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Enzymatic degradation of anthracene by the white rot fungus *Phanerochaete chrysosporium* immobilized on sugarcane bagasse

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ABSTRACT

Bagasse is a by-product of sugar milling and important fuel resource for that industry. It is a fibrous, low density material with a very wide range of particle sizes and high moisture content. The goal of this study is the development of a system based on the use of the ligninolytic enzyme manganese peroxidase (MnP) produced by *Phanerochaete chrysosporium* for the degradation of polycyclic aromatic hydrocarbons (PAHs), of which anthracene was selected as an example. The white rot fungus *P. chrysosporium* immobilized on bagasse was grown in both stationary and agitated cultures (rotary shaker, 80 rpm) using nitrogen limited growth medium to study the ability of the fungus to degrade anthracene in aqueous media. Production of MnP occurred simultaneously in nitrogen limited culture medium with the added $MnSO_4$ at 40 ppm. The MnP activity was at relatively high level (76 U l^{-1}) and in this condition, the residual anthracene concentration was 16%.

1. Introduction

Environmental pollution with hazardous wastes containing recalcitrant xenobiotic chemicals has become one of the major ecological problems. Unlike the naturally occurring organic compounds that are readily degraded upon introduction into the environment, some of these synthetic chemicals are extremely resistant to biodegradation by native microorganisms [1,2]. Many of these compounds are major environmental pollutants such as munitions waste, pesticides, organochlorines, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, wood preservatives and synthetic polymers [3,4].

PAHs in contaminated soils, waters, and sediments present a significant risk to the environment and human health. PAHs are hydrophobic compounds, and they persist mainly in ecosystems through their low water solubility [5].

Because of low molecular weight compared to most of the other PAHs, anthracene has a higher solubility and can be found at more significant levels in water. It can represent a threat to the environment [5]. Therefore, it is important to determine the impact of this molecule on living organisms from aquatic and soil ecosystems. For all of these reasons, and because its structure is found in carcinogenic PAHs such as benzo(a)pyrene and benzo(a)anthracene, we have chosen anthracene as a model compound of the PAH family [5,6].

A main problem of aromatic compounds such as anthracene is their poor solubility in aqueous media. Eibes et al. [7] showed that the addition of different water miscible organic solvent (such as acetone, methyl-ethyle-ketone, methanol and ethanol) increased the bioavailability of anthracene [7].

Among other possibilities, an environmentally friendly approach for PAHs degradation could be based on the use of white rot fungi, which are known to degrade a great variety of complex compounds due to their complex enzymatic system [8].

Ligninolytic fungi causing white rot of the wood have been shown to degrade and mineralize a large variety of recalcitrant compounds due to the nonspecifity of their enzyme machinery. Many of those compounds are major environmental pollutants [9,10].

White rot fungi such as *Phanerochaete chrysosporium* typically secrete one or more of the three principal ligninolytic enzymes, i.e. lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13) and phenol oxidase (Laccase) (LAC, E.C. 1.10.3.2) [11].

Regarding limitation of free cells of *P. chrysosporium* for use in biodegradation process, cell immobilization offers a suitable alternative. Good results for cell immobilization of *P. chrysosporium* on pinewood chip for phenol and chlorinated phenol degradation was shown [10]. Therefore, when a natural support material is used for cell immobilization, conducting a satisfactory pollution removal process becomes more feasible. Bagasse is a ligno-cellulosic residue

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(by-product) of sugar industry and is almost completely used by the sugar factories themselves as fuel for the boilers. It has low cost and exists at high value [12–14].

The research reported here was aimed at evaluating the importance of elevated level of extracellular, ligninolytic activities of *P. chrysosporium* immobilized on bagasse for rapid and efficient degradation of anthracene.

2. Experimental

2.1. Microorganism cultivation and inoculum preparation

P. chrysosporium (1557) was purchased from the Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ) and was maintained at low temperature 4 °C, on 2.5% malt extract agar slants. Subcultures were routinely made every 2 months. Inocula consisted of conidial suspensions and was diluted so that the absorbance at 650 nm was 0.5, in a 1 cm path-length cuvette. The number of spores was determined using thoma counting-cell under the optical microscope at ×1000 magnification (2.4×10^6 cells ml^{-1}). For preparation of conidial suspension, spores were taken from 6-day-old slants (stationary mode of the incubation at temperature 25 °C).

2.2. Anthracene biodegradation experiments

2.2.1. Culture media

Nitrogen-limited synthetic growth medium, which was used in the present study, had following composition (l^{-1} of distilled water): glucose 10 g; KH_2PO_4 2 g; $MgSO_4{\cdot}7H_2O$ 0.5 g; $CaCl_2$ 0.1 g; $MnSO_4$ 0.03 g; NaCl 0.06 g; $FeSO_4{\cdot}7H_2O$ 6 mg; $CoCl_2$ 6 mg; $ZnSO_4{\cdot}7H_2O$ 6 mg; $CuSO_4$ 6 mg; $AlK(SO_4)_2{\cdot}12H_2O$ 0.6 mg; H_3BO_3 0.6 mg; $Na_2MoO_4{\cdot}2H_2O$ 0.6 mg; yeast extract 0.012 g; diammonium tartarate ($C_4H_{12}N_2O_6$) 0.2 g; thiamine 1 mg; veratryle alcohol 0.07 g and Tween 80 0.5 g. The pH of the medium was adjusted to 4.5 using 20 mM sodium acetate. The growth medium composition was based on the culture composition given by Tien and Kirk [15] and Podgorink et al. [16], but preliminary work in our laboratory showed that some components could be reduced or completely eliminated without any major adverse effect. The above-mentioned formulation was thus used in the present study.

2.2.2. Bagasse preparation

Sugarcane bagasse was purchased from Dezful sugarcane industries located in south of Iran. It was washed twice with hot distillated water, dried in oven at 105 °C for 24 h, milled and sieved to 1–2 cm mesh for cell immobilization. Bagasse composition was shown in Table 1 [12].

2.2.3. Anthracene solubility

Eibes et al. showed that due to the maximal solubilization of anthracene and minimum loss of enzyme activity, acetone was selected as the optimal cosolvent. In this report the anthracene solution was prepared through Eibes et al. method with concentration of 1 mg l^{-1} and acetone concentration of 19% (v/v) [7].

2.2.4. Biodegradation of anthracene

For consideration of parameter effects on enzyme production and anthracene removal, each Erlenmeyer flask (250 ml) containing 45 ml of above mentioned solution (with different Mn^{2+} concentrations, 10, 30, 40 ppm) and 1 g bagasse [6] were autoclaved for 30 min at 8 psi.

Then, the spore suspension prepared as above was added at 10% level [8]. The mixture was incubated at 37 °C on a rotary shaker (80 rpm) for 2 days. After 2 days the anthracene solution with predetermined concentration was added to the bagasse on which the fungal cells were immobilized.

The anthracene biodegradation was carried out with incubation of the culture at 37 °C for 10 days. The effect of certain factors on the anthracene biodegradation were studied in static and shaken conditions. All tests in the present study were carried out at least in duplicate and the values given in each figure represent the means ± standard derivations.

Table 1
Sugarcane bagasse composition

Compositions	Cellulose	Hemi-cellulose	Lignin	Ash
%	41	24	18	2

2.2.5. Anthracene analysis

It was shown anthracene has flourecense property, 250 nm ($\lambda_{excitation}$) and 406 nm ($\lambda_{emission}$) [2]. In this report anthracene analysis was determined by fluoremeter, model EEL 244, made in England.

2.2.6. Enzyme assay

The manganese peroxidase (MnP) activity was measured according to the method described by Gandolfi-Boer et al. [17]. As it is pointed out elsewhere, complex formation of Mn^{2+} ions with malonate can be monitored spectophotometrically at 270 nm (extinction coefficient = 11,590 M^{-1} cm^{-1}) [18]. One unit (U) is the amount of the Mn^{3+} malonate complex (μmol min^{-1}).

3. Results and discussion

The biodegradation of recalcitrant pollutants (anthracene and pyrene) by ligninolytic fungal enzymes has been documented [9]. Our purpose was to provide information about the degradation rates and enzyme levels synthesized by degrading of fungal culture. As the synthesis of ligninolytic enzyme depends on cultivation conditions [9], various type of fungal cultures was selected. In this experiment glucose was used of an appropriate carbon source in all other similar conditions.

3.1. Effect of Mn^{2+} concentration on anthracene biodegradation

The major components of the lignin-degrading enzyme system of *P. chrysosporium* are LiP and MnP with the expression during the secondary phase of fungal growth [19]. This biochemical event is triggered by nitrogen limitation of the growth medium and the presence and activity of each of these two extracellular enzymes are highly dependent on culture conditions and composition of the growth medium [19]. Nitrogen limited culture medium was used in the present study and the results obtained are in agreement with the studies that growth media containing low nitrogen concentrations generally provide the best nutrient conditions for the expressing ligninolytic enzymes by the fungus. Literature surveys show that the Mn^{2+} in the growth medium of *P. chrysosporium* affects the formation of LiP and MnP differently [11]. The formation of MnP is dependent on Mn, which has enhancing effect on transcription of MnP. The suppressive effect of MnP on LiP formation in the cultures of *P. chrysosporium* has been widely described [19]. In the present study the biodegradation process was tested. At 10 and 30 ppm $MnSO_4$, the biodegradation process was significant and the anthracene remaining after 7 days of the culture incubation was negligible (Fig. 1).

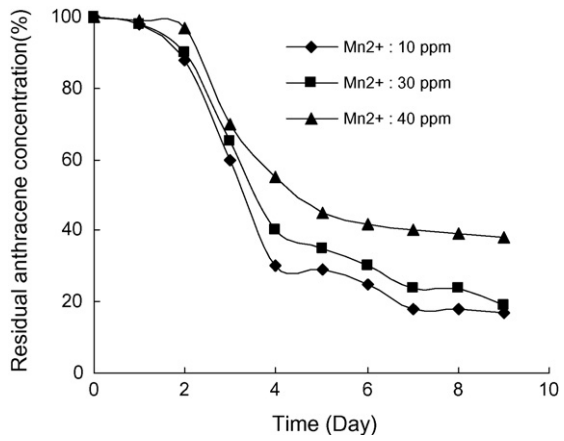

Fig. 1. Effect of Mn^{2+} concentration on anthracene biodegradation by bagasse immobilized *P. chrysosporium*.

3.2. Effect of immobilization on enzyme activity

A comparision of cultures of *Irpex lacteus* on free cells and immobilized cells on an inert substrate (polyurethane) and a lignocellulosic substrate (pinewood) showed differences between the production of extracellular ligninolytic activities, in free cells and immobilized cells. The results showed a significant reduction in the synthesis of MnP in free cells and immobilized cells [9]. Fig. 2 shows the activity of MnP produced by the bagasse-immobilized *P. chrysosporium* was highest at 7 days of the incubation (76 U l^{-1}).

3.3. Effect of the incubation mode on anthracene biodegradation

To determine the influence of agitation on the biodegradation process, the experiment with free mycelium and bagasse immobilized fungal cells was performed in static and shaken mode. Fig. 3 shows, the level of the anthracene biodegradation for bagasse immobilized fungal cells did not change after 7 days of the incubation under shaking conditions. The residual anthracene concentration for the free cells after 7 days of the incubation was 54%, while a significant decrease in residual anthracene was observed by day 7 of the incubation for immobilized cells.

Culture agitation improved the biodegradation ability of bagasse immobilized fungal cells considerably, although the repressive effect of culture agitation on the ligninase activity and lignin

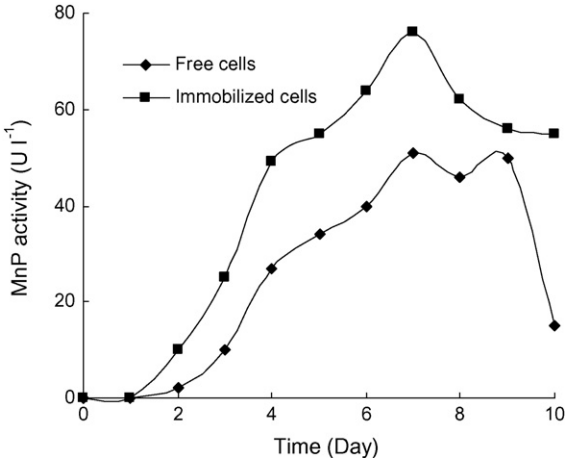

Fig. 2. MnP production by free and bagasse immobilized *P. chrysosporium* during anthracene biodegradation.

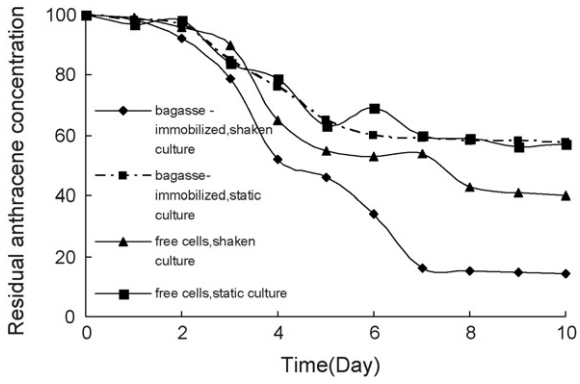

Fig. 3. Effect of the incubation mode on anthracene biodegradation by bagasse immobilized *P. chrysosporium*.

degradation by *P. chrysosporium* has been reported by many researchers [9].

4. Conclusion

From the results obtained in the present study, it can be concluded that bagasse appears to be a suitable support for *P. chrysosporium* immobilization and biodegradation capacity of the fungus was significant and related to production of MnP and its activity. This behavior can be due to high degree of porosity the bagasse cell support. Bagasse is a sugarcane industry wastes, in this study we used it as a support for increasing of enzyme activity and biodegradation of anthracene.

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