

1 **Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) by fungal enzymes: A**  
2 **review.**

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13 **Abstract**

14 Polycyclic aromatic hydrocarbons (PAHs) are a large group of chemicals. Their sources can  
15 be either natural or anthropogenic. They represent an important concern due to their  
16 widespread distribution in the environment, their resistance to biodegradation, their potential  
17 to bioaccumulate and their harmful effects. In fact, natural resources polluted with PAHs  
18 usually lead to mutagenic and carcinogenic impacts in fresh-water, marine-water and  
19 terrestrial species. Several pilot treatments have been implemented to prevent further  
20 economic consequences and deterioration of soil and water quality. As a promising option,  
21 fungal enzymes are regarded as a powerful choice for potential degradation of PAHs. Their  
22 rate of degradation depends on many factors, such as environmental conditions, fungal strain,  
23 nature of the fungal enzyme and nature and chemical structure of the PAH among others.  
24 *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Bjerkandera adusta* are most  
25 commonly used for the degradation of such compounds due to their production of ligninolytic  
26 enzymes as lignin peroxidase, manganese peroxidase and laccase. The rate of biodegradation  
27 depends on many culture conditions, such as temperature, oxygen, accessibility of nutrients  
28 and agitated or shallow culture. Moreover, the addition of biosurfactants can strongly modify  
29 the enzyme activity. The removal of PAHs is dependent on the ionization potential. The study  
30 of the kinetics is not completely comprehended, and it becomes more challenging when fungi

31 are applied for bioremediation. Degradation studies in soil are much more complicated than  
32 liquid cultures because of the heterogeneity of soil, thus, many factors should be considered  
33 when studying soil bioremediation, such as desorption and bioavailability of PAHs. Different  
34 degradation pathways can be suggested. The peroxidases are heme-containing enzymes  
35 having common catalytic cycles. One molecule of hydrogen peroxide oxidizes the resting  
36 enzyme withdrawing two electrons. Subsequently, the peroxidase is reduced back in two steps  
37 of one electron oxidation. Laccases are copper-containing oxidases. They reduce molecular  
38 oxygen to water and oxidize phenolic compounds.

39 **Keywords:** Polycyclic aromatic hydrocarbons (PAHs), Biodegradation, Fungi, Enzymes

40  
41 **Abbreviations**

42  
43 *P: Phanerochaete*

44 *C: Coriolus*

45 *T: Trametes*

46 *P: Pleurotus*

47 *I: Irpex*

48 LiP: Lignin peroxidase

49 MnP: Mn-peroxidase

50 VP: Versatile peroxidase

51 LAC: Laccase

52 ABTS: 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)

53 VA: 3,4-dimethoxybenzyl alcohol, veratryl alcohol

54 PAHs: Polycyclic aromatic hydrocarbons

55 ANT: Anthracene

56 PHE: Phenanthrene

57 FLU: Fluorene

58 PYR: Pyrene

59 FLA: Fluoranthene

60 CHR: Chrysene

61 B[a]P: Benzo[a]pyrene

62 B[a]A: Benzo[a]anthracene

63 IP: Ionization potential

64 WRF: White-rot fungi

## 65 **1 Introduction**

66 Polycyclic aromatic hydrocarbons (PAHs), or polyarenes, are a large group of chemicals with  
67 two or more fused aromatic rings in linear, angular or clustered arrangements. PAHs with less  
68 than six aromatic rings are often denominated as small PAHs and those containing more than  
69 six aromatic rings are often called large PAHs (Haritash and Kaushik, 2009). They occur as  
70 colorless, white/pale yellow solids with low solubility in water, high melting and boiling  
71 points and lower vapour pressure as seen in Table 1. With an increase in molecular weight,  
72 their solubility in water decreases; melting and boiling point increases and vapour pressure  
73 decreases (Patnaik, 2007).

74 The widespread occurrence of PAHs is due to their generation from the incomplete  
75 combustion or pyrolysis of numerous organic materials, such as coal, oil, petroleum gas, and  
76 wood. PAHs exhibit the most structural variety in nature compared to any other class of non-  
77 halogenated molecules in the eco- and biosphere. Moreover, with continued oil production  
78 and transport, the quantities of these hydrocarbons in water and sediment will keep increasing  
79 (Arun et al., 2008). Fate of PAHs in the environment includes volatilization, photo-oxidation,  
80 chemical oxidation, adsorption on soil particles and leaching (Haritash and Kaushik, 2009).  
81 They are difficult to degrade in natural matrices and their persistence increases with their  
82 molecular weight. Therefore, these compounds represent an important concern due to their  
83 widespread presence in the environment, their resistance towards biodegradation, their  
84 potential to bio-accumulate and their mutagenic and carcinogenic effects that occurs by  
85 breathing air containing PAHs in the workplace, or by coming in contact with air, water, or  
86 soil near hazardous waste sites, or by drinking contaminated water or milk etc. (Lei et al.,  
87 2007; Albanese et al., 2014; Wang et al., 2014a; Zhao et al., 2014).

88 Potential treatments have been implemented to prevent further economic consequences and  
89 deterioration of soil and water quality. Among such treatments, bioremediation initiatives  
90 promise to deliver long lasting and low cost solutions for PAHs degradation. Biodegradation  
91 of hydrocarbons was carried out either by bacteria (Cybulski et al., 2003; Arulazhagan and  
92 Vasudevan, 2011; Mao et al., 2012; Hamamura et al., 2013; Sun et al., 2014; Cébron et al.,  
93 2015; Darmawan et al., 2015; Ferreira et al., 2015; Okai et al., 2015; Singh et al., 2015), fungi  
94 (K. G. Wunch et al., 1999; Li et al., 2005; Chan et al., 2006; Elisabet Aranda, 2009;  
95 Hadibarata et al., 2009; Hadibarata and Kristanti, 2014; Bonugli-Santos et al., 2015; Cébron  
96 et al., 2015; Jové et al., 2015; Marco-Urrea et al., 2015; Mineki et al., 2015; Simister et al.,

97 2015; Young et al., 2015) or algae (Chan et al., 2006; Diaz et al., 2014; Luo et al., 2014). As a  
98 result of such a large experience, the fungi emerge as a powerful choice for degradation of  
99 polyaromatic hydrocarbons. They have advantages over bacteria due to their capability to  
100 grow on a large spectrum of substrates and at the same time, they produce extracellular  
101 hydrolytic enzymes, which can penetrate the polluted soil and remove the hydrocarbons  
102 (Balaji and Ebenezer, 2008; Messias et al., 2009; Venkatesagowda et al., 2012).

103 The rate of bioremediation of a pollutant depends on the environmental conditions, type of  
104 microorganism, as well as the nature and chemical structure of the compound to be removed.  
105 Therefore, to develop a bioremediation process, a number of factors are to be taken into  
106 account. The level and rate of biodegradation of PAHs by fungal enzymes relies upon growth  
107 factors, such as, oxygen, accessibility of nutrients, and enzyme optimum conditions like pH,  
108 temperature, chemical structure of the compound, cellular transport properties, and chemical  
109 partitioning in growth medium (Singh and Ward, 2004).

110 Enzymatic biodegradation of polycyclic aromatic hydrocarbons by fungal strains have not  
111 been significantly reviewed, to the best of our knowledge. The main objective of the present  
112 review is to understand the enzymatic biodegradation of PAHs using fungal strains. In this  
113 sense, rate and pathways of biodegradation of PAHs are strongly related to the environmental  
114 conditions for the enzymatic activity and also for the fungal growth., It is also dependent on  
115 the system where the degradation takes place either ex-situ or in-situ, and on the nature and  
116 chemical structure of the pollutant. Also, the mechanisms of enzymes degrading PAHs should  
117 be highlighted. Therefore, to build a complete scenario of enzymatic fungal bioremediation  
118 many factors need to be considered at the time.

## 119 **2 Fungal enzymes**

120 Davis et al., (1993) demonstrated that all fungi have innate efficiency to degrade PAHs.  
121 Ligninolytic fungi have been extensively studied for the past few years (Haritash and  
122 Kaushik, 2009) because they produce extracellular enzymes with extremely reduced substrate  
123 specificity. This evolved due to the irregular structure of lignin but resulted in the ability to  
124 also degrade and mineralize various organopollutants (Hatakka, 1994; Vyas et al., 1994a;  
125 Hammel, 1995). Latest research showed that extracellular peroxidases of these fungi are  
126 responsible for the initial oxidation of PAHs (Acevedo et al., 2011; Betts, 2012; Li et al.,  
127 2014; Zhang et al., 2015). Fungal lignin peroxidases oxidize a number of PAHs directly,  
128 while fungal manganese peroxidases co-oxidize them indirectly through enzyme-mediated

129 lignin peroxidation. (Vyas et al., 1994a) have tested several white rot fungi and suggested that  
130 all of them oxidize anthracene to anthraquinone. The ligninolytic system contains three  
131 principal enzyme groups, i.e. lignin peroxidase (LiP), Mn-dependent peroxidase (MnP),  
132 phenol oxidase (laccase, tyrosinase), and H<sub>2</sub>O<sub>2</sub> producing enzymes (Novotný et al., 2004a).  
133 Ligninolysis is oxidative, it is induced by high oxygen ranges in the culture medium, and is  
134 part of the organism secondary metabolism; it is expressed under nutrient limiting conditions,  
135 generally nitrogen (Haemmerli et al., 1986; Hammel et al., 1986; Sanglard et al., 1986;  
136 Novotný et al., 2004a) and their physiology has been broadly studied. Thus, there is an  
137 elaborated comprehension of the ligninolytic mechanisms of basidiomycetes (Hatakka, 1994;  
138 Thurston, 1994). Novotný et al., (2004b) studied the degradation amounts and enzymatic  
139 activities of MnP, LiP and laccase in different species of ligninolytic fungi cultivated in liquid  
140 medium and soil and their impact on some xenobiotics including PAHs. They showed that  
141 degradation of anthracene and pyrene in spiked soil by *Phanerochaete chrysosporium*,  
142 *Trametes versicolor* and *Pleurotus ostreatus* depends on the MnP and laccase levels secreted  
143 into the soil. Thus, fungal degradation of PAHs is not as fast or effective as bacteria, but they  
144 are very non-specific and have the capability to hydroxylate a large variety of xenobiotics.  
145 Furthermore, many fungi are naturally living in soil litter and could grow into the soil and  
146 propagate through the solid matrix to remove the PAHs. These criteria make the ecological  
147 role of ligninolytic fungi in bioremediation (Lee et al., 2014; Winqvist et al., 2014; Kristanti  
148 and Hadibarata, 2015; Lee et al., 2015). In addition to MnP, LiP and laccase other fungal  
149 enzymes, such as Cytochrome P450 monooxygenase, epoxide hydrolases, lipases, proteases  
150 and dioxygenases have been extensively studied for their ability to degrade PAHs (Bezalel et  
151 al., 1997; Balaji et al., 2014).

### 152 **3 Different species of fungus enzyme-degrading PAHs**

153 Since the effectiveness of bioremediation depends on the selection of species with matching  
154 properties, a vast range of fungi have been investigated for their capability to metabolize  
155 PAHs up to six rings and the metabolite secretion is strain-dependent. In fact, as reported by  
156 Pothuluri et al., (1992), *Cunninghamella elegans* degraded acenaphthene to its corresponding  
157 metabolites: 1-acenaphthenone, 1,2-acenaphthenedione, *cis*-1,2-dihydroxyacenaphthene,  
158 *trans*-1,2-dihydroxyacenaphthene, 1,5-dihydroxyacenaphthene, 6-hydroxyacenaphthenone,  
159 also anthracene is degraded to anthracene *trans*-1,2-dihydrodiol 1-anthrol, 9,10-  
160 anthraquinone, phthalate, glucuronide, sulfate and xyloside conjugates of hydroxylated  
161 intermediates using the strains: *Bjerkandera* sp, *Cunninghamella elegans*, *Naematoloma*

162 *frowardii*, *Phanerochaete chrysosporium*, *Phanerochaete laevis*, *Pleurotus ostreatus*,  
163 *Pleurotus sajor-caju*, *Ramaria* sp, *Rhizoctonia solani*, *Trametes versicolor* (Cerniglia, 1982;  
164 Cerniglia and Yang, 1984; Bezalel et al., 1996; Johannes and Majcherczyk, 2000) (Table 2).  
165 Recently, Jové et al., (2016) have conducted a comparative study on degradation efficiency of  
166 anthracene by three ligninolytic white-rot fungi (*P.chrysosporium*, *Irpex lacteus* and  
167 *P.ostreatus*) and three non-ligninolytic fungi, and have shown that *P. chrysosporium*  
168 exhibited higher degradation efficiency of 40% compared to 38% with *Irpex lacteus* and less  
169 than 30% of anthracene removal with *P. ostreatus*. Balaji et al., (2014) studied the capability  
170 of different fungal strains to secrete extracellular enzymes, such as lipase, laccase, peroxidase  
171 and protease, PAHs contaminated soil solution was used as the unique carbon source. The  
172 best lipase production was observed in *Penicillium chrysogenum* (112 U ml<sup>-1</sup>), followed by  
173 *Lasiodiplodia theobromae* VBE1 (100 U ml<sup>-1</sup>). However, *Colletotrichum gleosporioides* was  
174 unable to produce lipase enzyme during PAHs degradation, because of the toxic impact of  
175 PAHs in contaminated soil. The best laccase production was observed in *P. chrysogenum* (79  
176 U ml<sup>-1</sup>) and *Aspergillus fumigatus* (73 U ml<sup>-1</sup>), while moderate peroxidase activity (52 U ml<sup>-1</sup>)  
177 was noticed in *Mucor racemose* and *Rhizopus stolonifer*. Similar results were reported by  
178 Venkatesagowda et al., (2012) and Thiyagarajan et al., (2008) with a highest lipase production  
179 of 108 U ml<sup>-1</sup> observed by *L. theobromae* and peroxidase production of 516 U ml<sup>-1</sup> observed  
180 by *Coprinus* sp. The studies of Balaji and Ebenezer, (2008) and Banu and Muthumary, (2005)  
181 revealed highest lipase production by *C. gleosporioidies* in solid-state fermentation. Lee et al.,  
182 (2014) investigated the efficiency of 150 taxonomically and physiologically diverse white rot  
183 fungi in a variety of biotechnological procedures, such as dye decolorization which  
184 corresponds to the beginning of lignin metabolism and is considered as a prediction of its  
185 capability to remove recalcitrant organopollutants, such as PAHs (Antonella Anastasi, 2009;  
186 Barrasa et al., 2009), gallic acid reaction which can be carried out to rank the fungi by their  
187 capability to degrade the PAHs, ligninolytic enzymes, and tolerance to four different PAHs:  
188 phenanthrene, anthracene, fluoranthene, and pyrene. All the fungi in this study produced three  
189 ligninolytic enzymes, LiP, MnP, and laccase. Nevertheless, since the ligninolytic enzyme  
190 activities of the fungi were analyzed in a nitrogen-limited condition, higher enzyme activity  
191 did not correlate with higher efficiency in the dye decolorization and gallic acid tests.  
192 Moreover, marine-derived fungi such as *Aureobasidium pullulans*, *Mucor* sp., *Aspergillus* sp.  
193 AS 58, *Pichia guilliermondii* M-30, *Aspergillus niger* etc. can be considered as a source of  
194 enzymes of environmental interest. Bonugli-Santos et al., (2015) has reported that these  
195 strains produce hydrolytic and/or oxidative enzymes, such as alginate, lyase, amylase,

196 cellulase, chitinase, glucosidase, inulinase, keratinase, ligninase, lipase, nuclease, phytase,  
197 protease, and xylanase. These enzymes have an optimal temperature from 35 to 70 °C, and an  
198 optimal pH from 3.0 to 11.0. For marine-derived fungal strains, salinity has to be taken into  
199 account in screening and production.

200 Almost all the fungi produce LiP, MnP and laccase but at different rates under the same  
201 culture conditions. As a result, the efficiency of enzymatic bioremediation is strongly  
202 dependent on the type of the fungal strain. Among all these studied fungi displaying lignolytic  
203 activity, the white rot fungi *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and  
204 *Bjerkandera adusta* have been intensively studied and have shown higher potential to  
205 metabolize PAHs (Haritash and Kaushik, 2009).

### 206 **3.1 *Phanerochaete chrysosporium***

207 The potential of *Phanerochaete chrysosporium* fungi for use in PAHs bioremediation was  
208 first reported by Bumpus et al., (1985) who stated that this white-rot basidiomycete partly  
209 degraded benzo[a]pyrene to carbon dioxide. Accordingly, several studies have been carried  
210 out to degrade PAHs by *P. chrysosporium* under ligninolytic, nutrient-sufficient, or other  
211 induced culture conditions by the ligninolytic extracellular enzymes or intracellular  
212 catabolism (Syed and Yadav, 2012; Gu et al., 2015) Besides, *P. chrysosporium* was reported  
213 to be a potent candidate for PAHs degradation due to its special physiological characteristics  
214 and active adsorption sites. In fact, *P. chrysosporium* can adsorb PAHs compound in its  
215 mycelial pellets owing to its abundant conjugated structures (C=C and aromatic components),  
216 numerous chemical groups (-OH, -COO-, O-C=O, -NH<sub>2</sub>, CO-NH) and high carbon content  
217 (Gu et al., 2015). In fact, the capability to degrade PAH was attributed to the generation of an  
218 extracellular lignin degrading enzyme (ligninase) throughout secondary metabolism (Sanglard  
219 et al., 1986). Studies on xenobiotic degradation by *P. chrysosporium* implied a non-specific  
220 battery of enzymes produced by this fungus that degrades the lignin polymer as the main  
221 agent in pollutant metabolism. The key enzymatic constituents of the ligninolytic system of *P.*  
222 *chrysosporium* are thought to be lignin peroxidase (LiP) and manganese peroxidase (MnP)  
223 (Bogan and Lamar, 1995a). LiPs are ideal candidates to be the catalysts of preliminary PAH  
224 oxidation in *P. chrysosporium*, and for anthracene, which is a LiP substrate (Hammel et al.,  
225 1992a). Nevertheless, many of the organopollutants degraded by *P. chrysosporium* are not  
226 LiP substrates. As an example, phenanthrene was initially found not to be a LiP substrate  
227 (Hammel et al., 1986), but later was claimed to undergo degradation in nutrient limited *P.*  
228 *chrysosporium* cultures (Bumpus, 1989a). *P. chrysosporium* strains are also able to degrade a

229 large variety of PAHs even under nitrogen limiting conditions (Andreoni et al., 2004; Bumpus  
230 et al., 1985). Bumpus, (1989) demonstrated that *P. chrysosporium* is able to cleave 70 to  
231 100% of at least 22 PAHs by substrate disappearance. Most of them profuse in anthracene oil  
232 within a period of 27 days of incubation with nitrogen-limited cultures of the fungus.  
233 Actually, the radiolabeled carbon of [<sup>14</sup>C]phenanthrene, which was the prevalent compound of  
234 this mixture, was oxidized to <sup>14</sup>CO<sub>2</sub>. Moreover, HPLC experiments and mass balance analysis  
235 revealed the conversion of [<sup>14</sup>C]phenanthrene to more polar and water-soluble metabolites.  
236 These results were corroborated by other researchers who have shown that besides  
237 [<sup>14</sup>C]phenanthrene degradation, this fungus is able to oxidize [<sup>14</sup>C]2-methylnaphthalene,  
238 [<sup>14</sup>C]biphenyl, and [<sup>14</sup>C]benzo[a]pyrene to <sup>14</sup>CO<sub>2</sub> (Sanglard et al., 1986). Lee et al., (2010)  
239 demonstrated the potential of two strains of *Phanerochaete sordida* (KUC8369, KUC8370)  
240 among seventy-nine screened white rot strains to degrade considerably higher amount of  
241 phenanthrene and fluoranthene than the strains of *P. chrysosporium* and have also proven that  
242 the strain KUC8369 was the best degrader of fluoranthene despite the fact that it produced  
243 lower MnP than *P. chrysosporium*. Phenanthrene metabolism in ligninolytic *P.*  
244 *chrysosporium* was different from the pathway of most bacteria. The PAH was cleaved  
245 between positions 3 and 4, and also differed from the process in non-ligninolytic fungi and  
246 other eukaryotes, unable to cause PAH ring fission. In fact, the ligninolytic fungus, *P.*  
247 *chrysosporium*, oxidizes phenanthrene at its C-9 and C10 position to give 2,2'- diphenic acid  
248 as a ring cleavage product. On the other hand, the major site of enzymatic attack by most  
249 bacteria is at the C-3 and C-4 position of phenanthrene. Also, *P. chrysosporium*, under non-  
250 ligninolytic conditions, metabolizes phenanthrene to phenols and trans-dihydrodiols. This  
251 observation proves that several enzymatic mechanisms may occur in *P. chrysosporium* for the  
252 initial oxidative attack on PAHs (Cerniglia and Yang, 1984).

253 The potential of *P. chrysosporium* in PAHs degradation is regarded to their extracellular  
254 enzymes. Therefore LiP and MnP. LiPs are ideal candidates to be the catalysts of preliminary  
255 PAH oxidation in *P. chrysosporium*. Nevertheless, it is suggested that other LiP-independent  
256 mechanisms need to exist for the initial oxidation of PAHs which are not lignin peroxidase  
257 substrates. Also, different pathways for PAHs degradation by *P. chrysosporium* can be  
258 considered.

### 259 **3.2 *Pleurotus ostreatus***



260 *P. ostreatus*, considered as white-rot fungi has been well documented for its capacity to  
261 degrade PAHs (Bezalel et al., 1996, 1997; Tortella et al., 2015). Bezalel et al., (1996) reported  
262 that the white rot fungi, *P. ostreatus* cleaves a wide variety of PAHs, including phenanthrene,  
263 with small correlation between PAH degradation and extracellular laccase, manganese  
264 peroxidase, or manganese-independent peroxidase activities. The same authors demonstrated  
265 in a subsequent study that *P. ostreatus* is able to metabolize phenanthrene to phenanthrene  
266 trans-9,10-dihydrodiol and 2,2'-diphenic acid as well as mineralizing it to CO<sub>2</sub>. The  
267 formation of phenanthrene trans-9R,10R-dihydrodiol, in which only one atom of oxygen  
268 originated from molecular oxygen, this indicates that *P. ostreatus* initially oxidizes  
269 phenanthrene stereoselectively, via a cytochrome P-450 monooxygenase and an epoxide  
270 hydrolase rather than a dioxygenase intervenes to form the dihydrodiol (Bezalel et al., 1997).  
271 Schützendübel et al., (1999) studied the degradation of polycyclic aromatic hydrocarbons  
272 (PAHs) with *Pleurotus ostreatus* in liquid cultures for the duration of 7 weeks. It removed  
273 43% and 60% of fluorene and anthracene after only 3 days of incubation. Phenanthrene,  
274 fluoranthene and pyrene were degraded uniformly during the 7 weeks but to a lower level  
275 than fluorene and anthracene, and their degradation rate reached a maximum of 15%. The  
276 removal of anthracene at a different rate than fluorene implies the synchronized existence of a  
277 minimum of two different degradation pathways. In this study, an addition of milled wood to  
278 the culture increased the secretion of MnP and laccase, but no increase in the degradation of  
279 PAHs was recorded. Possibly, for MnP, this can be due to the lack of H<sub>2</sub>O<sub>2</sub> during the  
280 secretion time which would inhibit the oxidation process (Field et al., 1992). The same reason  
281 could explain the clear correlation between the degradation of PAHs and laccase activity since  
282 there is a limitation of co-substrates for the oxidation mediated by this enzyme.

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284

### 285 **3.3 *Bjerkandera adusta***

286 Other than *Pleurotus ostreatus*, Schützendübel et al., (1999) investigated the degradation of  
287 phenanthrene, fluoranthene, pyrene, fluorene and anthracene with *Bjerkandera adusta* in the  
288 same culture conditions. This fungus degraded 56% and 38% of fluorene and anthracene,  
289 while other PAHs were removed uniformly but to a lower rate. LiP and MnP activity was not  
290 detected during this oxidation and the ending quinones resulting from this oxidation were not  
291 observed as final products (Field et al., 1992). These results suggest a new pathway than the

292 typical extracellular ones, as described for the removal of phenanthrene by *Phanerochaete*  
293 *chrysosporium* (Sutherland et al., 1991) and *Pleurotus ostreatus* (Lea Bezael et al., 1996a),  
294 and the higher oxidative potential produced in latter phase by these enzymes can increase  
295 PAHs degradation.

296 Another study by Wang et al., (2002) investigated the usefulness of a chemically modified  
297 manganese peroxidase with cyanuric chloride-activated methoxypolyethylene glycol,  
298 produced by *Bjerkandera adusta*. The modified and native enzymes demonstrated identical  
299 catalytic properties in the oxidation of Mn(II) and other substrates including veratryl alcohol,  
300 guaiacol, 2,6-dimethoxyphenol, and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate).  
301 However, the modified enzyme exhibited higher level of resistance to denaturation by  
302 hydrogen peroxide and stability to organic solvents such as N,N-dimethylformamide,  
303 acetonitrile, methanol, ethanol and tetrahydrofuran. Likewise, the modified enzyme  
304 demonstrated better stability to higher temperatures and lower pH than the native enzyme.  
305 The same author showed in later study that the oxidation rate of PAHs, such as anthracene,  
306 pyrene, benzo[a]pyrene and PAHs with ionization potentials of 7.43 eV or lower, decreased in  
307 the presence of manganous ions in the purified manganese-lignin peroxidase (MnLiP) hybrid  
308 isoenzyme from *Bjerkandera adusta*. Therefore, chemical modification of manganese  
309 peroxidase from *B. adusta* improved its effectiveness.

#### 310 **4 Culture conditions**

311 Natural resources polluted with PAHs usually raise their mutagenic and carcinogenic impact  
312 in fresh-water, marine-water and terrestrial species. This leads to unfavorable conditions for  
313 growth of even indigenous microorganisms. To overcome this limitation and obtain the  
314 highest enzyme production, culture conditions and inducers should be optimized (Balaji et al.,  
315 2014). Generally, microorganisms require suitable growth conditions (e.g. carbon source,  
316 nutrients, temperature, pH, redox potential and oxygen content which, strongly affect their  
317 growth (Adams et al., 2015). White rot fungi, in turn, appear to act because of their ability to  
318 penetrate contaminated soils with their hyphae and the production of extracellular oxidases  
319 (Wang et al., 2009).

320 Hadibarata and Kristanti, (2014) reached higher rates of degradation of n-eicosane by adding  
321 glucose as a carbon source for *Trichoderma* S019 strain. In this regard, the incubation time  
322 was found to be critical parameter influencing the degradation rate, for instance, Zafra et al.,  
323 (2015b) have found that *Trichoderma asperellum* could degrade 74 % of phenanthrene, 63 %

324 of pyrene, and 81 % of benzo[a]pyrene after 14 days of incubation at concentration of 1000  
325 mg kg<sup>-1</sup>. Moreover, *Peniophora incarnata* KUC8836 was able to degrade up to 95.3 % of  
326 phenanthrene and 97.9 % of pyrene after 2 weeks of incubation (Lee et al., 2014) and showed  
327 higher degradation rate of creosote at higher concentration of 229.49 mg kg<sup>-1</sup> (Lee et al.,  
328 2015). Furthermore, the concentration of the contaminant represents a key point which  
329 influenced the degradation of PHAs. Zafra et al., (2015a) have shown that the concentration  
330 of contaminant had a selective pressure on hydrocarbon-degrading organisms, and higher  
331 PAHs is a growth-limiting for microorganisms which developed a response against PAHs  
332 regarding cell membrane structure, mycelia pigmentation, and sporulation alterations.  
333 Likewise, the growth of fungi is inhibited in highly contaminated soils and overall  
334 remediation by fungi is extremely slow, needing many days or even more than a month as  
335 reported by Drevinskas et al., (2016). In this sense Balaji et al., (2014) also tested different  
336 carbon sources for lipase production by *P. chrysogenum* and *L. theobromae* and cellulose in  
337 *M. racemosus* and sucrose induced the highest activity in these species. Similarly, nitrogen  
338 sources have to be taken into account, yeast extract was found to be the best inducer of  
339 maximum lipase production in the mentioned strains. Dharmsthiti and Kuhasuntisuk, (1998)  
340 also demonstrated that yeast extract, as a supplemental source increased lipase production by  
341 *Pseudomonas aeruginosa* LP602 in lipid-rich wastewater treatment. Moreover, Mineki et al.,  
342 (2015) investigated the degradation of PAHs with *Trichoderma/Hypocrea* genus which used  
343 pyrene as sole source of carbon, and found that the growth of the strain and pyrene-degrading  
344 activity was enhanced to 27% and 24-25% compared with the control after incubation for 7  
345 and 14 days, respectively, by adding 0.02% yeast extract, 0.1% sucrose, or 0.1% lactose.  
346 Garapati and Mishra, (2012) also reported the relevance of nutrients in biodegradation of  
347 hydrocarbon by a fungal strain Ligninolytic enzymes can be regulated by aromatic  
348 compounds, such as different dyes and PAHs, that way fungi can use these aromatic  
349 compounds as unique source of carbon (Yang et al., 2011). In fact, it is unwise to test  
350 ligninolytic enzyme activity when screening species with highest PAH removal in the absence  
351 of substrate as the use of xenobiotic as substrates can induce the enzyme activity (Lee et al.,  
352 2014). This technique is attainable for fungal species that produce ligninolytic enzymes with  
353 higher efficiency, such as *Bjerkandera adusta* KUC9107 and *Skeletocutis perennis* KUC8514  
354 for LiP production; *Phanerochaete velutina* KUC8366 and *Phanerochaete sp.* KUC9015 for  
355 MnP production; and *Cerrena consors* KUC8416 and 8421 for laccase production (Moreira et  
356 al., 2006). Hofrichter et al., (1998), found that a wide spectrum of aromatic compounds was in  
357 part mineralized by the manganese peroxidase (MnP) system of the white rot fungus

358 *Nematoloma frowardi* and that mineralization was enhanced by peptide glutathione GSH (a  
359 natural peptide produced by eukaryotic cells which protects cells against reactive oxygen  
360 species and free radicals) and depended on the ratio of MnP activity to concentration of GSH.  
361 This suggests that carbon and nitrogen are essential for enzyme activity and consequently  
362 affects PAHs degradation. Furthermore, PAHs themselves can be used as a substrate and at  
363 the same time, as a nutrient source for enzymes inducing their activity reducing costs of  
364 culturing.

365 Simultaneously, the effect of different surfactants can affect PAHs removal. Balaji et al.,  
366 (2014) showed that lipase production was maximized with Triton X-100 boosting activities to  
367  $68 \text{ U ml}^{-1}$  in *P. chrysogenum*,  $72 \text{ U ml}^{-1}$  in *M. racemosus* and  $62 \text{ U ml}^{-1}$  in *L. theobromae*  
368 VBE1. Also, Gopinath et al., (2013) highlighted the relevance of surfactant in lipase  
369 production and its numerous applications. The highest lipase activity by *Metarhizium*  
370 *anisopliae*, occurs when Tween 80 and SDS were applied (Ali et al., 2009). Likewise, Chen et  
371 al., (2006) investigated the effect of surfactants on PAHs degradation by white rot fungi in  
372 soil water system, and found that Triton X-100 and SDS restrained the removal of PAHs.  
373 Moreover, biosurfactants enhance the removal of PAHs (Arun et al., 2008). Thus, using  
374 surfactants is generally effective for the biodegradation process, but previous testing is needed  
375 to prevent the inhibition in some cases.

376 Different temperatures were detected for optimal enzymes activity. Most of the enzymes have  
377 highest activity at mesophilic temperatures and it declines with very high and reduced  
378 temperatures. Several enzymes are claimed to be active even at extreme temperatures. At a  
379 temperature of  $5 \text{ }^{\circ}\text{C}$ , only the laccase activity is detected. The optimum temperature for  
380 laccase activity is  $45 \text{ }^{\circ}\text{C}$ , but it declines to 30% at  $5 \text{ }^{\circ}\text{C}$ , and 31% at  $75 \text{ }^{\circ}\text{C}$ . However, the  
381 activity of Mn-dependent peroxidase was higher even at  $75 \text{ }^{\circ}\text{C}$  (Haritash and Kaushik, 2009).  
382 Farnet et al., (2000) have shown that the activity of fungus, *Marasmius quercophilus* laccase  
383 was the highest at  $80 \text{ }^{\circ}\text{C}$ .

384 The extracellular enzyme release and polycyclic aromatic hydrocarbons (PAHs) removal in  
385 agitated and shallow stationary liquid cultures of *Phanerochaete chrysosporium* requires the  
386 addition of two inducers of lignin peroxidase (LiP) and manganese peroxidase (MnP), veratryl  
387 alcohol and Tween-80, respectively (Ding et al., 2008). However, if shallow stationary  
388 cultures are used, they also produce enzyme since it increased the contacting area between  
389 cells and oxygen without shear stress, while agitated cultures increased biodegradation rate by  
390 aiding interphase mass transfer of PAHs into aqueous phase. Simultaneously, they are  
391 recognized as inhibitors to the production of ligninolytic enzymes due to shear stress on

392 mycelia. The use of a LiP stimulator, veratryl alcohol, did not increase PAH degradation but  
393 considerably improved LiP activity. In contrast, Tween-80 enhanced MnP secretion and PAH  
394 degradation in shallow stationary cultures. On the other side, high PAH degradation was  
395 noticed in agitated cultures in the absence of apparent LiP and MnP activities. Same results  
396 were proved by Schützendübel et al., (1999), who mentioned that degradation of fluorene,  
397 anthracene, phenanthrene, fluoranthene and pyrene are not associated with the production of  
398 extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. Similar results were  
399 described by Lea Bezalel et al., (1996a) et Verdin et al., (2004) who found degradation of  
400 PAH in fungal cultures in the absence of LiP and MnP activities. Mohammadi et al., (2009)  
401 have also tested the effect of the incubation mode on anthracene biodegradation and it was  
402 revealed that the culture agitation clearly increased the biodegradation capacity of bagasse  
403 immobilized fungal cells despite the repressive effect of culture agitation on the ligninase  
404 activity.

405 All these outcomes indicated that extracellular peroxidase activities are not directly associated  
406 with the PAH degradation, and the increased solubility may be essential in the enhancement  
407 of PAH degradation rather than enzyme activity and hence suggested the possibility of  
408 producing other oxidative and hydrolytic enzymes that were not analyzed but could probably  
409 have degraded PAHs. Another suggestion is the PAHs degrading role of intracellular  
410 enzymes, such as tyrosinases and dioxygenases secreted into the culture filtrate by different  
411 fungi (Milstein et al., 1983).

412 Enzyme immobilization allows an alternative procedure that enables an increase in the steady  
413 state of enzymes and significant environmental tolerance. Immobilized enzyme, which can be  
414 established by several methods, such as adsorption, entrapment, and covalent bonding based  
415 on chemical/physical mechanisms, has enhanced activity and stability. The immobilized  
416 enzyme can withstand a wider range of temperatures and pH, as well as significant substrate  
417 concentration changes; this makes the complex much more resistant to severe environments  
418 (Dodor et al., 2004). This results in a longer lifetime and higher productivity per active unit of  
419 enzyme. Immobilization enables the enzyme to be recycled, and such an approach is much  
420 better suited for hydrophobic PAHs-contaminated soil bioremediation (Chang et al., 2015).

## 421 **5 Ionization potential**

422 Bogan and Lamar, (1995a) defined the behavior of intact fungus and the MnP-based lipid  
423 peroxidation system with respect to a larger variety of creosote PAHs. The disappearance of  
424 three- to six-ring creosote PAH components from intact fungal cultures and throughout lipid

425 peroxidation in vitro was reported. In each of these cases, the approach is shown to be mainly  
426 dependent on IP, indicating that the contribution of one or more than one electron oxidants is  
427 included over the entire range of PAHs tested. One-electron oxidation of PAHs occurs by  
428 peroxidases ( $IP \leq 7.35$  eV), Mn dependent peroxidase ( $IP \leq 8.19$  eV), ligninase ( $IP \leq 7.55$  eV)  
429 and laccase ( $IP \leq 7.45$  eV) (Cavaliere et al., 1983). The IP values, referring to the energy  
430 needed to eliminate an electron and to form a cation radical are 8.12 for naphthalene, 8.03 for  
431 phenanthrene, 7.21 for benzo(a)pyrene, and 7.31 for benzo(g,h,i)perylene (Table 1) (Cavaliere  
432 et al., 1983). PAHs like benz[a]anthracene, pyrene, and anthracene, that have ionization  
433 potential  $< 7.35$  eV, are LiP substrates, whereas PAHs, such as phenanthrene and  
434 benzo[e]pyrene that have ionization potentials  $> 7.35$  eV, cannot be LiP substrates. The  
435 products of the enzymatic oxidation are PAH quinones. For example, benzo[a]pyrene is  
436 oxidized to its 1,6-, 3,6-, and 6,12-quinones, pyrene to its 1,6- and 1,8-quinones, and  
437 anthracene to 9,10-anthraquinone (Hammel, 1995). Vyas et al., (1994a) has suggested that *P.*  
438 *ostreatus* and *T. versicolor* produce enzyme(s) other than lignin peroxidase capable of  
439 oxidizing compounds with higher ionization potential, such as anthracene.

## 440 **6 Kinetics**

441 The characteristics of the soil determine the diversity and activity of its microflora which is  
442 responsible for the degradation of polycyclic aromatic hydrocarbons and any other compound.  
443 In addition, soil characteristics influence the strength of interactions between the PAHs and  
444 individual soil compounds. Cutright, (1995) indicated that *Cunninghamella echinulata* var.  
445 *elegans* efficiently degrades PAHs in the presence of these nutrients while any other  
446 indigenous microorganisms are not. Moreover, for a first-order reaction system, the rate of  
447 change in contaminant concentration is proportional to the contaminant concentration in the  
448 soil and time prediction tool in degradation depends on the microorganism, the contaminant  
449 type and its concentration.

450 The prediction of time for bioremediation of polluted soil is based mostly on the  
451 microorganisms, pollutant type and its concentration. Furthermore, the improvement of more  
452 appropriate kinetic model needs the monitoring of biomass, respiration studies, and  
453 investigation of interactions of different organisms. Although bioremediation has a larger rate  
454 of success than synthetic methods, still the kinetics is not completely understood, and the  
455 kinetics becomes more challenging when fungi are applied for bioremediation (Haritash and  
456 Kaushik, 2009). As described previously, the different enzymes involved in fungal

457 degradation have maximum activity at different temperatures and some of them are active  
458 even at extreme temperatures. Therefore, monitoring the kinetics for various fungal strains is  
459 complicated, but most of them have good degradation capacities in a mesophilic range. The  
460 degradation rate can be improved by pretreatment at a high temperature which results in  
461 volatilization and decrease in the soil–water partition coefficient, as a result dissolution of  
462 pollutants increases the degradation rate.

## 463 **7 Soil and liquid cultures**

464 Most research studies on the extracellular enzyme activity produced by fungi have primarily  
465 been focusing on experiments in liquid culture (Ruiz-Dueñas et al., 1999; Kwang Ho Lee,  
466 2004; Eibes et al., 2006a; Rodrigues et al., 2008; Mäkelä et al., 2009; Dashtban et al., 2010).  
467 Nevertheless, some interest has been given to the changes of the enzymes produced by fungal  
468 strains during PAHs degradation in different soil types (Wang et al., 2009). When fungi get  
469 into non-sterile soil, they must compete with indigenous soil microbes for nutrients and the  
470 mycelia of the fungi may be affected. As a consequence, the production of enzymes may be  
471 influenced by more complicated elements (McErlean et al., 2006; Wang et al., 2014a).

472 Boyle et al., (1998) showed that white rot fungi growing in soil presented low amounts of  
473 degraded polyaromatic hydrocarbons (PAHs), even though they did degrade some other  
474 organopollutants. Nevertheless, in liquid culture, they degraded several PAHs. The latter  
475 interpretation was supported by Novotný et al., (2004b) who have demonstrated that the  
476 importance of higher fungal enzyme levels for effective degradation of recalcitrant  
477 compounds was better revealed in liquid media in comparison to the same strains growing in  
478 soil. Liquid culture reports have documented the degradation and/or mineralization of an  
479 extensive range of PAHs with phenanthrene, fluorene, benzofluorene, anthracene,  
480 fluoranthene, pyrene, benz[a]anthracene, and benzo[a]pyrene, among them (Bogan and  
481 Lamar, 1995a).

482 PAHs existing in soil are largely differentiated because of the higher heterogeneity of the soil  
483 structure (Li et al., 2007). Furthermore, the forms of the sorbed contaminants in soils are an  
484 essential element that affects the degradation (Yang et al., 2009). A significant spectrum of  
485 PAHs is biodegradable in aqueous culture but they are not biodegradable in soil. This fraction  
486 is classified as persistent residue with highly reduced bioavailability (Cornelissen et al.,  
487 2005).

488 PAHs molecules can be divided into three categories considering the desorption and  
489 bioavailability: easily desorbing and available fraction; the hardly desorbing and available  
490 fraction; and the irreversible and completely unavailable fraction (Li et al., 2007). Therefore,  
491 at the beginning of degradation, PAHs are quickly desorbed, and the desorption could  
492 possibly not present a limitation for biodegradation. The sorbed forms or the bioavailability of  
493 a contaminant are controlled by the characteristics of the contaminant and the soil, along with  
494 the contact time between the contaminant and the soil (Wang et al., 2014b).

495 Wang et al., (2009) observed a degradation of phenanthrene, pyrene and benzo[a]pyrene in  
496 soils by *Phanerochaete chrysosporium*. The highest activity of LiP and MnP reached 1.92 U  
497 g<sup>-1</sup>. Their high molecular size with higher ring number, reduced aqueous solubility and large  
498 octanol/water partition coefficient ( $K_{ow}$ ) of these compounds, makes them firmly combined  
499 and entrapped in soil micropores or soil organic matter (SOM) matrix. This results in the  
500 limitation on their biodegradability by enzymes. Huesemann et al., (2003) confirmed that the  
501 low rates of PAHs biodegradation were due to their low bioavailability in soil and low mass  
502 transfer rate of hydrophobic organic contaminants to the aqueous phase rather than deficiency  
503 in microorganisms degrading them. Furthermore Wang et al., (2009) also demonstrated that  
504 the degradation of pyrene by *P. chrysosporium* decreased with increasing SOM content,  
505 confirming that the SOM content can negatively affect the bioavailability of PAHs (Gill and  
506 Arora, 2003). While the maximum of LiP and MnP activities increased.

507 Also sorption of contaminants makes them less bioavailable with elevated contact time  
508 (aging) in the soil (Antizar-Ladislao et al., 2006; Li et al., 2008). The decrease in  
509 bioavailability induced by aging fluctuates with the contaminants and soils and the  
510 mechanisms are still to be investigated in deep (Northcott and Jones, 2001; Nam and Kim,  
511 2002; Watanabe et al., 2005). Some studies suggested that the contaminants are slowly  
512 transported from easily desorbing and bioavailable sites to hardly desorbing and less  
513 bioavailable sites where they accumulate throughout aging, and even to irreversible and non-  
514 bioavailable sites, which leads to reduced decontamination rates (Northcott and Jones, 2001;  
515 Sun et al., 2008).

516 Furthermore, sterilization of the soil is typically employed when investigating biodegradation  
517 in laboratory experiments to ensure that the degradation capability is high. This situation is  
518 not applied in bioremediation field. Few studies have described the effects of soil sterilization  
519 on the bioavailability of sorbed contaminants and changes in SOM (Northcott and Jones,  
520 2001; Nam et al., 2003; Kelsey et al., 2010). Wang et al., (2014) observed that sterilization



521 increased the degradation of pyrene because of the removal of competition from indigenous  
522 microbes.

523

## 524 **8 Degradation Pathways**

525 An understanding of the process for PAHs degradation in fungal strains would be a crucial  
526 step in the clarification of the enzymatic mechanisms. Ligninolytic enzymes undergo a one  
527 electron radical oxidation, producing aryl cation radicals from contaminants followed by  
528 generation of quinones (Vyas et al., 1994b; Cerniglia, 1997a). As an example, the intact  
529 culture of *P. chrysosporium* degraded anthracene to anthraquinone (Hammel et al., 1991).  
530 Anthraquinone was further degraded to phthalic acid and carbon dioxide. Purified forms of  
531 lignin peroxidase and manganese peroxidase likewise were able to oxidize anthracene,  
532 pyrene, fluorene and benzo[a]pyrene to the corresponding quinones (Haemmerli et al., 1986;  
533 Hammel et al., 1986, 1991; Hammel, 1992; Bogan and Lamar, 1996) (Figure 1).

534 Also, the crude and the purified ligninase of *Phanerochaete chrysosporium* oxidize the  
535 benzo(a)pyrene into three soluble organic compounds, which are benzo(a)pyrene 1,6 -, 3,6-,  
536 and 6,12-quinones (Haemmerli et al., 1986). These facts support the suggestion that lignin-  
537 degrading enzymes were peroxidases, mediating oxidation of aromatic compounds through  
538 aryl cation radicals. The ligninase which was unstable in the presence of hydrogen peroxide  
539 could be stabilized by inclusion of veratryl alcohol to the reaction mixture. The oxidation of  
540 benzo(a)pyrene was stimulated by this alcohol. Likewise, Hammel et al., (1986) studied the  
541 oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins and found that the  
542 lignin peroxidase (ligninase) of *P. chrysosporium* catalyze the oxidation of a wide range of  
543 lignin-related compounds. Tests with pyrene as the substrate revealed that pyrene-1,6-dione  
544 and pyrene-1,8-dione are the main oxidation products (84% of total as determined by high  
545 performance liquid chromatography), and gas chromatography/mass spectrometry analysis of  
546 ligninase-catalyzed pyrene oxidations executed in the presence of H<sub>2</sub>O revealed that quinone  
547 oxygens occur from water. Whole cultures of *P. chrysosporium* oxidized pyrene to these  
548 quinones in transient step. Experiments with dibenzo[p]dioxin and 2-chlorodibenzo[p]dioxin  
549 revealed that they are also substrates for ligninase. The immediate product of  
550 dibenzo[p]dioxin oxidation was the dibenzo[p]dioxin cation radical, which was noticed in  
551 enzymatic reactions by its electron spin resonance and apparent absorption spectra. The cation  
552 radical mechanism of ligninase thus was applied besides lignin, to other environmentally  
553 important aromatics. Hammel, (1992) also studied the oxidation pathway of phenanthrene and

554 phenanthrene-9,10-quinone (PQ) by the ligninolytic fungus, *Phanerochaete chrysosporium* at  
555 their C-9 and C-10 positions to result in a ring-fission product, 2,2'-diphenic acid (DPA),  
556 which was identified in chromatographic and isotope dilution experiments. DPA formation  
557 from phenanthrene was relatively higher in reduced nitrogen (ligninolytic) cultures than in  
558 high-nitrogen (non-ligninolytic) cultures and was not present in uninoculated cultures. The  
559 oxidation of PQ to DPA included both fungal and abiotic process, and was not affected by the  
560 amount of nitrogen added, and cleaved rapidly than phenanthrene to DPA. Phenanthrene-  
561 trans-9,10-dihydrodiol, which was earlier shown to be the major phenanthrene metabolite in  
562 non-ligninolytic *P. chrysosporium* cultures, was not formed in the ligninolytic cultures.  
563 Therefore, phenanthrene degradation by ligninolytic *P. chrysosporium* proceeded in sequence  
564 from phenanthrene to PQ and then to DPA, involving both ligninolytic and non-ligninolytic  
565 enzymes, and is not initiated by a common microsomal cytochrome P-450. The extracellular  
566 lignin peroxidases of *P. chrysosporium* were not able to oxidize phenanthrene *in vitro* and  
567 consequently were also less likely to catalyze the first step of phenanthrene degradation *in*  
568 *vivo*. Both phenanthrene and PQ were mineralized to identical range by the fungus, which  
569 supported the intermediacy of PQ in phenanthrene degradation, but both compounds were  
570 mineralized considerably less than the structurally associated lignin peroxidase substrate  
571 pyrene. Hammel et al., (1991) reported that *Phanerochaete* was generally different from the  
572 bacteria, which proceeded through AC cis-1,2-dihydrodiol instead of AQ and has been  
573 suggested to yield salicylate rather than phthalate as a monocyclic cleavage product. The  
574 formation of quinone to prepare the aromatic ring for cleavage is an uncommon  
575 biodegradation approach and was showed to be of general significance in *P. chrysosporium*.  
576 LiPs have also been involved in the degradation of polychlorinated phenols by this organism.  
577 The monooxygenase system of cytochrome P-450 producing epoxides can also be included in  
578 degradation of PAHs. The epoxides can be rearranged into hydroxyl derivatives or can be  
579 hydrolyzed to vicinal dihydrodiols. Ligninolytic fungus, *Irpex lacteus* degraded anthracene  
580 and phenanthrene and the main degradation products were anthraquinone and phenanthrene-  
581 9,10-dihydrodiol, respectively as shown in Figure 2. The study also suggested the degradation  
582 pathway of anthracene and phenanthrene (Cajthaml et al., 2002). Thus, several systems are  
583 involved in the degradation of PAHs with fungal enzymes including intracellular cytochrome  
584 P450 and extracellular lignin peroxidase, manganese peroxidase and laccase.

585 The biodegradation of PAHs was studied under aerobic and anaerobic conditions. Fungal  
586 strains were grown on PAHs under static aerobic conditions for 6 and 10 days. The highest

587 degradation of naphthalene (69%) was performed by a strain that had MnP activity, followed  
588 by strain that showed lignin peroxidase and laccase activities. Likewise, it was found that  
589 highest degradation of phenanthrene (12%) was observed with the strain that contained MnP  
590 and laccase activities (Clemente et al., 2001). Soil fungi *Aspergillus* sp., *Trichocladium*  
591 *canadense*, and *Fusarium oxysporum* degrade polycyclic aromatic hydrocarbons low-  
592 molecular-weight PAHs (2-3 rings) and produce ligninolytic enzymes also under  
593 microaerobic and highly reduced oxygen conditions, but ligninolytic enzyme activities can  
594 vary among fungi and PAHs. Under microaerobic conditions, the 3 species demonstrated at  
595 least one of the assayed ligninolytic activities (LiP, MnP, laccase). In contrast, under very-  
596 low-oxygen conditions, ligninolytic enzyme activity was frequently not observed (Silva et al.,  
597 2009).

598 Marco-Urrea et al., (2015), described the biodegradation pathways of PAHs using non-  
599 ligninolytic fungi. These non-ligninolytic strains showed a particular type of resistance to  
600 different amounts of PAHs. The most common mechanism of PAHs transformation is the  
601 intracellular accumulation followed by the degradation, akin to benzo[a]pyrene degradation  
602 using intracellular enzymes of the strain *Fusarium solani* (Fayeulle et al., 2014). Likewise,  
603 extracellular enzymes, such as laccase, can also be produced by some of these non-  
604 ligninolytic fungi. but they are not as effective as intracellular enzymes degrading PAHs. The  
605 phase I of degradation pathway includes the formation of oxidized metabolites, such as  
606 hydroxyl-, dihydroxy-, dihydrodiol- and quinone-derivatives followed by the phase II which  
607 includes the conjugation with sulfate-, methyl-, glucose-, xylose- or glucuronic acid groups.  
608 These metabolites are less harmful than the original PAHs (Cerniglia and Sutherland, 2010).

## 609 **9 Mechanism of degradation with enzymes**

### 610 **9.1 Characteristics of ligninolytic enzymes**

#### 611 **9.1.1 Characteristics of peroxidases**

612 A couple of extracellular oxidative enzymes are responsible for lignin degradation:  
613 peroxidases and laccases (phenol oxidases). Both peroxidases were initially found in *P.*  
614 *chrysosporium* (Tien and Kirk, 1983). A number of other fungi also possess these enzymes,  
615 while others have either one or the other (Mester and Tien, 2000). In the majority of species,  
616 peroxidases are generally recognized to be families of isozymes occurring as extracellular  
617 glycosylated proteins which may enhance their stability (Thurston, 1994). The ratio between

618 the isozymes varies with the culture age and the culture conditions (Leisola et al., 1987;  
619 Bogan and Lamar, 1995b). They need hydrogen peroxide to oxidize lignin and lignin-related  
620 compounds. Their molecular weights and isoelectric points range from 35 to 47 kDa and 2.8  
621 to 5.4 kDa, respectively (Leisola et al., 1987; Bogan and Lamar, 1995b; Johansson et al.,  
622 2002). The peroxidases are single heme-containing enzymes (protoporphyrin IX) so that the  
623 absorption spectrum of the native enzyme has a very particular absorbance maximum at 406-  
624 409 nm (ten Have and Teunissen, 2001). The peroxidases are divided into two different types  
625 depending on their different substrate spectra: manganese peroxidase (MnP), for which  
626 Mn(II) is best reducing substrate and the lignin peroxidase (LiP). LiP oxidizes non-phenolic  
627 and phenolic aromatic compounds (ten Have and Teunissen, 2001).

### 628 **9.1.2 Characteristics of laccase**

629 Laccase belongs to the copper oxidase family that is able to catalyze the oxidation of phenols,  
630 polyphenols and anilines, which are largely dispersed in higher plants, fungi and bacteria  
631 (Tavares et al., 2006). The enzyme is typically larger than peroxidases, having a molecular  
632 weight around and above 60 kD and have acidic isoelectric points (ten Have and Teunissen,  
633 2001). As with other extracellular enzymes, laccases are glycosylated. As an alternative to  
634 H<sub>2</sub>O<sub>2</sub>, laccases use dioxygen as an oxidant, reducing it by four electrons to water. These types  
635 of enzymes have four copper per enzyme, that represents three different types, and  
636 consequently, every type has a different role in the oxidation of laccase substrates  
637 (Messerschmidt and Huber, 1990). The type 1 copper is suggested to be included in the  
638 reaction with the substrates. It has an absorption maximum at the wavelength of 610 nm  
639 which gives the enzyme the typical blue color. The type 2 copper and the two type 3 coppers  
640 cluster in a triangular form which is involved in the binding and in the reduction of O<sub>2</sub> as well  
641 as the storage of electrons coming from the reducing substrates. The type 2 copper does not  
642 present visible absorbance, while the type 3 coppers have an absorption maximum at 330 nm  
643 (Mester and Tien, 2000; ten Have and Teunissen, 2001). It was demonstrated that, in the  
644 presence of suitable mediators, laccase is capable to oxidize a considerably larger range of  
645 compounds, such as PAHs (Peng et al., 2015).

646 **9.1.3 Mediators of laccase** Laccase has been well studied for its capacity to oxidize PAHs,  
647 xenobiotic and phenolic lignin model compounds (Majcherczyk et al., 1998; Peng et al.,  
648 2015). Earlier, its application was limited because of the low oxidation potential, thus, in the  
649 presence of an appropriate mediator; laccases show higher oxidation capability resulting in

650 numerous biotechnological applications involving oxidation of non-phenolic lignin  
651 compounds and detoxification of various environmental pollutants (Upadhyay et al., 2016;  
652 Khambhaty et al., 2015). Recently, laccase has found applications in other sectors, such as in  
653 the design of biosensors and nanotechnology (Li et al., 2014; Upadhyay et al., 2016). Besides,  
654 they are used in the decolourization and detoxification of industrial effluents and the treatment  
655 of wastewater (Viswanath et al., 2014; Chandra and Chowdhary, 2015). These mediators  
656 include 1-hydrobenzotriazole (1-HBT) (Majcherczyk et al., 1998a), 2,2'-azino-bis-(3-  
657 ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Bourbonnais et al., 1997), and violuric acid  
658 (Xu et al., 2000), but also natural mediators have been explored, such as phenol, aniline, 4-  
659 hydroxybenzoic acid, 4-hydroxybenzyl alcohol (Johannes and Majcherczyk, 2000), 3-  
660 hydroxyanthranilate (Eggert et al., 1996). These natural mediators were as effective as the  
661 synthetic compounds (Johannes and Majcherczyk, 2000). Other natural compounds  
662 including cysteine, methionine, and reduced glutathione, containing sulfhydryl groups, were  
663 also efficient as mediator compounds (Johannes and Majcherczyk, 2000).

664 Many reports have studied the oxidation of PAH by purified fungal laccases (Table 3). LACs  
665 of *T. versicolor*, *C. hirsutus*, *P. ostreatus*, and *Coriolopsis gallica* were the most studied.

666 For example, *T. versicolor* LAC, in combination with HBT, was capable to oxidize two  
667 PAHs, acenaphthene and acenaphthylene; ABTS did not clearly affect the oxidation rate.  
668 LAC without mediator oxidized about 35% of the acenaphthene and only 3% of  
669 acenaphthylene. The principal products obtained after incubation were 1,2-acenaphthenedione  
670 and 1,8-naphthalic acid anhydride (Johannes et al., 1998). The purified LAC of *T. versicolor*  
671 did not transform PHE. The supplementation of a redox mediator, ABTS or HBT, to the  
672 reaction mixture improved the degradation of PHE by LAC about 40% and 30%, respectively  
673 (Han et al., 2004). LAC produced by *C. hirsutus* catalyzed The oxidation of five PAHs: FLA,  
674 PYR, ANT, B[a]P, and PHE in the presence of the redox mediators ABTS and HBT. B[a]P  
675 was the most effectively oxidized substrate In the system mediated by ABTS, , while ANT  
676 was the most effectively oxidized substrate in the one mediated by HBT. There was no clear  
677 correlation between the IP and the oxidation of the substrates. The rate of oxidation by LAC  
678 of *C. hirsutus* varied from 10.9 to 97.2% depending on the PAHs examined. The oxidation of  
679 FLA and PYR by *C. hirsutus* LAC was effective and ranged from 37.9 to 92.7%. PYR which  
680 is one of the least oxidizable PAHs was still oxidized until 40% in the presence of all the  
681 mediators.

682 LAC from *T. versicolor* catalyzed the *in vitro* oxidation of ANT and B[a]P which have IPs  
683  $\leq 7.45$  eV. The presence of ABTS improves the oxidation of ANT while it is crucial for the

684 oxidation of B[a]P. Anthraquinone was recognized as the major end product of ANT  
685 oxidation (Sutherland et al., 1991). Consequently, the PAH-oxidizing abilities of LAC differ,  
686 depending on the fungal species from which it was produced (Cho et al., 2002).

687 ANT was entirely degraded by the LAC of *Ganoderma lucidum* fungus in the absence of a  
688 redox mediator. At the same time and in the presence of the mediator, this same LAC  
689 degraded B[a]P, FLU, acenaphthene, acenaphthylene, and B[a]A at a rate ranging from 85.3%  
690 to 100% (Pozdnyakova, 2012; Pozdnyakova et al., 2006).

691 The degradation of B[a]P by purified LAC of *Pycnoporus cinnabarinus* was investigated. The  
692 reaction required the presence of the exogenous ABTS as a mediator. Almost all of the  
693 substrate (95%) was transformed within 24 hours. The enzyme principally oxidized the  
694 substrate to benzo[a]pyrene-1,6-, 3,6- and 6,12-quinones (Rama et al., 1998).

695 The effect of different mediators on LAC action was investigated by Pickard et al., (1999).  
696 Different PAHs were oxidized by *Coriolopsis gallica* LAC such as B[a]P, 9-  
697 methylanthracene, 2-methylanthracene, ANT, biphenylene, acenaphthene, and PHE. 9-  
698 methylanthracene was the most rapidly oxidized substrate. There was no apparent correlation  
699 between the ionization potential of the substrate and the first-order rate constant for substrate  
700 degradation *in vitro* by adding ABTS. The effects of mediating substrates were studied  
701 furthermore by applying ANT as a substrate. A synergistic effect of HBT and ABTS was  
702 detected. In fact, HBT supported approximately one-half ANT oxidation rate that ABTS  
703 supported, whereas HBT with ABTS enhanced the oxidation rate nine-fold, compared with  
704 the oxidation rate supported by only ABTS (Pickard et al., 1999).

705 The white-rot fungi generate yellow form of LAC during solid-state fermentation of a  
706 substrate containing natural lignin. The active center of this enzyme is transformed by the  
707 products of lignin degradation. Consequently, LAC becomes capable to catalyze the oxidation  
708 of nonphenolic compounds in the absence of mediators (Pozdnyakova et al., 2006a). The rate  
709 of degradation using the yellow LAC produced by *P. ostreatus* was also detected. The  
710 naphthalene derivatives  $\alpha$ - and  $\beta$ -naphthols,  $\alpha$ -nitroso- $\beta$  naphthol,  $\alpha$ -hydroxy- $\beta$ -naphthoic  
711 acid, and  $\alpha$ - naphthylamine were all appropriate LAC substrates despite yellow LAC did not  
712 catalyze the degradation of the two-ring PAH naphthalene. Yellow LAC oxidized all the  
713 PAHs of three to five rings such as ANT, PYR, FLU, FLA, PHE, and perylene, with rates of  
714 degradation ranging from 40% to 100%. The efficiencies were greater than that observed for a  
715 blue LAC from the same fungus without and with ABTS and HBT mediators.. The same  
716 product of ANT oxidation and several unknown products of FLU oxidation were noticed in  
717 solutions of various solvents (Pozdnyakova, 2012; Pozdnyakova et al., 2006).

## 718 **9.2 Catalytic cycle of peroxidases**

719 LiP and MnP have a common catalytic cycle, as also observed for other peroxidases (ten  
720 Have and Teunissen, 2001). One molecule of H<sub>2</sub>O<sub>2</sub> oxidizes the native enzyme by  
721 withdrawing two electrons, creating compound I. The latter could be reduced back to two  
722 single-electron oxidation steps to the native form through an intermediate compound II. In the  
723 case of LiP, reduction of compound II is the rate-limiting step in the catalytic cycle. For this  
724 reason, this compound is regarded to be less effective than LiP compound I. As the reduction  
725 of compound II is relatively slow, it is available for longer time for a reaction with H<sub>2</sub>O<sub>2</sub>  
726 resulting in inactive enzyme, identified as compound III which is characterized to be a  
727 complex between LiP and superoxide (Cai and Tien, 1992). Other fungal enzymes could  
728 provide the needed hydrogen peroxide for peroxidase activity. As part of their ligninolytic  
729 system, white-rot fungi produce H<sub>2</sub>O<sub>2</sub>-generating oxidases (Kirk and Farrell, 1987), such as  
730 glucose oxidases, glyoxal oxidase, and aryl alcohol oxidase. White-rot fungi that lack H<sub>2</sub>O<sub>2</sub>-  
731 generating oxidases depend on the oxidation of physiological organic acids, such as oxalate  
732 and glyoxylate which indirectly results in H<sub>2</sub>O<sub>2</sub> (ten Have and Teunissen, 2001). Also, the  
733 reduction of quinones to their equivalent hydroquinones and the subsequent autoxidation or  
734 enzymatically catalyzed oxidation may generate H<sub>2</sub>O<sub>2</sub> due to the involvement and reduction  
735 of O<sub>2</sub> (Muñoz et al., 1997).

### 736 **9.2.1 MnP**

737 MnP is distinct from the other peroxidases due to the framework of its binding site. MnP  
738 oxidizes Mn<sup>2+</sup> to Mn<sup>3+</sup>, which cannot be substituted by other metals at physiological  
739 concentrations (Glenn et al., 1986). At the time of the discovery of MnP, it was revealed that a  
740 number of aliphatic organic acids including lactate and oxalate induced Mn<sup>2+</sup> oxidation rate  
741 (Glenn et al., 1986; Matsubara et al., 1996). These organic acids, e.g., oxalate and to a lower  
742 degree malonate and glyoxylate were demonstrated to be produced as *de novo* metabolites by  
743 white-rot fungi (Dutton and Evans, 1996). These acids are able to chelate Mn<sup>3+</sup> resulting in a  
744 comparatively stable complex. The complexed Mn<sup>3+</sup> can then oxidize phenolic lignin model  
745 compounds and many phenols via phenoxy radical configuration (Jensen et al., 1994). Beside  
746 phenolic structures, the MnP system has also been observed to oxidize nonphenolic lignin  
747 model compounds (Hofrichter et al., 1998).

748 PAH degradation studies revealed that MnP from *I. lacteus* was capable to effectively degrade  
749 three- and four-ring PAHs, including phenanthrene, anthracene and fluoranthene. MnP

750 produced by *Anthracophyllum discolor*, degraded pyrene (>86%), and anthracene (>65%)  
751 alone or in mixture, and also degraded fluoranthene and phenanthrene but less effectively  
752 (<15.2% and <8.6%, respectively) (Acevedo et al., 2011). MnP-catalyzed oxidation of PAHs  
753 resulted in respective quinones. Anthrone, which is an expected intermediate was formed  
754 during the degradation of anthracene by MnP, and it was followed by the production of 9,10-  
755 anthraquinone. Anthraquinone has earlier been revealed as the typical oxidation product in *in*  
756 *vitro* reactions of peroxidases. More oxidation resulted in the production of phthalic acid, as it  
757 was shown in ligninolytic cultures of *P. chrysosporium* (Hammel et al., 1991). The  
758 characteristic ring-cleavage product 2-(2-hydroxybenzoyl)-benzoic acid shows that MnP is  
759 capable to cleave even the aromatic ring of a PAH molecule. One single report was found  
760 suggesting that MnP does not oxidize anthracene in the presence of  $Mn^{2+}$  (Vazquez-Duhalt et  
761 al., 1994).

762 Since the high hydrophobicity of PAHs significantly inhibits their degradation in liquid  
763 media, MnP degraded anthracene, dibenzothiophene, and pyrene in the presence of acetone  
764 (36% v/v), which is a miscible organic solvent. Anthracene was degraded to phthalic acid and  
765 had the highest degradation rate, followed by dibenzothiophene and then pyrene (Eibes et al.,  
766 2006b).

767 Degradation of PAHs by crude MnP produced by *Nematoloma frowardii* was experimented  
768 on separate PAHs: PHE, ANT, PYR, FLA, and B[a]A and then on a mixture of different  
769 PAHs: PHE, ANT, PYR, FLA, CHR, B[a]A, B[a]P, and benzo[b]fluoranthene. The oxidation  
770 of PAHs was enhanced in the presence of glutathione which is a mediator substance capable  
771 to generate reactive thiyl radicals. Products of glutathione-mediated MnP mineralization were:  
772 14C-PYR (7.3%), 14C-ANT (4.7%), 14C-B[a]P (4.0%), 14C-B[a]A (2.9%), and 14C-PHE  
773 (2.5%) (Sack et al., 1997c). The induction effect of reduced glutathione (GSH) was also  
774 investigated by Thomas Günther, (1998) and showed an increase of the oxidative strength of  
775 MnP. As a consequence anthracene was fully reduced and 60% of pyrene was degraded after  
776 only 24h.

777 Therefore, alternative redox mediators, increasing the oxidative effect of MnP have been  
778 investigated. MnP was capable to oxidize FLU which has a high IP value (8.2 eV) and  
779 creosote which is a complex PAHs mixture in the presence of Tween-80. Also, Tween-80  
780 enable MnP produced by *Stropharia coronilla* to oxidize a large amount of B[a]P into polar  
781 fragments (Steffen et al., 2003).

## 782 **9.2.2 LiP**



783 LiP is able to oxidize several phenolic and non-phenolic substrates with calculated ionization  
784 potential, a measure for the ease to abstract an electron from the highest filled molecular  
785 orbital, up to 9.0 eV (ten Have and Teunissen, 2001). LiP has been revealed to entirely  
786 oxidize methylated lignin and lignin model compounds as well as several polyaromatic  
787 hydrocarbons (Hammel et al., 1992a). Among the oxidation reactions catalyzed by LiP are the  
788 cleavage of C $\alpha$ -C $\beta$  and aryl C $\alpha$  bond, aromatic ring opening, and demethylation (Kaal et al.,  
789 1995). One secondary metabolite, veratryl alcohol (VA), has been the focus of many studies.  
790 VA is a rich substrate for LiP and increases the oxidation of otherwise weak or terminal LiP  
791 substrates (Ollikka et al., 1993). Three main roles of VA have been recommended so far. As  
792 defined earlier, VA could behave as a mediator in electron-transfer reactions. Secondly, VA is  
793 a good substrate for compound II; for that reason, VA is important for completing the  
794 catalytic cycle of LiP through the oxidation of terminal substrates.127 Thirdly, VA prevents  
795 the H<sub>2</sub>O<sub>2</sub>-dependent inactivation of LiP by reducing compound II back to native LiP. In  
796 addition, if the inactive LiP compound III is established, the intermediate VA<sup>+</sup> is able to  
797 reduce LiP compound III back to its native form (ten Have and Teunissen, 2001).

798 Purified LiP from *P. chrysosporium* had been shown to attack B[a]P using one-electron  
799 abstractions, causing unstable B[a]P radicals which undergo further spontaneous reactions to  
800 hydroxylated metabolites and many B[a]P quinones (Haemmerli et al., 1986).  
801 benzo[a]pyrene-1,6-, 3,6-, and 6,12-quinones were detected as the products of B[a]P  
802 oxidation by *P. chrysosporium* LiP. At the same time with the appearance of oxidation  
803 products, LiP was inactivated. Similarly to all peroxidases, LiP is inhibited by the presence of  
804 hydrogen peroxide (Valderrama et al., 2002); the addition of VA to the reaction mixture could  
805 stabilize the enzyme. The oxidation rate is ameliorated more than 14 times in the presence of  
806 VA, and the most of the enzyme activity was retained during the B[a]P oxidation (Haemmerli  
807 et al., 1986).

808 Most of reports on the oxidation of PAHs with LiP concentrated on LiP from *P.*  
809 *chrysosporium* as shown in Table 3. Anthraquinone is the major product of anthracene  
810 oxidation by LiP produced by *P. chrysosporium* (Field et al., 1996). Hammel et al., (1986a)  
811 demonstrated that LiP produced by *P. chrysosporium* catalyzes the degradation of certain  
812 PAHs with IP < 7.55 eV. As a consequence, H<sub>2</sub>O<sub>2</sub>-oxidized states of LiP are more oxidizing  
813 than the analogous states of standard peroxidases.

814 Studies on pyrene as a substrate showed that pyrene-1,6-dione and pyrene-1,8-dione are the  
815 principle oxidation products. Gas chromatography/mass spectrometry analysis of LiP-

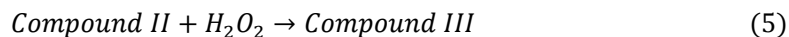
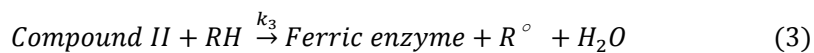
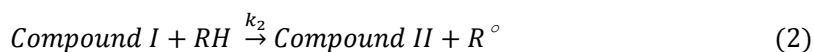
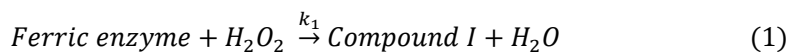
816 catalyzed pyrene oxidation done in the presence of H<sub>2</sub>O<sub>2</sub> revealed that the quinone oxygens  
817 come from water. The one-electron oxidative mechanism of LiP is relevant to lignin and  
818 lignin-related substructures as well as certain polycyclic aromatic and heteroaromatic  
819 contaminants. The oxidation of pyrene by entire cultures of *P. chrysosporium* also generated  
820 these quinones. As a result, it can be concluded that LiP catalyzes the first step in the  
821 degradation of these compounds by entire cultures of *P. chrysosporium* (Hammel et al.,  
822 1986).

823 Vazquez-Duhalt et al., (1994) utilized LiP from *P. chrysosporium* to investigate the oxidation  
824 of anthracene, 1-, 2-, and 9- methylanthracenes, acenaphthene, fluoranthene, pyrene,  
825 carbazole, and dibenzothiophene. Among the studied compounds, LiP was able to oxidize  
826 compounds with IP<8 eV. The greatest specific activity of PAHs oxidation was shown when  
827 pHs are between 3.5 and 4.0. The reaction products involve hydroxyl and keto groups. The  
828 product of anthracene oxidation was 9,10-anthraquinone. The products of LiP oxidation of 1-  
829 and 2-methylanthracene were 1- and 2-methylanthraquinone, respectively.

830 9,10-anthraquinone, 9-methyleneanthranone, and 9-methanol-9,10- dihydroanthracene were  
831 the products detected by from the oxidation of 9-methylanthracene (Vazquez-Duhalt et al.,  
832 1994). Anthraquinone resulting from carbon-carbon bond cleavage of 9-methylanthracene,  
833 was also observed. The mass spectra of the two products resulting from acenaphthene  
834 correspond to 1-acenaphthenone and 1-acenaphthenol. The comparison of the GC-mass  
835 spectrometry analysis of dibenzothiophene oxidation by LiP with a sample of authentic  
836 dibenzothiophene sulfoxide resulted in sulfoxide. The UV spectrum of the product of pyrene  
837 oxidation most closely fitted that of 1,8- pyrenedione. In spite fluoranthene and carbazole  
838 were oxidized, their products were not established (Vazquez-Duhalt et al., 1994).

839 Torres et al., (1997) studied LiP, cytochrome *c*, and hemoglobin for oxidation of PAHs in the  
840 presence of hydrogen peroxide and demonstrated that LiP oxidized anthracene, 2-  
841 methylanthracene, 9- hexylanthracene, pyrene, acenaphthene, and benzo[a]pyrene; the  
842 unreacted compounds included chrysene, phenanthrene, naphthalene, triphenylene, biphenyl,  
843 and dibenzofuran. The oxidation of the aromatic compounds by LiP matched with their IPs;  
844 only those compounds that had IPs<8 eV were transformed. The reaction products from the  
845 three hemoproteins (LiP, cytochrome *c*, and hemoglobin) were principally quinones, which  
846 suggest that the three biocatalysts have the same oxidation mechanism. The resulting product  
847 from anthracene was anthraquinone, and the resulting product from 2-methylanthracene was  
848 2-methylanthraquinone. The ending products for pyrene and benzo[a]pyrene oxidation were  
849 pyrenedione and benzo[a]pyrenedione, respectively. The mass spectra results of the products

850 from acenaphthene degradation catalyzed by LiP correlated well with 1-acenaphthone and  
 851 1-acenaphthol (Torres et al., 1997).  
 852 Experiments on the catalytic properties of ligninolytic enzymes demonstrates that degradation  
 853 by LiP is restricted to certain range of compounds according to their IP values. Furthermore,  
 854 the catalytic activities of MnP and LAC are extended to the following factors (a) the presence  
 855 of some natural and synthetic mediators such as ABTS for LAC and glutathione for MnP and  
 856 LAC; (b) the modification of the active center of LAC during fermentation of a fungi on  
 857 lignin-containing natural substrates; (c) the combination of PAH oxidation with lipid  
 858 peroxidation (MnP and LAC). Therefore, MnP and LAC can be considered as the most  
 859 effective in PAHs oxidation since their role extends to the initial oxidation and production of  
 860 quinones (Pozdnyakova, 2012).  
 861



862  
 863 \*RH represents the reducing substrate and R<sup>°</sup> represents the reducing substrate after one  
 864 electron oxidation

### 865 9.2.3 Catalytic cycle of laccase

866 Laccases are known to catalyze the oxidation of a significant variety of phenolic compounds  
 867 and aromatic amines (Peng et al., 2015). When certain substrates can potentially provide two  
 868 electrons such as ABTS, laccases carry out one-electron oxidation. As a result, radicals are  
 869 produced which undergo subsequent non-enzymatic reactions as seen in Equation 6.



870

871 Hundreds of studies have been done on the characteristics of fungal laccases. And most of the  
872 research has been investigating tree laccases or other copper-containing oxidases (Tollin et  
873 al., 1993).

874 Even though, the redox potential of laccases (0.5-0.8 V) does not favor the oxidation of non-  
875 phenolic compounds, numerous studies have demonstrated that laccases are capable of  
876 oxidizing compounds which have redox potentials higher than that of the enzyme. In these  
877 studies, ABTS, 1-hydroxybenzotriazole (HOBt) or 3-hydroxyanthranilate were applied as a  
878 cooxidant/mediator, and non-phenolic lignin, veratryl alcohol, and PAH were oxidized  
879 (Collins and Dobson, 1996; Eggert et al., 1996; Bourbonnais et al., 1997; Majcherczyk et al.,  
880 1998a). The enzyme kinetic background of these reactions is still not identified.

## 881 **10 Conclusions**

882 Enzymatic bioremediation is the tool to convert PAHs to less harmful/non-harmful forms with  
883 less chemicals, energy, and time. It is a solution to degrade/remove contaminants in an eco-  
884 friendly way. From the early to the current research, vast range of fungi have proved their  
885 efficiency in the bioremediation of PAH-contaminated wastes through enzymes, such as MnP,  
886 LiP, laccase and other fungal enzymes, such as Cytochrome P450 monooxygenase, epoxide  
887 hydrolases, lipases, protease and dioxygenases.

888 The enzymatic bioremediation of a pollutant and the rate at which it is reached relies upon  
889 the environmental conditions, number and type of the microorganisms, characteristics of the  
890 chemical compound to degrade. Hence, to improve the degradation rate and develop a  
891 bioremediation system, various factors are accountable which need to be dealt with and are to  
892 be investigated, such as pretreatment at high temperature.

893 Powerful and cost-effective bioremediation should involve either entire mineralization of the  
894 PAHs or at minimum biotransformation to less harmful compounds. Generally, fungal rates of  
895 degradation of PAHs are slow and inefficient compared to bacteria; however, since numerous  
896 fungi have the ability to hydroxylate a wide variety of PAHs, their ecological role could be  
897 significant since these polar intermediates can be mineralized by soil bacteria or detoxified to  
898 simpler non-hazardous compounds. Additionally, fungi have an advantage over bacteria since  
899 the fungal mycelium could grow into the soil and spread itself through the solid matrix to  
900 degrade the PAHs. To improve and empower biodegradative potential of fungi, substantial

901 research on the enzymes included in PAH degradation pathways and on the molecular  
902 genetics and biochemistry of catabolic pathways is required.

903

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905

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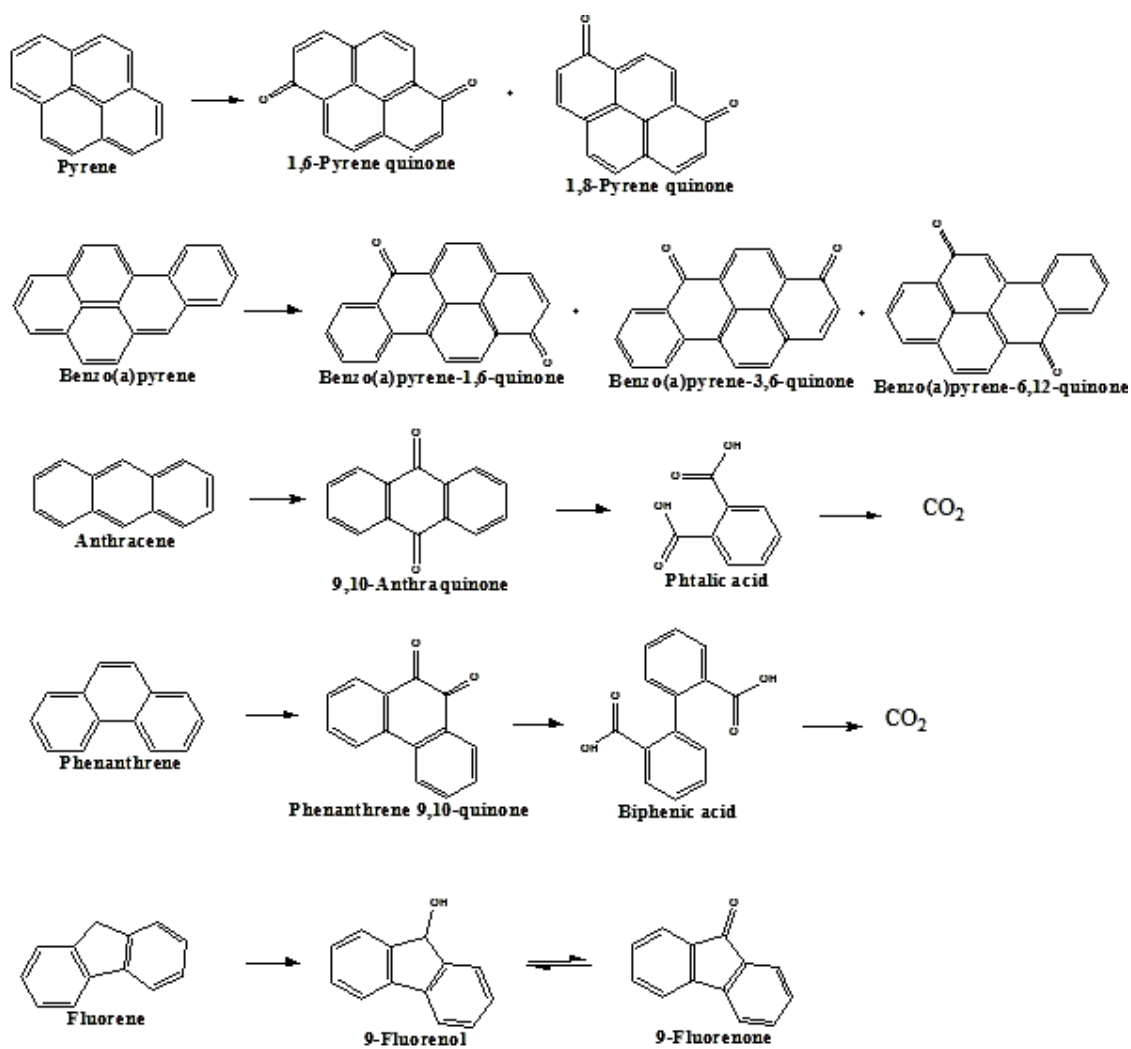


Fig.1. Oxidation of polycyclic aromatic hydrocarbons by ligninolytic fungi

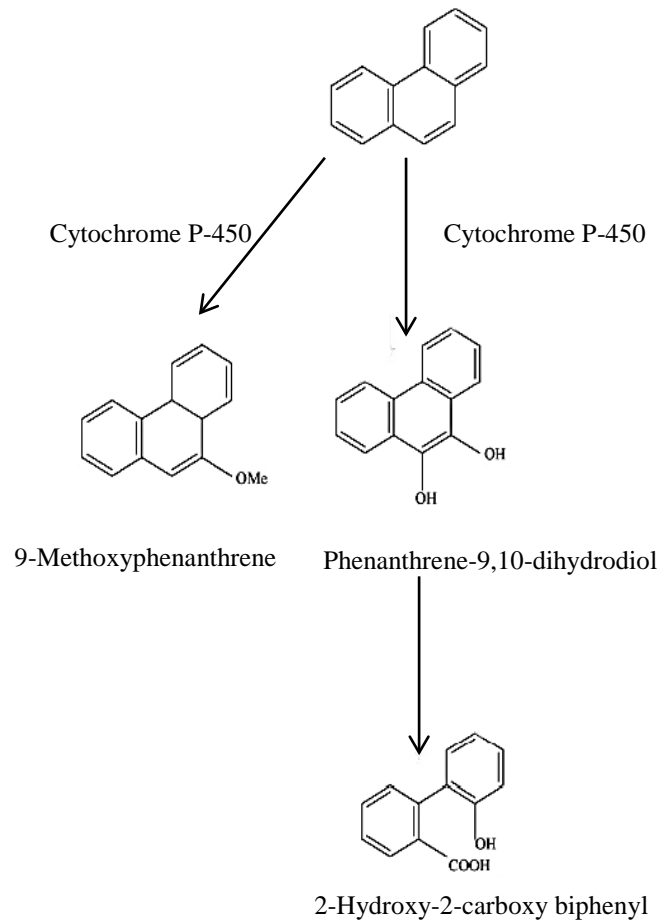


Fig.2. Degradation pathway of phenanthrene using the fungus, *Irpex lacteus* (Modified from Cajthaml et al., 2002)

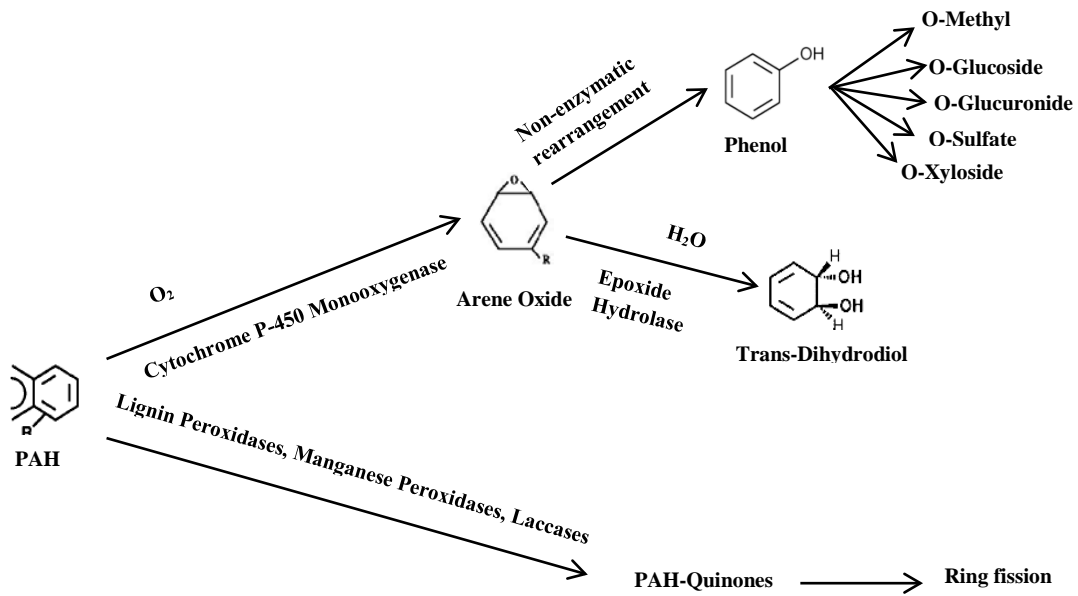






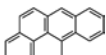
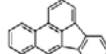




Fig.3. Different pathways for the fungal metabolism of polycyclic aromatic hydrocarbons

**Table 1:** Physical-chemical characteristics of different polycyclic aromatic hydrocarbons

Compound	formula	Mol. wt. (g mol <sup>-1</sup> )	CAS registry No.	Vapour Pressure (Pa at 25°C)	Boiling Point (°C)	Melting Point (°C)	Aqueous solubility (mg/l)	Ionization Potential(eV) <sup>a</sup>	Structure
Naphthalene	C10H8	128	91-20-3	11.9	218	80.2	30	-	
Anthracene	C14H10	178	120-12-7	$3.4 \times 10^{-3}$	340	216.4	0.015	7.43	
Phenanthrene	C14H10	178	85-01-8	$9.07 \times 10^{-2}$	339-340	100.5	1-2	8.03	
Fluoranthene	C16H10	202	206-44-0	$1.08 \times 10^{-3}$	375-393	108.8	0.25	7.90	
Pyrene	C16H10	202	129-00-0	$5.67 \times 10^{-4}$	360-404	393	0.12-0.18	7.53	
benz[a]anthracene	C18H12	228	56-55-3	$14.7 \times 10^{-3}$	438	162	0.0057	<7.35	
benz[a]pyrene		252	50-32-8	$0.37 \times 10^{-6}$	495	179	0.0038	<=7.45	
Benzo[b]fluoranthene	C20H12	252	205-99-2	$1.07 \times 10^{-5}$	168	168.3	-	7.70	
Benzo[k]fluoranthene	C20H12	252	207-08-9	$1.28 \times 10^{-8}$	217	215.7	-	7.48	
Benzo(ghi)perylene	C22H12	276	191-24-2	$1.33 \times 10^{-8}$	525	277	-	7.31	

<sup>a</sup>IPs for all the PAHs except benzo[b]fluoranthene and benzo[k]fluoranthene are from (Pysh and Yang, 1963). The IPs were determined by the polarographic oxidation method. IPs for benzo[b]fluoranthene and benzo[k]fluoranthene are from the modified neglect of diatomic overlap calculations of (Simonsick and Hites, 1986).

**Table 2:** Polycyclic aromatic hydrocarbons oxidized by different species of fungi and their corresponding metabolites

Compounds	Microorganisms	References	Metabolites	References
Acenaphthene	<i>Cunninghamella elegans</i>	(J V Pothuluri et al., 1992)	1-Acenaphthenone, 1,2-Acenaphthenedione, <i>cis</i> -1,2-Dihydroxyacenaphthene, <i>trans</i> -1,2-Dihydroxyacenaphthene, 1,5-Dihydroxyacenaphthene, 6-Hydroxyacenaphthenone	(J V Pothuluri et al., 1992)
Anthracene	<i>Bjerkandera</i> sp, <i>Cunninghamella elegans</i> , <i>Naematoloma frowardii</i> , <i>Phanerochaete chrysosporium</i> , <i>Phanerochaete laevis</i> , <i>Pleurotus ostreatus</i> , <i>Pleurotus sajor-caju</i> ,  <i>Ramaria</i> sp, <i>Rhizoctonia solani</i> , <i>Trametes versicolor</i>	(L. Bezalel et al., 1996; Bogan and Lamar, 1995; Cerniglia and Yang, 1984; Hammel et al., 1992a; Johannes and Majcherczyk, 2000; Kotterman et al., 1998; Sack and Günther, 1993)	Anthracene <i>trans</i> -1,2-Dihydrodiol 1-Anthrol, 9,10-Anthraquinone, Phthalate, Glucuronide, Sulfate and Xyloside conjugates of hydroxylated intermediates	(Lea Bezalel et al., 1996a; Cerniglia, 1982; Cerniglia and Yang, 1984; Collins and Dobson, 1996; Field et al., 1992; Hammel et al., 1991; Johannes et al., 1996; Sutherland et al., 1992)
Phenanthrene	<i>Aspergillus niger</i> , <i>Cunninghamella elegans</i> , <i>Naematoloma frowardii</i> , <i>Phanerochaete chrysosporium</i> , <i>Phanerochaete laevis</i> , <i>Pleurotus ostreatus</i> , <i>Syncephalastrum racemosum</i> , <i>Trametes versicolor</i>	(L. Bezalel et al., 1996; Bogan and Lamar, 1996; Bumpus, 1989; Cerniglia, 1997; Hammel et al., 1992a; Kotterman et al., 1998; Sack and Günther, 1993)	Phenanthrene <i>trans</i> -1,2-dihydrodiol Phenanthrene <i>trans</i> -3,4-dihydrodiol Phenanthrene <i>trans</i> -9,10-dihydrodiol Glucoside conjugate of 1-phenanthrol 1-,2-,3-,4-, and 9-phenanthrol 1-methoxyphenanthrene, Phenanthrene-9,10-quinone 2,2'-Diphenic acid	(Lea Bezalel et al., 1996b; Casillas et al., 1996; Cerniglia et al., 1989; Cerniglia and Yang, 1984; Hammel et al., 1992b; Sack et al., 1997a, 1997b; Sutherland et al., 1991)

Fluorene	<i>Cunninghamella elegans, Laetiporus sulphureus, Phanerochaete chrysosporium, Pleurotus ostreatus, Trametes versicolor</i>	(L. Bezalel et al., 1996; B W Bogan et al., 1996; Bogan and Lamar, 1996; Sack and Günther, 1993)	9-Fluorenone 9-Fluorenol 2-Hydroxy-9-fluorenone	(Lea Bezalel et al., 1996a; Bill W. Bogan et al., 1996; Pothuluri et al., 1993)
Fluoranthene	<i>Cunninghamella elegans, Naematoloma frowardii, Laetiporus sulphureus, Penicillium sp, Pleurotus ostreatus</i>	(Sack and Günther, 1993)	Fluoranthene <i>trans</i> -2,3-dihydrodiol, 8 and 9-Hydroxyfluoranthene <i>trans</i> -2,3-dihydrodiols, Glucoside conjugates of hydroxylated intermediates	(JAIRAJ V. Pothuluri et al., 1992; Pothuluri et al., 1990)
Pyrene	<i>Aspergillus niger, Agrocybe aegerita, Candida parapsilopsis, Crinipellis maxima, Crinipellis perniciosa, Crinipellis stipitaria, Crinipellis zonata, Cunninghamella elegans, Fusarium oxysporum, Kuehneromyces mutabilis, Marasmiellus ramealis, Marasmius rotula, Mucor sp, Naematoloma frowardii, Penicillium janczewskii, Penicillium janthinellum, Phanerochaete chrysosporium, Pleurotus ostreatus, Syncephalastrum racemosum, Trichoderma harzianum</i>	(L. Bezalel et al., 1996; Hammel et al., 1986; Manilla-Pérez et al., 2011)	1,6-Pyrenequinone 1,8-Pyrenequinone Glucoside conjugates 1-Pyrenol 1,6-dihydroxypyrene 1,8-dihydroxypyrene 1-Pyrene sulfate 1-Hydroxy-8-pyrenyl sulfate 6-Hydroxy-1-pyrenyl sulfate Pyrene <i>trans</i> -4,5-Dihydrodiol	(Lea Bezalel et al., 1996a; Cerniglia et al., 1986; Hammel et al., 1986; Lange et al., 1996; Launen et al., 1995; Sack et al., 1997a)
Benzo[a]anthracene	<i>Candida krusei, Cunninghamella elegans, Phanerochaete chrysosporium Phanerochaete laevis,</i>	(Bill W. Bogan et al., 1996;	Benz[a]anthracene <i>trans</i> -3,4-dihydrodiol, Benz[a]anthracene <i>trans</i> -8,9-dihydrodiol,	(Cerniglia et al., 1994; C. E. Cerniglia et al., 1980)

	<i>Pleurotus ostreatus</i> , <i>Rhodotorula minuta</i> , <i>Syncephalastrum racemosum</i> , <i>Trametes versicolor</i>	Cerniglia, 1984)	Benz[ <i>a</i> ]anthracene <i>trans</i> -10,11-dihydrodiol, Phenolic and tetrahydroxy derivatives of benz[ <i>a</i> ]anthracene, Glucuronide and Sulfate  conjugates of hydroxylated intermediates	
Benzo[ <i>a</i> ]pyrene	<i>Aspergillus ochraceus</i> , <i>Bjerkandera adusta</i> , <i>Bjerkandera</i> sp, <i>Candida maltosa</i> , <i>Candida maltosa</i> , <i>Candida tropicalis</i> , <i>Chrysosporium pannorum</i> , <i>Cunninghamella elegans</i> , <i>Mortierella verrucosa</i> , <i>Naematoloma frowardii</i> , <i>Neurospora crassa</i> , <i>Penicillium janczewskii</i> , <i>Penicillium janthinellum</i> , <i>Phanerochaete chrysosporium</i> , <i>Phanerochaete laevis</i> , <i>Pleurotus ostreatus</i> , <i>Ramaria</i> sp, <i>Saccharomyces cerevisiae</i> ,  <i>Syncephalastrum racemosum</i> , <i>Trametes versicolor</i> , <i>Trichoderma</i> sp, <i>Trichoderma</i>  <i>viride</i>	(L. Bezalel et al., 1996; Bogan and Lamar, 1996; Bumpus et al., 1985; Haemmerli et al., 1986; Sack and Günther, 1993)	Benzo[ <i>a</i> ]pyrene <i>trans</i> -4,5-dihydrodiol Benzo[ <i>a</i> ]pyrene <i>trans</i> -7,8-dihydrodiol Benzo[ <i>a</i> ]pyrene <i>trans</i> -9,10-dihydrodiol Benzo[ <i>a</i> ]pyrene-1,6-quinone Benzo[ <i>a</i> ]pyrene-3,6-quinone Benzo[ <i>a</i> ]pyrene-6,12-quinone 3-Hydroxybenzo[ <i>a</i> ]pyrene 9-Hydroxybenzo[ <i>a</i> ]pyrene 7b,8a,9a,10b-tetrahydrobenzo[ <i>a</i> ]pyrene, 7b,8a,9a,10b-tetrahydroxy-7,8,9,10-tetrahydrobenzo[ <i>a</i> ]pyrene, Benzo[ <i>a</i> ]pyrene 7,8-dihydrodiol-9,10-epoxide, Glucuronide and Sulfate  conjugates of hydroxylated intermediates	(Carl E. Cerniglia et al., 1980; Cerniglia and Gibson, 1980a, 1980b, 1979; Haemmerli et al., 1986; Launen et al., 1995)
Chrysene	<i>Cunninghamella elegans</i> , <i>Penicillium janthinellum</i> , <i>Syncephalastrum racemosum</i>	(Kiehlmann et al., 1996; Pothuluri et al., 1995)	2-Chrysenyl sulfate  2-Hydroxy-8-chrysenylsulfate  Chrysene <i>trans</i> -1,2-dihydrodiol	(Kiehlmann et al., 1996; Pothuluri et al., 1995)



Benzo[e]pyrene

*Cunninghamella elegans*

(Pothuluri et al., 1996)

3-Benzo[e]pyrenyl sulfate

(Pothuluri et al., 1996)

10-Hydroxy-3-benzo[e]pyrenyl  
sulfate

Benzo[e]pyrene-3-0-b-  
glucopyranoside

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**Table 3:** Polycyclic aromatic hydrocarbons oxidation by different enzymes

Enzymes	Microorganisms	PAHs	Products	References
LiP	<i>P. chrysosporium</i>	B[a]P	B[a]P-1,6-quinone B[a]P-3,6-quinone B[a]P-6,12-quinone	(Haemmerli et al., 1986; Torres et al., 1997)
		ANT	9,10-anthraquinone	(Field et al., 1996; Torres et al., 1997; Vazquez-Duhalt et al., 1994)
		PYR	PYR-1,6-dione; PYR-1,8-dione	(Hammel et al., 1986; Torres et al., 1997; Vazquez-Duhalt et al., 1994)
		FLA	ND	(Vazquez-Duhalt et al., 1994)
		1-methylanthracene	1-methylanthraquinone	(Vazquez-Duhalt et al., 1994)
		2-methylanthracene	2-methylanthraquinone	(Torres et al., 1997; Vazquez-Duhalt et al., 1994)
		9-methylanthracene	9-anthraquinone; 9-methyleneanthranone; 9-methanol-9,10-dihydroanthracene	(Vazquez-Duhalt et al., 1994)
		Acenaphthene	1-acenaphthenone; 1-acenaphthenol	(Torres et al., 1997; Vazquez-Duhalt et al., 1994)
		Dibenzothiophene	dibenzothiophene sulfoxide	(Vazquez-Duhalt et al., 1994)
		MnP	<i>Anthracophyllum discolor</i>	PYR; ANT; FLA; PHE
PHE; ANT; FLA; PYR	9,10-anthraquinone			(Baborová et al., 2006)
<i>I. lacteus</i>	ANT		anthrone; 9,10-anthraquinone; 2-(2-hydroxybenzoyl)-benzoic acid; phthalic acid	(Eibes et al., 2006; Field et al., 1996; Hammel et al., 1991; Moen and Hammel, 1994)
	<i>P. chrysosporium</i>		FLU	9-fluorenone

		PHE	PHE-9,10-quinone; 2,2_- diphenic acid	(Moen and Hammel, 1994)
		dibenzothiophene	4-methoxybenzoic acid	(Eibes et al., 2006)
	<i>Nematoloma frowardii</i> ( <i>Phlebia</i> sp.)	PHE; ANT; PYR; FLA; CHR; B[a]A; B[a]P; benzo[b]fluoranthene	CO2 from PHE; ANT; PYR; B[a]A; B[a]P	(Sack et al., 1997c; Thomas Günther, 1998)
	<i>Stropharia coronilla</i>	ANT; B[a]P	9,10-anthraquinone; CO2; B[a]P-1,6-quinone	(Steffen et al., 2003, 2002)
	<i>C. hirsutus</i>	ANT; PHE; PYR; FLA; B[a]P B[a]P; ANT; PHE; FLU;	ND	(Cho et al., 2002)
		9-methylanthracene;		
	<i>Coriolopsis gallica</i>	2-methylanthracene; Acenaphthene;	9-fluorenone; dibenzothiophene sulfone	(Bressler et al., 2000; Pickard et al., 1999)
		carbazole; N-ethylcarbazole;		
		Dibenzothiophene ANT; FLU; B[a]A; B[a]P;		
LAC	<i>Ganoderma lucidum</i>	Acenaphthene; Acenaphthylene	ND	(Hunsa Punnapayak, 2009)
	<i>P. ostreatus</i>	ANT; PHE; FLU; PYR; FLA; perylene	9,10-anthraquinone; 9- fluorenone	(Pozdnyakova et al., 2006)
	<i>Pycnoporus cinnabarinus</i>	B[a]P	B[a]P-1,6-quinone; B[a]P- 3,6-quinone; B[a]P-6,12-quinone	(Rama et al., 1998)
		Acenaphthene; PHE; ANT;	1,2-acenaphthenedione 1,8- naphthalic acid anhydride;	(Binková and Šrám, 2004; Böhmer et al., 1998; Cañas et al., 2007; Collins et al., 1996; Johannes et al., 1998;
	<i>T. versicolor</i>	Acenaphthylene, B[a]P; ANT; FLA;	9,10-anthraquinone; PHE- 9,10-quinone, 2,2_-diphenic	Johannes and Majcherczyk, 2000; Majcherczyk et al., 1998)
		PYR; B[a]A; CHR; perylene;		

benzo[b]fluoranthene; acid; B[a]P-1,6-quinone;  
benzo[k]fluoranthene; FLU B[a]P-3,6-quinone;  
B[a]P-6,12-quinone

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