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Use of two-surfactants mixtures to attain specific *HLB* values for assisted TPH-diesel biodegradation

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Abstract: In a surfactant assisted biodegradation process, the choice of surfactant(s) is of crucial importance. The question is: does the type of surfactant(i.e. chemical family) affect the biodegradation process at fixed hidrophillic-lypofillic balance(*HLB*) values? Microcosm assessments were developed using contaminated soil, with around of 5000 mg/kg of hydrocarbons as TPH-diesel. Mixtures of three nonionic surfactants were employed to get a wide range of specific *HLB* values. Tween20 and Span20 were mixed in the appropriate proportions to get *HLB* values between 8.6 and 16.7. Tween/Span60 mixtures reached *HLB* values between 4.7 and 14.9. Finally, Tween/Span80 combinations yielded *HLB* values between 4.3 and 15. TPH-diesel biodegradation was measured at the beginning, and after 8 weeks, as well as the FCU/gr_{soil}, as a measure of microorganisms' development during the biodegradation period. A second aim of this work was to assess the use of guar gum as a biodegradation enhancer instead of synthetic products. The conclusions of this work are that surfactant chemical family, and not only the *HLB* value clearly affects the assisted biodegradation rate. Surfactant's synergism was clearly observed. Regarding the use of guar gum, no biodegradation enhancement was observed for the three assessed concentrations, i.e., 2, 20, and 200 mg/kg, respectively. On the contrary, TPH-diesel removal was lower as the gum concentration increased. It is quite possible that guar gum was used as a microbial substrate.

Keywords: aged soils; enhanced bioremediation; HLB; mixtures; Tween; Span; surfactants

Introduction

Aged soils are difficult to treat by biological methods, since contaminants i.e, hydrocarbon compounds, can be tightly adsorbed into the soil particles. This problem can be solved by using small quantities of specific surfactants to increase compounds' bioavailability. Selection of the right surfactant and dose is of crucial importance to the biodegradation process, but frequently the selection process is based on a trial an error method. Surfactant hidrophillic-lypofillic balance (*HLB*) value, is an expression of the surfactant affinity molecule to the organic matter and water phases. This parameter can be a helpful tool in the right surfactant selection. In a previous work(Torres, 2004), the combined effect of temperature, and surfactant *HLB* and dose effects over the TPH-diesel removal in a Mexican aged soil was investigated. A statistical design was used in order to minimize the number of experiments needed for achieving that purpose. The results of that work indicated that the parameter that mostly affected biodegradation process was temperature, followed by *HLB* surfactant value, and surprisingly, surfactant dose at the end.

It is well known that certain mixtures of surfactants can provide better performance than pure surfactants for a wide variety of applications and thus is expected that enhanced solubilization of water in water-in-oil (w/o) microemulsions will also be achieved with certain surfactant mixtures (Huibers, 1997).

Huibers and Shah(Huibers, 1997) defined synergism in surfactants as any situation where mixtures of surfactants have superior properties when compared to properties of any of the single components alone. They stated that strong synergic effects in mixtures of nonionic surfactants would not be expected, as synergism in anionic-nonionic surfactant mixtures has been attributed to Couloumbic, ion-dipole, or hydrogen-bonding interactions among the polar groups. Nonionics, ,which have minimum intermolecular interactions, should have, by comparison, the lowest synergism of all mixtures. After the experimental section, they showed that even nonionic surfactant mixtures show evidence of synergism. Different authors have measured by different methods, the size of those interactions. Palous *et al*. (Palous, 1998) employed cross-differentiation relations in the identification of interactions between non-ionic and ionic surfactants. Kunieda *et al*. (Kunieda, 1998) used phase diagrams and small-angle X-ray scattering for the characterization of mixed ionic-nonionic surfactant systems. Finally, Rosen and Zhou used surface tension measurements and theoretical equation to describe the interaction parameter for mixed monolayer formation at the aqueous solutions interface, β^{σ}.

Theoretical *HLB* value for a given mixture of surfactants is given by Equation (1)(ICI, 1992):

$$HLB_{mixture} = (HLB_{surfactant\ A})(X_A) + (HLB_{surfactant\ B})(X_B) + \cdots, \quad (1)$$

where, $HLB_{surfactant\ A}$, $HLB_{surfactant\ B}$, $HLB_{mixture}$ are the *HLB* values for surfactant A, B, and the mixture X_A, and X_B are the weight fraction of every surfactant present in the mixture.

Some natural gums, including phytogenic and microbial products have been reported because of their rheological and stabilizing properties. Most high-molecular-weight water-soluble polymers are known as stabilizing agents, viscosity builders, and gellifying agents (Garti, 1994). Particularly,

the phytogenic surfactants *quillaya saponin* and *soya lecithin* (Soeder, 1996), and the plant-based surfactant obtained from the fruit pericarp of *Sapindus mukuross* (Roy, 1997), have been reported as natural surfactants (*quillaya saponin* and *soya lecithin*) and biodegradation enhancers (*Sapindus mukurossi* surfactant) for contaminated soils treatment.

Guar gum is part of the galactomanannans family, similar to locust bean gum consisting of a (1—4)-linked -dmannopyranose backbone with branchpoints from their 6-positions linked to a D-galactose (Chaplin, 2004) (Fig. 1). Guar gum emulsification activity has been reported. It has been stated that guar gum reduced surface tension of water to approximately 55 mmol/(L · m), and adsorb/precipitate on oil-water interfaces, reducing their interfacial tensions (Garti, 1994). As far as we know, guar gum has not been reported in enhanced biodegradation applications.

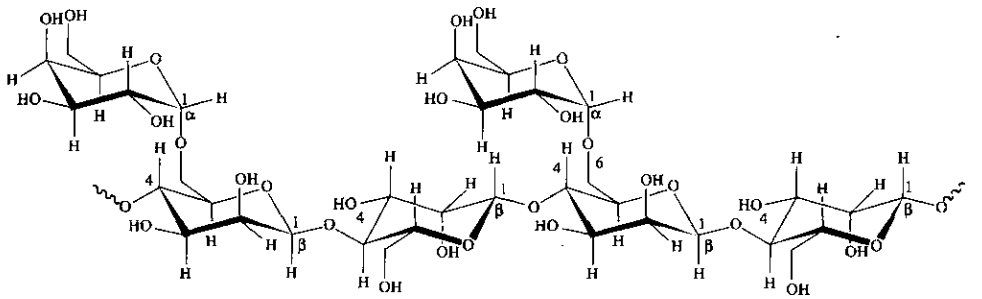

Fig. 1 Guar gum structure

With these antecedents, the aims of this work are: (a) to investigate weather or not the chemical family, besides *HLB* value can affect the enhanced biodegradation process; (b) to investigate the possibility of using guar gum as an enhancer of biodegradation process, and (c) to determine the effect of humidity over biodegradation process in presence of surfactants.

1 Materials and methods

1.1 Contaminated soil

The soil employed in this work is a contaminated soil from an old oil storage and distribution station located in northern Mexico(Iturbe, 2004). The main analytes found in the site were TPH-diesel, TPH-gasoline, PAHs and metals. Most of the organic compounds are contained in the TPH-diesel fraction. A given sample soil contained 3970 mg/kg of TPH-diesel fraction and 4.71 mg/kg of the TPH-gasoline fraction(Fig.2). Table 1 shows physical, chemical and microbiological characteristics of the soil sample employed in this work.

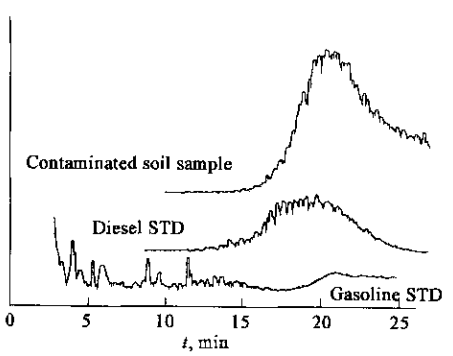

Fig. 2 Contaminated soil chromatogram

1.2 Microbial counts

Total heterotrophic bacteria count was developed as follows. One gram of soil was dissolved in 9 ml of peptonated solution (1 g of peptone in 1000 ml of water) and so consecutively until reaching a 1×10^{-7} dilution. Three of those dilutions were plated on agar plate count (Merk 5463) Petri dishes, prepared as the manufacturing indications. 0.1 ml of the fixed dilution was placed on every Petri dish and incubated during 48 h at 25℃. After that period, colonies were counted and reported as FCU/g soil.

Table 1 Contaminated soil physical, chemical and microbiological characteristics

Parameter	Values	Unit	Parameter	Values	Unit
Physical properties			Metals content		
Porosity	0.37	–	Na	272	mg/kg
Sand	92	%	K	332	mg/kg
Fines	7.9	%	Ca	24289	mg/kg
Bulk density	1.82	mg/cm^3	Mg	619	mg/kg
pH, 1 mol/L KCl	6.1	–	Mn	90	mg/kg
Microbiological issues			Cd	1	mg/kg
Heterotrophic bacteria	4.5E + 08	FCU/g soil	Cr	10	mg/kg
Total nitrogen	439	mg/kg	Cu	23	mg/kg
Phosphorus	63.7	mg/kg	Fe	5734	mg/kg
Organic matter	0.00536	mg/kg	Ni	12	mg/kg
			Pb	224	mg/kg
Humidity	0.45	%	Zn	1444	mg/kg

1.3 Microbial genera and species identification

One gram of soil was diluted in peptonated solution. Different dilutions were prepared as described above. Dilutions were platted on Petri dishes prepared with BHI media(Merk). Colonies were selected because of their color and/or morphologies. Colonies were re-platted in fresh BHI media Petri dishes. Pure colonies were characterized using the Gram technique for separation of Gram-positive and Gram-negative bacteria. Two miniaturized biochemical systems were employed. API 20 E (Biomeraux S. A.,

France) for Gram negative and BBL CRYSTAL GP ID (Becton Dickinson S. A., France) for Gram-positive bacteria.

1.4 Microcosm assessments

Wide mouth glass flasks (0.1 m high × 0.06 m diameter) were used. 30 g of soil were conditioned with the amount of $(NH_4)_2SO_4$ required to keep a C/N/P ratio of about 100∶15∶1. The desired amount of surfactant and the water necessary to get a humidity of 20% were added and the soil was thoroughly mixed, except for the 30% and 13.5% assessments. A surfactant(s) solution containing the amount necessary to get a value of 2 mg surfactant/kg soil was added. This value was employed, as in our previous work (Torres, 2003) showed that 2 mg/kg is enough for biodegradation enhancement. Flasks were tightly closed with Teflon lined plastic caps. A strip of Parafilm was used around the flask necks in order to assure no air interchange. Two blanks were run together with the surfactant assessments. The first blank is a sterile blank. The flask was sterilized at 121℃ for 15 min in a laboratory sterilizer. The second blank is a soil sample with $(NH_4)_2SO_4$, but no surfactant added. All assessments were run at 28℃.

1.5 Surfactants employed and their mixtures

Surfactants employed in this work were Span20, Span 60, and Span80 (sorbitan monolaurate, monoestearate and monooleate; Fig. 3), as well as Tween20, Tween60 and Tween80(the corresponding etoxilated Span products, P_{oe} = 20(Fig. 4). Mixtures of the two nonionic surfactants were employed to get a wide range of specific *HLB* values. Tween20 and Span20 were mixed in 100%—0%, 75%—25%, 50%—50%, 25%—75%, and 0%—100% proportions to get *HLB* values of 8.6, 10.6, 12.6, 14.7, and 16.7, in accord to Equation (1). Tween/Span60 mixtures reached *HLB* values of 4.7, 7.2, 9.8, 12.3, and 14.9. Finally, Tween/Span80 combinations yielded *HLB* values of 4.3, 7.0, 9.6, 12.3, and 15. *HLB* for single surfactants were in Italics. Table 2 shows the surfactants combinations and the theoretical *HLB* value, in accord to Equation (1). *HLB* values for the single surfactants are on the same table.

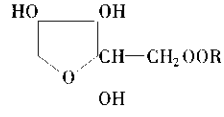

Fig. 3 Molecular structures for Span (sorbitan monocarboxylate) family members
R = laurate(20), stearate(60), or oleate(80)

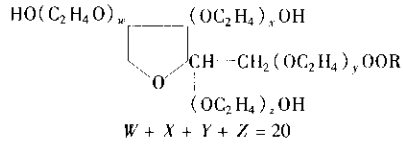

Fig. 4 Molecular structure for Tween[polyoxyethylene(20) sorbitan monocarboxylate] family members
R = laurate(20), stearate(60), or oleate(80)

Table 2 Description and results of the 22 biodegradation assessments

Test	Surfactant		Theoretical *HLB*	Final humidity, %	Final TPH-diesel, mg/kg	Final FCU/ g soil
	Span family, %	Tween family, %				
0	Initial sample		-	22.3	4156	4.5×10^5
1	20 (100)	20 (0)	8.6	21.9	3120	9.0×10^5
2	20 (75)	20 (25)	10.6	22.6	3206	6.7×10^6
3	20 (50)	20 (50)	12.6	20.3	2742	5.6×10^6
4	20 (25)	20 (75)	14.7	21.6	3105	3.4×10^6
5	20 (0)	20 (100)	16.7	18.7	2826	3.9×10^6
6	60 (100)	60 (0)	4.7	21.1	3266	9.4×10^5
7	60 (75)	60 (25)	7.2	21.4	3976	2.6×10^6
8	60 (50)	60 (50)	9.8	20.6	3057	2.7×10^6
9	60 (25)	60 (75)	12.3	20.6	3012	1.3×10^7
10	60 (0)	20 (100)	14.9	20.7	3180	3.1×10^6
11	80 (100)	80 (0)	4.3	20.7	3573	8.5×10^6
12	80 (75)	80 (25)	7.0	20.5	3505	2.4×10^6
13	80 (50)	80 (50)	9.6	21.0	3296	2.3×10^6
14	80 (25)	80 (75)	12.3	20.1	3896	7.6×10^5
15	80 (0)	80 (100)	15.0	21.6	3432	5.3×10^6
16	No-treatment blank		—	21.4	3255	1.6×10^5
17	Sterile blank		—	17.4	4036	ND
18	Guar gum, 2 mg/kg			20.9	3821	4.3×10^6
19	Guar gum, 20 mg/kg		—	21.8	3913	1.9×10^5
20	Guar gum, 200 mg/kg		—	21.3	4106	2.6×10^4
21	Humidity 13.5 %		4.3	11.8	2685	3.4×10^5
22	Humidity 31 %		4.3	32.4	3838	1.4×10^6

1.6 Statistical analysis

SPSS Program, version 11 (SPSS Inc., USA) was employed for the analysis of raw data.

2 Results and discussion

2.1 Application of surfactants mixtures-TPH-diesel removals

Table 2 shows the results of the 22 biodegradation assessments. Note that biodegradation values were not very high, since a period of only 8 weeks was selected for the biodegradation assessment. Values between 6.25% and 30.8% were obtained for the experiments with surfactant mixtures, including both blanks. Fig.5 shows the biodegradation values for the single surfactants assessments, in comparison with the sterile blank and the no-surfactant test. All assessments were carried out with 2 mg/kg of surfactant. As it can be seen, the tendency of the biodegradation or removal value is that etoxilated products (Tween family i.e., high *HLB* values) are more effective that non-etoxilated ones (Span family i.e, low *HLB* values). In contrast, Doong and Lei(Doong, 2003), reported that a *Pseudomonas putida* strain showed better PAHs(naphthalene, phenanthrene and pyrene) mineralization values in the presence of surfactants with low *HLB* values(Brij 30 = 9.7) in comparison than medium-high *HLB* values surfactants (Triton X - 100 = 13.5, Tween80 = 15.0, SDS = 40). Surfactant Brij 35 (*HLB* = 16.9) even inhibited biomass growth. All assessments were carried out at liquid medium flasks level.

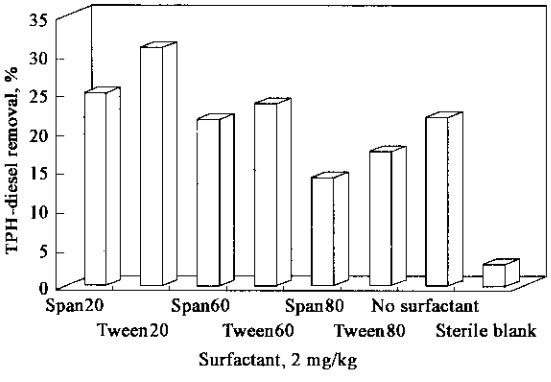

Fig. 5 TPH-diesel removal for Span and Tween single surfactants

On the other hand, it seems that monolaurates family showed higher removals than the correspondent monoestearates, and monooleates families. Note that only monolaurates and monoestearates products reached TPH-diesel removals higher than that reached at the no-surfactant test(21.7%). For comparison purpose, the sterile blank assessment removal value (2.9%) is plotted in the same figure. This means that surfactant chemical family affects the TPH-diesel removal values.

Fig.6 shows that the TPH-diesel removal values are plotted as a function of the *HLB* value of every employed surfactant mixture. As observed, for every chemical family, different TPH-diesel removal values were obtained, but maximum values do not correspond with the line extremes i.e., the single surfactant assessment. For family 20 (monolaurates), the maximum value was achieved with the 50%—50% mixture (*HLB* = 12.6). For family 60 (monoestearates), the maximum corresponds to the mixture 75%—25% (*HLB* = 7.2). Finally, for the family 80 (monooleates), the maximum corresponds to the 50%—50% mixture (*HLB* = 9.6). These facts clearly indicate that *HLB* is not the only one factor responsible for the biodegradation success. Both *HLB* value and chemical family are responsible of the biodegradation enhancement. A statistical analysis (p = 0.05) showed that there are significant differences among surfactants families, but not among *HLB* surfactant values.

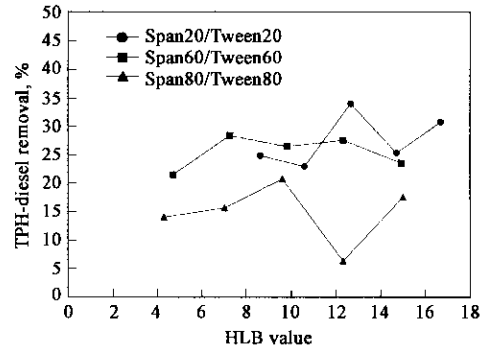

Fig. 6 TPH-diesel removal as a function of *HLB* value for the three-surfactant families

In resume, using surfactant mixtures of Span20/Tween20, it is possible to get TPH-diesel removals in the range of 22.9% to 34% (average = 27.6% ± 4.63%, median = 25.3%). With Span60/Tween60 surfactants, removals from 21.4% to 28.4% (average = 25.4% ± 2.9%, median = 26.4%) can be reached. Finally, for Span80/Tween80 surfactant mixtures, TPH-diesel removals between 6.25% and 20.7% (average = 14.8% ± 5.4%, median = 15.6%) can be obtained.

This behavior can be explained in terms of the solubilization level promoted for every surfactant or surfactant-mixture. Huiber and Shah (Huiber, 1997) measured the water-to-oil volume ratio as a solubilization index for nonylphenol surfactant containing 1.5 ($C_9Poe_{1.5}$), and 12 (C_9Poe_{12}) polyethylene oxide molecules. They found that the best solubilization index was not for the $C_9Poe_{1.5}$ (*HLB* = 4.6), nor for the C_9Poe_{12} (*HLB* = 14.2) surfactants, but for a mixture of them, with an intermediated *HLB* value of 9, very similar results were observed for C_9Poe_4 + $C_9Poe_{7.5}$ mixtures.

2.2 Application of surfactants mixtures-biomass growth

Table 2 shows the final FCU/g soil values for the 22 assessments, including two blanks. Note that all values are on the 10^5—10^7 interval. Values are the average for a triplicate test. FCU/g soil value for sample 0 has a value of 4.5×10^5. From this point, values as low as 1.6×10^5. FCU/g soil can be obtained(no surfactant blank). The sterile blank, as expected, showed no viable biomass present. The maximum FCU/g soil value corresponds to the test 9 (1.3×10^7). Fig. 7 shows the FCU/g soil values as a function of the *HLB* values for the three employed surfactant families. As

noted, FCU/g soil values do not show a consistent trend regarding the surfactant *HLB* value. For example, in the case of the monooleates family(Span80/Tween80), there seems to be a diminution in the FCU/g soil value as the *HLB* value is augmented for the last point (*HLB* = 15).

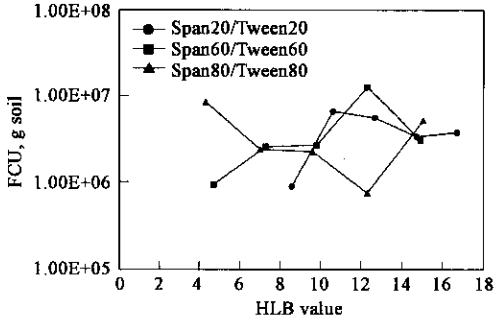

Fig. 7 Microbial growth as a function of *HLB* values

Regarding the monoestearates family, the trend is the opposite: there is an increase on the FCU/g soil value as the *HLB* value increases up to the *HLB* = 12.3 point. The last *HLB* value(14.9), a diminution on the FCU/g soil value is observed. Finally, for the monolaurates family, there is an increase on the FCU/g soil value from *HLB* = 8.6 to *HLB* = 10.6, and the FCU/g soil is kept more or less constant later on. In general, it can be said that more bacterial growth was observed when using *HLB* values higher than 10, in combination with family Span60/Tween80, and Span20/Tween20. In a *HLB* value vs. FCU/g soil value plot (figure not shown), no correlation between the two parameters was obtained. A big dispersion for the points was observed for the three families together or even for every single-family analysis. It has been previously reported that biodegradation patterns are not necessarily linked to biomass growth patterns.

2.3 Microbiological characterization of some soil samples

Table 3 shows genera (and in some cases the species) of bacteria found on the three analyzed soil samples. The first one corresponds to the original contaminated soil (sample 0). On this soil, Gram-positive and Gram-negative bacteria were identified. In the first group, *Corynebacterium* sp., *Clavibacter* sp., and *Streptococcus* sp. can be mentioned. Regarding the second group, *Pseudomonas* sp., specifically *P. fluorescens* and *P. putida* were detected.

On soil number three, corresponding to the best biodegradation assessment value, *Corynebacterium* sp. was the only one gram-positive identified bacterium. *P. putida* and *P. fluorescens*, as well as *Yersinia pestis* were identified too. On soil number 16(no surfactant assessment), no Gram-positive bacteria were found. *Pseudomonas* sp., *P. Fluorescens*, *P. Putida*, *Stentrophomonas* sp., and *Yersinia pestis* were identified among the Gram-negative bacteria.

Table 3 Bacteria genus and specie found in soils 0(initial sample), 3 (best biodegradation test) and 16 (no surfactant assessment)

Soil sample	Gram positive	Gram negative
0	*Corynebacterium* sp.	*Pseudomonas fluorescens*
	Clavibacter sp.	*Pseudomonas putida*
	Streptococcus sp.	-
3	*Corynebacterium* sp.	*Yersinia pestis*
	-	*Pseudomonas putida*
	-	*Pseudomonas fluorescens*
16	-	*Pseudomonas* sp.
	-	*Pseudomonas fluoresecens*
	-	*Pseudomonas putida*
	-	*Stenotrophomonas* sp.
	-	*Yersinia pestis*

As reported in many other works, biodegradation process selects the microorganisms with the required degradation capabilities or toxicity resistances. It seems that *Pseudomonas* species as well as *Corynebacterium* sp. are responsible for TPH-diesel biodegradation in presence of surfactants i.e, Span20-Tween20. When no surfactant was present, besides *Pseudomonas* and *Corynebacterium* species, *Stentrophomonas*, *Xanthomonas* sp. and *Yersinia pestis* (both Gram-negative) were also predominant. On the initial soil sample, only *Corynebacterium*, *Clavibacter*, and *Streptococcus* (Gram-positive), as well as *Pseudomonas fluorescens* and *P. putida* (Gram-negative) were predominant.

Regarding these genera and species, it can be highlighted that *Corynebacterium* sp. are bacteria widely distributed in soil, water, and skin and mucous of both men and animal. There are many species, divided in those that require lipids(potentially harmful to men), and those who do not require lipids (low harmful potential to men). They are aerobic or anaerobic facultative, most are capable of glucose fermentation and most are catalase-positive. One of the most studied *Corynebacterium* genera, regarding its biodegradation capabilities is *C. glutamicum*. *C. hoagii* has been mentioned in literature because of its Cr(VI) resistance (Viti, 2003). *Clavibacter* sp. are quite related with *Corynebacterium* sp. In fact, some years ago, they were included in that species. Recently, a species of *Clavibacter* ALA2 has been reported because of this capability in the transformation of linoleic acid to a novel trihydroxy unsaturated fatty acid(Hou, 1997). *Streptococcus* sp. are facultative anaerobic bacteria. They are cocci capable of glucose fermentation very related to *Enterococcus* sp. (Bergey, 1994). They are always associated as human and animal pathogens. They have been reported because of their potential to produce exopolysaccharides, especially *S. thermophilus*(Ruijssenaars, 2000).

Pseudomonas sp. are slightly curved rods. They are aerobic, oxidase positive/negative and catalase positive. They are ubiquitous in soils and water. Main *Pseudomonas* species are *P. aeruginosa*, *P. fluorescens*, *P. chlororafis*, *P.*

putida, *P. aureofaciens* and *P. syringae*. Very often, *P. fluorescens* and *P. putida* presence in soils has been mentioned, because of their interesting degradation capabilities(Bergey ,1994).

Yersinia pestis are rod-coccobacilli, facultative anaerobic and present oxidase-negative, catalase positive characteristics. They are closely related to *Enterobacteria*, *Hafnia*, *Citrobacter*, *Escherichia*, *Klebsiella*, *Proteus* and *Pasteurella* (Bergey, 1994). *Yersinia pestis* is one of the seven species reported(Krieg, 1984). It is not reported as ubiquitous soil bacteria, but in some studies *Yersinia* has been mentioned. *Xanthomonas* sp. before classified as *Stenotrophomonas* sp., are straight rods, obligately aerobic bacteria. Most of species are phytopatogens, found in cabbage, lettuce, nuts, and other cultures. Main *Xanthomonas* genera are *X. campestris*, *X. fragariae*, *X. albineans*, *X. ampelina* and probably *X. populi*(Bergey , 1994).

2.4 Use of guar gum as biodegradation enhancer

A very simple test was developed in order to check the a priori suitability of guar gum as a biodegradation enhancer. One gram of soil was weighted on glass test tubes and 10 ml of distilled water containing the equivalent amount of guar gum to reach solution concentrations of 0, 1, 2, 3, 5, and 10 g/L were added. Test tubes were sealed and mixed at the same time and allowed to settle for 5 min. Turbidity on every tube was visually compared. This parameter resulted higher as the gum concentration was higher, if compared with the only water assessment. After that, biodegradation assessment as described in material and methods section was developed for 2, 20 and 200 mg/kg soil concentrations. Results are presented at Fig. 8. As shown, none of the three guar gum concentrations enhanced the biodegradation process, if compared to the no-surfactant test. In fact, biodegradation rate was lower as the gum concentration increased. This fact would have two meanings. On one hand, it is well known that natural gums are biodegradable by different consortia and single microorganisms. Specifically, organisms containing endo-β-mannanase are capable of guar gum degradation. This enzyme has been reported as present in some microorganisms, such as *Aspergillius niger* (Cheng, 2000). The same was observed for three different bacteria employed for bio-elimination of phenanthrene and fluoranthene in presence of quillaya saponin and soya lecithin(Soeder, 1996). On the other hand, this fact would mean that guar gum inhibited TPH-diesel biodegradation. At high concentrations, even these natural surfactants can inhibit bacterial growth. In the work reported by Soeder *et al*. (Soeder, 1996), quillaya saponin resulted slightly toxic to one of the three strains assessed. The CFU/g soil number for every guar gum concentration was 3.2×10^6(0 g/kg), 4.3×10^6(2 mg/kg), 1.9×10^5(20 mg/kg), and 2.3×10^4(200 mg/kg). These values suggested that biomass growth was inhibited as the guar gum concentration increased. With data developed on this work, it is difficult to say which of the two mechanisms is the responsible for the low biodegradation rates in presence of guar gum.

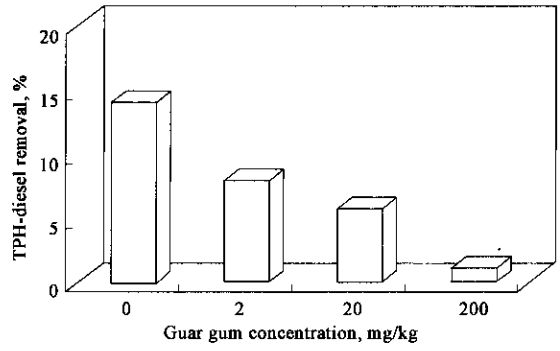

Fig. 8 THP-diesel removal for guar assessments

2.5 Effect of humidity over the biodegradation process in presence of a fixed surfactant amount

The soil humidity did affect TPH-diesel removal biodegradation assessments(Table 4). The three tests were carried out using Span80(2 mg/kg), which in turn gives a 4.3 *HLB* value. As shown in Table 4, the higher the initial soil humidity, the lower the TPH-diesel removal. A plot of TPH-diesel removal vs. soil humidity gave the following adjust to a line:

$$\text{TPH - diesel removal} = 50.87 - 1.4824\ (\text{humidity}), \quad \text{with } R^2 = 0.8113. \qquad (2)$$

Even when experimental points are a few, this equation could help to decide which is the best humidity level for future biodegradation assessments. Note in Table 2 that final humidity values are quite near from the hypothetical water content (20%), except 17, 21 and 22 tests, corresponding to the sterile blank (17.4%), and the 13.5% and 31% initial humidity assessments.

Table 4 TPH-diesel removals as a function of soil initial humidity

Assessment	Humidity, %	TPH-diesel removal, %	FCU/g soil
11	13.5	35.4	3.4×10^5
21	20	14	8.5×10^6
22	31	7.6	1.4×10^6

3 Conclusions

For every chemical family, different TPH-diesel removal values were obtained, but maximum values do not correspond with the line extremes i.e., the single surfactant assessment. For monolaurates, the maximum value was achieved with the 50%—50% mixture (*HLB* = 12.6). For monoestearates, the maximum corresponds to the mixture 75%—25% (*HLB* = 7.2). Finally, for monooleates, the maximum corresponds to the 50%—50% mixture (*HLB* = 9.6). These facts clearly indicated that *HLB* is not the only one factor responsible for the biodegradation success. Both *HLB* value and chemical family are responsible of the biodegradation

enhancement.

FCU/g soil was very variable depending on the *HLB* value and the chemical family. In general, it can be said that more bacterial growth was observed when using *HLB* values higher than 10, in combination with family Span60/Tween80, and Span20/Tween20. In a *HLB* value vs. FCU/g soil value plot, no correlation between the two parameters was obtained. A big dispersion for the points was observed for the three families together or even for every single-family analysis.

On the original contaminated soil (sample 0), *Corynebacterium* sp., *Clavibacter* sp., and *Streptococcus* sp., as well as *Pseudomonas* sp., specifically *P. fluorescens* and *P. putida* were detected. On soil number three, corresponding to the best biodegradation assessment value, *Corynebacterium* sp., *P. putida*, *P. fluorescens*, as well as *Yersinia pestis* were identified. On soil number 16 (no surfactant assessment), *Pseudomonas* sp., *P. Fluorescens*, *P. Putida*, *Stentrophomonas* sp., and *Yersinia pestis* were identified.

None of the three guar gum concentrations enhanced the biodegradation process, if compared to the no-surfactant test. In fact, biodegradation rate was lower as the gum concentration increased. This fact would have two meanings: (a) Guar gum was employed by bacteria as a substrate, or (b) gum displayed toxicity over the biomass growth.

Regarding the effect of water content over the biodegradation process in presence of surfactants, it was observed that the higher the initial soil humidity, the lower the TPH-diesel removal. A plot of TPH-diesel removal vs. soil humidity gave the following adjust to a line: TPH-diesel removal = 50.87 − 1.4824 (humidity), with R^2 = 0.8113.

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