



## Environmental Microbiology

# Ligninolytic fungus *Polyporus* sp. S133 mediated metabolic degradation of fluorene



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## ABSTRACT

This study aimed to investigate the impact of nonionic surfactants on the efficacy of fluorine degradation by *Polyporus* sp. S133 in a liquid culture. Fluorene was observed to be degraded in its entirety by *Polyporus* sp. S133 subsequent to a 23-day incubation period. The fastest cell growth rate was observed in the initial 7 days in the culture that was supplemented with Tween 80. The degradation process was primarily modulated by the activity of two ligninolytic enzymes, laccase and MnP. The highest laccase activity was stimulated by the addition of Tween 80 (2443 U/L) followed by mixed surfactant (1766 U/L) and Brij 35 (1655 U/L). UV-vis spectroscopy, TLC analysis and mass spectrum analysis of samples subsequent to the degradation process in the culture medium confirmed the biotransformation of fluorene. Two metabolites, 9-fluoreneol ( $\lambda_{\max}$  270,  $t_R$  8.0 min and  $m/z$  254) and protocatechuic acid ( $\lambda_{\max}$  260,  $t_R$  11.3 min and  $m/z$  370), were identified in the treated medium.

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## Introduction

Fungal biotransformation is considered as one of the most important methods for removing pollutant from the ecosystem. In case of organic pollutants, the primary issue that impedes their clearance is their characteristically low solubility in water. The inherent hydrophobic nature of these organic pollutants results in their selective partitioning in the soil matrix such that their bioavailability is seriously

compromised. This in turn jeopardizes the success of the bioremediation treatment. There is an urgent need for the development of alternative treatment modalities which will help remove these pollutants from the environment by making them more available for the degradation by microorganisms. Surfactant-mediated biodegradation is one such promising process. This method has the potential to increase the solubility of hydrophobic compounds, such as PAHs, thereby increasing their bioavailability and expediting their removal from the environment.<sup>1–3</sup> Previous studies have

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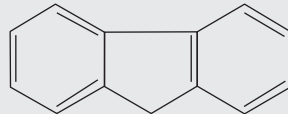
shown that depending on the surface of the substance and the type of the surfactant used, the process of surfactant sorption to microorganisms can result in either an increase or a decrease in adhesion.<sup>4</sup>

Micelles are the solution aggregates formed by surfactant molecules in water when their concentration is above the critical micelle concentration or CMC. Micelles have dual nature: their hydrophilic outer surface is exposed to the solvent whereas the core is composed of hydrophobic moieties. Several researchers are actively conducting studies in an attempt to investigate the effect of surfactants on microbial transformation of pollutants; all these studies are based on surfactant utilization at concentrations above CMC. Hydrocarbons are known to be partitioned into the hydrophobic core of the micelles.<sup>5,6</sup> But, it is well established that extent of solubilization is influenced by a multitude of factors such as type and concentration of the surfactant, the interaction between the surfactant and soil, the contact time between the contaminant and soil as well as the hydrophobicity of the compound in question.<sup>7,8</sup>

Fluorene is a three-ring PAH found ubiquitously in the environment.<sup>9</sup> It is produced as a result of an incomplete combustion of certain products. Fluorene leaches into the groundwater either as a result of direct contamination from polluted surface waters or indirectly through the soil. Fluorene is not considered as a genotoxic agent but the carcinogenic aspect of its molecular structure has been popularly used as an indicator for the evaluation of PAH-containing pollutants.<sup>10</sup> White-rot fungi have been frequently employed for the degradation of various organic environmental pollutants. Their exceptional capacity to degrade and biotransform organic contaminants can be attributed to the production of ligninolytic enzymes such as MnP, LiP, laccase, and versatile peroxidase.<sup>11–13</sup> The ligninolytic enzymes are responsible for the depolymerization of PAHs, which results in the production of various types of phenol. They also oxidize substrates in the presence of a suitable co-substrate.<sup>14</sup> Extensive studies have been undertaken in order to investigate the potential of white-rot fungus to degrade PAHs.<sup>15,16</sup> However, the impact of solubilization of fluorene with single and mixed nonionic surfactants on the degradation rate remains unexplored.

This study attempts to fill the above mentioned lacuna by evaluating the biodegradation of fluorene by the *Polyporus* species in liquid cultures in the presence of two synthetic surfactants. Our study also quantifies the resultant biomass produced, the extracellular enzymes and the glucose consumption involved in the process. Furthermore, in an attempt to comprehensively study the degradation process, metabolites formed during fluorene degradation have been identified and characterized using a variety of chromatographic techniques. Two nonionic surfactants, Tween 80 and Brij 35, were chosen to study the influence of the surfactant structure on the biodegradation and biotransformation of fluorene by the fungal biomass. Studies have established that nonionic surfactants are more effective on account of their higher adsorption capacity in clay fractions as compared to anionic or cationic surfactants.<sup>6</sup> The relationship between enzyme activity, glucose consumption, fluorene utilization and biomass production in the presence of Tween 80 and Brij 35 was also established. The data generated by this study will contribute

**Table 1 – Structure and physical–chemical characters of fluorene.**

Molecular structure	
Molecular formula	C <sub>13</sub> H <sub>10</sub>
Appearance	White crystal
Molecular weight	166.22
Density	1.202 g/mL
Melting point	117 °C
Boiling point	295 °C
Aqueous solubility (20–25 °C)	1.98 mg/L
Octanol–water partition coefficient (logL/kg)	4.0155

toward defining a new approach for biotransformation of PAHs by improving our understanding about the influence of surfactants in the process. It is hoped that the results of our study will be a significant contribution to the field of biodegradation especially surfactant enhanced biodegradation (Table 1).

## Materials and methods

### Chemicals and microorganisms

Fluorene and an internal standard, 4-chlorobiphenyl, were obtained from Sigma–Aldrich (Milwaukee, WI). Both were supplied at >97% purity. Nonionic surfactants such as Brij 35 and Tween 80, and extraction solvents such as ethyl acetate and dichloromethane were obtained from Acros Chemicals. Malt extract as well as other chemicals required for liquid culture were procured from Difco (Detroit, USA). All the solutions obtained or prepared utilized analytical grade and high-purity reagents. The fungus used in the study was isolated from Matsuyama, Japan. The *Polyporus* sp. S133 was cultivated in a 100-mL Erlenmeyer flask containing mineral salt medium (MSM). The composition of the growth medium MSM is given in Table 2. The sterility of MSM was ensured by autoclaving the growth medium prior to use. Fluorene (10 mg/L), Tween 80 and Brij 35 solutions were sterilized by vacuum filtration in order to prevent any conversion of the compounds.<sup>15,17</sup> For estimation of enzyme activity, the culture was supplemented with the desired amount of fluorene and incubated for specific time intervals. All experiments were conducted in triplicate.

### Enzyme assays and biomass determination

The enzyme analysis of the fungus was conducted as per the protocol described earlier.<sup>18</sup> Laccase activity was determined by ABTS oxidation and assayed spectrophotometrically at 420 nm. MnP activity was determined by the oxidation of malonate and dimethoxyphenol in an MnSO<sub>4</sub> solution and assayed by monitoring changes in absorbance at 270 nm. The enzyme production was expressed in U L<sup>-1</sup>. Biomass production was estimated by centrifuging the culture followed by filtration through a Whatman No. 1 filter paper. The biomass of the fungi was expressed in g L<sup>-1</sup>.

**Table 2 – Concentrations of mineral salt media (MSM) constituents.**

MSM constituents	Concentration (g/L)	Trace element	Concentration (mg/L)
Malt extract	10	FeSO <sub>4</sub> ·7H <sub>2</sub> O	12
Glucose	10	MnSO <sub>4</sub> ·7H <sub>2</sub> O	3
KH <sub>2</sub> PO <sub>4</sub>	2	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	3
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1	CoSO <sub>4</sub> ·7H <sub>2</sub> O	1
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.5	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	1
Ammonium tartrate	0.5		
Trace element	10 mL		

### Solubilization test

The solubilization test was performed as described previously.<sup>19,20</sup> Single nonionic surfactants were used in the following concentrations: 0.1, 0.5, 1, 2, 4 mM. Mixed surfactant solutions (Tween 80 and Brij 35) were prepared at same concentration in a 1:1 ratio.

### Instrumental analysis

Agilent 5975E FID GC-MS (DB-1 capillary column; 0.25 μm ID; 0.25 mm diameter; 30 m length) was used to identify and characterize the metabolites present in the incubated culture. As described in our previous study, trimethylchlorosilane was used in the silylation procedure employed for detecting samples containing hydroxyl and carboxyl groups.<sup>18</sup> The GC temperature was maintained as follows: initial 70 °C (1 min), increased at the rate of 18 °C min<sup>-1</sup> till 150 °C, increased at the rate of 28 °C min<sup>-1</sup> till 330 °C, held at 330 °C for 10 min. The injector and interface temperatures were set at 260 °C with a splitless time of 2 min. The injection concentration was 1 μL in the splitless condition and flow rate of the helium was adjusted to 1 mL/min. The EI ionization mode was used with electron energy of 1.3 eV and a mass range of 50–500 amu. The mass spectra of the samples were compared with authentic standard compounds as well as the database library (Wiley 275L).<sup>21</sup>

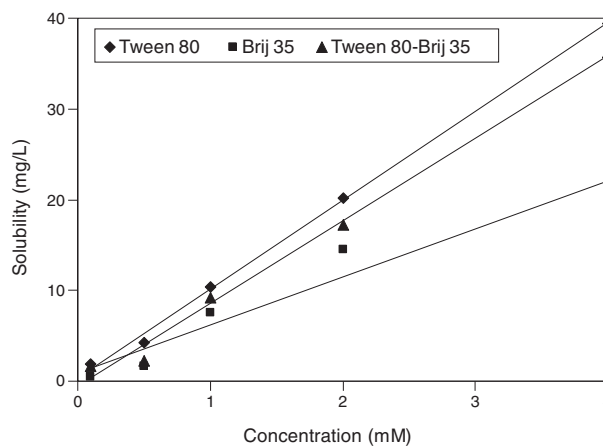
## Results and discussion

### Solubilization of fluorene

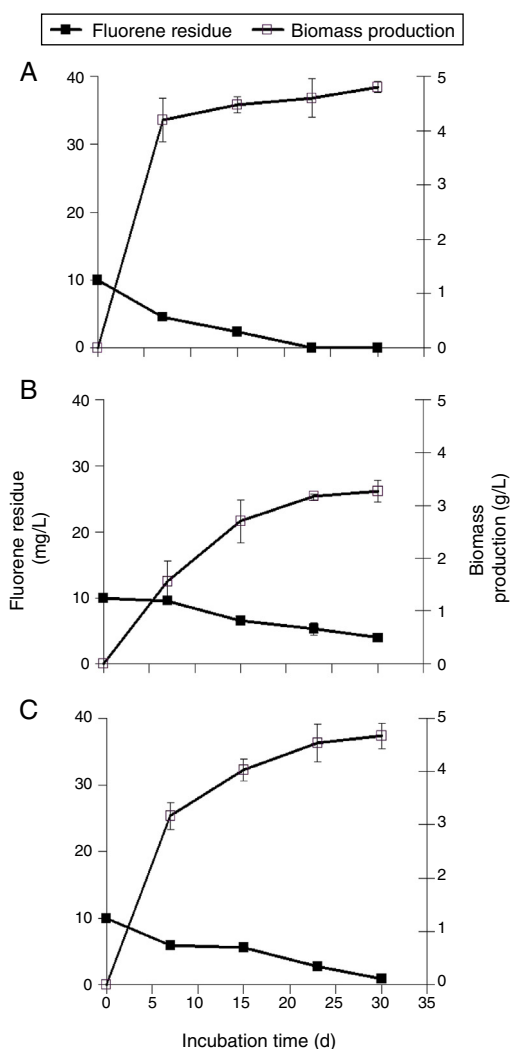
Fig. 1 shows the change in the solubility of fluorene as affected by the addition of single and mixed nonionic surfactants. It was observed that the solubility of fluorene was significantly enhanced by addition of surfactants and that the solubility of the pollutant was directly proportional to the surfactant concentrations. The highest solubility noted in this study was achieved by the addition of Tween 80 (39.4 mg L<sup>-1</sup>); the addition of mixed solutions resulted in a moderate rise in the solubility, whereas the lowest solubility values were observed upon addition of Brij 35 (20.4 g L<sup>-1</sup>). It is hypothesized that the addition of fluorene in combination with the nonionic surfactant solution facilitates the formation of mixed micelles which in turn improves the contact between microorganisms and surfactant.

### Biodegradation of fluorene by *Polyporus* sp. S133

It is evident from the data available in the literature that fluorene is likely to be absorbed onto the fungal biomass surface prior to being accumulated, degraded, and metabolized by the fungus. In order to identify the impact of single and mixed surfactants on fluorene removal, the growth medium of *Polyporus* sp. was supplemented with the addition of Tween 80, Brij 35, and mixtures of Tween 80 and Brij 35. S133 culture was undertaken in order to estimate both biomass production as well as fluorene degradation. Results indicated that both the cell growth as well as the degradation of fluorene was influenced by the presence of surfactants (Fig. 2). As is shown in Fig. 2, the presence of Tween 80 significantly enhanced the removal of fluorene as well as the biomass production. Fluorene was found to be degraded in entirety by *Polyporus* sp. S133 after an incubation period of 23 d. The fastest growth rate was experienced in the initial 7 days (0.6 g L<sup>-1</sup> per day) in the culture that was supplemented with Tween 80. Tween 80 increased the sorption extent and no distinction was found between surfactant and mycelia. Fig. 2 suggests that the addition of Tween 80 and mixed surfactants enhanced the fluorene degradation and biomass production by *Polyporus* sp. S133. It was observed after the 23-day incubation period that the cellular production and generation slowly decreased largely because most of the fluorene had already been degraded. The results show that the maximum fluorene degradation was observed for the Tween 80 culture (0.4 mg per day), which 0.2 and 0.27 mg/d compared to was mixed surfactant and Brij 35.



**Fig. 1 – Solubilization of fluorene by single and mixed surfactant: Tween 80; Brij 35; Tween 80–Brij 35.**



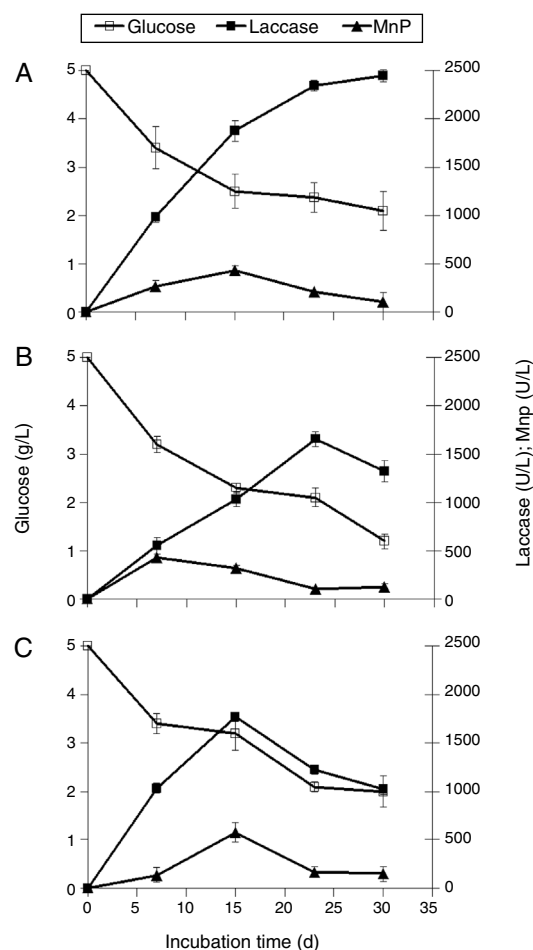
**Fig. 2 – Utilization of fluorene and biomass production in single and mixed surfactant: Tween 80 (A); Brij 35 (B); Tween 80–Brij 35 (C).**

Dioxygenase, peroxidase and laccase enzymes play a critical role in the biotransformation of fluorene by *Polyporus* sp. S133. Enzymes such as 1,2-dioxygenase, 2,3-dioxygenase, MnP, LiP, and laccase were studied with the aim of gaining additional insight into the mechanism of decolorization (Table 3). It was noted that the degradation process was primarily determined by the activity of two ligninolytic enzymes:

**Table 3 – Enzyme detection of *Polyporus* sp. S133 at initial degradation (10 d).**

Enzyme	Activity (U/L)
Manganese peroxidase	45.4
Lignin peroxidase	1.3
Laccase	113.8
1,2-Dioxygenase	0.2
2,3-Dioxygenase	0.5

Values are means of three experiments.



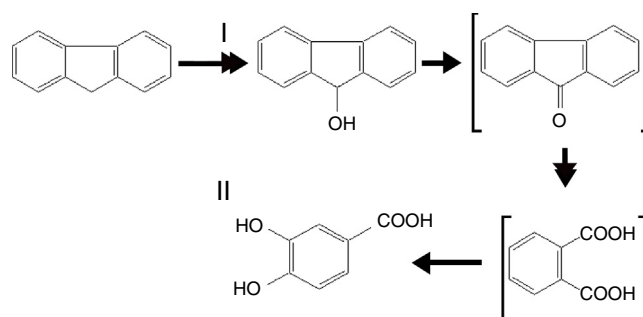
**Fig. 3 – Effect of non-ionic surfactant on enzyme activities and glucose consumption. Tween 80 (A); Brij 35 (B); Tween 80–Brij 35 (C).**

laccase and MnP. In order to assay the impact of nonionic detergents on the enzyme activity as well as on the fluorene degradation process, activity of the enzyme was plotted against glucose consumption for different concentrations of the surfactants (Fig. 3).

It was observed that for all surfactants, the maximum laccase production was seen on day 30 of the incubation; it was also seen that the activity declined significantly at day 15 for Brij 35 and at day 23 for mixed surfactants. Growth medium supplemented with Tween 80 exhibited the highest activity ( $2443 \text{ U/L}^{-1}$ ) for laccase followed by the mixed surfactant ( $1766 \text{ U/L}^{-1}$ ) and lastly by Brij 35 ( $1655 \text{ U/L}^{-1}$ ). A similar analysis of the MnP activity with varying combinations of the surfactant proved that MnP activity decreased after 7–15 days of incubation and the downward trend continued till the end of the experimental period. The glucose consumption was significantly high in the first 7 days of incubation but was observed to decrease slowly after that till the end of the experimental period. The highest consumption of glucose was seen in the growth medium supplemented by Brij 35 ( $3.8 \text{ g/L}^{-1}$ ) after 30 days of incubation. From this result it can be concluded that when the growth medium is supplemented by Brij 35, glucose is the preferred source of carbon for

fungal growth. The function of glucose in the culture was to induce the enzymatic system of the fungus. From the results obtained above, we theorize that the production of ligninolytic enzymes such as laccase and peroxidase was suppressed by addition of fluorene and surfactant. Our results are in accord with a previous study that analyzed PAH degradation by various white-rot fungi. The authors of the study reported that the degradation rates were found to be associated both with the activity of ligninolytic enzymes as well as with the type of surfactant used. The authors also proposed that the enzymes that play an important role in PAHs degradation, namely laccase and MnP, are also implicated in the metabolism of some PAHs. Laccase is also considered to be an important enzyme for oxidizing PAHs in liquid culture.<sup>15,16</sup> The function of MnP in the fluorene removal process is similar to that of laccase in the early weeks of incubation and it sustains till the end of the incubation period.

It was observed that the microorganisms grew at a faster rate when the liquid culture was supplemented with a carbon source such as glucose. Glucose was supplemented into the fungal culture to fulfill the function of a co-metabolic substrate in the degradation process. Previous studies have established that glucose plays an important role in the depolymerization of long chain fatty acids in the biodegradation process. Also, several reports in literature claim that many organic pollutants were more quickly transformed when glucose was supplemented into the culture as a co-substrate for growth.<sup>22,23</sup> Other researchers have proved that for most white-rot fungi, simple carbon sources, such as glucose, provide nutrients that are crucial for the production of extracellular enzymes and biomass production. Although ligninolytic enzymes are produced during the secondary metabolism and are strongly influenced by nutrient limitation, the actual pollutant



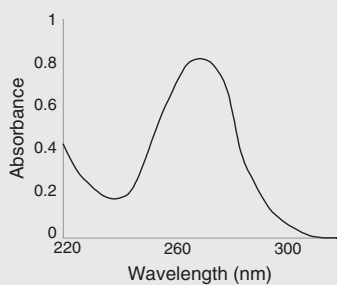
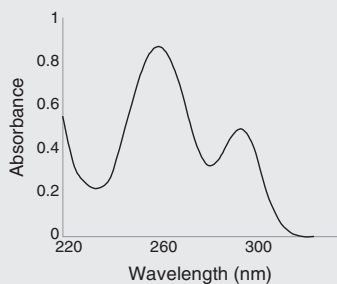
**Fig. 4 – Proposed pathway of fluorene metabolism by *Polyporus sp. S133*. Bracket-compounds were unidentified in our culture extract.**

degradation process occurs after the production of enzymes.<sup>24</sup>

#### Fluorene metabolites

The culture was acidified with HCl in order to stop the fungal growth following which the sample was extracted with ethyl acetate. TLC and UV absorbance of the sample extracts revealed the presence of three fluorene metabolites (Table 4). GC-MS analyses of the ethyl acetate-extracted samples were found to have two major chromatographic peaks (Table 4). Metabolite I ( $R_f$  0.35) exhibited  $\lambda_{max}$  at 270 nm which corresponds to the 9-fluorenone standard. The spectrum of Compound I ( $t_R$  of 8.0 min) show maxima  $m/z$  254,  $M^+$ , a fragment ion at  $m/z$  239 ( $M^+ - 15$ ) representing loss of methyl, as well as the expected fragment ion at  $m/z$  165 ( $M^+ - 89$ ) which could be accounted for by the sequential loss of  $OSi(CH_3)_3$ , and

**Table 4 – UV absorbance and mass spectral analysis of the metabolic product produced from fluorene by *Polyporus sp. S133*.**

Metabolites	Retention time (min)	$m/z$ of fragment ions (% relative abundance)	Possible structure	UV absorbance
I	8.0	73 (62), 104 (6), 139 (12), 152 (19), 165 (100), 178 (17), 193 (5), 221 (5), 223 (6), 239 (82), 254 (92, $M^+$ ), 255 (31)	9-Fluorenone-BSTFA (confirmed with a standard)	
II	11.3	73 (100), 74 (12), 165 (17), 181 (14), 193 (98), 194 (28), 223 (15), 311 (33), 355 (54), 370 (73, $M^+$ ), 371 (29)	Protochaeteuic acid-TMS derivative (confirmed with a standard)	

at 73, 178, and 254. Further metabolite characterization was done by growing *Polyporus* sp. S133 in a 9-fluoreneol acid culture. In the culture, we identified metabolite II ( $t_R$  of 11.3 min), whose spectra was found to have a main ion at  $m/z$  370 ( $M^+$ ), a fragment ion at  $m/z$  355 ( $M^+ - 15$ ) representing loss of methyl along with expected fragment ions at  $m/z$  181 [ $M^+ - 89$ ;  $-OSi(CH_3)_3$ ], 73 [( $CH_3$ ) $_3$ Si], 165 and 193. Based on the mass spectral data obtained above, we identified compound II as protocatechuic acid.

*Polyporus* sp. S133 is a white-rot fungus that has the capacity to eliminate and transform many types of PAHs. In this study, we established that the fungus succeeded in producing the ligninolytic enzyme important for the degradation of fluorene. Two major enzymes, laccase and MnP, were presumed to be vital for the transformation of the fluorene. During the laccase-mediated oxidation of PAHs, quinone is formed during the initial step of degradation while 9-fluoreneol-1-carboxylic acid is formed at a later stage. In the next step the carboxylic acid compound undergoes a ring cleavage to form 9-fluoreneol and protocatechuic acid which is finally converted to tricarboxylic acid. Unfortunately, fluorene quinone and 9-fluoreneol-1-carboxylic acid was not identified in any of the extracts obtained in this study (Fig. 4). It is known that certain metabolites produced as a by-product of PAHs metabolism are hazardous in nature. For this reason fragmentary metabolism of fluorene has the potential to pose a grave threat to the ecosystem because the characteristic of intermediate metabolites may actually be more harmful than that of the parent compound.<sup>25–28</sup>

## Conclusions

From the results presented above it can be confidently concluded that fluorene, a three-ring PAH, was degraded in its entirety by a fungus *Polyporus* sp. S133 following a 23-day incubation in the liquid culture format. The positive relationship between biomass, enzyme and degradation established that the fluorene bioavailability was significantly increased by surfactant mediated bioaccumulation enhancement especially that by Tween 80. Nonionic surfactants were observed to increase the sorption rate of fluorene on the biomass and thus improve the removal process. Two important ligninolytic enzymes, laccase and MnP, were found to play a significant role in the complex process of fluorene transformation by *Polyporus* sp. S133. To conclude, our study makes a powerful argument in support of the utility and importance of Tween 80 in the PAHs-polluted soil bioremediation process.

## Conflicts of interest

The authors declare no conflicts of interest.

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