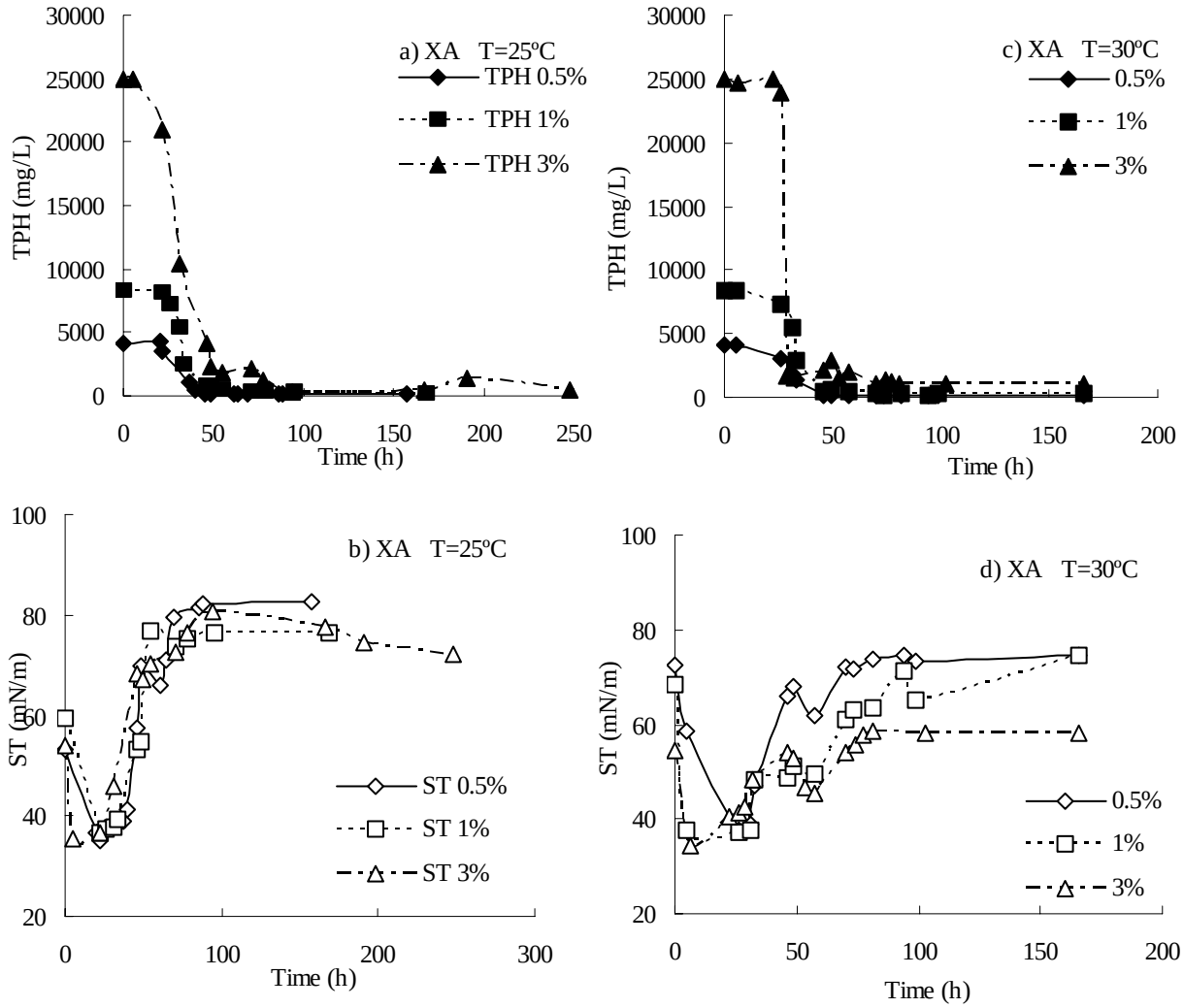

Fig. 2. XB VS.XC

Table 3. Summary of the diesel biodegradation results and evolution of surface tension

Consortium	T(ºC)	C_0(% v/v)	Diesel-removal efficiency (%)	Time to degrade diesel(h)	ST-reduction rate(mN m^{-1} h^{-1})	ST-reduction percentage (%)
		0.5	98	52.1	0.826	34
XA	25	1	98	57.6	1.026	38
		3	91	35.1	3.756	35
		0.5	95	51.7	1.334	48
	30	1	95	50.8	1.193	45
		3	95	54.8	3.080	37
	35	1	27	--	0.519	15
		0.5	98	55.2	0.826	34
XB	25	1	92	48.8	0.279	11
		3	81	47.3	0.743	30
		0.5	97	52.9	0.874	29
	30	1	98	43.6	0.578	23
		3	98	38	0.774	33
	35	1	23	--	0.822	27
		0.5	93	50.9	0.591	15
XC	25	1	95	48.8	0.556	21
		3	94	41.5	1.176	15
		0.5	97	55.3	0.973	31
	30	1	95	51.2	1.195	45
		3	94	40.2	1.031	39
	35	1	30	--	0.139	4

the curve. Regardless of the surfactant concentration, a further reduction in the ST will not be observed with further addition of surfactant once the CMC has been reached, rather, more micelles are formed (Fox &Bala, 2000).

From the results in Fig. 3, the CMCs values and the maximum yields of biosurfactants were determined. The yield of biosurfactants(g/g) was expressed as the ratio between the production of biosurfactants (g/L) and the initial diesel concentration (10 g/L). The CMC values were 2.72 g/L, 0.42 g/L and 0.45 g/L for XA, XB and XC respectively; and the yield values were 6.9 g/g, 1.3g/g and 1.8 g/g for XA, XB and XC respectively.Similar values were reported by Pornsunthorntawee *et al*. (2008).

Fig. 3shows that the XA consortium yielded the highest concentration of biosurfactants, much larger than the others although the value of the CMC was elevated. For the other two consortia studied, the values of the CMC and the biosurfactants yield were similar and lower than the values of the XA consortium. Microbial candidates for biosurfactant production are expected to decrease ST to around 35 mN/m (Desai & Banat, 1997). It can be observed that in this work, we achieved reduction in ST to more than that value with the three different consortia, it could be attributed to that we are working with mixed bacterial consortia and presumably with a mixture of biosurfactant (though this last point has not been tested).

Finally, some other differences can be found by performing a comparative global analysis (regardless of the consortium used) linking the biosurfactants production with the biodegradation efficiency, the maximum rate of biodegradation (mg/(L h))in the exponential stage and the average rate of biodegradation (mg/(L h)) for each consortia at 25 ºC and 1 % (v/v) initial diesel concentration. For that purpose, Fig. 4 shows the average biodegradation rate remained approximately constant, but the maximum biodegradation rate (during the exponential growth stage) clearly increased. The average biodegradation efficiency slightly increased versus the biosurfactants production. In a previous study, Whang *et al*. (2008) observed similar results. They found that the increased biosurfactant addition enhanced the diesel solubility, the biomass growth andthe diesel biodegradation percentage and also, a higher estimated values of specific grow rate (μ) were observed.In this study, despite of that similar efficiencies have been obtained in the previous study of the diesel biodegradation; attending to the biosurfactants production, the consortium XA, from the soil with the lower content of hydrocarbons, has reported the best results in the rate of the diesel biodegradation.

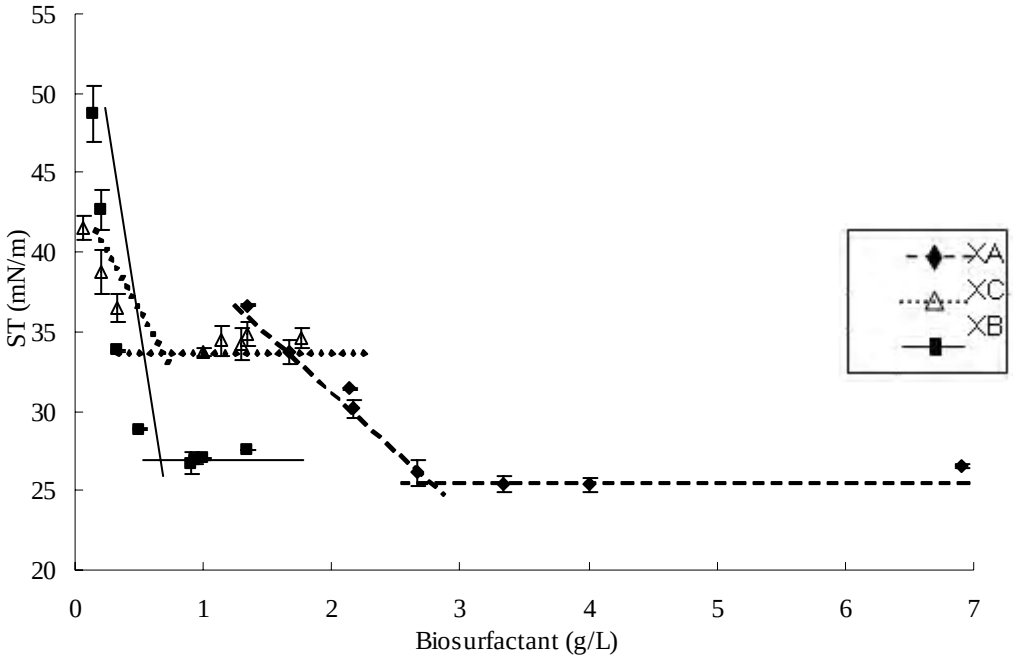

Fig. 3. XA Consortium

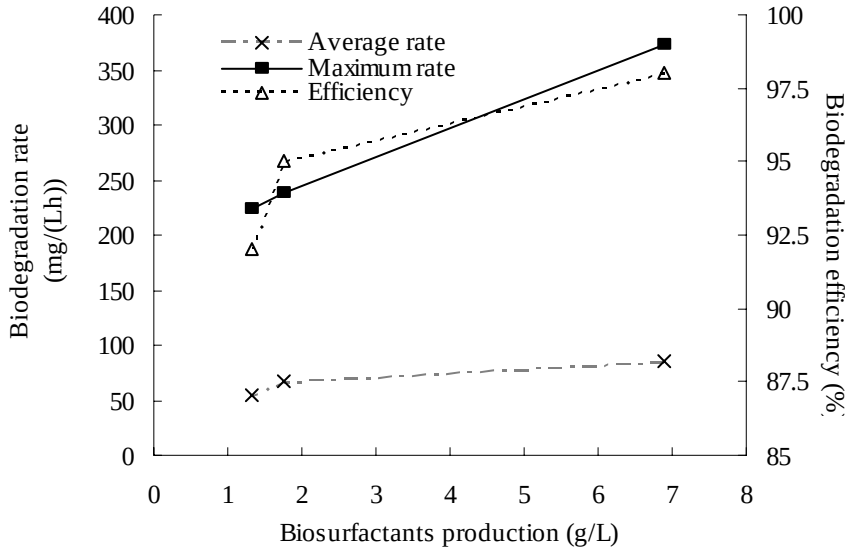

Fig. 4. Average biodegradation rate

CONCLUSION

Although consortia were obtained from three differentsoils, similar bacterial genera were detected after the enrichment process, and the result in the biodegradation of diesel in all experiments was entirely satisfactorywith good average efficiencies (approximately 90 %) and no important differences were observed between them in this regard. Thermal inhibition was observed at 35 ºC. The evolution of the ST was similar in all cases: an initial fast reduction followed by an increase once the diesel had been consumed. All the three consortia were found to be efficient biosurfactants producers. Consortia XB and XC offered similarlybiosurfactants yields and CMC values, while the XA consortium generated a greater quantity of biosurfactants but with a higher CMC. It was detected that the maximum biodegradation rate increased with the biosurfactants yield.

While there were some differences between the three consortia due to their different origins, the dieselenrichment process resulted in consortia with very similar efficiencies for diesel biodegradation. This conclusion might assume that in prolonged presence of contaminants (hydrocarbon in this case),edaphic consortia could be adapted to use the hydrocarbons as substrate and could be able to generate enough biosurfactantswhich would allow them to metabolizethe hydrocarbons.

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