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Full Length Research Paper

Biodegradation of phenol by free and encapsulated cells of a new *Aspergillus* sp. isolated from a contaminated site in southern Brazil

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The aim of this study was to compare the biodegradation performance of phenol by using free and encapsulated cells of a new *Aspergillus* sp. strain isolated from a crude oil contaminated soil in southern Brazil. In batch cultures, maximum degradation rates were not significantly different between free and encapsulated cells, but a decrease in adaptation time for encapsulated ones was observed. This fact indicates the presence of a microenvironment that is more favorable to biodegradation inside encapsulated cells, because of the protector effect of gel matrix, which reduces abiotic stress. Encapsulated filamentous fungus *Aspergillus* sp. LEBM2 showed a promising application in bioaugmentation processes, reaching maximum phenol degradation rate of 7.71 ± 0.21 mg/l.h for an initial phenol concentration of 500 mg/l.

Key words: Bioremediation, bioaugmentation, immobilization, phenol, filamentous fungi.

INTRODUCTION

Phenols and phenolic compounds are widely distributed as environmental pollutants due to their common presence in the effluents of many industrial processes, including oil refineries, ceramic plants, coal conversion process, phenolic resins, pharmaceutical and food Industries. These compounds represent a serious ecological problem due to their toxicity and widespread occurrence in the environment. Once wastewater containing phenol is discharged into the receiving body of water, it endangers fish life, even at relatively low concentration of 5 - 25 mg/l (Yan et al., 2005). Due to their high inhibitory and antibacterial activity, phenols may create problems in the operation of biological treatment plants (Rigo and Alegre, 2004). They also add odor to drinking and food processing water (Adav et al., 2007) and have mutagenic and carcinogenic effects (Bolaños et al., 2001).

In spite of phenolic toxic properties, a number of microorganisms can utilize phenol under aerobic conditions as sources of carbon and energy (Chen et al., 2002; Hidalgo et al. 2002; Riso and Alegre, 2004; Santos and Linardi, 2004; Yan et al., 2005; Adav et al., 2007; Juang and Wu, 2007; Nair et al., 2007).

Bioremediation technologies are well established for the clean up of chemically contaminated sites, and many technologies are applied commercially in large scale (Jorgensen et al., 2000). Bioaugmentation is the technique for improving the capacity of a contaminated matrix to remove pollution by the introduction of specific strains or microbial consortia. The basic premise for this intervention is that the metabolic capacities of the indigenous microbial community already present in the biotope slated for cleanup will be increased by an exogenously enhanced genetic diversity, thus leading to a wider repertoire of productive biodegradation reactions (Fantroussi and Agathos, 2005).

Success of a bioaugmentation procedure depends on the survival of the inoculated cells. Their survival can be promoted by the encapsulation in a carrier, as poliacrilamide (Chen et al., 2002), gellan gum (Moslesmy et al., 2002) and alginate (Santos et al., 2003), which protects them against the natural competition with the native

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micro-organisms (Moslemy et al., 2002).

Among all encapsulating materials, alginate is the most widely used one, which is a linear heteropolysaccharide of D-mannuronic and α-guluronic acids extracted from various species of algae. The functional properties of alginate as a supporting material are strongly associated with the composition and sequence of L-guluronic and mannuronic acids. Divalent cations such as Ca^{2+} bind preferentially to the polymer of L-guluronic acid (Chen et al., 2006).

The use of encapsulated cells for environmental applications has many advantages over free cell formulations, including protection from biotic stresses such as predation by protozoa and bacteriophage, protection from abiotic stresses such as the inhibitory effect of toxic compounds, enhanced survival and improved physiological activity, supply of co-encapsulated nutritional additives, increased cell densities and preferential cell growth in various internal aerobic and anaerobic zones of encapsulating gel. Encapsulation may also increase transport distances of degrading cells in subsurface bioaugmentation schemes. Microbial attachment to soil grain surfaces followed by formation of extracellular polymers (exopolymers) is a major impediment to the successful distribution of free active cells (Moslemy et al., 2002). By preventing washout, immobilization allows maintenance of a high cell density in a bioreactor at any flow rate. Catalytic stability can be greater for encapsulated cells and some encapsulated microorganisms tolerate higher concentrations of toxic compounds than do their non-encapsulated counterparts (Santos et al., 2003).

The degradation of phenol by immobilized cells had been investigated for several microorganisms such as *Pseudomonas* (González et al., 2001), *Candida* (Chen et al., 2002), *Graphium* (Santos et al., 2003) and *Alcaligenes* (Nair et al., 2007). However, the use of encapsulated *Aspergillus* cells for phenol biodegradation is scanty.

This study has the results obtained from the biodegradation of phenol by free and encapsulated cells of *Aspergillus* sp. LEBM2. Batch experiments were carried out in order to obtain the maximum phenol degradation rates by analyzing the influence of the immobilization in calcium-alginate gel beads on biodegradation performance.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade.

Synthetic wastewater

Sterile synthetic wastewater composition (mg/l) was proposed by Passos et al. (2009): 400 KH_2PO_4; 200 $MgSO_4.7H_2O$; 100 NaCl; 25 $CaCl_2.2H_2O$; 3 $MnSO_4.H_2O$; 500 $NH_4NO_3.H_2O$; 500 glucose; phenol (250 or 500).

Microorganism

Aspergillius sp. LEBM2 was previously isolated from a contaminated soil at Rio Grande harbor area, Southern Brazil, and was capable of using phenol as carbon source (Santos et al., 2008). Strain was maintained on potato dextrose agar (PDA) at 4°C.

Spores suspension

The strain was propagated in 500 ml flasks containing 100 ml of PDA agar. Flasks were maintained at 25°C for 5 days until sporulation. Spores were diluted in sterile distilled water with Tween 80 0.2%. After removing mycelium, the resulting suspension was filtered to remove the hyphal filaments and spores were counted in a Neubauer chamber.

Fungal Spores encapsulation

Spores were encapsulated in calcium alginate according to Ellaiah et al. (2004). About 20 ml of sterile sodium alginate solution (3%, w/v) and 5 ml of spore suspension (5 x 10^6spores/ml) were mixed fully and the slurry was dripped into 0.2 M $CaCl_2.2H_2O$ solution at room temperature. The beads (about 3 mm of diameter) were maintained in $CaCl_2$ solution for 1 h at 4°C, and then the beads were washed three times with sterile distilled water.

Inoculum

The free and encapsulated spores were inoculated in 500 ml flasks with cotton plugs containing 50 ml of synthetic wastewater (250 mg/l of phenol) and were incubated at 25°C for 5 days to spores germination.

Phenol degradation experiments

Experiments in triplicate were carried out for initial phenol concentrations of 250 and 500 mg/l. Flasks containing synthetic wastewater were inoculated with free and encapsulated cultures, resulting in a total volume of 150 ml. Temperature and agitation were controlled at 25°C and 200 rpm on a rotary shaker. Control assays (without inoculum addition) were performed under the same experimental conditions in order to verify abiotic losses. Samples were withdrawn at regular intervals for phenol determination.

Phenol determination

For determination of phenol content, the Folin-Ciocalteau phenol reagent was used, involving the successive addition of 1 ml sodium carbonate (200 g/l) and 0.5 ml Folin-Ciocalteau phenol reagent to 10 ml sample. After 60 min at 20°C, the absorbance was measured at 725 nm against a distilled water and reagent blank (García et al., 2000).

Statistical analysis

The results were evaluated statistically by Variance Analysis and Tukey test at 95% confidence level ($p < 0.05$) with Statistica® 5.0 software.

RESULTS AND DISCUSSION

Figure 1 shows the time course of phenol biodegradation

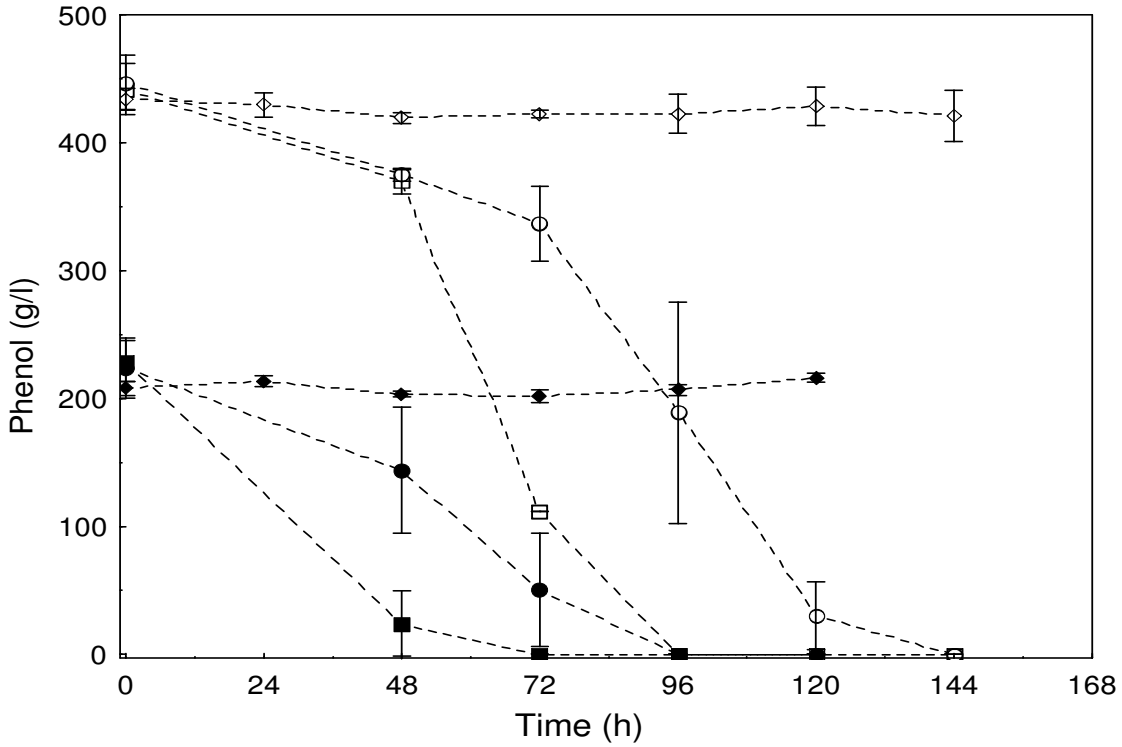

Figure 1. Batch cultures for phenol removal on a rotary shaker at 200 rpm and 30°C. ■ (encapsulated cells, 250 mg/l); ● (free cells, 250 mg/ml); ◆ (control, 250 mg/l); □ (encapsulated cells, 500 mg/l); O (free cells, 500 mg/l); ◇ (control, 500 mg/l).

in flask culture by free and encapsulated cells of *Aspergillus* sp. LEBM2. According to control assays, abiotic losses could be neglected.

The period for complete degradation varied according to the initial phenol concentration. For 250 mg/l, complete removal was reached in 72 and 96 h for encapsulated cell and free-cell cultures, respectively. For 500 mg/l, degradation occurred in 96 and 144 h, respectively.

The maximum degradation rates could be calculated based on data shown in Figure 1 by using Microcal Origin® 6.0 software. Results are in Figure 2. For a phenol concentration of 250 mg/l, maximum degradation rates were not significantly different at 95% confidence level. The maximum degradation rate was found as 3.48 ± 0.21 mg/l.h by free mycelium and 4.27 ± 0.68 mg/l.h by encapsulated mycelium. A similar behavior was observed when initial concentration increased to 500 mg/l (7.71 ± 0.21 and 6.38 ± 0.34 mg/l.h, respectively).

Apparently, the internal and external mass transfer resistances of substrates (phenol and oxygen) were not the determinant factors controlling the biodegradation process; otherwise, the maximum phenol degradation rates for encapsulated cells should be lower than the free ones. Adav et al. (2007) observed the same behavior for immobilized *Acinetobacter* in this level of phenol. On the order hand, Santos et al. (2003) reported higher maximum degradation rates for free *Graphium* FIB4 up to 12 mM, due to the resistance for diffusion of substrates through alginate matrices.

However, encapsulated cells of *Aspergillus* sp. resulted in a better performance than the free cells in batch process by reducing the adaptation phase and, consequently, the time for complete phenol degradation. As shown in Figure 1, when the initial concentration was 250 mg/l, after 48 h of incubation, the culture with cell beads reached 90% of phenol removal, whereas free cells reached 35%. As the inhibitory effect of phenol was less intensive in this concentration, encapsulated cells rapidly degraded phenol, and an adaptation phase was not clearly observed, while for free cells an adaptation time occurred (48 h). At 500 mg/l, during the first 48 h, both free and encapsulated cells were in the adaptation phase, because of the strong inhibitory effect of phenol. Thus, in this period, phenol concentration decreased slowly and no differences were observed between the cultures. However, for encapsulated cells, the decrease in phenol concentration was too fast after this time, reaching 75.8% in 72 h, while for free cells this adaptation period extended up to 72 h (degradation reached 24.7% in this time), and then phenol concentration decreased rapidly. In encapsulated-cell culture, the carrier material acts as a protective cover against the toxicity of phenol. By forming networks of the beads, a diffusion barrier for phenol is built up, which is not

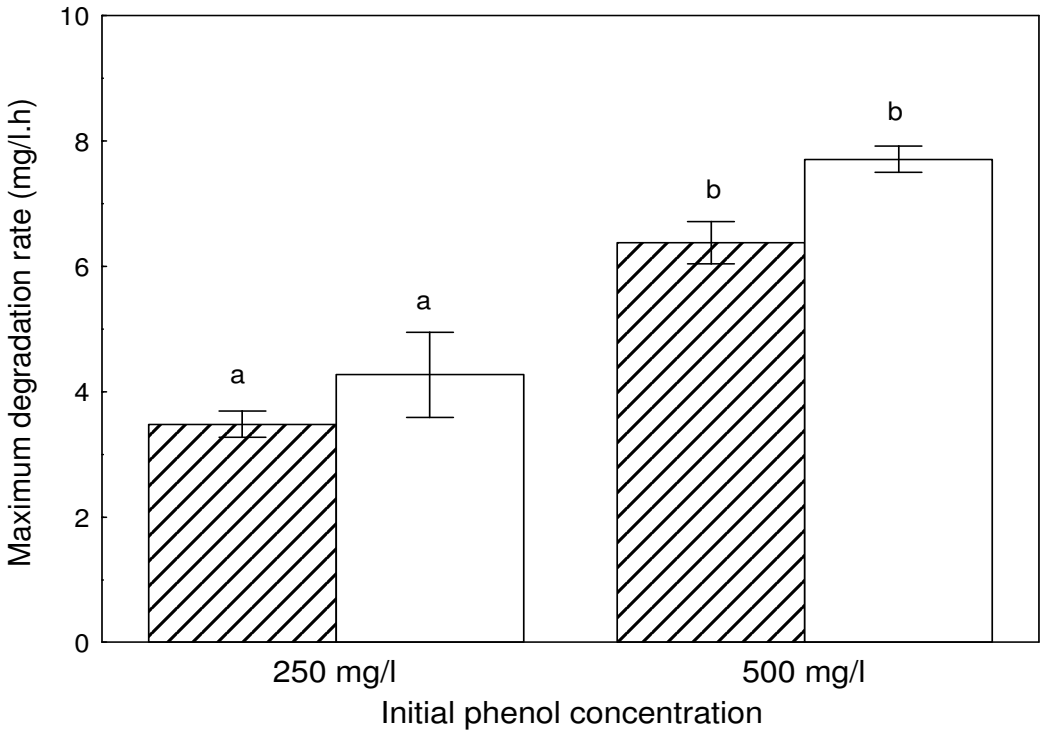

Figure 2. Mean values and standard deviations for maximum phenol degradation rates for free and encapsulated Aspergillus sp. LEBM2 cells. Same letters indicate that there was no significant difference at 95% confidence level ($p<0.05$). Striped bars (free cells); blank bars (encapsulated cells).

present in free-cell culture (Chen et al., 2002).

Moreover, the change in initial phenol concentration from 250 to 500 mg/l led to an increase in maximum degradation rate for both free and encapsulated cells (Figure 2). Similar behavior was observed by Santos and Linardi (2004) for *Graphium* sp. FIB4 of up to 10 mM of phenol and can be related to a high phenol tolerance of the strain.

Conclusion

Our work shows that *Aspergillus* sp. LEBM2 encapsulated cells in calcium alginate is promising for application in bio-augmentation schemes in order to degrade phenol and possibly other related aromatic compounds at high concentrations in superficial water, groundwater and wastewater which leads to a reduction in time for complete phenol removal in relation to free cells.

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