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Microbial Biodegradation of Aromatic Compounds in a Soil Contaminated with Gasohol

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Research Article

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ABSTRACT

The studies developed in this work aimed to find alternatives to biodegradation or bioremediation of soils contaminated with gasoline or gasohol. So, the biodegradation of benzene, toluene and *o*-xylene (BTX) in soil samples contaminated with gasoline or gasohol by a bacterial consortium was studied. Four bacterial strains were selected for the consortium based on their growth capacity in gasoline, gasohol and BTX as sole carbon sources, and on the production of biosurfactants in mineral medium containing gasohol as the sole carbon source. The reduction of TX concentrations in soil slurries in a multi-cell bioreactor system was used as the criterion to evaluate biodegradation efficiency. BTX removal was highly stimulated by air injection and mineral nutrients, and was significantly increased by the presence of the bacterial consortium. Addition of a proprietary oxygen release compound did not stimulate the biodegradation of BTX.

Keywords: Bioremediation; Biodegradation; Hydrocarbons; Bacterial consortium; ORC;

1. INTRODUCTION

The release of petroleum products (e.g., gasoline, diesel, fuel oil) from above-ground and underground storage tanks or transport pipelines are the major causes of groundwater pollution. Benzene, toluene, ethylbenzene, and xylene isomers (BTEX), the major

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components of petroleum products, are toxic chemicals (Chen et al., 2010). BTEX are typical hazardous organic compounds that are present in almost any petrol or gasoline spill on the soil's surface or in the subsurface. These pollutants have been found to cause many serious health side effects in humans (e.g. skin and sensory irritation, central nervous system depression, respiratory problems, leukemia, cancer, as well as disturbances of the kidney, liver and blood systems) and, therefore, their removal from groundwater and surface waters is essential (Aivalioti et al., 2010). Furthermore, 20-25% anhydrous ethanol is added to gasoline (gasohol) in Brazil, and this promotes a co-solvency effect that increases the solubility of hydrocarbons in water (Prantera et al., 2002). Ethanol enhances the solubilisation and migration of BTX into aquifers and, since the alcohol is a preferential substrate for soil bacteria, interferes with the aerobic biodegradation of BTX in the aquifer environment (Powers et al., 2001), unless oxygen is actively supplied.

There exist numerous methods for the removal of these pollutants from soils. They involve both physico- chemical and biological approaches. Although the former are more effective than biological methods they are expensive and require a high energy demand and consumption of many chemical reagents. For this reason, the use of microorganisms capable of degrading toxic compounds, known as bioremediation, has become an attractive technology (Hamdi et al. 2007).

Depending on the nature, volume and location of the contaminated material, bioremediation can be carried out *in-situ* or *ex-situ. In-situ* treatment systems aim at eliminating the contaminant at the contaminated site, but the introduction of the additives required to create the ideal conditions for such biotreatment poses a substantial challenge. On the other hand, *ex-situ* treatment systems, especially those that make use of bioreactors, allow the establishment of appropriate and controlled conditions in order to maximize the microbial activity. A further advantage of *ex-situ* systems is that the time required for treatment is lower than that required by *in-situ* systems (Schacht and Ajibo, 2002).

Since petroleum hydrocarbons (PHCs) are of natural origin and are also ubiquitous in the environment, many different micro-organisms have the ability to effectively degrade them (Atlas and Bartha, 1998). On the other hand, no single microbial species can degrade all of the monoaromatic components of gasoline and, thus, a successful bioremediation of complex contaminants would require the simultaneous action of different populations within a mixed culture (Provident et al., 1995). Moreover, the availability of the contaminating species within the environment can also exert a marked effect on the efficiency of the biodegradation process. For example, PHCs tend to bind to soil components, making their removal or degradation more difficult (Atlas and Cerniglia, 1995; Barathi and Vasudevan, 2001). In this context, some micro-organisms produce amphiphilic biosurfactants that can emulsify hydrocarbons, thus enhancing their solubility in water, by decreasing interfacial tension and increasing the displacement of oily substances from soil particles (Banat, 1995).
The presence of oxygen and mineral nutrients can also control the rate of microbial degradation of PHCs (Von Wedel et al., 1988). Interest has recently been shown in the use of oxygen-release compounds (ORCs) to promote the direct oxidation of pollutants and, at the same time, to increase aerobic microbial degradation (Arienzo, 2000). The availability of nitrogen and phosphorus is also essential for the effective biodegradation of hydrocarbons. Numerous studies have demonstrated that maintenance of appropriate concentrations of nitrogen and phosphorus to stimulate the degradation of organic pollutants in soils (Bragg et al., 1994; Cleland et al., 1997; Breedveld and Sparrevik, 2000; Margesin et al., 2000).

In view of the fact that information concerning the use of aerobic bioreactors and of ORCs for the treatment of gasohol-contaminated soils in Brazil is extremely limited, the present study was conducted with the aim of evaluating bioremediation efficiency using a multi-cell bioreactor system. The effects of a proprietary ORC, air injection, mineral nutrients and inoculation with a bacterial consortium on the degradation of BTX present in gasohol were studied.

2. MATERIALS AND METHODS

2.1 MICROORGANISMS

A total of 52 bacterial strains, obtained from hydrocarbon-contaminated soil and water or from oily sludge, were provided by the culture collection of the Laboratório de Biotecnologia e Biodiversidade para o Meio Ambiente (LBBMA). The cultures were activated in R2A broth (Reasoner and Geldreich, 1985), centrifuged and resuspended in mineral medium (Margesin and Schinner, 1997) containing ammonium nitrate (0.148 g/l) named as MMN. Appropriate volumes of bacterial suspensions were inoculated into tubes containing 4 ml of the same medium, supplemented with gasoline or gasohol (2%, v/v) as the sole carbon source, to give a final optical density (OD) of 0.05 at 600 nm. The tubes were incubated at 30ºC and 150 rpm, and microbial growth was evaluated from OD_{600} values determined every 72 h over a 15-day period.

Bacterial strains with satisfactory growth (OD_{600} > 0.2) in the previous step were activated in R2A medium, centrifuged and resuspended in MMN. Appropriate volumes of bacterial suspensions were inoculated into tubes containing 4 ml of the same medium, supplemented with benzene (1%, v/v), toluene (3%, v/v) or xylene isomers (6%, v/v), to an OD_{600} of 0.05. The tubes were incubated at 30ºC and 150 rpm, and microbial growth was evaluated from OD_{600} values, determined every 72 h over a 15-day period. These strains were checked for biosurfactant production in gasohol-supplemented (2%, v/v) MMN. Biosurfactant production was evaluated at 7-day intervals for 21 days by surface tension reduction of the growth medium, using a Fischer (Pittsburgh, PA, USA) Surface Tensiomat Model 21 tensiometer.

The microbial consortium used in the bioremediation experiments comprised the bacterial strains chosen on the basis of their growth in MMN with BTX as the sole carbon source, and on their ability to produce biosurfactants. These bacterial strains were identified by their fatty acid methyl esters (FAME) profiles, using the Sherlock (Newark, DE, USA) Microbial Identification System®. The fatty acid profiles of the bacterial strains were compared with those in the reference library ITSA 1.0® (MIDI, Newark, DE, USA).

2.2 EFFECT OF ORC ON MICROBIAL GROWTH

The growth of each member of the bacterial consortium was evaluated individually in MMN supplemented with gasohol (5%, v/v) and a proprietary formulation of phosphate-intercalated magnesium peroxide (30 g/l; Regenesis ORC®, San Clemente, CA, USA). Cultures were incubated at 30ºC and 150 rpm, and microbial growth was evaluated from OD_{600} values determined 7 days after inoculation.

2.3 BIOREMEDIATION OF GASOHOL-CONTAMINATED SOIL SLURRIES

The soil used in the bioremediation experiments was collected at a depth of 3 m in Viçosa, Minas Gerais, Brazil, and was sterilised prior to use (except where otherwise stated) by autoclaving at 121^{o}C for 30 min. MMN and water used to prepare soil slurries were sterilised separately under similar conditions. Studies of the biodegradation of gasohol in soil slurries were conducted in a proprietary multi-cell bioreactor system (Fig. 1).

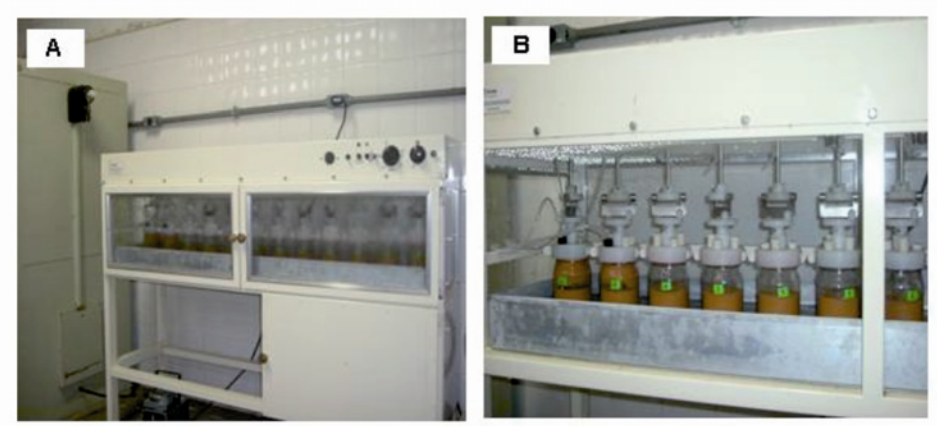

Fig. 1. Multi-cell bioreactor system used in the bioremediation tests: A) Front view of the equipment; B) interior view showing the air-feed tubes and the mechanism of rotation of the flasks

The instrument comprised 12 cylindrical glass flasks immersed in a water bath, the temperature of which was maintained at 30ºC by a central water-heating system. The axis of each flask was fitted with a stainless steel rod, bearing a stirring plate connected ¾ of the way along the rod, and this was coupled, through further rods and a gearing system, to the central electric motor. The stirring speed was maintained at 150 rpm. Each flask contained 100g of soil, 3 ml of gasohol and either 300 ml of MMN or 300 ml of water. Where appropriate, flasks were inoculated with the selected microbial consortium in the proportion of 10^{8} cfu/ml of slurry for each member of the consortium. The oxygen effect was evaluated by applying three different treatments to the cultures: (i) no oxygenation; (ii) injection of filtered (0.45 µm membrane; Millipore, Billerica, MA, USA) atmospheric air into the flask by compression pump; and (iii) incorporation of Regenesis ORC® into the medium at the rate of 3% w/w based on the hydrocarbon content. The 13 treatments utilised are specified in Table 1.

Following 15 days of incubation, 10 ml aliquots of the soil slurries were removed aseptically and the residual concentrations of BTX determined by the headspace method and the analyses were performed on a Shimadzu (Osaka, Japan) model QP5000 GS MS equipped with a Class 5000 data acquisition system and a Nukol® fused-silica capillary column (100 m x 0.20 mm i.d.; film thickness 0.25 µm; Supelco, Bellefonte, PA, USA) (Serrano and Galego, 2004). The column oven temperature was programmed from 40 to 240°C at 5 ºC/min; the injector and transfer line temperature was maintained at 250°C, and the carrier gas used was helium at 1.3 ml/min.

Table 1. Residual concentrations of benzene, toluene and *o*-xylene in gasohol-contaminated soil slurries submitted to biodegradation treatment in a multi-cell bioreactor system

Treatment	Soil slurry[a]	Medium	Oxygenation[b]	Residual concentration (mg/l)[c]		
				Benzene	Toluene	*o*-Xylene
1	non-sterile	water	none	$5.47^{b} \pm 0.05$	$2.73^{bc} \pm 0.12$	$0.99^{b} \pm 0.03$
2	sterile	water	none	$6.73^{a} \pm 0.13$	$3.28^{a} \pm 0.05$	$1.07^{a} \pm 0.04$
3	sterile	water	ORC	$6.76^{a} \pm 0.06$	$3.20^{a} \pm 0.06$	$1.03^{ab} \pm 0.07$
4	sterile	water	air injection	$5.86^{b} \pm 0.13$	$2.89^{b} \pm 0.06$	$0.91^{c} \pm 0.10$
5	inoculated	water	none	$2.17^{e} \pm 0.05$	$1.33^{e} \pm 0.06$	$0.45^{e} \pm 0.01$
6	inoculated	water	ORC	$2.02^{ef} \pm 0.89$	$1.28^{ef} \pm 0.56$	$0.39^{e} \pm 0.02$
7	inoculated	water	air injection	$1.71^{fg} \pm 0.04$	$1.05^{f} \pm 0.02$	$0.23^{f} \pm 0.01$
8	sterile	MMN	none	$4.41^{c} \pm 0.02$	$2.03^{cd} \pm 0.04$	$0.58^{d} \pm 0.02$
9	sterile	MMN	ORC	$3.98^{c} \pm 0.02$	$1.99^{d} \pm 0.03$	$0.59^{d} \pm 0.01$
10	sterile	MMN	air injection	$3.46^{d} \pm 0.01$	$1.74^{e} \pm 0.01$	$0.41^{e} \pm 0.01$
11	inoculated	MMN	none	$1.33^{gh} \pm 0.03$	$0.69^{g} \pm 0.12$	$0.29^{f} \pm 0.02$
12	inoculated	MMN	ORC	$1.21^{h} \pm 0.05$	$0.68^{g} \pm 0.05$	0.24f ± 0.07
13	inoculated	MMN	air injection	$0.344^{l} \pm 0.24$	$0.144^{h} \pm 0.29$	$0.12^{g} \pm 0.02$

[a] Soil samples were sterilised (except for treatment 1) and slurred in either distilled water or mineral medium (Margesin and Schinner, 1997) with 0.148 g/l ammonium nitrate (MMN).

[b] Oxygenation was applied (where indicated) by application of a proprietary oxygen release compound (ORC) or by injection of filtered atmospheric air by compression pump.

[c] Mean values ± standard deviations (n = 3) correspond to residual concentrations after treatment at 30ºC with agitation at 150 rpm for 15 days.

Mean values bearing the same superscript letter, in the same column, are not significantly different at the 5% probability level (Duncan test).

Samples were manually injected at a split ratio of 69:1, and mass spectra were recorded in the *m/z* range 40 to 500 amu. Identification of the separated compounds was carried out by comparing retention times with those of a standard mixture. Quantification was conducted by selective ion monitoring (SIM).

3. RESULTS AND DISCUSSION

3.1. SELECTION OF BACTERIAL STRAINS

Sixteen of the 52 strains were able to grow successfully (OD_{600} > 0.2 after 15 days incubation) in MMN with gasoline as the sole carbon source. Among these, 10 were strongly stimulated (OD_{600} > 0.8) and 6 were strongly inhibited (OD_{600} < 0.1) when incubated in MMN supplemented with gasohol. The presence of ethanol as a component of gasohol increases the solubility of petroleum hydrocarbons in water, where the solubilities of benzene, toluene and *o*-xylene in water are increased by 20, 40 and 50%, respectively, by ethanol at 10% (v/v) compared with pure water (Fernandes and Corseuil, 1999). The increased solubility of BTX in the aqueous phase of the gasohol-MMN may increase the toxicity of these compounds to micro-organisms, and this could explain the inhibition in growth observed for some strains. On the other hand, primary alcohols such as ethanol can be preferentially biodegraded, thus stimulating the growth of some microbial species (Corseuil and Marins, 1997), as observed in the present study. Unfortunately, however, ethanol can also inhibit hydrocarbon degradation by catabolic repression. Experiments with pure cultures revealed that ethanol repressed catabolic genes and reduced the metabolic flow of toluene, hence affecting its biodegradation (Lovanh et al., 2002). Moreover, Hunt et al. (1997) reported that, under aerobic conditions, ethanol was preferentially degraded by *Pseudomonas putida* (PpF1) as a result of the repression of the synthesis of enzymes involved in benzene degradation. Such preferential degradation of alcohol may also lead to the exhaustion of the oxygen required for the biodegradation of hydrocarbons (Corseuil and Hunt, 1998). Barker at al. (1990) concluded that the persistence of benzene, toluene, ethylbenzene and xylene (BTEX) in the presence of high concentrations of methanol was caused by inhibition of the biodegradation of aromatic contaminants, along with oxygen exhaustion during the preferential biodegradation of the alcohol.

The 16 bacterial strains able to use gasoline as the sole carbon source were further screened for growth in MMN supplemented with BTX. Strains LBBMA 105A (OD_{600}> 0.2 in the presence of benzene), LBBMA 193 (OD_{600} > 0.2 in the presence of benzene or xylenes; OD_{600} > 0.1 in the presence of toluene) and LBBMA B1 (OD_{600} > 0.2 in the presence of toluene) were selected as members of the consortium for gasohol bioremediation. These strains were identified as *Stenotrophomonas maltophilia* (LBBMA 105A) and *Pseudomonas balearica* (LBBMA 193). The FAME profile of LBBMA B1 did not match any of the entries in the Midi´s ITSA 1.0 Library.

Strains LBBMA 53A, 88A, 115 and 178 produced biosurfactants (surface tension < 40 N/m) using gasohol as the sole carbon source. Cunha et al. (2004) reported biosurfactant production by a strain of *Serratia* sp. in culture medium containing gasohol as the sole carbon source, although few other examples of this trait are available in the literature. Biosurfactant production could be an advantageous characteristic in micro-organisms used in the bioremediation of soils contaminated with gasohol or other petroleum derivatives, since the *in-situ* production of such agents could help to increase the bioavailability of PHCs. Among the abovementioned strains, *Pseudomonas aeruginosa* LBBMA 53A and

Pseudomonas sp. LBBMA 178 were chosen as additional members of the microbial consortium for gasohol bioremediation. *P. aeruginosa* is known to produce rhamnolipid biosurfactants (Arino et al., 1996) that may inhibit the growth of some species of bacteria (Guerra-Santos et al., 1984; Lang, 2002). The potential toxic or inhibitory effect of rhamnolipids on the candidates of the consortium was thus evaluated, and growth inhibition was only evident for the *Pseudomonas* sp. LBBMA 178 strain (Fig. 2). This strain was hence excluded from the consortium selected for gasohol bioremediation.

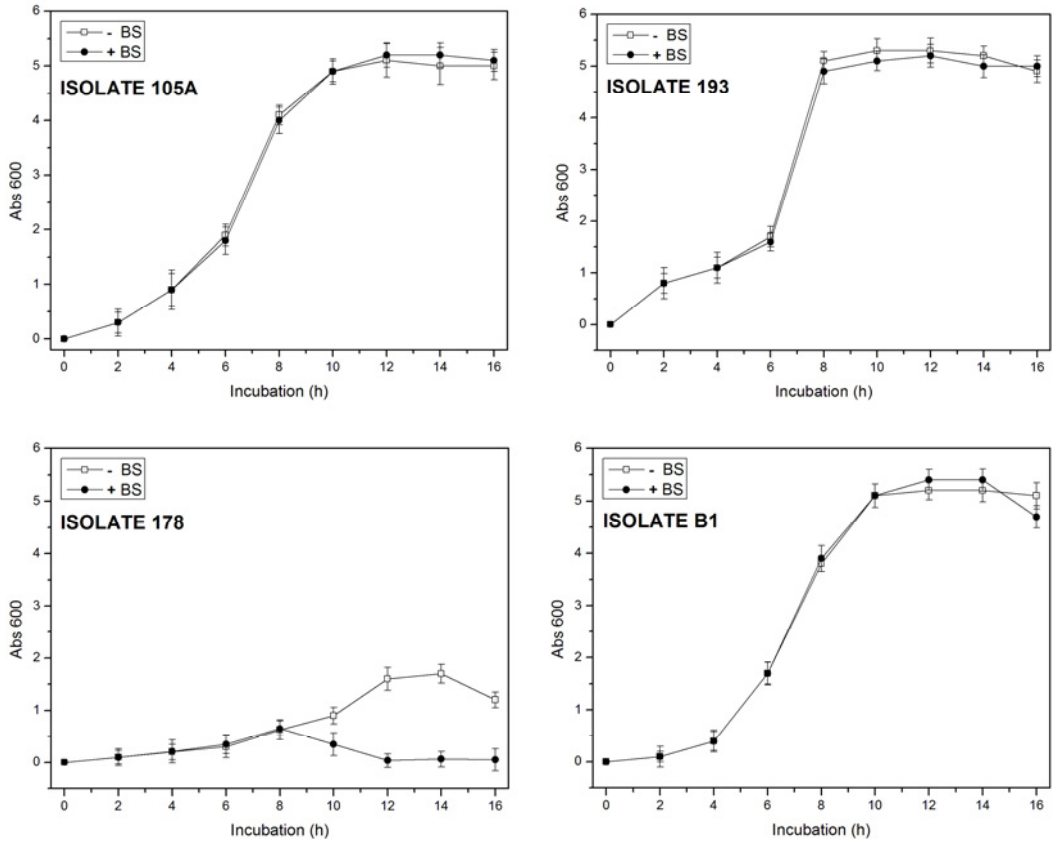

Fig. 2. Effect of the rhamnolipid biosurfactant on the growth of isolates, LBBMA 105A, 178, 193 and B1.

The micro-organisms were inoculated into culture tubes containing 4 ml mineral medium with 0.148 g/l ammonium nitrate (MMN) supplemented with glucose (2%, w/v) and rhamnolipid biosurfactant in 3CMC (critical micelar concentration). Microbial growth was determined by measuring optical densities at 600 nm (-BS = absence of biosurfactant; +BS = presence of biosurfactant).

3.2 EFFECT OF ORC ON MICROBIAL GROWTH

Addition of Regenesis ORC® to the culture medium inhibited the growth of all components of the consortium. This effect could be attributed to the increase in pH of the culture medium caused by the production of $Mg(OH)_2$, following the reaction of the MgO_2 with water. Gallizia et al. (2004) also reported a significant increase in pH during a study of the biostimulation of

enzymatic activities by ORC in sea environment microorganisms. It may, thus, be concluded that the utilisation of ORC in bioremediation should be limited to environments that are characterised by high buffering capacity or in situations where the pH can be readily controlled.

3.3 BIOREMEDIATION OF GASOHOL-CONTAMINATED SOIL SLURRIES

The levels of BTX in soil slurries inoculated with the microbial consortium (treatments 5 - 7 and 11 - 13) were significantly lower than those obtained in equivalent treatments involving uninoculated sterile soil (treatments 2 – 4 and 8 – 10, respectively) (Table 1). These results indicate that a previous screening of BTX degradation in the mineral medium may be useful for selecting microbial strains for bioremediation of BTX-contaminated soil, especially when adopting a controlled ex-situ bioremediation procedure. The presence of mineral nutrients, together with the microbial consortium (treatments 11 – 13), caused a significant reduction in BTX levels, compared with equivalent treatments carried out in the absence of nutrients (treatments 5 - 7, respectively). Thus, biodegradation of petroleum hydrocarbons in nutrient-limited subsoil could be somewhat restricted, as also noted by Bartha (1986).

The BTX concentration was significantly higher in uninoculated sterile soil (treatment 2) than in the equivalent treatment with non-sterile soil (treatment 1). This result suggests the presence of autochthonous microbial populations that have the capacity to degrade BTX. The existence of micro-organisms with the physiological potential to degrade hydrocarbons, including BTX, in the subsoil has been widely reported (e.g., Cavalca et al., 2000).

The application of Regenesis ORC® (treatments 3, 6, 9 and 12) did not result in any increase in the efficiency of BTX degradation, in comparison with the respective controls (treatments 2, 5, 8 and 11). This result may be attributed to the inhibition of microbial growth caused by increased pH (8.4 – 9.1) in the presence of ORC, in comparison with the controls (pH values close to 7.0).

Among the treatments involving uninoculated sterile soil slurries (treatments 2 – 4 and 8 – 10), significantly lower residual BTX concentrations were observed in those in which air injection was applied (treatments 4 and 10). These reductions were probably caused by the volatilisation of the aromatic compounds. Air injection in the presence of the bacterial consortium caused a further reduction in BTX concentration. In treatments that did not receive mineral nutrients, the reduction of *o*-xylene concentration was higher than for benzene and toluene. In comparison with the control (treatment 5), the reduction in *o*-xylene attributable to ventilation (treatment 7) was 49%, whereas for benzene and toluene, the contribution of ventilation was only 21%. In the presence of mineral nutrients, ventilation (treatment 13 cf. to 11) contributed significantly to the removal of benzene (74%), toluene (79%) and, to a lesser extent, *o*-xylene (59%). These results indicate that mineral nutrient availability was more limiting for the biodegradation of benzene and toluene than for the biodegradation of *o*-xylene.

In summary, the microbial consortium constituted of *P. aeruginosa* (LBBMA 53A), *S. maltophilia* (LBBMA 105A), *P. balearica* (LBBMA 193) and the unknown sp. LBBMA B1, effectively promoted the biodegradation of BTX in gasohol-contaminated soil. Cavalca et al. (2004) reported the isolation of bacteria belonging to the genera *Pseudomonas, Azoarcus, Bradyrhizobium, Microbacterium* and *Mycobacterium* using an air injection system in a BTEX-contaminated aquifer. Additionally, the degradation of monoaromatic hydrocarbons by *S. maltophilia* has also been described (Lee et al., 2002). To the best of our knowledge,

there are no reports employing *P. balearica* when using monoaromatic hydrocarbons as the sole carbon source.

4. CONCLUSION

Sixteen of 52 bacterial strains tested showed satisfactory growth using gasoline as the sole carbon source, and 10 of these used gasohol as the sole carbon source. Strains LBBMA 53A (*P. aeruginosa*) and LBBMA 178 (*Pseudomonas* sp.) produced biosurfactants using gasohol as the sole carbon source. Regenesis ORC® inhibited the growth of all of the bacterial strains selected for the microbial consortium. Air injection proved to be a better technique for supplying oxygen to the bioremediation process in the multi-cell bioreactor system. Application of the microbial consortium resulted in a significant increase in the biodegradation of BTX present in gasohol.

The results obtained are promising and open up future perspectives for the use of the bacterial consortium in the bioremediation of gasohol-contaminated soils.

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