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Evaluation of Physical Parameters Involved in Mycoremediation of Benzo[*a*]Pyrene by *Pleurotus ostreatus*

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Benzo[*a*]pyrene (B*a*P) is a highly toxic organic pollutant widely distributed in terrestrial and aquatic environments and is often referred as a xenobiotic compound. The present investigation was carried out to determine the physical factors affecting the biodegradation of B*a*P by the PO-3 isolate of *Pleurotus ostreatus*. When tested for the effect of media pH on the extent of B*a*P degradation, pH 6.0 facilitated both highest degradation (63.1%) and fungal biomass accumulation. Mesophilic range of temperature in general supported good fungal growth and subsequent degradation of the toxin. However, the highest degradation (64.2%) was observed when the inoculated media was incubated at 30°C. Subjecting the mineral salt broth to different agitation speeds showed different levels of B*a*P removal, wherein 180 rpm resulted in the highest level of B*a*P degradation (66.3%). Eventually, increasing the agitation speed beyond this decreased both the biomass and the extent of B*a*P degradation. Thus, the biodegradative potential of *Pleurotus ostreatus* PO-3 is strongly affected by these physical factors. A better understanding of the other parameters may result in effective decontamination of the polluted sites using *Pleurotus ostreatus* PO-3 isolate.

Key words: Benzo[*a*]pyrene, *Pleurotus ostreatus*, Degradation, pH, Temperature, Agitation.

Benzo[*a*]pyrene (B*a*P) is a five ring high molecular weight polycyclic aromatic hydrocarbon (HMW PAH) that is produced by the incomplete combustion of biological material and organic compounds[1]. B*a*P has been listed by the US Environmental Protection Agency (USEPA) as a priority pollutant because besides its recalcitrant nature, it also behaves as a teratogen, mutagen and carcinogen[2].

Although B*a*P may undergo chemical oxidation and photolysis, microbial degradation has been reported as the major process affecting B*a*P persistence and disappearance in nature[3]. PAH oxidizing microbes are present in the environment everywhere, which includes soil, sediments and lignin containing wood material[4].

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Investigations of the microbial bioconversion of HMW PAHs have shown that in contrast to bacteria and soil fungi, wood and litter-decay fungi are efficient degraders of these organopollutants, where they have the ability to mineralize PAHs with four and more condensed aromatic rings such as BaP. White rot basidiomycetes fungi like *Phanerochaete chrysosporium, Trametes versicolor, Stropharia coronilla* and *Pleurotus ostreatus* have been reported for their ability to degrade B*a*P[5-8].

Researchers suggested that the ability of these fungi to degrade PAHs is due to the synthesis of extracellular lignin modifying enzymes like lignin peroxidases (LiP), manganese peroxidases (MnP), laccases and other oxidases[9].

Considering that a number of environmental factors such as pH, temperature and level of oxygen availability may influence the rate and extent of B*a*P degradation, the subject of our

present investigation is to determine the various physical parameters involved in the B*a*P degradation by the selected PO-3 strain of *P. ostreatus*.

MATERIALS AND METHODS

The present study was conducted during the period from 30.08.2011 to 24.05.2012 at the Department of Microbiology, Genohelix Biolabs, Chamarajpet, Bangalore, Karnataka, India.

Chemicals and reagents

All the fine chemicals used were purchased from SRL Chemicals, India and were of the highest purity and analytical grade. HPLC grade B*a*P standard (98% pure) was procured from Spectrochem Pvt. Ltd., Mumbai, India

Effect of different pH, temperature and agitation speed

Results from our previous study showed that amending the mineral salt medium with co-metabolic substrate, nitrogen and surfactant such as glucose (0.01% w/v), yeast extract (0.1% w/v) and Tween 80 (0.05% w/v) greatly enhanced the level of B*a*P degradation by the PO-3 isolate of *P. ostreatus*[1].

To study the effect of several physical factors on the degradation of B*a*P (10 µg /ml of the mineral salt medium) by the same fungal isolate, the above nutrients and the surfactant were added to the mineral salt medium of the following composition (g/L): $(NH_4)_2HPO_4$; 05, KH_2PO_4; 0.8, K_2HPO_4; 0.3, $MgSO_4.7H_2O$; 0.3, $CaCl_2.2H_2O$; 0.055, $ZnSO_4.6H_2O$; 0.004, $CuSO_4$; 0.07, Thiamine; 1ml (2mg/ml).

Effect of pH was studied by adjusting the initial pH of the media from 4 to 9 with a gradual increment of 1 unit. Temperature for the maximal B*a*P degradation was determined by subjecting the inoculated broth to different incubation temperatures (20, 25, 30, 35, 40, 45 and 50°C). To study the level of aeration required, agitation speed of the inoculated broth was set at 50, 100, 120, 150, 180 and 200 rpm respectively.

Analytical methods

Extraction of residual BaP

The B*a*P extraction for fungal cultures was performed using a modified method proposed by Capotorti *et al.,* 2004[10]. Once the concentrated extract was obtained it was subjected to High Performance Liquid Chromatography analysis.

High Performance Liquid Chromatography analysis

HPLC analysis was carried at Genohelix Biolabs, Bangalore, India following the procedure of Bhattacharya *et al*., 2012[1], wherein the condensed sample was filtered through 0.25 µ nitrocellulose membrane filter. 80:20 (v/v) of acetonitrile: water was used to prepare the working standard solution of B*a*P with concentration of 10 µg /ml. 20 µl of the eluate containing 0.1 µg of the standard B*a*P was injected into the reverse phase HPLC system (Waters, USA, model number- 2487, with Dual λ absorbance UV detector and binary pump system, model number-1525). The mobile phase used was acetonitrile: water (80:20 v/v); with a flow rate of 1 ml/min. A reverse phase, YMCA Triart C-18 column of 3 µm (150 x 4.6 mm) was used.

The concentration of the B*a*P stock standard solution was determined at 254 nm. Area under the absorbance peak was used to estimate the percentage of degradation using a formula: $[(C_i-C_f)/C_i]*100$, where C_i is the initial concentration of B*a*P and C_f is the final concentration of B*a*P.

Statistical analysis

Effect of each parameter was studied in triplicate and the data are graphically presented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

PAH are xenobiotic compounds with two or more fused benzene rings generated from natural as well as anthropogenic sources. Widely distributed as environmental contaminants, PAH possess detrimental biological effects, toxicity, mutagenecity and carcinogenicity[11].

Mycoremediation refers to fungal degradation or transformation of hazardous organic contaminants to less toxic compounds[12]. *P. ostreatus* is a potential white-rot fungus that can be used for the treatment of B*a*P contamination not only in liquid culture but also in contaminated soil[13].

At the same time, biodegradation of HMW PAH such as B*a*P and the rate of its metabolism can be inhibited or decreased by several critical

factors, which includes deficient electron acceptor like O_2, nutrients, water content, pH and temperature of the surrounding environment[14, 15].

Effect of initial pH of media on BaP degradation

Every microorganism has a minimal, a maximal and an optimal pH for growth and metabolism. Microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH[16]. Thus studying the effect of the media pH on the degradation potential of the fungal isolate was an important criterion of this paper. To the best of our knowledge this is the first study that reports the effect of media pH on the level of B*a*P degradation by *P. ostreatus.*

From our investigation it was found that slightly acidic to neutral pH favoured both extensive fungal biomass formation and degradation of the xenobiotic (Fig. 1), with the highest activities been supported at pH 6 (63.1% and 49 mg of biomass/50 ml of broth). Fungi being acidophiles, the membranes and cell wall were more stabilized under slightly acidic condition. This prevented the charge imbalance inside the cells and on the surface at pH 6. As a result, *P. ostreatus* PO-3 did not have to spend much of the available energy for maintaining the homeostasis inside the cell for its survival. At extremes of pH, the proper functioning of the membranes along with the enzymatic machinery involved in PAH degradation

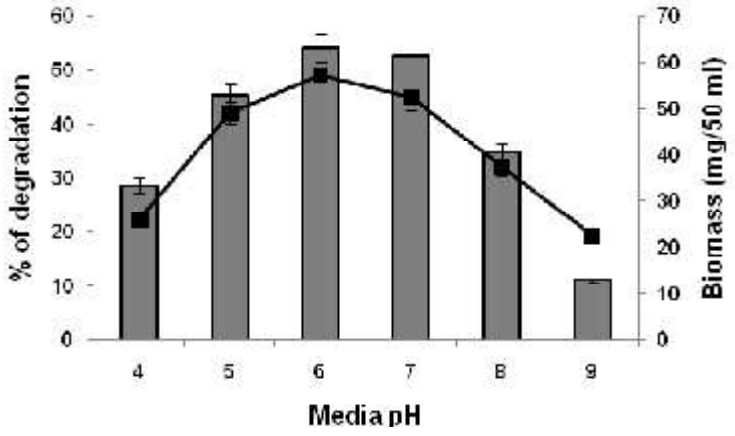

Fig. 1. Effect of initial pH of the media on BaP degradation and fungal biomass production. Data represent mean ± S.D. (n=3); $P < 0.05$

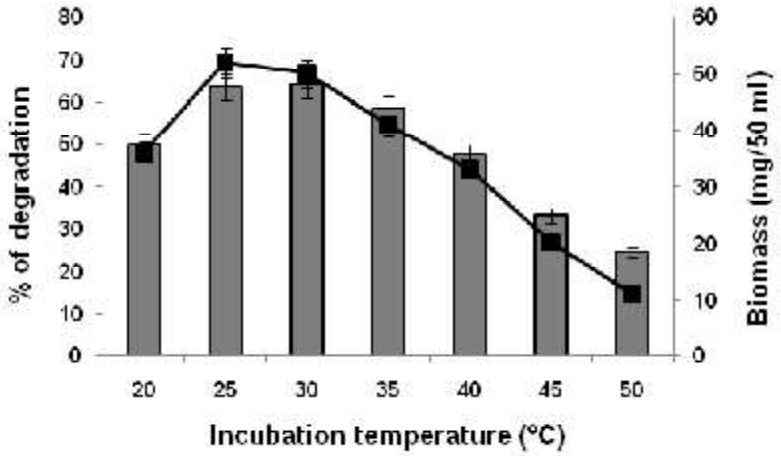

Fig. 2. Effect of incubation temperature on B*a*P degradation and fungal biomass production Data represent mean ± S.D. (n=3); $P < 0.05$

might have been affected because of which the rate of degradation and fungal biomass decreased.

An earlier study states that disappearance of phenanthrene and pyrene was significantly inhibited when pH increased from 5 to 7 and decreased to 7 to 9[17]. Phenanthrene removal was 40% at pH 5.5 after 16 days, whereas at neutral pH value of phenanthrene removal was more than 80% with *Burkholderia cocovenenans*[18]. *Sphingomonas paucimobilis* strain BA2 was however more sensitive to the pH of the growth media, with the degradation of PAHs significantly inhibited at pH 5.2 relative to pH 7[19].

Effect of incubation temperature on B*a*P degradation

Temperature has a considerable effect on the ability of the microorganisms to degrade PAHs and in general most contaminated sites will not be at the optimum temperature for bioremediation. The solubility of PAHs increases with an increase in temperature[20], which increases the bioavailability of the PAH molecules. In addition, oxygen solubility decreases with increasing temperature, which reduce the metabolic activity of aerobic microorganisms[21].

Biodegradation of PAHs can occur over a wide temperature. However, most studies tend to focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures.

According to our study, temperature influenced the degradation of B*a*P. The level of B*a*P degradation was significant in the mesophilic range (Fig. 2) with the highest degradation occurring at 30°C (64.2%). Earlier, *Fusarium* sp degraded 100 µg/l of B*a*P to a significant level with reciprocatory shaking at 180 rpm for 30 days at 32°C[2].

Similar to our results, the maximum utilization of phenanathrene by *Pseudomonas paucimobilis* was obtained at temperature 30°C[22].

Effect of agitation speed on B*a*P degradation

As degradation of PAHs is generally reported to be an oxidative reaction, optimization of the agitation speed was required to correlate whether aeration plays a significant role in the fungal biomass production and the level of biodegradation.

Our study reveals that aeration significantly affected the biomass formation of *P.ostreatus* PO-3 and the highest biomass formation and the biodegradation of B*a*P was observed at the higher shaking speeds (Fig. 3). Faster the agitation speed, higher was the biodegradation of B*a*P, since it facilitated better aeration. 180 rpm resulted in the highest level of B*a*P degradation (66.3%). However at the highest agitation speed there was both decrease in biomass and degradation, which may be due to the less attachment of the cells to that of the B*a*P.

This result is in agreement with previous reports where agitation conditions for PAH degradation not only increased oxygen availability but also increased PAH solubility into the aqueous phase for uptake by the organism[23].

Fungi being aerobic in their requirement for oxygen, aeration of the broth facilitated better hyphal formation due to better distribution of

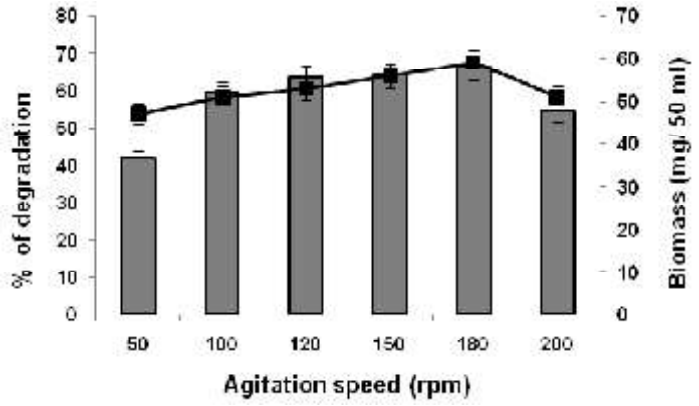

Fig. 3. Effect of agitation speed on B*a*P degradation and fungal biomass production. Data represent mean ± S.D. (n=3); $P < 0.05$

nutrients, temperature and less oxygen tension. Since, degradation of B*a*P by *P.ostreatus* is mediated by oxygenase enzyme like laccase, the level of the enzyme production and activity was enhanced in the presence of molecular oxygen which resulted in better degradation[24]. When oxygen was available in plenty, the fungi could enzymatically incorporate atmospheric oxygen more efficiently in the aromatic nucleus of the B*a*P and bring about better initial ring oxidation, which is usually the rate limiting step in the biodegradation of PAHs[25, 26].

CONCLUSION

The study revealed that with the use of a liquid medium, physical parameters significantly affected the B*a*P degradation by *P. ostreatus* isolate PO-3. Adjustment of the medium pH to slightly acidic value of 6.0 and incubating the mineral salt medium at 30°C at 180 rpm greatly enhanced the degradation of the PAH. The current findings clearly denote that *P. ostreatus* isolate PO-3 has a remarkable potency for the degradation of B*a*P.

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REFERENCES

1. Bhattacharya, S., Angayarkanni, J., Das, A., Palaniswamy, M. Mycoremediation of Benzo[a]Pyrene by *Pleurotus ostreatus* Isolated from Wayanad District in Kerala. India. *Int J Pharm Bio Sci.,* 2012; **2**(2): 84-93.
2. Chulalaksananukul, S., Gadd, G. M., Sangvanich, P., Sihanonth, P., Piapukiew, J., Vangnai, A. S., Biodegradation of benzo(a)pyrene by a newly isolated *Fusarium* sp. *FEMS Microbiol. Lett.,* 2006; **262**: 99-106.
3. Vidali, M., Bioremediation: an overview. *Pure Appl. Chem.,* 2001; **73**: 1163-1172.
4. Tam, N. F. Y., Guo, C. L., Yau, W. Y., Wong, Y. S. Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong. *Mar. Pollut. Bull.* 2002; **45**: 316-24.
5. Bogan, B.W., Lamar, R.T. Polycyclic aromatic hydrocarbon degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Appl. Environ. Microbiol.,* 1996; **62**: 1597-03.
6. Steffen, K.T., Hatakka, A., Hofrichter, M. Removal and mineralization of polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. *Appl. Microbiol. Biotechnol.,* 2002; **60:** 212-17.
7. Han, M.J., Choi, H.T., Song, H.G. Degradation of phenanthrene by *Trametes versicolor* and its laccase. *J. Microbiol.,* 2004; **42**: 94-8.
8. Pozdnyakova, N.N., Nikiforova, S.V., Makarov, O.E., Chernyshova, M.P., Pankin, K.E., Turkovskaya, O.V. Influence of cultivation conditions on pyrene degradation by the fungus *Pleurotus ostreatus* D1. *World J. Microbiol. Biotechnol.,* 2010; **26**: 205-11.
9. Baldrian, P., Der Weische, C. W., Gabriel, J., Nerud, F., Zadrazil, F. Influence of cadmium and mercury on activities of ligninolytic enzymes and degration of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in Soil. *Appl. Environ. Microbiol.,* 2000; **66**(6): 2471 – 78.
10. Capotorti, G., Digianvincenzo, P., Cesti, P., Bernardi, A. and Guglielmetti, G. Pyrene and benzo (a) pyrene metabolism by an *Aspergillus terreus* strain isolated from a polycyclic aromatic hydrocarbons polluted soil. *Biodegradation,* 2004; **15**(2): 79-85.
11. Haritash, A. K., Kaushik, C. P. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater*., 2009; **169**(1-3):1-15.
12. Sasek, V., Cajthaml, T. Bhatt, M. Use of fungal technology in soil remediation: a case study. *Water Air Soil Pollut. Focus.,* 2003; 3(3): 5-14.
13. Hou, H., Zhou, J., Wang, J., Du, C., Yan, B. Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Proc. Biochem.,* 2004;

39(11): 1415-19.

14. Rolling, W. F., Milner, M. G., Jones, D. M., Daniel, F., Swannel, R. J., Head, I. M. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. *Appl. Environ. Microbiol.* 2002; 68(11): 5537-48.
15. Kim, S. H., Kang, S. M., Oh, K. H., Kim, S. I., Yoon, B. J. Kahng, H. Y. Characterization of PAH-degrading bacteria from soils of the reed rhizosphere of Sunchon Bay using PAH consortia. *Kor. J. Microbiol.* 2005; 41: 208-15.
16. Bhattacharya, S., Das, A., Mangai, .G, Vignesh, K, Sangeetha. J. Mycoremediation of congo red dye by filamentous fungi. *Braz. J. Microbiol.,* 2011; 42: 1526-36.
17. Park, K. S. Sims, R. C., Dupont, R. Transformations of PAHs in soil systems. *J. Environ. Eng -ASCE,* 1990; **116**: 632-40.
18. Wong, J. W. C., Lai, K. M., Wan, C. K., Ma, K. K., Fang, M., Isolation and optimisation of PAH-degradative bacteria from contaminated soil for PAH bioremediation. *Water Air Soil Pollut*., 2002; **139**: 1-13.
19. Kastner, M., Breuer, J. M., Mahro, B., Impact of inoculation protocols, salinity and pH on degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Appl. Environ. Microbiol.,* 1998; **64**: 359-62.
20. Margesin, R., Schinner, F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *App. Microbiol. Biotechnol.,* 2001; **56**: 650-63.
21. Bamforth,S. M., Singleton, I., Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *J.Chem. Technol. Biotechnol.,* 2005; **80**:723-36.
22. Bishnoi, K., Sain, U., Kumar, R., Singh, R., Bishnoi, N. Distribution and biodegradation of polycyclic aromatic hydrocarbons in contaminated sites of Hisar (Indai). *Indian J. Exp. Biol.,* 2009; **47**: 210-17.
23. Johnsen, A. R., Wick, L. Y., Harms, H. Principles of microbial PAH-degradation in soil. *Environ Pollut.,* 2005; **133**: 71-84.
24. Novotný, C., Erbanová, P., Sasek, V., Kubatova, A., Cajthaml, T., Lang, E., Krahl, J., Zardazil, F. Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation,* 1999; **10**: 159-68.
25. Cerniglia, C. E., Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation,* 1992; **3**: 351-68.
26. Albert, L. J., Naidu, R. Bioremediation of high molecular weight polycyclic aromatic hyreocarbons: a review of the microbial degradation of benzo[α]pyrene. *Int. Biodeter. Biodegr.,* 2000; **45**: 57-88.