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Degradation of Pyrene in Soils by Free and Immobilized Yeasts, *Candida tropicals*

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Abstract The aim of this research was to ascertain the efficacy of immobilized yeasts for the degradation of pyrene in soil. Pyrene degradation by individual yeasts and their mixtures was clearly enhanced when the yeasts were immobilized with chemical and physical entrapments, and cross-shaped beads from physical entrapment were more promising than spherical beads from chemical entrapment. Immobilized yeast mixture *Candida tropicals* Y 219 + Y 220 showed the best degradation of pyrene, exhibiting collaboration in pyrene removal. Scanning electron micrographs demonstrated good macroporous structure for mass transfer of substrates and immobilized cell growth.

Keywords Pyrene · Immobilization · Soil · Yeast

As widely distributed contaminants, polycyclic aromatic hydrocarbons (PAHs) are of great concern because they can be carcinogenic as well as toxic to many organisms. The contamination of soils by PAH compounds is common in many locations (Hill and Ghoshal, 2002). Thus, an understanding of their removal from the environment is important (Fasnacht and Blough, 2002). Immobilized cell technology has been successfully used to degrade contaminants (Cassidy et al., 1996). This technology offers several advantages over the application of free cells in the destruction of xenobiotics (Nawaz et al., 1998). Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional treatment systems due to their high mechanical strength and high cell concentration (Jianlong et al., 2001; Beshay et al., 2002). Among the several immobilization methods reported, entrapment, which enclose cells in a proper support matrix, has been applied widely (Song et al., 2005). An immobilization matrix based on entrapment can be achieved by means of cross-linking using a chemical agent or by iterative freezing and thawing (Cunningham et al., 2004; Chen et al., 2002; Karigar et al., 2006).

In this study, polyvinyl alcohol (PVA) and activated carbon were added to sodium alginate and then cross-linked by a chemical agent or by the freezing and thawing method. In addition, before cross-linking three types of yeasts were added separately, in pairs, or in a triad to increase pyrene degradation in a soil slurry bioreactor. The aim of this research was to ascertain the efficacy of immobilized yeasts for the degradation of pyrene in a slurry bioreactor and the optimum support matrix of the immobilized cells. The degradation of pyrene by yeasts immobilized by means of a chemical agent or by freezing and thawing was compared. Furthermore, interactions between different yeasts in the matrix were determined to indicate whether a synergistic degradation existed.

Materials and Methods

A soil sample was collected from the agricultural field of the Ecological Station of the Institute of Applied Ecology, Chinese Academy of Sciences, at a depth of 0 to 20 cm. Soil characteristics are shown in Table 1. After the removal of stones and visible plant material, the soil was air-dried, grounded and sieved through 1 mm mesh. About 30 g of

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Table 1 Characteristics of the experimental soil

PH	Total organic carbon (%)	Texture (%)			Bulk density (g/cm^2)
		Sand	Silt	Clay	
6.8	1.78	21.4	46.5	32.1	2.53

soil treated by the above procedure was homogeneously mixed in a 250-mL Erlenmeyer flask with 15 mL of a pyrene/methanol solution (200 mg L^{-1}) to prepare a contaminated soil sample with a known mass of pyrene. The spiked soil was kept in the dark for days to completely evaporate the methanol. A concentration of pyrene in the soil sample of 100 mg kg^{-1} was therefore assumed, which is within the reported rang for soils (Witt and Trost, 1999). The spiked soil in the Erlenmeyer flask was then mixed with 90 mL of medium A to form slurry after shaking. The minimal medium composition (medium A) for cultivation of yeast contained (per liter): 20 g glucose, 200 g potato, and a pH adjusted to 7.0. The Erlenmeyer flasks with the slurry inside were sterilized by an autoclave for 30 min.

Three yeasts (*Candida tropicals* MY, Y 219, and Y 220), proven to be capable of efficient pyrene degradation, were selected for this study. They were identified through morphological, biochemical and physiological methods. The yeasts were maintained in batch cultures in 250-mL Erlenmeyer flasks containing 100 mL of medium A. Batch cultures of yeasts were grown in an orbital incubator at 28°C in the dark.

The yeasts were harvested during their logarithmic growth phase to ensure the highest yeast activities and numbers. The yeasts were immobilized alone, in pairs or in a triad with either a physical or chemical entrapment method. To prepare the matrix solution, 10 g of polyvinyl alcohol (PVA), 0.5 g of sodium alginate, and 5 g of activated carbon were first carefully dissolved by slow stirring in 60 mL of distilled water. The solution was kept overnight, autoclaved for 30 min, cooled to 40°C, mixed with each yeast alone (20 mL), in pairs (10 mL of each), or in a triad (6.6 mL of each), and made up to a final volume of 100 mL. In the physical entrapment method, the matrix solution with yeasts was poured into trays with cross-shapes inside, frozen at –15°C in a freezer for 22 h, thawed under sterilized aerated conditions and air-dried, frozen again at –15°C in a freezer for 17 h , and eventually thawed at under sterilized aerated conditions and air-dried to form cross-shaped beads. In the chemical entrapment method, the matrix solution with yeasts was added to 300 mL of saturated boric acid solution for cross-linking. The final mixture was extruded drop by drop into the cation solution, from a height of approximately 15 cm and at a rate of approximately one drop per second to form spherical gel beads with a diameter of 2.5 mm. After formation, the

Fig. 1 Comparison of bead sizes and shapes during immobilization. A. Spherical bead from chemical entrapment; B. Cross-shaped bead from physical entrapment

spherical or cross-shaped beads were cultured in fermentation medium (medium B, containing 2% glucose, 0.3% leavening, 20% potato, and a pH adjusted to 7.0 followed by sterilization of the medium at 121°C for 15 min) in three cycles on a rotary shaker at 28°C for 30 h. After each cycle of cultivation, the used fermentation medium was decanted and the beads were washed with sterile water and transferred into fresh fermentation medium. The beads formed from chemical and physical entrapments are shown in Fig. 1.

Free yeasts or the equivalent of immobilized cells were added to 250-mL Erlenmeyer flasks containing the soil slurry described above. The degradation processes were carried out at room temperature (28°C) on a rotary shaker at 160 rounds per minute for four days. Samples from the soil slurry were taken under sterile conditions at incubation periods of 24, 48, 72 and 96 h for the analysis of residual pyrene. Control experiments were carried out in parallel on the sterile slurry with the same PAH loadings.

A dried soil sample was transferred into 100 mL Teflon tubes, and mixed with dichloromethane (1 g of soil: 5 mL of dichloromethane). Each sample was extracted for 2 h in an ultrasonic bath, in which the water temperature was lower than 40°C. The mixtures were then centrifuged to separate the supernatant from the soil. A half milliliter of the extract was passed through a glass column containing 1 g of silica gel wetted with hexane. The extract was eluted with 2 mL of a mixture of hexane: dichloromethane (1:1, v/v). The eluate was completely dried under a gentle stream of nitrogen. The solid residue was dissolved in 1 mL of methanol for high-performance liquid chromatography (HPLC) analysis. Quantification of pyrene and benzo(a)pyrene in methanol solutions was done using an HPLC (Hewlett-Packard 1090-Series) with a diode array detector. The mobile phase used was methanol:water (85:15, v/v) at a flow rate of 0.8 mL min^{-1}, and the detector wavelength was 240 nm for pyrene. The injection volume was 10 μL. The extraction recovery of pyrene and under the given conditions was 91%.

The means of degradation were compared using one-way analysis of variance (ANOVA), and the homogeneity

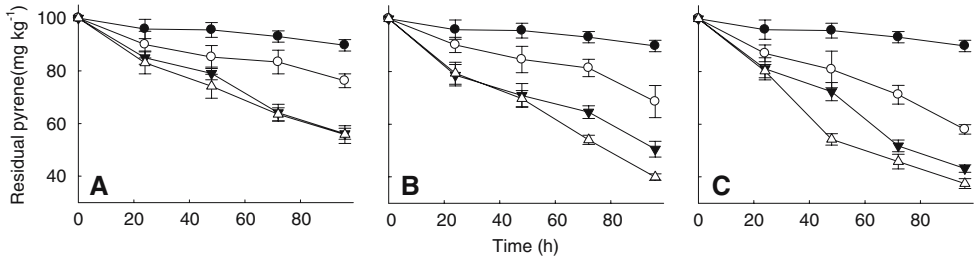

Fig. 2 Degradation of pyrene in slurry reactors by yeasts. A, *Candida tropicals* MY; B, *Candida tropicals* Y219; C, *Candida tropicals* Y220. •, ○, ▼, and □ are sterile control, free cells, immobilized cells with chemical entrapment, and immobilized cells with physical entrapment, respectively

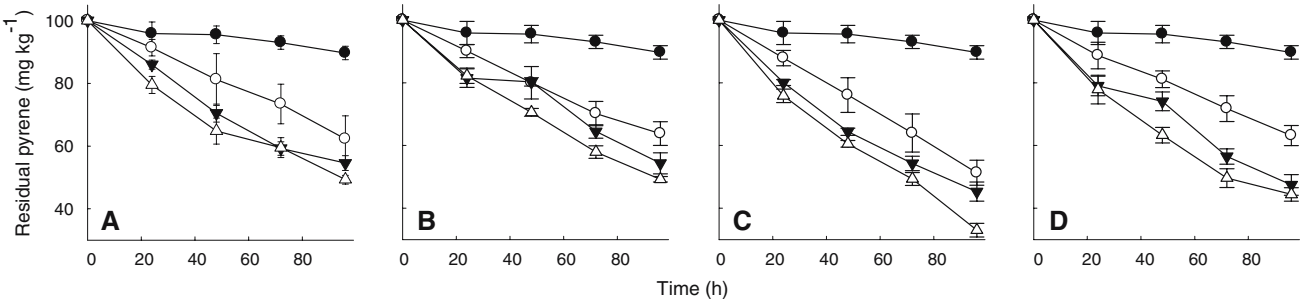

Fig. 3 Degradation of pyrene in slurry reactors by mixed yeast cultures. A, MY+Y219; B, MY+Y220; C, Y219+Y220; D, MY+Y219+Y220. •, ○, ▼, and □ are sterile control, free cells, immobilized cells with chemical entrapment, and immobilized cells with physical entrapment, respectively

of the variances was tested with Levene's test. If the values were statistically different, post comparisons were performed using the Duncan test. All statistical analyses were conducted using the statistical software package SPSS 11.5, at 95% confidence.

Results and Discussion

The degradation kinetics of pyrene by free and immobilized *Candida tropicals* MY, Y 219, and Y 220 are shown in Fig. 2. It can be seen that *Candida tropicals* Y 220 had better pyrene degradation capabilities than the other yeasts. Pyrene degradation by the yeasts was obviously enhanced when the yeasts were immobilized with chemical or physical entrapments. Yeasts immobilized with chemical entrapments degraded 44, 50, and 57% of pyrene, respectively, and immobilized yeasts with physical entrapment degraded 44, 60, and 62% of pyrene, respectively, after 96 h of incubation from an initial 100 mg kg^{-1} concentration of pyrene. Under identical condition, only 24, 31, and 42% of pyrene was degraded by the free cells, respectively. Figure 2 demonstrates that physical entrapment was better than chemical entrapment for the degradation of pyrene.

The individual yeasts were also mixed together in all possible combinations to study the feasibility of consortium immobilization and cell interactions. The degradation kinetics of pyrene by immobilized yeast mixtures are compared in Fig. 3. It demonstrates that immobilization technology was also applicable to a consortium of yeasts in the soil slurry reactor, as immobilized yeast mixtures showed advantages in degrading pyrene over free cells. On the other hand, physical entrapment was more promising than chemical entrapment for the yeast mixtures. Degradation of pyrene by yeast mixtures with chemical entrapment was 45, 46, 55, and 53%, respectively after 96 h of incubation, and that by yeast mixtures with physical entrapment was 51, 51, 67, and 56%, respectively.

To determine the interactions of the cells in the matrix, the pyrene removal after 96 h biodegradation by individual immobilized yeasts and their mixtures was compared in Fig. 4. It can be seen that both inhibitory and synergistic effects on the degradation of pyrene existed. Yeast mixture *Candida tropicals* Y 219 + Y 220 immobilized with physical entrapment showed the best degradation of pyrene, exhibiting collaboration in pyrene removal. The second best pyrene degradation was from *Candida tropicals* Y 220 with physical entrapment.

Figure 5 shows scanning electron micrographs of immobilized *Candida tropicals* Y220 from the bead surface to the center. At the surface of the beads with physical entrapment, the cells grew very well due to enough oxygen

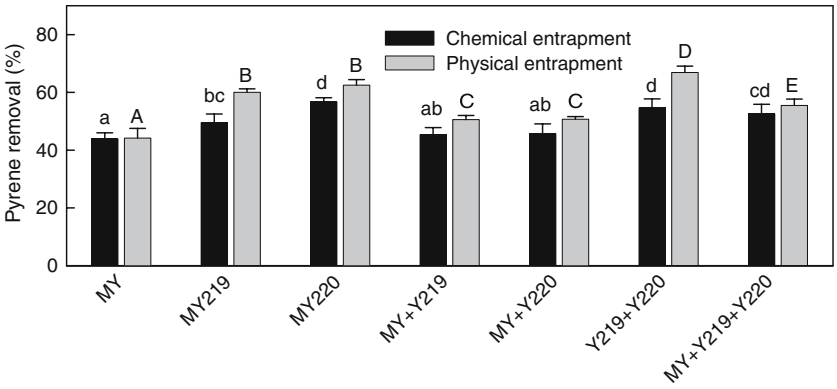

Fig. 4 Comparison of pyrene degradation by immobilized yeasts and their mixtures with chemical and physical entrapments. Error bar indicates one standard deviation (SD). Different letters means significant differences between plots ($p < 0.05$, one-way ANOVA)

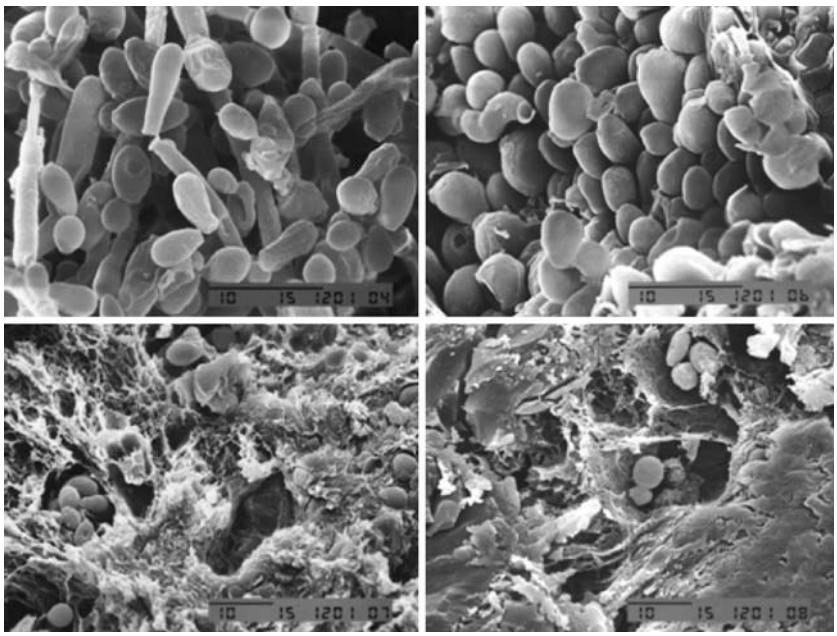

Fig. 5 Scanning electron micrographs of immobilized *Candida tropicals* Y220 with physical entrapment

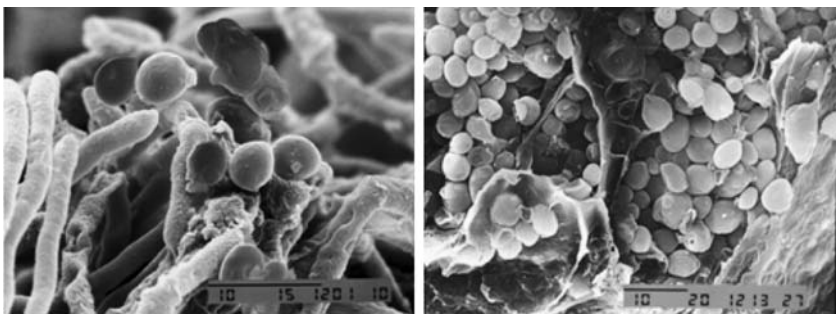

Fig. 6 Scanning electron micrographs of immobilized *Candida tropicals* Y219+Y220 with physical entrapment

supply, while in the center of the beads, the cell number was smaller than at the bead surface. Figure 6 presents scanning electron micrographs of immobilized yeast mixture *Candida tropicals* Y219 + *Candida tropicals* Y220. Good growth of the two yeasts can also be clearly seen.

Most of the studies performed were aimed at degrading organic contaminants in water. The potential of immobilized microorganisms for the cleaning of contaminated soils is scarcely described in the literature. Exotic microorganisms can not always survive well in soil because of environmental stress and competition with autochthonic organisms. Immobilization is known to reduce competition with autochthonic microorganisms and offer protection from both the extremes of pH and the presence of toxic compounds in

the contaminated soil. It is envisaged that immobilization cell technology could be applicable to a wide variety of bioremediation scenarios for a particular suite of contaminants (Cunningham et al., 2004). This study provides new insight into its application in bioremediation.

Microorganisms can be immobilized in the support matrix as isolates, in pairs, or as consortia, and the interactions of the immobilized microorganisms in the matrix may be different. Some synergistic effects may occur between the organisms for a better performance of the bioremediation system (Chung et al., 2003; Wang et al., 2004; Ionata et al., 2005; Prpich et al., 2005). This study shows that both synergistic and inhibitory effects existed in the experiment, demonstrating that it is essential to examine the relationships of the cells before using them together in the immobilization. It is expected that an immobilized system using the appropriately selected microorganisms may be applicable to certain bioremediation scenarios (Cunningham et al., 2004).

Good diffusivity of the support matrix is essential in the application of immobilization technology in remediation. In this study physical entrapment showed its advantages in degradation of pyrene in a soil slurry reactor, because the freeze-thaw procedure produced macroporous matrices, which was advantageous in the mass transfer of substrates. A cross shape was applied in the physical entrapment as we assumed this shape had a large surface area which may be beneficial for oxygen transfer. Scanning electron micrographs proved that the macroporous structure of the immobilized beads was very good for both mass transfer and cell growth.

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References

Beshay U, Abd-El-Haleem D, Moawad H, Zaki S (2002) Phenol biodegradation by free and immobilized *Acinetobacter*. Biotechnol Lett 24:1295–1297

Cassidy MB, Lee M, Trevors JT (1996) Environmental applications of immobilized microbial cells: a review. J Ind Microbiol 16:79–101

Chen KC, Lin YH, Chen WH, Liu YC (2002) Degradation of phenol by PAA-immobilized Candida tropicalis. Enzyme Microb Tech 31:490–497

Cunningham CJ, Ivshina IB, Lozinsky VI, Kuyukina MS, Philp JC (2004) Bioremediation of diesel-contaminated soil by microorganisms immobilised in polyvinyl alcohol. Int Biodeter Biodegr 54:167–174

Fasnacht MP, Blough NV (2002) Aqueous photodegradation of polycyclic aromatic hydrocarbons. Environ Sci Technol 36:4364–4369

Hill AJ, Ghoshal S (2002) Micellar solubilization of naphthalene and phenanthrene from non-aqueous-phase liquids. Environ Sci Technol 36:3901–3907

Jianlong W, Liping H, Hanchang S, Yi Q (2001) Biodegradation quinoline by gel immobilized *burkholderia* sp. Chemosphere 44:1041–1046

Karigar CK, Mahesh A, Nagenahalli M, Yun DJ (2006) Phenol degradation by immobilized cells of *Arthrobacter citreus*. Biodegradation 17:47–55

Nawaz MS, Billedeau SM, Cerniglia CE (1998) Influence of selected physical parameters on the biodegradation of acrylamide by immobilized cells of *Rhodococcus* sp. Biodegradation 9:381–387

Prpich GP, Daugulis A (2005) Enhanced biodegradation of phenol by a microbial consortium in a solid–liquid two phase partitioning bioreactor. Biodegradation 16:329–339

Song SH, Choi SS, Park K, You YJ (2005) Novel hybrid immobilization of microorganisms and its applications to biological denitrification. Enzyme Microb Tech 37:567–573

Witt G, Trost E (1999) Polycyclic aromatic hydrocarbons (PAHs) in sediments of the Baltic Sea and of the German coastal waters. Chemosphere 38:1603–1614

Wang Y, Fan Y, Gu JD (2004) Dimethyl phthalate ester degradation by two planktonic and immobilized bacterial consortia. Int Biodeter Biodegr 53:93–101