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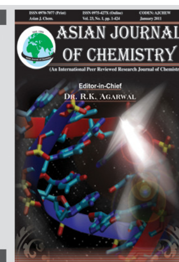

Fluorene Removal from Synthetically Concocted Marine Water by *Trametes versicolor*

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In this study; sub-tropical white rot fungi, *Trametes versicolor* was investigated for its ability to degrade fluorene in the mediums containing glucose and different concentrations of fluorene (5.0-50.0 mg/L) in batch systems. *T. versicolor* was also studied for the ability to degrade fluorene in a medium has high saline concentrations. (20, 15, 10, 5, 3.5, 3, 2.5, 1.5 %). Samples were collected at weekly intervals for 6 weeks and fluorene was detected by high performance liquid chromatography. Laccase and Mn peroxidase enzyme activities were also monitored and not detectable at the end of the incubation in higher saline conditions (except 1.5 % salinity). The greatest biodegradation was obtained by *T. versicolor* with 30.0 mg/L fluorene concentration in 3.5, 3.0, 2.5 and 1.5 % salinity medium.

Key Words: White rot fungi, *Trametes versicolor*, Fluorene, HPLC.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are pollutants, found in most terrestrial ecosystems, which arise from industrial operations and from natural events such as forest fires[1,2]. Anthropogenic inputs of polycyclic aromatic hydrocarbons from atmospheric deposition, industrial stack emission, oil spills, ship traffic, urban runoff and illegal discharge of industrial effluents have caused significant accumulation polycyclic aromatic hydrocarbons in marine environments[3]. Polycyclic aromatic hydrocarbons are often carcinogenic, mutagenic, or toxic and several of these, including phenanthrene, fluorene and fluoranthene, are listed by the US Environmental Protection Agency (USEPA) as priority pollutants[4-7]. Polycyclic aromatic hydrocarbon contamination can be remediated by physical, chemical or biological methods. Physical/chemical methods typically include extraction and/or destruction after removal. Microbial degradation is the major means for environmental decontamination[8,9]. Most biological approaches considered for the restoration of polycyclic aromatic hydrocarbon-contaminated sites depend on the activity of bacteria. A possible alternative studied for degradation of this type of compound is the use of ligninolytic fungi[10,11]. Fluorene (FL), a tricyclic polycyclic aromatic hydrocarbon. This xenobiotic and its derivatives are commonly found in petroleum and oil spills, vehicle exhaust emissions, waste incinerations, industrial effluents and smoked foods. Because fluorene is present in various polycyclic aromatic hydrocarbons mixtures and its structure is found in several mutagenic and/or carcinogenic polycyclic aromatic hydrocarbons. Both bacteria and fungi play an important role in the degradation of polycyclic aromatic hydrocarbons in ecosystems and contaminated sites. Bacterial removal of fluorene has been extensively studied[11-13]. But less is known about the fungal removal of fluorene. *Cunninghamella elegans*[14], *Penicillium* sp.[15] and white rot fungi such as *Pleurotus ostreatus*[16-18] were tested for biodegradation of fluorene. In this study, fluorene removal by *T. versicolor* was studied in different fluorene and saline concentrations. To our best of knowledge, there is no another study on fluorene removal by *T. versicolor* in with/without salinity medium.

EXPERIMENTAL

Fluorene and common chemicals and substrates were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and Sigma (St. Loui, MO, USA) (Fig. 1). Growth medium components were obtained from Merck (Darmstadt, Germany).

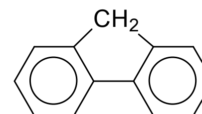

Fig. 1. Chemical structure of fluorene

Organism and culture conditions: The white rot fungus *T. versicolor* (ATCC 11 235) was maintained on 2 % (w/v) malt agar slants at 4 ºC and the fungus was activated at 26 ºC, for three days. The mycelium were harvested with sterile 0.9 % NaCl solution and then inoculated into 100 mL of 2 % malt

extract broth (pH 4.5) in 250 mL erlenmeyer flasks at 26 ºC and 175 rpm for 4 days. Pellets were inoculated into the medium consisting of 10.0 g glucose, 1.0 g of $NH_4H_2PO_4$, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, 0.025 g of yeast extract. Cultivation was carried out in an orbital shaker incubator, at 26 ºC, 175 rpm[19]. At the beginning of the fourth day of incubation, fluorene was added to the flasks aseptically at the desired concentrations. Aliquots were assayed for the laccase and Mn-peroxidase activity. *T. versicolor* was investigated for its ability to degrade fluorene in the mediums containing different saline concentrations (20, 15, 10, 5, 3.5, 3, 2.5 and 1.5 %) in batch systems. Experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of liquid medium plus fluorene dissolved in acetone to 1 mL.

Sampling: Samples (1 mL) were taken every week (total 6 week) and centrifuged to remove suspended biomass and then ligninolytic (laccase and Mn peroxidase) activities in supernatants were determined. All experiments were performed in triplicate.

Analytical methodology

Extraction procedure: Fluorene extraction and analysis was performed as described in Yuan *et al*.[20] and Chang *et al*.[21]. Briefly, 1 mL of *n*-hexane was added 1 mL sample. The organic phase was collected. The combined crude extracts of organic phase were dried. The residue was dissolved in acetonitrile (1.5 mL), filtered through a 0.45 µm membrane filter and an aliquot of 5 µL was taken for HPLC analysis.

HPLC analysis: Fluorene concentrations were determined by HPLC, was performed with a liquid chromatograph (Shimadzu) equipped with Diode array detector. The separation column, Supelcosil™ column LC_{18}, was 4.6 mm inside diameter × 250 mm (Supelco, Bellefonte, PA). The mobile phase was acetonitrile:water (70:30, v/v). The flow rate was 0.5 mL/min and detection was made at 254 nm. Each sample was injected at least three times and the mean calculated.

Enzyme assays: Laccase (Lac) production was assessed by measurement of enzymatic oxidation of 2,2^2-azinobis-(3-ethylbenzothiazoline- 6-sulphonic acid) (ABTS) at 420 nm ($\varepsilon = 3.6 \times 10^4$ cm^{-1} M^{-1})[22]. The reaction mixture contained 300 mL of extracellular fluid, 300 mL of 1 mM ABTS and 0.1 M Na Acetate buffer (pH 4.5). One Unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 mmol ABTS in 1 min.

Mn peroxidase (MnP) (EC 1.11.1.13) activity was measured by monitoring the increase in absorbance at 270 nm due to the oxidation of malonic acid at the present of H_2O_2, at pH 4.5 ($\varepsilon = 11590$ M^{-1} cm^{-1})[23]. The activities were expressed in U/L.

Removal rate and efficiency of removal (η): The removal efficiency, percentage and rate were measured with these equations.

The removal efficiency, η

$$\eta = 1 - \left(\frac{C}{C_0}\right)$$

Removal, R (%)

$$R = \left[\frac{(C_0 - C)}{C_0}\right] \times 100$$

C_0: Initial concentration, C: Last concentration.

RESULTS AND DISCUSSION

Removal of fluorene: Fluorene removal by *T. versicolor*, previously shown to possess high ligninolytic degradation activities (produce manganese peroxidase and laccase) as the major ligninolytic enzymes[19], was studied in a liquid medium including 10.0 g/L glucose and 20.0 mg/L fluorene at 26 ºC (Fig. 2). It was studied in a liquid medium not to contain cell as control and prepared calculation.

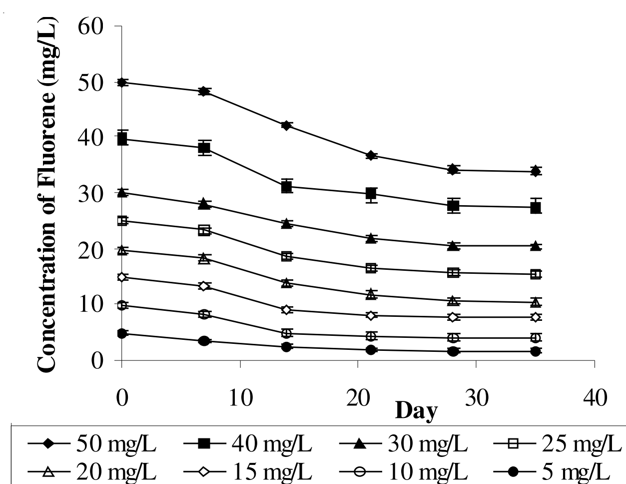

Fig. 2. Biodegradation of fluorene in different concentrations

When fluorene concentration increased, growth diminished with appearance of sterile mycelia and sometimes for the highest concentration (50 mg/L). Removal of fluorene was also practiced between 5.0-50.0 mg/L concentrations in batch systems at 26 ºC. Results were exhibited in Table-1. It was observed that the removal percentage of fluorene by *T. versicolor* was higher at lower concentration of fluorene and it is likely due to the toxicity of aromatic hydrocarbons. This results allowed us to select a final concentration of fluorene (20.0 mg/L) for removal studies. Afterwards, the removal efficiency was calculated and results were given in Fig. 3. Removal efficiency increased with low fluorene concentrations. Laccase and Mn peroxidase enzymes were inhibited from fluorene.

TABLE-1
PERCENTAGE BIODEGRADATION OF DIFFERENT FLUORENE CONCENTRATIONS

Fluorene concentration (ppm)	Biodegradation (%)
5	54.22
10	44.18
15	39.09
20	37.44
25	32.65
30	27.82
40	27.69
50	27.80

In order to determine the role of ligninolytic enzymes on fluorene degradation, laccase and Mn peroxidase activities were monitored during the removal of this compound. Maximum enzyme activities at all studied fluorene concentrations were observed on14th day of incubation. At higher concentrations levels than 20.0 mg/L fluorene concentrations, observed enzyme

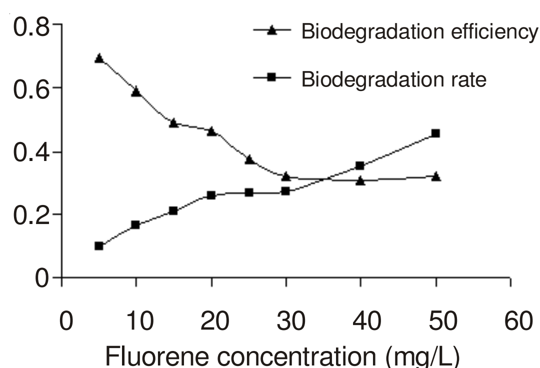

Fig. 3. Biodegradation efficiency and rates of fluorene in different concentrations

activities were decreased sharply it is likely due to the toxicity of aromatic hydrocarbons, as mentioned before (Fig. 4). Among the enzymes secreted by white-rot fungi during degradation of lignin, LiP and Mn peroxidase have been acknowledged to be especially significant in the degradation of polycyclic aromatic hydrocarbons[24,25]. The white rot fungus *T. versicolor* has been used as a model organism because of the implication of laccase and Mn peroxidase in the oxidation of fluorene[26,27]. The laccase seems merely involved in the oxidation of fluorene[28] but the transcription of a manganese peroxidase was correlated with fluorene reduction[29]. Kolomytseva *et al*., has been reported that laccase of *Rhodococcus rhodochrous* VKM B-2469 was able to oxidize *in vitro* most of the fluorene tested. Fluorene has been removed to 13.6 % from the reaction mixture during the 192 h incubation[30]. Extracellular Mn peroxidase activities were compared in discrete concentrations of fluorene. The graph was pointed in Fig. 4. Mn peroxidase activity increased in the 14th day of incubation in samples and then decreased. The results revealed that a high level of extracellular laccase was detected from the first week then declined to about 50 % in the second week and then no activity in last week. Mn peroxidase and Laccase enzymes inhibited.

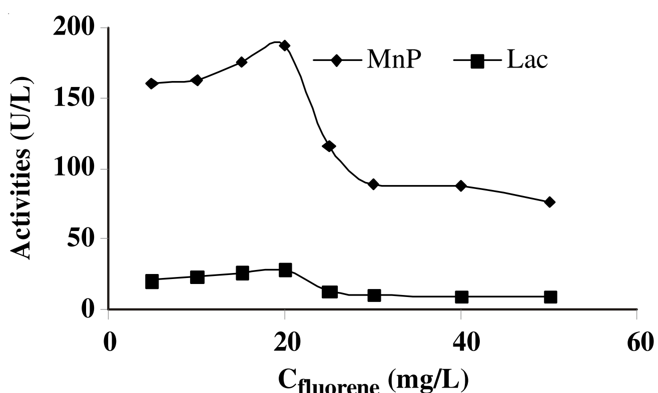

Fig. 4. Laccase and Mn peroxidase activities in different fluorene concentrations

In this study, laccase and Mn peroxidase activities were not detected during removal of fluorene by *T. versicolor* in last week. Similar results were described by Schtzendubel *et al*., 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* and Ding *et al*., who reported while LiP activity was not directly related with polycyclic aromatic hydrocarbon degradation role of Mn peroxidase in the degradation of polycyclic aromatic hydrocarbon could not be confirmed in *Phanerochaete chrysosporium*[31].

Effect of saline conditions: The evaluation of the possible inhibitory effect of salinity is essential for the selection of the most suitable strains for the remediation of shorelines and coastal areas polluted with oil spills. The concentrations of salts in the medium and the fluorene concentrations being major factors that could affect degradation capacity, the objective of our experiments was to determine the levels of salinity and fluorene concentration which affect the degradation capability of the selected fungi, *T. versicolor*. The effect of concentration of the salts was determined in 20, 15, 10, 5, 3.5, 3, 2.5, 1.5 %. It can be seen from 20.0 mg/L fluorene removal assay (Fig. 5), used to assess the extracellular ligninolytic enzyme (laccase and Mn peroxidase) activity because fungal bioremediation potential is often attributed to these enzymes. Control conditions aren't include cell, only contain fluorene in saline medium.

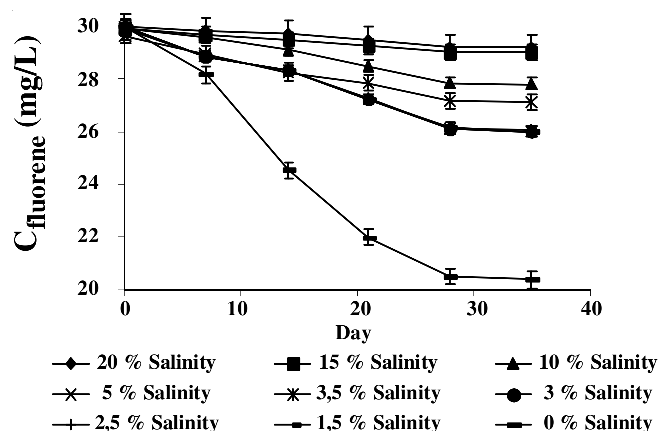

Fig. 5. Biodegradation of 30.0 mg/L fluorene in different salinity medium

The results in saline medium of fluorene removal were exhibited in Table-2. Saline of water is between 3.5-3.0 *T. versicolor* could achieve the removal of fluorene in saline per cent of sea and ocean. *T. versicolor* was degraded to 5.0 mg/L fluorene in saline medium and was able to grown in fluorene at concentrations as high as 50.0 mg/L. Compared to the well-studied white-rot fungi, *I. lacteus* and *L. tigrinus* and *B. adusta*,

TABLE-2
PERCENTAGE BIODEGRADATION OF 30.0 mg/L FLUORENE IN DIFFERENT SALINITY MEDIUM

Per cent of salinity in medium	Biodegradation (%)
20.0	2.15
15.0	2.57
10.0	6.51
5.0	7.57
3.5	12.69
3.0	12.44
2.5	12.40
1.5	12.46

were inhibited by salinity levels of 0.32 %[11]. In this study, fluorene (20.0 mg/L) was removed 12.46 % by *T. versicolor* in 1.5 % salinity medium. Present study showed that there was no inhibition of fungal growth but that the saline conditions might either interact physico-chemically with substrate transport in the cell or interact with the activity of enzymes and other membrane proteins of the cell.

Ligninolytic Mn peroxidase and enzyme activities were determined in saline medium samples. The enzyme was inhibited in high saline concentrations. The results were given in Fig. 6. Both saline and salt free medium, laccase enzyme activity didn't change a lot. But Mn peroxidase activity decreased 18 % in saline medium. While laccase enzyme activity in salt free medium of seventh day is 12.57 U/L, laccase enzyme activity in saline (1.5 % salinity level) medium of seventh day is 17.44 U/L. Since Mn peroxidase enzyme activity in salt free medium of seventh day is 109.53 U/L, this enzyme activity in saline medium of same day is 89.88 U/L. Mn peroxidase activity of fluorene removal was measured in different saline concentrations (Fig. 6). Fluoren was not reduced high salinity level, because laccase and Mn peroxidase enzymes were inhibited. Compared to salinity characteristic of sea water had minimal effect on ligninolytic activity of *I. lacteus* and *L. tigrinus*, while in *B. adusta*, activity was inhibited by salinity levels of 0.32 %[11]. Fluorene removal seems to occur independently of the level of tested extracellular ligninolytic enzymes production in saline and non-saline conditions. *T. versicolor* could achieve the reduction of high concentration fluorene in sea and ocean conditions. In the light of these results, future research should be focused on the selection of the most appropriate bioreactor configuration for removal. This study demonstrates that surfactants such as saline can negatively affect the depletion of fluorene by *T. versicolor*. The evaluation of the possible inhibitory effect of salinity is essential for the selection of the most suitable strains for the remediation of shorelines and coastal areas polluted with oil spills. The concentrations of salts in the medium and the fluorene concentrations being major factors that could affect removal capacity. The objective of present experiments was to determine the levels of salinity and fluorene concentration which affect the removal capability of the selected fungi, *T. versicolor*.

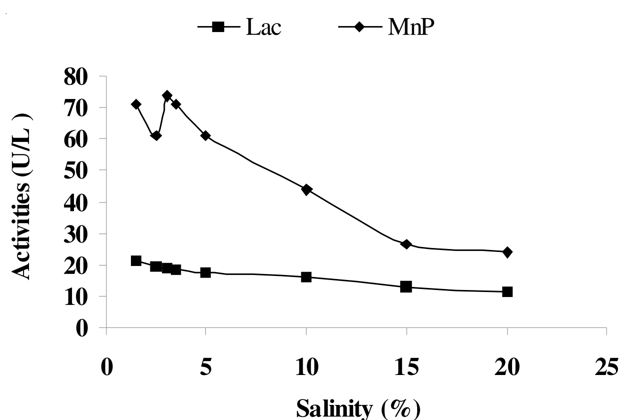

Fig. 6. Laccase and Mn peroxidase activities in different salinity medium

Conclusion

T. versicolor is used to remove fluorene in defined media with and without saline to simulate ocean and sea water. It was shown that laccase and manganase peroxidase contributes to fluorene reduction. Although laccase activities didn't affect from high saline conditions significantly, manganese peroxidase activities were decreased to almost half-level comparing to without saline conditions. Fluorene removal efficiency and degradation rates at different fluorine concentrations were also calculated and it was found that, as expected, removal efficiency is decreasing with increasing fluorene concentrations. Different white rot fungal isolates can contribute differently to polycyclic aromatic hydrocarbon bioremediation as shown by the differences in polycyclic aromatic hydrocarbon reduction. *T. versicolor* is one of them had the highest degradative capabilities of the initial fluorene while cultures of *T. versicolor* were most efficient in degrading the fluorene in salinity conditions. This information contributes to enhance the bioremediation efficiency of polycyclic aromatic hydrocarbon in real environment. To our best of knowledge, this is the first study to reveal the importance of white rot fungi involved in saline cycling for the degradation of ocean-sea-bound fluorene. Besides, removal of fluorene by *T. versicolor* is undoubtedly a most promising technique to remediate contaminated seas. However, a number of problems remain to be solved, before they can be successfully used in the marine. Two requirements appear to be essential *i.e.*, development of standard procedures for benchmarking fungal degradative potential and their optimal conditions and further investigation on real marine water, where possible, under field conditions. Only when this is done, can the full potential of using *T. versicolor* in fluorene reduction and remediation be evaluated.

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