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Full Length Research Paper

Pre-screening of filamentous fungi isolated from a contaminated site in Southern Brazil for bioaugmentation purposes

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Four *Aspergillus* sp. strains were isolated from contaminated soil in Rio Grande, Southern Brazil. The biodegradation potential of these strains was evaluated using a simple method involving the determination of colony growth rates on plates containing a specific hydrocarbon or petroleum derivative as the only carbon source. The LEBM1 strain presented a high tolerance level to BTX. It was the only strain capable of growth on all the media, with growth rates varying from 1.3 to 2.2 mm/day. The LEBM2 strain presented the potential for phenol degradation, while the LEBM3 strain could be used for gasoline, diesel oil, hexane and chlorobenzene.

Key words: Bioremediation, bioaugmentation, biodegradation, hydrocarbons, filamentous fungi, colony growth rate.

INTRODUCTION

The petroleum industry is responsible for the generation of large amounts of organic residues, as well as for the pollution of soils, rivers and seas. One of the best approaches to restoring contaminated environments is to make use of the physiological potential of microorganisms able to degrade the pollutants in a bioremediation process. It is an attractive approach to cleaning up hydrocarbons because it is simple to maintain, applicable over large areas, cost-effective and leads to the complete destruction of the contaminant (Bento et al., 2005).

Bioremediation involves either stimulating indigenous microbial populations by environmental modifications (biostimulation), or introducing exogenous microbial populations known to be efficient degraders into a contaminated site, a process also known as bioaugmentation (Bento et al., 2005).

Numerous microorganisms are known for their ability to degrade hydrocarbons. The biodegradation capabilities of bacteria have been recognized, but fungi have been the subject of recent research (Colombo et al., 1996; Krivobok et al., 1998; Salicis et al., 1999; García et al., 2000; Garon et al., 2000; Baheri and Meysami, 2002; Romero et al., 2002; Chaillan et al., 2004; Santos and Linardi, 2004; Potin et al., 2004), due to their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin degradation, which are capable of degrading high molecular weight, complex or more recalcitrant compounds, including aromatic structures.

The full potential of biodegradation by filamentous fungi for bioremediation purposes has not been fully investigated. The use of filamentous fungi isolated from contaminated soil may offer advantages for several reasons. Owing to their ability to extend through the soil by hyphal elongation, fungi can access xenobiotics. In addition fungi are capable of growing under stressful environmental

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conditions, such as environments with low pH or low water activity (Potin et al., 2004).

It would appear that special attention should be paid to bioremediation processes taking place in tropical and subtropical regions, since little information is available on work in these areas in comparison with other geographical areas. In such studies, it would be interesting to use simple methods to make a preliminary selection of potential strains for bioaugmentation purposes.

Therefore, the main goal of this work was to evaluate the colony growth rates of filamentous fungi isolated from contaminated soil in the Rio Grande harbor area in Southern Brazil, using different petroleum hydrocarbons or derivatives as the only carbon source, with a view to selecting strains for future employment in bioaugmentation schemes.

MATERIAL AND METHODS

Chemicals

Phenol, hexane, chlorobenzene, benzene, toluene and xylene were of analytical grade. Gasoline and diesel oil were purchased from a local petroleum refinery.

Soil samples

Soil samples were collected from the top 10 cm of contaminated soil in the Rio Grande harbor area, located in the Patos Lagoon estuary, Southern Brazil. The samples were transported in 500 ml sterile flasks and stored at 4℃ until used. This soil had the following characteristics: oil and grease 9.5%; phenols 4.6 mg/Kg; moisture content 8.96%; pH 9.95.

Enrichment

The soil was inoculated into 125 ml flasks containing 50 ml of mineral medium supplemented with 2% glucose. The flasks were incubated at 30^0C and 150 rpm for 144 h, according to Cunha et al. (2001).

Isolation, identification and maintenance

Fungal strains were isolated from these cultures by spreading onto Petri dishes containing potato dextrose agar (PDA), according to Taniwaki (1996).

The isolated strains were identified using the microculture technique and according to the general principles of fungal classification (Barnett and Hunter, 1998). The strains were maintained on 2% malt extract agar at 4℃.

Growth assessment

The mineral medium proposed by Cunha et al. (2001) with the following composition was used (g/l): 0.4 KH_2PO_4; 0.2 $MgSO_4.7H_2O$; 0.1 NaCl; 0.025 $CaCl_2.2H_2O$; 0.003 $MnSO_4.H_2O$; 0.5 $NH_4NO_3.2H_2O$; 12 agar.

Test dishes were prepared by adding 0.25% or 0.5% (v/v) of a sterile specific hydrocarbon or petroleum derivative as the only carbon source [gasoline, diesel oil, hexane, chlorobenzene, a mixture of benzene, toluene and xylene (BTX)]. For phenol-based media, the concentrations were 0.025 and 0.05% (w/v). The solutions were thoroughly mixed with a magnetic stirrer just before adding to the plates (Meysami and Baheri, 2003).

Isolated filamentous fungi were previously cultivated on Saboraud Agar. For the evaluation of colony growth an adapted version of the technique of Sanzo (1999) was used. An inoculation loop was used to aseptically scrape off sporulating mycelia from the surface of the slants. The scraped mycelia were used to inoculate an agar suspension (0.1 ml), the agar being added to avoid the spores spreading over the plate. The suspension was used to centrally inoculate the surface of Petri plates containing hydrocarbon-based media. Five replicates were assessed for each set of plates.

The plates were incubating for 14 days at 25^oC and examined periodically for changes in colony diameter. The growth, assessed as the change in diameter of the approximately circular growing colony, was determined by measuring at least two diameters per plate. The average of the diameters was used as the colony diameter at that particular time of measurement. Colony growth rates (mm/day) were calculated by regressing the colony diameter against the days after inoculation.

Statistical analysis

The results were evaluated statistically through Variance Analysis and Tukey test at 95% confidence level ($p < 0.05$), using Statistica 6.0 software.

RESULTS AND DISCUSSION

Fungi identification

Four strains were isolated from the contaminated soil. They were identified as *Aspergillus* sp. and were denominated LEBM1, LEBM2, LEBM3 and LEBM4. According to Chaillan et al. (2004), *Aspergillus* and *Penicillium* are the most commonly encountered genera of hydrocarbon degraders in oil contaminated tropical soils, in agreement with the present results.

Culture growth evaluation

Colony growth rate evaluation has been used to investigate the growth of filamentous fungi, for example to determine the effects of water activity and temperature (Samapundo et al., 2007), the antifungal activities of plant extracts (Quiroga et al., 2001) and avian excreta (Osono et al., 2006) and the toxicity of pentachlorophenol (Dritsa et al., 2007) and crude oil (Meysami and Baheri, 2003). In this work, we suggest this methodology as a tool to establish the biodegradation potential of fungal strains.

The growth curves of the isolates, based on colony diameters as a function of time, were typical of fungal growth. The correlation coefficients (r^2) were greater than 0.95 (data not shown), indicating that the linear regression adequately explained the variation of the colony diameter as a function of time.

The mean values and standard deviations for the growth rates of each strain in the different hydrocarbons

Table 1. Average colony growth rates ± standard deviations (mm/day) and statistical analyses of the data*.

Pollutant	LEBM1		LEBM2		LEBM3		LEBM4	
	0.25%	0.5%	0.25%	0.5%	0.25%	0.5%	0.25%	0.5%
Gasoline	$2.1\pm0.1^{A,ac}$	$2.0\pm0.3^{A,ac}$	$2.1\pm0.3^{A,ac}$	$1.8\pm0.1^{B,a}$	$2.2\pm0.2^{A,ac}$	$2.3\pm0.2^{A,c}$	$1.1\pm0.1^{A,b}$	$0.9\pm0.1^{AB,b}$
Diesel Oil	$2.1\pm0.2^{A,a}$	$2.1\pm0.2^{A,a}$	$1.8\pm0.1^{B,a}$	$1.8\pm0.2^{B,a}$	$2.1\pm0.2^{A,a}$	$2.1\pm0.3^{A,a}$	$1.0\pm0.3^{AB,b}$	$0.8\pm0.1^{ABD,b}$
Hexane	$2.1\pm0.1^{A,a}$	$2.1\pm0.1^{A,ad}$	$1.6\pm0.2^{B,b}$	$1.8\pm0.1^{B,db}$	$2.3\pm0.2^{A,a}$	$2.2\pm0.1^{A,a}$	$0.8\pm0.2^{ABD,c}$	$0.5\pm0.1^{D,c}$
Chlorobenzene	$2.1\pm0.1^{A,ad}$	$2.2\pm0.2^{A,ad}$	$1.7\pm0.1^{B,b}$	$1.9\pm0.2^{AB,ab}$	$2.2\pm0.2^{A,ad}$	$2.3\pm0.2^{A,d}$	$0.7\pm0.2^{BD,c}$	$0.9\pm0.1^{AB,c}$
BTX	$1.3\pm0.3^{B,a}$	$0.0^{C,b}$	$0.0^{C,b}$	$0.0^{C,b}$	$0.0^{B,b}$	$0.0^{B,b}$	$0.0^{C,b}$	$0.0^{C,b}$
Phenol**	2.1 ± 0.1^{a}	0.6 ± 0.2^{b}	1.8 ± 0.2^{a}	1.1 ± 0.2^{c}	1.3 ± 0.1^{c}	0.6 ± 0.2^{b}	0.5 ± 0.1^{b}	0.0^{d}

*Arithmetic means within the same column with the same uppercase letter are not significantly different from each other at the 95% confidence level ($p < 0.05$). Also, arithmetic means within the same row with the same lowercase letter are not significantly different from each other at the 95% confidence level ($p < 0.05$).
**0.025 and 0.05% (w/v), respectively.

or oil derivatives are presented in Table 1. An analysis of variance and the Tukey test were performed to establish if there were any differences between the growth rates.

There were no significant differences between the colony growth rates of *Aspergillus* sp. LEBM1 on gasoline, diesel oil, hexane and chlorobenzene, reaching values of about 2.1 mm/day. The exception was BTX (0.25%), in which a significantly different grow rate (1.3 mm/day) was obtained.

The growth rates for *Aspergillus* sp. LEBM2 in media containing 0.25% gasoline and 0.5% chlorobenzene were significantly greater, reaching 2.2 mm/day. The growth rates of this strain in media containing diesel oil and hexane were statistically equal, at both concentrations.

The colony growth rates of *Aspergillus* sp. LEBM3 in media containing gasoline, diesel oil, hexane and chlorobenzene were not significantly different at either concentration (2.3 mm/day).

For *Aspergillus* sp. LEBM4, a significant difference in growth rate was observed between gasoline (1.1 mm/day) and chlorobenzene (0.7 mm/day) at the 0.25% level. At 0.5%, the growth rate in media containing chlorobenzene (0.9 mm/day) differed from that containing hexane (0.5 mm/day). Thus the LEBM4 strain showed significantly lower growth rates in all the media (from 0.5 to 1.1 mm/day).

Growth in the mineral medium containing BTX was not observed at the concentrations studied, with the exception of *Aspergillus* sp. LEBM1. This strain grew in media containing 0.25% BTX. This behavior can probably be explained on the basis of the inhibition of microbial growth at these concentrations. However, the LEBM1 strain showed high tolerance to BTX, pollutants showing high toxicity and water solubility. Similar performance has not been reported in the literature.

Using media containing 0.25% gasoline, *Aspergillus* sp. LEBM4 presented lower growth rates than the other strains, the same occurring with diesel oil at the same concentration. *Aspergillus* species have frequently been detected in fuels in Brazil (Bento and Gaylarde, 2001).

When hexane or chlorobenzene was used, *Aspergillus* sp. LEBM1 and LEBM3 presented significantly greater growth rates. According to Spigno et al. (2003), there is a lack of publication in the literature on fungi capable of metabolizing these solvents. Moreover, chlorobenzene is a chemical solvent extensively used in industrial processes, for which biodegradation processes associated with bacteria are more frequently used (Wang et al., 2007).

With respect to phenol at concentration of 0.025%, *Aspergillus* sp. LEBM1 and LEBM2 presented greater growth rates than the LEBM3 and LEBM4 strains. However, at a concentration of 0.05%, *Aspergillus* sp. LEBM2 differed from the others, presenting the best performance for phenol degradation (1.1 mm/day). The LEBM4 strain had no growth capacity at this concentration.

Comparing the different concentrations, with the exception of BTX and phenol, all the fungi gave statistically equal growth rates with the increase in concentration. For gasoline, diesel oil, hexane and chlorobenzene, the change in concentration did not inhibit mycelial growth.

For phenol, all the strains showed better performance at the lowest concentration, indicative of the strong inhibitory effect of phenol. *Aspergillus* species have been selected as phenol degraders (García et al., 2000; Santos and Linardi, 2004), and considering that the majority of microorganisms are sensitive to 0.02%, the performance of *Aspergillus* sp. LEBM2 was significant.

Conclusion

It could be observed that the biodegradation potential of the fungal strains varied within the species, as mentioned by Oudot et al. (1993). All the differences observed can be explained on account of the fact that the degradation capability of a compound is related to the activity of required catabolic enzymes and to the inhibitory effect on microbial growth (Colombo et al., 1996).

Chaillan et al. (2004) observed that the biodegradation activity of fungi was greater with saturated than with aro-

Table 2. Potential strains for bioaugmentation purposes.

Hydrocarbon or petroleum derivative	Recommended strains
Gasoline	*Aspergillus* sp. LEBM1 and LEBM3
Diesel Oil	*Aspergillus* sp. LEBM1, LEBM2 and LEBM3
Hexane	*Aspergillus* sp. LEBM1 and LEBM3
Chlorobenzene	*Aspergillus* sp. LEBM1 and LEBM3
BTX	*Aspergillus* sp. LEBM1
Phenol	*Aspergillus* sp. LEBM2

matic compounds. Colombo et al. (1996) verified that the degradation capability varied according to the fungi. *Aspergillus terreus* and *Penicillium chrysogenum* degraded aliphatic hydrocarbons more efficiently than aromatic ones, the opposite occurring with *Fusarium solani*. The present results are in agreement with these reports. It was observed that *Aspergillus* sp. LEBM1 exhibited degradation activity on both saturated and aromatic hydrocarbons. It was the only strain that grew in the BTX medium.

Based on statistical analysis of data, we suggest in Table 2 the potential strains to bioaugmentation purposes for removing specific pollutants. Therefore, colony growth rate analysis was a satisfactory tool to evaluate the potential of a filamentous fungi strain to degrade different pollutants.

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