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EFFECT OF pH ON THE MANGANESE DEPENDENT PEROXIDASE ACTIVITY OF *PENICILLIUM FREII* AND *ASPERGILLUS NIGER*

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ABSTRACT

White rot fungi have been implicated in the bioremediation of oil contaminated sites. Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant organic compounds formed by the fusion of two or more benzene rings. They are also important components of petroleum hydrocarbons associated with oil-contaminated environments. Ligninolytic enzymes of white rot fungi are involved in the biodegradation of PAHs and other structurally similar organic compounds. Fungal cultures were isolated by enrichment culture collected from road-side soil in College Lane Campus of University of Hertfordshire, Hatfield, United Kingdom and identified as *Penicillium freii* and *Aspergillus niger*. After identification, *Penicillium freii* and *Aspergillus niger* were cultured in malt extract broth adjusted to range of broth pH 5.5, 7.0 and 8.5, incubated and harvested after seven days. Manganese dependent peroxidase (MnP) activity (µmol/ml/min) was determined using oxidation of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride + DMAB (3-dimethylaminobenzoic acid). MnP activity of *Aspergillus niger* was optimum at pH 5.5, while that of *Penicillium freii* was optimum at pH 8.5.

1.0 INTRODUCTION

All over the world, there is an emerging concern amongst members of the public on environmental issues, such as; gas flaring, emission of carbon monoxide from the exhaust pipes of vehicles and motorbikes, oil spillages into soil, underground water and marine bodies from vandalized pipelines, bush burning, inappropriate disposal of toxic waste, which are the main cases of environmental pollution caused by human activities (Balba et al.,1998; Peter, 1994).

Petroleum hydrocarbons are important examples of toxic organic compounds that infiltrate environment consistently in large amounts via different routes (Balba et al., 1998). The activities of petroleum refineries and transportation of petroleum products; including accidents and leakage of petroleum tankers are contributory factors to the introduction of polycyclic aromatic hydrocarbons into the environment (Balba et al., 1998; Kanaly & Harayama, 2000).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic compounds that consists of two or more fused benzene rings and they are formed by the fusion of benzene rings in a linear, angular or cluster pattern through a pyrolitic process during the partial combustion of organic compounds such as coal, tar, wood, gas, garbage, fossil fuels, cigarette smoke and exhausts of trucks, vehicles and motorcycles (Abou-Arab et al., 2010; Balba et al., 1998; Cerniglia, 1997; Habe & Omori, 2003; Mugica et al., 2010).

PAHs have access to the environment via the above-mentioned routes and are distributed to water, atmosphere, soil and food (Abou-Arab et al, 2010). PAHs are present naturally as components of fossils fuels (Kanaly & Harayama, 2000; Obayori & Salam, 2010). PAHs are also a group of relevant environmental pollutants associated with oil spillage in aquatic and terrestrial ecosystems, which have known and suspected mutagenic and carcinogenic potentials (Balba et al., 1998; Boonchan, Britz & Stanley, 2000). Benzo (a) pyrene is known to be the most toxic PAH (Balba et al., 1998).

There is a wide distribution of petroleum hydrocarbon degrading bacteria, yeast and fungi in salt water, fresh water and soil environments (Balba et al, 1998). The physical and chemical methods of remediation of contaminated soils such as soil vapour extraction, soil washing and thermal desorption are costly due to the equipment and personnel involved and also there might be incomplete elimination of the contaminants; while bioremediation which is the biological method of remediating contaminated soil is environmental friendly and much more cost effective and can facilitate total destruction of harmful pollutants, but can be time consuming (Balba et al., 1998; Habe & Omori, 2003).

White rot fungi belongs to the division of basidiomycetes within the fungi kingdom that feeds on wood, degrading the lignin component of the wood in the process through the activities of their lignin degrading enzymes/ligninolytic enzymes, which culminates in the white rotting of wood (Novotny et al., 1999). White rot fungi are used in bleaching of wood pulp and are also capable of degrading and mineralising some persistent organic environmental pollutants such as munitions, pesticides, chlorophenols, polychlorinated biphenyls, synthetic

dyes, bleach-plant effluents, synthetic polymers, organic wood preservatives and other structurally similar pollutants due to the non-specificity of their different enzymes (Kalmis et al., 2008; Urairuj et al., 2003; Novotny et al., 2004; Pointing, 2001). pH, temperature, oxygen level, water availability, microbes nutrient requirements and PAHs bioavailability are parameters that influences PAHs degradation in oil polluted soils (Balba et al., 1998)

The focus of this study was the effect of pH on the manganese dependent peroxidase activity of *Penicillium freii* and *Aspergillus niger.* This involved the culturing of *Penicillium freii* and *Aspergillus niger* in malt extract broth at pH range of 5.5, 7.0 and 8.5, extraction of extracellular ligninolytic enzymes from *Penicillium freii* and *Aspergillus* niger and Manganese dependent peroxidase assays using the required substrates and reagents.

2.0 MATERIALS AND METHODS

Soil samples were collected from road-side soil in College Lane campus of the University of Hertfordshire, Hatfield, United Kingdom and fungal cultures were isolated by enrichment culture. They were identified microscopically and with molecular techniques using PCR amplification and sequencing of 18S rDNA and identified as *Penicillium freii* and identified as *Aspergillus niger*.

2.1 Fungal Growth

900mL Malt extract broth solution was prepared the solution was divided equally in 3 parts of 300ml each and the pH of 300ml each of the solution was adjusted to pH of broth set particularly at 5.5, 7.0 and 8.5 each. 100ml Malt extract broth solutions was added to nine conical flasks; with each set of three conical flasks containing the different pH ranges, inoculated with fungal cultures and incubated in a rotary shaker at 25^0C and 120 rpm. The fungal cultures were harvested after seven days

2.2 Chemicals and Reagents

3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and 3-dimethylaminobenzoic acid (DMAB) Sigma-Aldrich, UK were used as enzyme substrates. Other reagents of analytical grade include sodium phosphate, sodium chloride, sodium bicarbonate, manganese sulphate, hydrogen peroxide and succinate-lactate were used.

2.3 Extraction of Fungal extracellular enzymes

The procedure with modified steps was used with reference to Vyas et al., 1994. Conical flasks containing fungal cells were harvested after seven days of growth. 100ml sodium phosphate buffer (0.1mol/L, pH 6.5) containing 0.1mol/L NaCl, was added to each flask, the contents beaten gently with a glass rod and agitated on a rotary shaker at room temperature for 45 min. the enzyme extracts were filtered through a nylon cloth and spun (15 000g, 20 min, 4^0C). Supernatants were collected and stored at -20^0C

2.4 Manganese (Mn^{2+}) dependent peroxidase (MnP) assay

The manganese dependent peroxidase assay of Vyas et al, 1994 with modifications was used. MnP activity was determined by spectrophotometric measurement of the oxidation of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride + DMAB (3-dimethylaminobenzoic acid) as chromogen. The reaction mixture (2ml) contained 0.5ml of 100 µmol succinate – lactate buffer (pH 4.5), 0.25ml of 0.1 µmol H_2O_2, 0.25ml of 0.1µmol MBTH, 0.25ml of 5 µmol DMAB, 0.25ml of 0.2 µmol $MnSO_4$ and 0.5ml of fungal samples supernatant. MnP activity was terminated using 1ml of cold $NaHCO_3$ and 100 µl of test samples were pipetted onto a micro titre plate in five replicates. An increase in absorbance was measured with a spectrophotometer at 590nm. The enzyme activity is defined as the amount of enzyme capable of oxidizing one µmol of MBTH per minute. Enzyme activity rate is calculated as shown below (see equation 1) and expressed as µmol/ml/min.

$$\mu mol/ml/min = \frac{\text{O D value X Total reaction volume}}{\text{E X ml of supernatant X incubation time}} \quad \ldots\ldots\ldots (1)$$

Where OD value = Optical density of MBTH oxidized by colour change
Total reaction volume = (2ml)
E = extinction coefficient of MBTH (0.0012)
incubation time = 45 minutes

3.0 RESULTS

Table 1. Absorbance at 590nm in five replicates, average absorbance at 590nm and standard deviation of *Aspergillus niger* cultured at different pH for MnP activity

ASPERGILLUS SPP								
SAMPLES	CONTENTS	1	2	3	4	5	Average	Standard deviation
pH 5.5	Supernatant + substrates	0.056	0.056	0.053	0.051	0.047	0.053	0.004
pH 7.0	Supernatant + substrates	0.047	0.046	0.046	0.046	0.046	0.046	0.000
pH 8.5	Supernatant + substrates	0.046	0.045	0.045	0.043	0.044	0.045	0.001
Control 1 - pH 5.5	Supernatant only	0.04	0.037	0.038	0.036	0.037	0.038	0.002
Control 2 - pH 7.0	Supernatant only	0.039	0.037	0.038	0.036	0.037	0.037	0.001
Control 3 - pH 8.5	Supernatant only	0.038	0.037	0.038	0.036	0.038	0.037	0.001
Control 4	Buffer + Substrates	0.03	0.03	0.031	0.03	0.032	0.031	0.001

Table 2. MnP Activity (µmol/ml/min) of *Aspergillus niger*

pH	MnP Activity (µmol/ml/min)
5.5	3.926
7.0	3.407
8.5	3.333

Table 3. Absorbance at 590nm in five replicates, average absorbance at 590nm and standard deviation of *Penicillium freii* cultured at different pH for MnP activity

PENICILLIUM SPP								
SAMPLES	CONTENTS	1	2	3	4	5	Average	Standard deviation
pH 5.5	Supernatant + substrates	0.045	0.051	0.042	0.042	0.045	0.0450	0.004
pH 7.0	Supernatant + substrates	0.045	0.041	0.04	0.041	0.04	0.0414	0.002
pH 8.5	Supernatant + substrates	0.043	0.04	0.038	0.066	0.039	0.0452	0.012
Control 1 - pH 5.5	Supernatant only	0.036	0.036	0.033	0.033	0.035	0.0346	0.002
Control 2 - pH 7.0	Supernatant only	0.038	0.037	0.036	0.035	0.033	0.0358	0.002
Control 3 - pH 8.5	Supernatant only	0.038	0.037	0.038	0.034	0.035	0.0364	0.002
Control 4	Buffer + substrates	0.03	0.03	0.03	0.028	0.029	0.0294	0.001

Table 4. MnP Activity (µmol/ml/min) of *Penicillium freii*

pH	MnP Activity (µmol/ml/min)
5.5	3.333
7.0	3.067
8.5	3.348

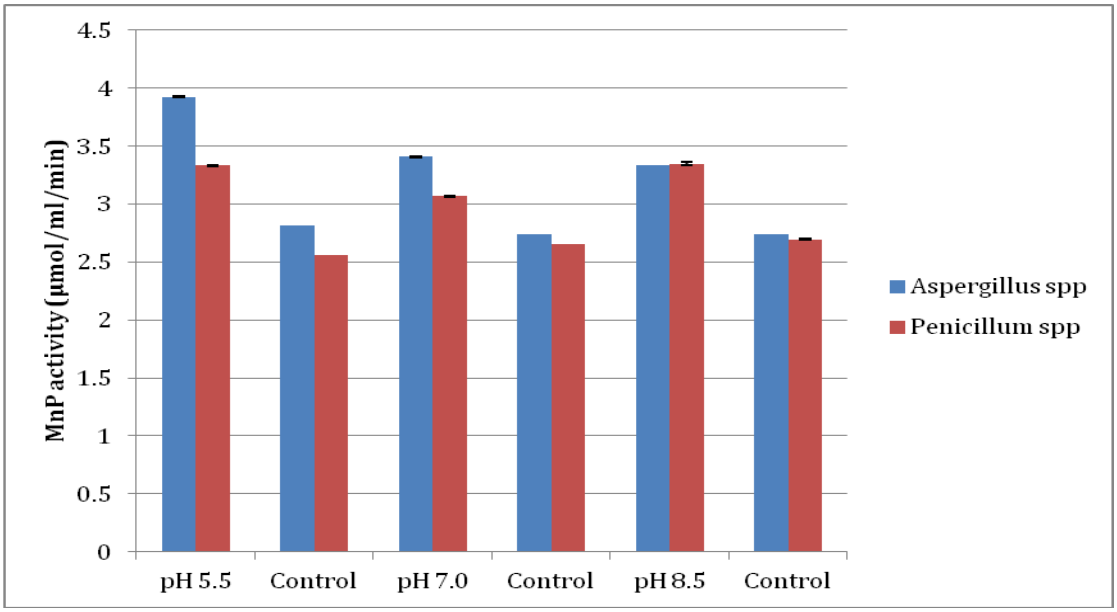

Figure 1. MnP activity (µmol/ml/min) of fungal samples at pH 5.5, 7.0 and 8.5. Colour symbols represent the different fungal samples. Vertical bar represents standard deviation of mean.

DISCUSSION

Table 2 depicts the calculated manganese dependent peroxidase (MnP) activity of *Aspergillus niger* obtained from table 1, while table 4 depicts the calculated MnP activity of *Penicillium freii* obtained from table 3. Table 2 showed a consistent decrease in MnP activity of *Aspergillus niger* from pH 5.5 to 8.5. MnP activity for *Aspergillus niger* was optimum at pH 5.5. In table 4, MnP activity for *Penicillium freii* was lowest at pH 7.0 and optimum at pH 8.5. The study of Wang, Vazquez-Duhalt & Pickard, 2001 on purified MnP oxidation of substrates other than MBTH had optimum activity at pH 5. The oxidation of 2,6-dimethoxylphenol (DMP) by MnP purified from *Aspergillus terreus* was found to have optimum MnP activity at alkaline pH of 12.5 and maintained stability between the pH range of 11.0 to 12.5 in the study of Kanayama et al., 2002. In another study conducted by Ruttimann-Johnson, Cullen & Lamar 1994, MnP isozymes activity of *Phanerochaete sordida* assayed with vanillylacetone was optimum between pH 4.0 and 4.5. The optima activity of two MnP isozymes purified from the white rot basidiomycete, *Dichomitus squalens* and assayed with 2,6-dimethoxylphenol (DMP) was at pH 4.5 and 5.0 respectively in the study of Perie, Sheng and Gold, 1996. In the study of Bonnen, Anton & Orth, 1994, the oxidation of guaiacol by MnP of *Agaricus bisporus* was optimum at pH 5.4 to 5.5. The difference in the optimum pH of MnP activity between fungal samples used in this study and the above documented studies cited could be due to the difference in substrates used, however, the MnP activity results of the fungal samples of this study is in agreement with the studies cited above indicating that optimum MnP activity can occur in both alkaline and acidic pH ranges.

CONCLUSION

In pulp and paper industries, pulp biobleaching occurs in alkaline conditions. Apart from biodegradation of PAHs, MnP of *Penicillium freii* can be employed in pulping and pulp bleaching processes, since this enzyme reacts under alkaline pH ranges.

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