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Polycyclic aromatic hydrocarbon biodegradation and extracellular enzyme secretion in agitated and stationary cultures of *Phanerochaete chrysosporium*

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Abstract

The extracellular enzyme secretion and biodegradation of polycyclic aromatic hydrocarbons (PAHs) were studied in agitated and shallow stationary liquid cultures of *Phanerochaete chrysosporium*. Veratryl alcohol and Tween80 were added to cultures as lignin peroxidase (LiP) and manganese peroxidase (MnP) inducer, respectively. Shallow stationary cultures were suitable for the production of enzyme, whereas agitated cultures enhanced overall biodegradation by facilitating interphase mass transfer of PAHs into aqueous phases. The use of a LiP stimulator, veratryl alcohol, did not increase PAH degradation but significantly enhanced LiP activity. In contrast, Tween80 increased both MnP secretion and PAH degradation in shallow stationary cultures. On the other hand, high PAH degradation was observed in agitated cultures in the absence of detectable LiP and MnP activities. The results suggested that extracellular peroxidase activities are not directly related to the PAH degradation, and the increased solubility rather than enzyme activity may be more important in the promotion of PAH degradation.

Key words: lignin peroxidase (LiP); manganese peroxidase (MnP); *Phanerochaete chrysosporium*; polycyclic aromatic hydrocarbons (PAHs); solubilization

Introduction

Polycyclic aromatic hydrocarbons (PAHs) have been found to have a variety of toxic, mutagenic and carcinogenic effects on microorganisms, plants and animals and are classified as compounds with significant risks for human health. Consequently, the US Environmental Protection Agency (EPA) has listed 16 PAHs among the priority pollutants to be monitored in aquatic and terrestrial ecosystems. PAHs are produced during incomplete combustion of fossil fuels, organic wastes and various industrial processes. The inertness of these compounds, their low water solubility and strong lipophilic character lead to very high accumulation levels and persistence in the environment, so their elimination is considered as a priority in many countries (Verdin *et al*., 2004).

Bioremediation, the use of living organisms to decontaminate polluted soil or water, is an important pollution control tool and is a practical alternative to traditional remediation technologies. The effectiveness of bioremediation in the detoxification of potential hazardous organic chemicals will depend in part on the chemical structure of the pollutants and on a better understanding of microbial biodegradation of PAH. A wide variety of microorganisms, e.g., bacteria, algae and fungi, have been shown to metabolize PAHs (Cerniglia, 1992; Juhasz and Naidu, 2000). Among these, the white rot fungi have attracted particular attention and have exhibited significant potential to metabolize PAHs by means of their extracellular, nonspecific and non-stereoselective enzymes produced during their secondary metabolize stage stimulated by carbon or nutrient starvation (Tekere *et al*., 2005). Among the enzymes secreted by white rot fungi, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) were acknowledged to be especially significant in the degradation of PAH (Bumpus *et al*., 1985; Sanglard *et al*., 1986; Hammel *et al*., 1986), they are also the major ligninolytic enzymes secreted by *Phanerochaete chrysosporium*. It has been shown that LiP can oxidize PAH up to an ionization potential (IP) of 7.55 eV, and MnP in manganic acetate-acetic acid systems is capable of oxidizing compounds with IPs ⩾7.8 eV, while MnP-dependent lipid peroxidation has been identified as the catalyzing mechanism for the oxidization of phenanthrene, a compound with a higher IP (i.e., IP = 8.03 eV). To improve the biodegradative potential of fungi, extensive research on optimizing the enzyme activity needs to be carried out.

Previous work showed that enzyme activity was in-

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fluenced by incubating cultures with enzyme substrates. A stimulation of enzyme activity *in vivo* was observed following the addition of certain natural or synthetic mediators. It was reported that veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA), a secondary metabolite produced by white rot fungi in response to nitrogen limitation, stimulates the development of ligninase activity in cultures and stimulates lignin degradation to CO_2 by *P. chrysosporium* (Faison and Kirk, 1985; Faison *et al*., 1986). The non-ionic surfactant Tween80 has a regulatory effect on the production of LiP and MnP, especially for MnP. Several authors have shown an improvement in enzyme excretion in the presence of Tween80 in immobilized and submerged cultures of *P. chrysosporium* (Asther *et al*., 1987; Ürek Pazarlioğlu, 2005), although the mechanism involved has not been established. It have been suggested that Tween80 transformed the cell membrane structure and promoted the permeation of MnP from the cell into the medium (Asther *et al*., 1987).

Furthermore, the biodegradation of PAHs is often limited by their bioavailability due to their low water solubility. Synthetic surfactant such as Tween80 can be used to enhance PAH solubility, but its negative influence on microorganism growth should be taken into consideration. In this study, the secretion of LiP and MnP by *P. chrysosporium*, was monitored simultaneously, and their ability to degrade PAH, phenanthrene, anthracene, pyrene, benz[a]anthracene and perylene, was also studied. VA and Tween80 as enzyme inducers were added to the media, their influences on enzyme activity and PAH degradation was investigated as well. Experiments were carried out both in shallow stationary conditions and in agitated conditions to determine the effects of enzyme activity and solubilization on the biodegradation of PAH.

1 Materials and methods

1.1 Microorganism

P. chrysosporium (CGMCC 5.776) was obtained from the Chinese General Microorganism Conservation Center, the strain were maintained on potato dextrose agar (PDA) slants (potato extract 20% (v/v), dextrose 20 g/L, KH_2PO_4 3 g/L, $MgSO_4$ 1.5 g/L, VB_1 8 mg/L, agar 20 g/L, pH 6.0) at 4°C.

Prior to use, strain were cultivated on the same medium at 35°C for 6–7 d, then spores were harvested and prepared by suspension in sterile water followed by passing through sterile glass wool to free it of mycelia. Spore concentration is determined by measuring absorbance at 650 nm on UV-Vis spectrometer (Tien *et al*., 1988).

1.2 Culture conditions

1.2.1 Effect of VA and Tween80 on production of extracellular enzymes

Liquid culture experiments were conducted in 150 ml Erlenmeyer flasks containing 10 ml of Kirk's medium with 1 ml inoculum of spore suspensions (described above) (Tien *et al*., 1988), VA and Tween80 were added to obtain a serial concentration in medium. Each flask was loosely capped with cellulose stoppers to permit passive aeration and the cultivation proceeded statically/agitated (150 r/min) under normal atmosphere in the dark at 30°C and 1 ml sterile water was added to each flask every 3 d to supplement the evaporated water. Cultures were sacrificed periodically by centrifugation (15,000×*g*, 15 min, 4°C) (Biofuge Stratos, Heraeus, German) and the supernatants were used for enzymes activity assay.

1.2.2 Oxidation of PAHs

Phenanthrene, anthracence, pyrene, benz[a]anthracence and perylene dissolved in chloroform as a stock solution at a total concentration of 1.67 g/L, and 1 ml of this solution were added to 150 ml flasks, The solvent was allowed to evaporate overnight, leaving a thin coating of mixed PAHs covering the bottom of the flasks, then medium with a serial concentrations of VA and Tween80 as described in Section 1.2.1 were added to the flasks, followed by inoculation of 1 ml of spore suspensions and the cultures were incubated in the dark at 30°C, in agreement with the conditions used to produce enzymes. For control experiments, 1 ml of sterile water was added to the medium instead of inoculum and then carried out in the same way as for the fungal degradations above. Samples were sacrificed periodically, the cultures were homogenized and used for PAH extractions.

1.3 PAH extraction and analysis

The whole contents of each flask were extracted three times with 50 ml of chloroform before concentrated with a rotavapor evaporation system to 1 ml, after which, the volume was adjusted to 10 ml with HPLC-grade methanol and quantified by high performance liquid chromatography (Agilent 1100, USA). The analyses were performed isocratically using a mobile phase of 11% water and 89% methanol and a reversed-phase column (Discovery C18, 5 μm, 150 mm × 4.6 mm). The flow rate was 1 ml/min and the wavelength used for UV detection was 254 nm.

1.4 Extracellular enzyme assays

The lignin peroxidase (LiP) and manganese peroxidase (MnP) activities of culture supernatant were assayed spectrophotometrically described in the literature (Archibald, 1992; Arora and Gill, 2001; Akamatsu and Shimada, 1996). LiP was determined at 651 nm based on the reduction of dye Azure B (pH 3.0, 35°C). The reaction mixture (2.5 ml) contained 50 mmol/L tartrate buffer (pH 3.0), 0.032 mmol/L Azure B, 0.5 ml culture supernatant and 0.4 mmol/L H_2O_2. MnP was assayed at 240 nm using $MnSO_4$ as substrate (pH 4.5, 37°C). The reaction mixture (4 ml) contained 95 mmol/L lactic buffer (pH 4.5), 1 mmol/L $MnSO_4$, 0.4 ml culture supernatant and 0.04 mmol/L H_2O_2. One unit of enzyme activity was defined as an OD transformation of 0.1 units per minute per ml of the culture supernatant.

1.5 Solubilization of PAHs with Tween80

An excess amount of individual PAHs (phenanthrene,

anthracence, pyrene, benz[a]anthracence and perylene) was added to 20 ml glass vials. A 10-L aliquot of aqueous Tween80 solution was then added to each vial to a final concentration in the range of 0–2000 mg/L. The samples was equilibrated by mixing for 48 h at 30°C on an orbit shaker (150 r/min), followed by centrifuged at 15000 g/min for 15 min. Solution 1 ml was then carefully withdrawn with a volumetric pipette and diluted to 5 ml with a methanol-water solution (1:1). The PAH concentration was then determined by HPLC as described in Section 1.3. The experiment was performed in duplicate and data shown were calculated from the mean value of samples.

1.6 Chemicals

Phenanthrene (>98%) was obtained from Acros, pyrene, benz[a]anthracence and perylene (>99%) were purchased from Aldrich, and anthracence (chemical pure) was purchased from Czhch. Veratryl alcohol (96%) was purchased from Acros, Dye Azure B was obtained from Fluka. Other chemicals used were analytical grade.

2 Results

2.1 Effect of VA on production of LiP

The effect of adding various concentrations of VA to shallow stationary cultures of *P. chrysosporium* is shown in Fig.1. The addition of VA to the cultures dramatically improved LiP activities. Significant enzyme activities were detected on the day 7 and 8, increased rapidly to a peak on the day 9. In the experiments, the highest enzyme activity was obtained as 3893 U/L with 5 mmol/L VA, compared to only 587 U/L in cultures without VA addition. The peak activity of LiP in cultures with a higher concentration of VA (i.e., 10 mmol/L) occurred later than that of cultures with low concentration of VA, but still effectively stimulate the LiP production. However, the further increase in a concentration up to 20 mmol/L completely suppressed the LiP activity.

2.2 Oxidation of PAH in cultures with different concentration of VA

The recovery rates of PAHs measured after 16 d successive incubations are shown in Fig.2. The culture conditions were in agreement with the conditions used to produce LiP, except that PAH were added. Better degradation of PAH were measured in cultures with low concentration of VA (i.e. 3 mmol/L) and without VA. High concentration of VA (⩾ 5 mmol/L) inhibited the degradation of PAH. A significant stimulation of LiP activity by 5 mmol/L VA did not result in a increase of PAH degradation rate.

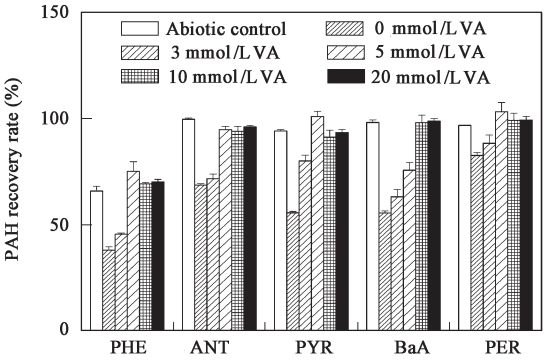

Fig. 2 PAH recovery rates after 16 d incubation in shallow stationary cultures of *P. chrysosporium* supplemented with different concentrations of VA. The experiments were run in triplicate, and the data shown represent mean ± SD.

2.3 Effect of Tween80 on MnP activity

Figure 3 shows the effect of C_{Tween80} on the production of MnP. Detectable levels of MnP activity was detected on the day 6, reaching a maximum on the day 12 and then the activities were slightly declined but still maintain on a high level. Fig.3 shows that Tween80 was a strong stimulator of MnP activity. MnP activity was steadily increased with the increase of Tween80 concentration, and the highest MnP activity was obtained as 2658.4±22.0 U/L in cultures with 2.0 g/L Tween80. The results further indicated that high concentration of Tween80 (> 0.5 g/L) in the test range did not suppress the activity of MnP.

2.4 Effect of Tween80 concentration on the oxidation of phenanthrene, pyrene, benz[a]anthracence and perylene

The time course disappearance profiles of four PAHs in the shallow stationary cultures of *P. chrysosporium* under

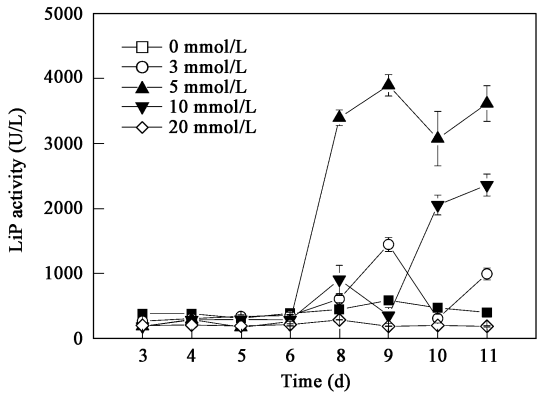

Fig. 1 LiP activity variation depending on concentrations of VA with respect to incubation time in shallow stationary cultures at 30°C.

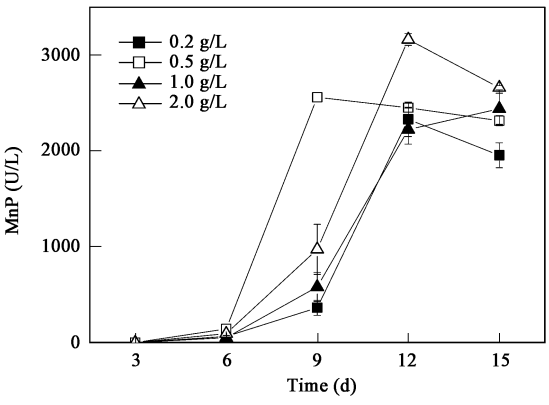

Fig. 3 Effect of Tween80 concentration on production of MnP in shallow stationary cultures of *P. chrysosporium* at 30°C. The experiments were run in triplicate, and the data shown represent mean values ± SD.

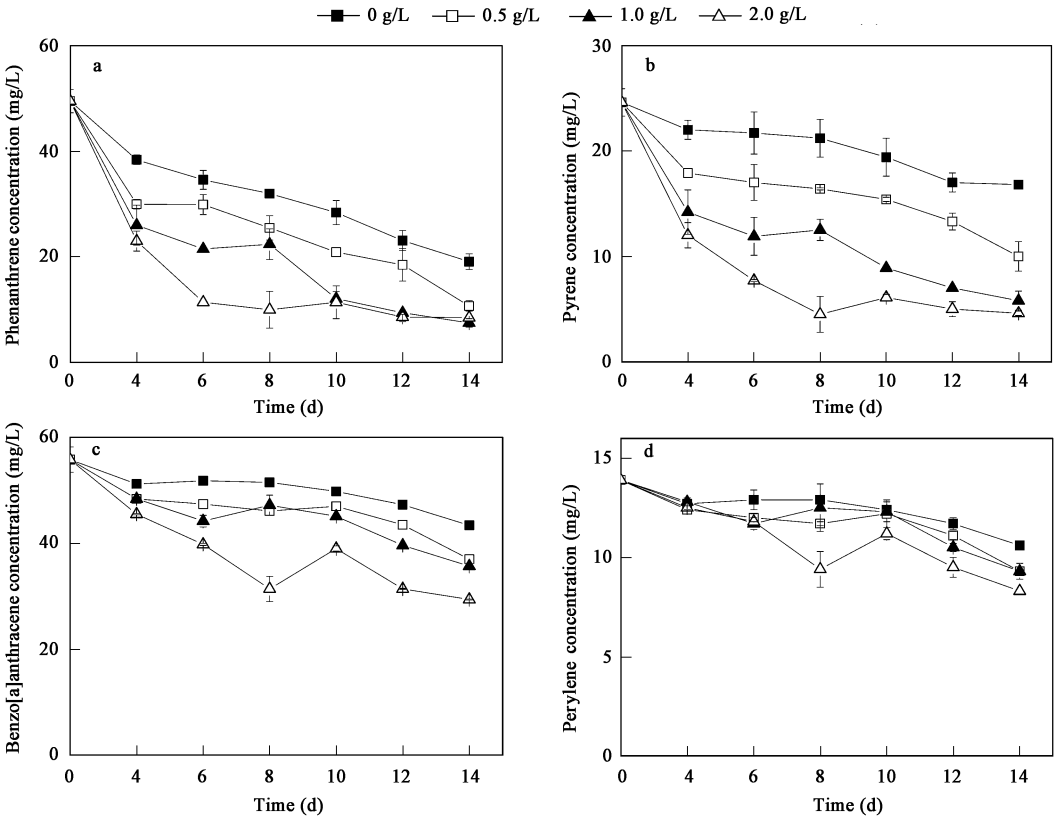

Fig. 4 PAH oxidation in shallow stationary cultures of *P. chrysosporium* in the presence of different concentration of Tween80. (a) phenanthrene; (b) pyrene; (c) benzo[a]anthracene; (d) perylene. The experiments were run in triplicate, and the data represent mean ± SD.

different concentrations of Tween80 are presented in Fig.4. The oxidation percentage of all the PAHs tested increased as Tween80 concentration increased. For example, the oxidation rate of pyrene was the highest (1.14 mg/(L·d)) at 2.0 g/L Tween80, lower at 1.0 and 0.5 g/L Tween80, for 1.06 and 0.76 mg/(L·d), respectively, and was the lowest (0.27 mg/(L·d)) without Tween80. Generally, after 14 d incubation, the maximum PAH oxidation percentage was obtained in the presence of 2.0 g/L Tween80 as 36.6%, 65.0%, 36.4%, 28.8%, compared to 7.1%, 15.4%, 11.3% and 12.2% in the culture without Tween80 for phenanthrene, pyrene, benz[a]anthracence, and perylene, respectively (The data were calculated from the difference between the PAH (%) oxidized by isolate and the PAH (%) disappeared in control).

2.5 Influence of Tween80 on the solubility and degradation of PAHs in agitated cultures

Surfactant molecules above their critical micelle concentration (CMC) form aggregates in water, which are called micelles. These aggregates have a hydrophobic core and a hydrophilic outer surface. Micelles are capable of dissolving hydrophobic PAHs in their hydrophobic core, which results in an increased apparent aqueous solubility of PAH (Zhao *et al*., 2005). The relationship between the Tween80 concentration and the PAH apparent solubility is represented in Fig.5. It demonstrates that Tween80 significantly enhanced the apparent solubility of all PAHs evaluated. A good linear relationship between the apparent solubility of PAH in Tween80 solution and the concentration of Tween80 from 0 to 2000 mg/L was observed. The solubility of phenanthrene was enhanced more than 20-fold in 2000 mg/L Tween80 solution and the solubilities of other PAHs were also enhanced to different degrees.

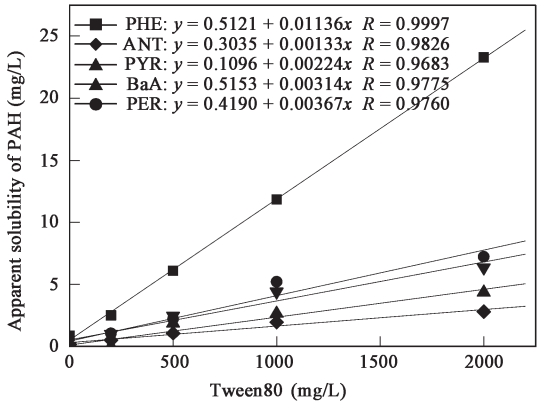

Fig. 5 Effect of Tween80 on the apparent solubility of PAHs. Data points represent the average of duplicate experiments.

Table 1 shows PAH degradation rates after a 14-d agitated incubation in the presence of different concentration of Tween80 and the measured enzyme activities. In contrast to shallow stationary cultures, degradation rates for all PAHs were significant in agitated cultures, where about 32.3%, 57.7%, 43.4% and 53.2% were degraded for phenanthrene, pyrene, benz[a]anthracence and perylene, respectively. Although no LiP or MnP activities were detected during the whole incubation period, with the

Table 1 PAH degradation and enzyme activities in 14 d agitated cultures of *P. chrysosporium* with addition of Tween80

$C_{Tween80}$ (g/L)	PAH degradation rate (%)				Enzyme activities (U/L)	
	Phenanthrene	Pyrene	Benz[a]anthracence	Perylene	LiP	MnP
0	32.3	57.7	43.4	53.2	ND	ND
0.5	41.8	76.4	87.5	89.9	ND	ND
1.0	36.1	48.7	46.3	57.5	ND	ND
2.0	31.3	49.5	85.9	89.9	ND	ND

ND: no activity detected. The data shown was calculated from the difference between the PAH (%) oxidized by isolate and the PAH (%) disappeared in abiotic control.

addition of low concentration of Tween80 (i.e. 0.5 g/L), a significant increase in the degradation rate of all PAHs tested in this study was observed. The degradation rate were increased more than 30% for phenanthrene and pyrene, and degradation rate was even doubled for benz[a]anthracence and perylene. However, a distinct inhibitory effect of all PAH degradation was observed in the presence of 1.0 g/L Tween80, which was probably due to the preferential degradation of Tween80, as microorganisms could presumably utilized Tween80 as carbon source. As for the cultures with addition of 2.0 g/L Tween80, the degradation rates of benz[a]anthracence and perylene were almost as the same as that obtained in 0.5 g/L of Tween80 culture.

3 Discussion

The biodegradation of recalcitrant pollutants by ligninolytic fungal enzymes *in vitro* has been documented (Tekere *et al*., 2005; Baborová *et al*., 2006). However, under *in vivo* conditions, other biochemical systems, processes, and interactions can also contribute to the degradation of the pollutants (e.g., fungal cytochrome P450 monooxygenase system, hydroxyl radical formation by the fungus) or limit the degradation rate (e.g., low bioavailability of the pollutant due to hydrophobicity of the pollutant molecule). The purpose in this study was to provide information about the degradation rates and enzyme activities secreted during incubation.

Agitated and shallow stationary cultures of *P. chrysosporium*, a white rot fungus producing LiP and MnP but not laccase, were compared with respect to extracellular enzyme synthesis and the capability of degrading PAH. Shallow stationary cultures are suitable for the production of enzyme because it increased the contacting area between cells and oxygen without shear stress, whereas agitated cultures are known to have an inhibitory effect on ligninolytic enzyme production due to shear stress on mycelia. We found that the use of LiP inducer, veratryl alcohol, in the range of 3–10 mmol/L concentrations distinctly stimulated LiP activity, but did not notably increase PAH degradation in shallow stationary cultures. This result suggested that LiP activity was not directly related with PAH degradation. In contrast, Tween80 increased both MnP secretion and PAH degradation in shallow stationary cultures. However, PAHs were degraded at a constant rate during all incubation time, although no activity of MnP was detected in young cultures (before 6 d), and the very high activity of this enzyme, observed after 12 d incubation, caused no increase in the degradation of PAH during this period. The supposed role of MnP in the degradation of PAH could not be confirmed. On the other hand, high PAH degradation was observed in agitated cultures in the absence of detectable LiP and MnP activities, either in the presence or absence of Tween80, which provided some evidence to support that extracellular peroxidases may not be directly involved in the PAH degradation. Similar results were described by Schtzendübel *et al*. (1999), who reported that degradation of fluorene, anthracene, phenanthrene, fluoranthene and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. Our results also agree with several reports on degradation of PAH in cultures of fungi without detectable activities of LiP and MnP activities (Bezalel *et al*., 1996a; Verdin *et al*., 2004). It is possible that *P. chrysosporium* could produce other oxidative and hydrolytic enzymes that were not analysed, but which could potentially have degraded PAH. The role of tyrosinases and dioxygenases, intracellular enzymes which were also secreted into the culture filtrate by various fungi may play a role in the degradation of PAH (Ferm and Coeling, 1972; Milstein *et al*., 1983).

Agitated cultures enhanced overall biodegradation by facilitating interphase mass transfer of PAH into aqueous phases. Solubilization and lowering of the surface and interfacial tension were thought to be main reasons for facilitating the transport and degradation of pollutants. We could speculate that the increased solubility rather than enzyme activity might be more directly involved in the promotion of PAH degradation.

4 Conclusions

The results indicated that the activities of LiP and MnP could be significantly enhanced by veratryl alcohol and Tween80, respectively, in shallow stationary cultures. PAHs were more sufficiently degraded in agitated cultures although no LiP or MnP activities were detected. It can be concluded that extracellular peroxidase activities are not directly related to the PAH degradation, and the increased solubility rather than enzyme activity may be more important in the promotion of PAH degradation.

Acknowledgements

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