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Use of Extracellular Enzyme Manganese Peroxidase in Enhancing Bioremediation

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Bioremediation refers to the use of biological systems, such as bacteria, fungi, and enzymes, to degrade environmental pollutants. During the secondary metabolism of plant life, white rot fungi produce and secrete extracellular enzymes like LiP, manganese peroxidase (MnP), and laccase. These extracellular ligninolytic enzymes of white rot fungus have the ability to degrade a wide spectrum of recalcitrant organo pollutants like PAHs, phenols, PCBs, and TNT and various types of dyes because of their non-specific characteristics. The objective of the study was to achieve the use of the extracellular ligninolytic enzyme, Manganese Peroxidase (MnP) for the degradation of polycyclic aromatic hydrocarbons (PAHs), of which Anthracene was selected as an example and also decolorization of textile azo dye of which orange II was selected as an example, thereby useful for enhancing bioremediation.

Key words: Bioremediation, White rot fungi, Ligninolytic enzymes, Organopollutants.

A wide variety of enzymes can be used for bioremediation, depending on what reactions need to occur and which contaminant needs to be degraded. The use of extracellular enzymes for industrial processes began in the 1960s and has since become a staple in such industries as textiles, food production, and detergent[1-3]. Extracellular enzymes refer to those enzymes that are either secreted by the microbes, such as lignin peroxidase (LiP) and manganese peroxidase (MnP) from white rot fungi, or those that enter the aqueous phase during anaerobic submerged fermentation process[4-6]. Such enzymes are naturally produced by the microbes and then harvested. In most cases, each enzyme has only one specific function, such as to lower the activation energy for the degradation of an intramolecular bond, but some are able to affect a wide range of different substrates[7,2,3]. It is the latter type of enzyme that is most useful for bioremediation. Even though these enzymes were originally created for a substrate that the microbe would normally encounter in nature, they are also able to react with synthetic and xenobiotic compounds[8,3]. Such reactions can transform a compound from a recalcitrant state to one that is more biodegradable[3]. In other cases, extracellular enzymes are able to increase the degradation rate of already biodegradable substances, such as activated sludge, allowing for more efficient treatment processes[9].

Enzymes from white rot fungi have been found to be effective degraders of different contaminants like PAHs, dyes, phenols, PCBs, and

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TNT; enzymes such as protease, lipase, and cellulase have demonstrated the ability to reduce pathogen counts, reduce the solids content, and increase deflocculation in sludge. In nature, white rot fungi degrade dead wood and other plants and are unique among eukaryotes because they are able to cleave the carbon–carbon bonds in contaminants such as PAHs[10]. Before this discovery, it was believed that only bacterial processes could break this bond[11]. During the secondary metabolism of plant life, white rot fungi produce and secrete LiP, manganese peroxidase (MnP), and laccase[12].

The extracellular ligninolytic enzymes of white rot fungus have the ability to degrade a wide spectrum of recalcitrant organo pollutants and various types of dyes because of their non-specific characteristics. An increased solubilisation of polyaromatics in aqueous media would have beneficial effects on the potential degradation of these compounds[13-15].

Anthracene, a three-ring PAH, was chosen due to its low aqueous solubility (0.07 mg/L[16]) and this compound has been proved to be a substrate of ligninolytic peroxidases[17]. Enzymatic degradation was selected as an alternative to bacterial processes because the biological degradation usually requires long periods of treatment (from 2 to 4 weeks) and presents lag phases (2 days) till the degradation begins[18,19]. The initial stage of the process was the selection of the most appropriate cosolvent from a list of four relatively safe, easily available and fairly inexpensive chemicals: acetone, methyl-ethyl-ketone (MEK), methanol and ethanol. The influence of the solvent on MnP activity was used as a criterion for this selection. In a second stage, the optimisation of the degradation process was conducted taking into account specific physico-chemical factors which may directly affect the activation of the MnP catalytic cycle and the degradation rate of anthracene.

Although enzymes have been used for decades in the textile industry as detergents, only recently have extracellular enzymes been examined for their ability to decolor and degrade dyes[3]. In order for dyes to be degraded in a wastewater treatment plant, the chromophores in the dyes must be oxidized and cleaved[20]. Decolorization of azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes and partial mineralization of azo dyes by white rot fungus have been reported. Two enzymes, laccase and MnP, are proven to be quite effective in this function. MnP oxidizes MnP (II) to MnP (III) which is responsible for the oxidation of phenolic compounds[21]. In this study decolorization of orange II dye by the extracellular enzyme manganese peroxidase was reported and thereby proving the ability of the enzyme in enhancing bioremediation.

The goal of this study is the development of a system based on the use of the extracellular ligninolytic enzyme, Manganese Peroxidase (MnP) for the degradation of polycyclic aromatic hydrocarbons (PAHs), of which Anthracene was selected as an example and also decolourization of textile azo dye of which Orange II was selected as an example, thereby enhancing bioremediation. Research was done in form of two case studies and thereby finally confirming that the extracellular enzyme, Manganese Peroxidase (MnP) had proved its ability in enhancing bioremediation.

MATERIAL AND METHODS

Fungal strains

MnP was obtained from metabolically distinct white-rot fungi, *Phanerochaete chrysosporium* (MTCC 4955) culture which was obtained from IMTECH, Chandigarh, India. Lyophilized culture was brought in laboratory and maintained on plates of Potato Dextrose Agar and stored at 4°C.

Chemicals

Anthracene and anthraquinone were obtained from Qualigens Chemicals (99% purity). Acetone, methanol and ethanol were purchased from Qualigens Chemicals; methyl-ethyl-ketone was supplied by Sigma-Aldrich (99.5% purity).

Dyestuff

Azo dye Orange II was procured from Nahar Fabrics, Lalru (Punjab) India. Orange II with three aromatic rings and one sulphonic group has complex structure & it is very difficult to degrade such dye in higher concentrations.

MnP production

P. chrysosporium was cultured in 250-mL Erlenmeyer flasks on the N-limited BIII

medium[22]. Once the peak production of MnP was detected, the fermentation was stopped. Crude enzyme was concentrated by ultrafiltration using a 10-kDa cut-off type YM-10 membrane (Amicon), and then it was centrifuged for 10 min at 20,000×*g*.

MnP activity assays

MnP activity was determined by monitoring the oxidation of 2, 6-dimethoxyphenol (DMP) spectrophotometrically at 469nm at 30°C. The reaction mixture contained 50mM sodium malonate (pH 4.5), 1mM DMP, 1mM $MnSO_4$, and up to 600 µL of supernatant in a total volume of 1 mL. The reaction was initiated by adding 0.4mM H_2O_2. One MnP activity unit was defined as the amount of enzyme transforming 1µmol of oxidative compound (DMP) produced per minute[23].

Case study i: Degradation of anthracene

Anthracene solubility assays

The solubility of anthracene was determined in 20-mL aliquots containing 25 mg anthracene (final concentration of 1.25 mg/L) with different concentrations of solvent ranging from 1 to 100%. The aliquots were placed in 100-mL Erlenmeyer flasks sealed with teflon plugs, in triplicate, equilibrated for 24 h on a shaker (150 rpm) at 20 or 30°C.

Afterwards, the 20-mL assays were filtered through a Millex- LCR13 cartridge (Millipore Corp.), with a pore diameter of 0.45µm and analysed by high-pressure liquid chromatography (HPLC).

Anthracene biodegradation assays

Oxidation of anthracene was carried out in 100-mL Erlenmeyer flasks, sealed with teflon plugs, with magnetic stirring at room temperature, i.e. 23 °C (except when indicated). The reaction mixture (50 mL) consisted of acetone 36% (v/v), anthracene (5 mg/L) and MnP (200 U/L) with different concentrations reported for MnP. The possible effects of environmental parameters, such as temperature, light and oxygen atmosphere, on the degradation of anthracene were investigated. The influence of temperature was evaluated in assays performed at 23, 30 and 40 °C. An oxygen atmosphere was also investigated by flushing industrial oxygen at periodic intervals (3 min every 30 min). Samples were withdrawn periodically to determine anthracene and anthraquinone concentrations by HPLC as described below, and the evolution of MnP activity was spectrophotometrically determined. To verify that degradation took place only due to an enzymatic oxidation, controls were run in parallel using boiled MnP. No change in anthracene concentration after 6–8 h of incubation was observed in any controls (data not shown).

Analytical determinations

A HP 1090 HPLC, equipped with a diode array detector monitoring the absorbance at 253 nm, a 4.6mm×200mm Spherisorb ODS2 reverse phase column (5 µm; Waters) and a HP ChemStation data processor were used for determining the anthracene and anthraquinone concentrations. The injection volume was set at 10 µL and the isocratic eluent (80% acetonitrile and 20% water) was pumped at a rate of 0.4 mL/min.

Case study ii: Decolourization of dyes

Treatment of the dye with MnP

The orange II dye (500mg/l) was treated in a reaction mixture (5ml) containing 50mM sodium malonate (pH 4.5), 1mM DMP, 1mM $MnSO_4$, and up to 600 µL of supernatant in a total volume of 1mL separately. The reaction was initiated by adding 0.4mM H_2O_2.

Decolorization Assay

Effect of pH (4.0-7.0) on biodegradation was studied and pH was maintained using 0.1N HCl or 0.1N NaOH. Effect of temperature was studied for 24, 26, 28, 30, 32 and 34°C. Different concentrations of dyes (50,100,150,200 and 250 mg/l) were prepared and studied. In all experiments, agitated liquid cultures were grown for 7 days in an incubator shaker. Samples were withdrawn at alternate days, centrifuged at 4000rpm and supernatant was scanned at 486nm (λmax for Orange II) for absorbance in a UV-Visible spectrophotometer. All experiments were performed in duplicates. Controls were maintained without dye. Decolorization was calculated as

$$\text{Decolorization (\%)} = \frac{(C_o - C_e)}{Co} \times 100$$

where, Co is initial concentration of dye (mg/l) and Ce is residual dye concentration (mg/l) at different time intervals.

RESULTS

Case study I & II

Solubility of anthracene in water: solvent mixtures

The solubility of anthracene in four water miscible solvents: acetone, methyl-ethyl-ketone (MEK), ethanol and methanol, was determined at 20°C and 30°C. Table 1 shows the solvent concentrations for the solubilisation of 1, 10 and 100 mg/L of anthracene at 20 and 30 °C. The solubilisation at 30 °C was slightly more beneficial for all co solvents since it implied a reduction between 7 and 12% of the total addition of the organic solvent in comparison with that required for 20 °C. The minimum concentrations of the organic solvents required to attain a solubility of 10 mg/L, which is a 140-fold increase of the anthracene solubility in water at 20 °C (0.07 mg/L (16)), were: 27% MEK, 36% acetone, 44% ethanol and 55% methanol (Table 1).

In vitro degradation of anthracene

To maximize in vitro degradation of anthracene by MnP, different environmental parameters were evaluated.

Table 1. Solvent concentration required for the solubilisation of 1, 10, 100mg/L of anthracene

Solvent	T(ºC)	Solvent concentration (%)		
		1mg/L	10mg/L	100mg/L
MEK	2030	1714	**27***24	NDND
Acetone	2030	2119	**36***33	5349
Ethanol	2030	3128	**44***41	6460
Methanol	2030	3732	**55***51	7667

ND: Not determined; *Bold values represent the values selected for the following experiment.

Table 2. Effect of parameters on the anthracene degradation by MnP in 2-h reactions

Parameters	Anthracene degradation (%)	Anthraquinone production (%)	Activity loss (U/L h)
Air	43.3	11.8	70
Oxygen	50.5	19.0	73
23°C	43.3	11.8	70
30°C	34.9	12.4	83
40°C	5.5	0	200

Table 3. Degradation of anthracene in organic solvents: water mixtures

Solvent	Mediating agent	Degradation rate of anthracene (µmol/L h)	Reference
40% acetone	-	0.96	Vazquez-Duhalt *R et al.*,1994[28]
5% DMF[a]	-	0.33	Laane C *et al.,*1987[29])
5% DMF[a]	-	0.70	Gorjup B *et al.,*1999[30]
5% DMF	5mM GSH[b]	1.15	Laane C *et al.,*1987[29]
5% DMF	5mM GSH[b]	2.34	Gorjup B *et al.,*1999[30]
36% acetone	-	4.40	Present study

[a]Dimethylformamide; [b]Glutathione

Effect of environmental parameters

Different parameters such as temperature and working under air or oxygen atmosphere were evaluated. As it can be seen in Table 2, dissolved oxygen (up to 25 mg/L in the reaction media) improved the anthracene degradation (50.5%) and anthraquinone production (19.0%) whereas the enzymatic activity loss was not affected. On the other hand, the increase of temperature to 30°C led to a reduction of the anthracene degradation (34.9%), as well as a greater activity loss (83 U/L h). Operation at 40°C provoked a very severe activity loss (MnP was totally inactivated after 1 h reaction), being therefore the oxidation of anthracene very small (5.5%).

Complete degradation of anthracene

In order to quantify the maximum extent of anthracene degradation, the operation was prolonged until complete oxidation. The degradation profile of 5mg anthracene/L (28µM) in a medium containing 36% acetone (v/v), malonic acid 20 mM, Mn^{2+} 20µM, continuous addition of H_2O_2 at 5µmol/L min working under oxygen atmosphere is shown in Fig. 1. The anthracene degradation was nearly complete after 6 h. During the first 2 h of the experiment, marked activity loss and anthracene degradation were observed.

Effect of pH on Decolorization of Orange II

Effect of pH (3.0 – 7.0) was investigated, keeping other parameters constant (temp 30°C and dye conc. 50mg/l). As pH increased from highly acidic conditions (pH 3 to pH 4), decolorization increased from 39.84 to 71.56%. Optimum decolorization of dye (86.34%) was found at pH 5.0, with further decrease 69.56% and 51.42% for pH 6.0 and 7.0 respectively. Maximum removal of color was observed at 7th day for all studied pH (Fig. 2). Since no significant change in removal of Orange II by studied enzyme was observed after 5th day, thus 5th day was optimum decolorization.

Effect of Temperature on Dye Removal

To explore temperature effect, experiments were performed at different temperatures (24-34°C), keeping other conditions constant (pH 5.0 and dye conc. 50mg/l). Initially there was an increase in dye degradation rate up to 30°C and afterward dye degradation decreased. Maximum degradation was observed between 28-30°C (Fig. 3).

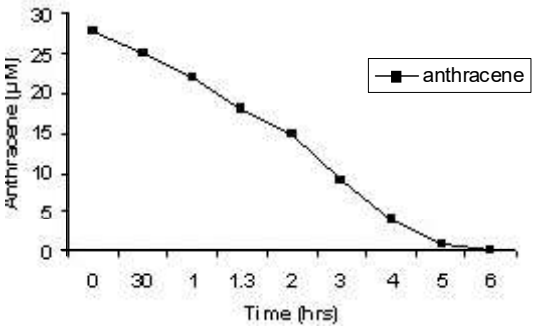

Fig. 1. Complete degradation of anthracene

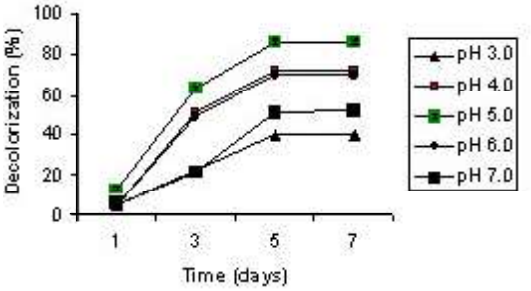

Fig. 2. Effect of pH on Decolorization of Orange II

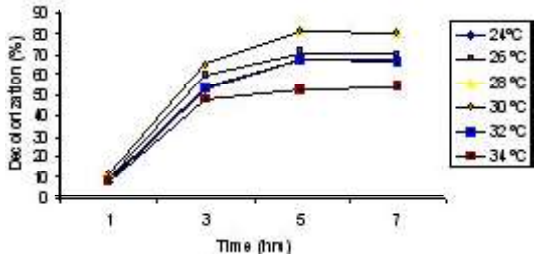

Fig. 3. Effect of Temperature on Dye Removal

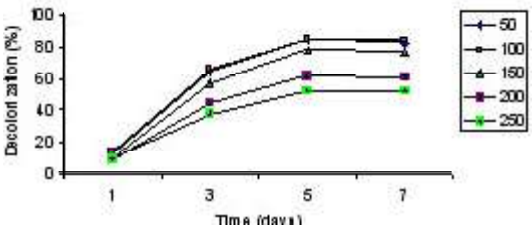

Fig. 4. Effect of Initial Dye Concentration (mg/L) on Decolorization of Orange II

Effect of Initial Dye Concentration on Decolorization of Orange II

Effect of varying dye concentration on dye degradation was investigated, keeping other parameters constant (temp 30°C and pH 5.0). For 50 and 100 mg/l concentration on dye, removal was almost the same but as concentration increased, removal of dye started decrease (Fig. 4). About 85.09% of dye was removed on 5th day of 100mg/l of dye.

DISCUSSION

Case study I & II

Degradation of Anthracene

The development of an efficient degradation system for polyaromatics based on the use of Peroxidases in vitro requires their increased bioavailability by using co solvents. The use of water miscible organic solvents was preferred to hydrophobic ones considering that enzymes in nearly dry conditions have to be solubilised by modification with amphipathic compounds, lipids or surfactants[24]. Furthermore, diffusional resistance for substrates and products across the water–organic solvent interface in organic solvents may be a major problem[25]. The choice of an organic solvent for a given reaction is based on two factors: (i) the ecological toxicity of the solvent; and (ii) the effects of the solvent on the reaction (including solubility of the substrate).

There are several studies of in vitro oxidations of polyaromatics (anthracene, phenanthrene, pyrene) with crude or purified MnP[26,27]. The reported assays were performed on a very small scale (1–5 mL) and only a limited degradation yield was achieved (Table 3). Our results compare very favourably with those of the cited reports. The lower efficiencies achieved may be due to either some compounds lacking in the amounts required or to the non-optimized physicochemical conditions, both of which are necessary for the enzymatic action.

Oxygen atmosphere increases the anthracene oxidation. This fact which has been observed in degradation of Azo dyes in water may be attributed to the Catalase-type activity of MnP (31). MnP releases atomic oxygen which could be directly used for the degradation of anthracene. In this case, it is interesting to see that the maximum degradation rate is coincident with the highest dissolved oxygen concentration in the medium (27.9 mg/L).

The degradation of anthracene outcomes into its total decomposition to the dead-end product: anthraquinone[32]. The degradation mechanism, probably arising via one-electron oxidative pathway, has a large complexity with the generation of intermediate compounds such as anthrol and anthrone[33]. Moreover, future work will be also focused on the biological degradation of anthraquinone by bacterial populations. Therefore, the overall process will be considered as a combination of an in vitro enzymatic system in the initial stage of degradation and a bacterial biological treatment to complete the process.

Decolorization of Orange II

MnP proved highly effective at reducing dyes as well. In present case study II, decolorization of Orange II was found dependent on temperature and pH. The enzyme showed maximum decolorization in slightly acidic conditions (pH 5.0). In highly acidic conditions, a decrease in decolorization rate may be due to decrease in enzymatic activity when pH changed from optimum levels. Dye concentration (50 – 100 mg/l), showed little change in decolorization rate, but higher dye concentration had inhibitory effect on enzyme thereby reducing color removal. Orange II with three aromatic rings and one sulphonic group has complex structure & it is very difficult to degrade such dye in higher concentrations. Enzymatic activity of selected fungus is possible mechanism involved in removal of dye[34]. Thus any bioprocesses based dye removal system using MnP enzyme should be design on the basis of these parameters for successful operation.

CONCLUSION

From the above case studies it was shown that the extracellular enzyme, Manganese peroxidase (MnP) is proven to be an effective means of enhancing bioremediation by degrading polycyclic aromatic hydrocarbons like anthracene and also decolorizing of various dyes like orange II. The use of microbes for these purposes is just beginning. Also it was evident from the results,

that a single enzyme produced by same organism can act on different compounds and degrade them. But as a given field site may contain any number of different compounds ranging from the easily biodegradable compounds to extremely recalcitrant ones, and using one type of enzyme may not be effective for the whole system. So studies must be done using multiple enzymes to degrade many different compounds concurrently. Further research is to be carried out on the use of various other extracellular enzymes like Lignin peroxidase (LiP), laccase etc. in enhancing bioremediation. Most importantly, more field studies must be done in order to prove how effective enzymes are in enhancing bioremediation. This is the only way to prove that enzymatic bioremediation is an effective and desirable alternative to current remediation strategies. In general, studies have shown that extracellular enzymes can be a valuable option for the remediation of certain recalcitrant compounds like PAHs, dyes, PCBs, OPs, phenols, wastewater and sludge, but this technology must be validated in the field.

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