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Cellular Uptake and Metabolism of High Molecular Weight Polycyclic Aromatic Hydrocarbons by the White-rot Fungus *Phanerochaete chrysosporium*

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are lipophilic molecules that generally partition into lipid rich tissues in living organisms where they can be actively metabolized by cell-associated enzymes. In this study, cellular uptake and accumulation of triphenylene, benzo(a)pyrene, and coronene in lipid vesicles of the fungus *Phanerochaete chrysosporium* ATCC 34541 was investigated. Numerous bodies that stained with the lipid specific dye Sudan III were observed in fungal hyphae of *P. chrysosporium* grown with the high molecular weight (HMW) PAHs, implying that they could be taken in by the fungus and stored in lipid vesicles. Furthermore, the subsequent metabolism of the HMW PAHs by the fungus was investigated. This fungus degraded benzo(a)pyrene significantly and showed poor degradation activities for triphenylene and coronene suggesting that fungal intracellular accumulation could not essentially accompany degradation, therefore evaluating transport of compounds in cells may help to discern lack of substrate metabolism due to enzyme specificity or insufficient enzyme contact.

Keywords High molecular weight polycyclic aromatic hydrocarbons, Cell accumulation, Intracellular enzymes, Lipid vesicles, Ionization potential

1. Introduction

The white-rot fungus *Phanerochaete chrysosporium* shown in figure 1 has been shown to be an ubiquitous degrader of an extensive array of xenobiotics compounds ([4, 5, 7, 24, 32, 39, 56, 61]) including polycyclic aromatic hydrocarbons (PAHs).

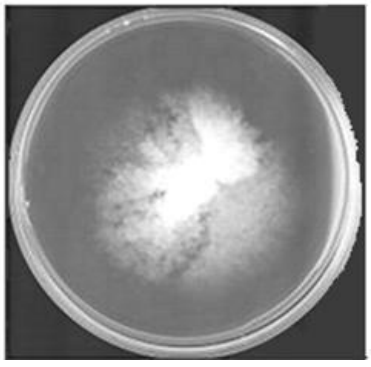

Figure 1. *P. chrysosporium* maintained on PDA media

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A series of PAHs are considered to be organo-pollutants consisting of benzene analogs having two or more aromatic rings in various alignments ([27, 48]). They can be classified into low-molecular-weight (LMW) PAHs with up to three aromatic rings that are known to be very toxic [37], and high-molecular-weight (HMW) PAHs consisting of four and more aromatic rings recognized as highly mutagenic, teratogenic, and carcinogenic for both humans and animals [40]. Over the years, numerous authors have associated the ability of *P. chrysosporium* and other related fungi to be ubiquitous PAH degraders with the extracellular ligninolytic enzyme system, which includes lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and laccase (LAC) ([4, 8 17, 30, 44, 58]). However, the rates of degradation do not seem to always correlate with the activities of extracellular ligninolytic enzymes ([23, 55, 61]). An analysis of the catalytic properties of extracellular ligninolytic enzymes suggests that their role in PAH degradation is limited to a narrow range of compounds according to their ionization potentials (IPs) ([20, 26, 52, 53]). Under optimized conditions, the extracellular ligninolytic enzymes are capable of oxidizing PAHs with an IP <7.6. On the other hand, it has been known that cytochrome P450 monoxygenase (P450) activity would play important roles ([2, 3, 28]) in degradation of PAHs with a higher IP such as triphenylene, benzo(a)pyrene, and

coronene. The earlier studies on fungal metabolism by *P. chrysosporium* ([15, 16, 38, 61]) suggest that the P450-mediated oxidation of PAHs might proceed under ligninolytic conditions.

The P450 enzymes are cell-associated enzymes capable of metabolizing substrates accumulated intracellularly. Many researchers have made an effort to elucidate cytochrome P450-dependent metabolic processes of fungi ([2, 3, 34, 35, 50, 61]). However, these processes are inevitably preceded by cellular uptake of the hydrophobic hydrocarbon *in vivo*. In the event of cellular uptake in living organisms, PAHs generally partition into lipid-rich tissues where they may be actively metabolized by the organisms' cell-associated enzymes, or simply accumulated in fatty tissues ([1, 6, 27, 36, 43, 47]). However, the transport of PAHs inside fungal cells can be limited by the size and solubility of these compounds ([11, 27]) and subsequent metabolism impacted by the ionization potentials ([20, 26, 52, 53]). Therefore, it is of interesting to elucidate accumulation and location of PAHs in the fungal cells to better understand fungal metabolism of PAHs. Triphenylene (TRI), benzo(a)pyrene (B(a)P, and Coronene (COR) with properties listed in table 1 are large molecules that may not easily traverse cellular membranes and to become potential substrates for the cell-associated enzymes.

Table 1. Selected Physicochemical Properties of High Molecular Weight Polycyclic Aromatic Hydrocarbons Used in this Study

Hydro-carbon	[a]Molar mass (g/mol)	[a]Water solubility (g/m^3)	[a]MP (℃)	[a]BP (℃)	[b]log k_{OW}	[c]IP
TRI	228	0.043	199.0	402.0	5.49	8.15
B (a)P	252	0.0038	175.0	495.0	6.04	7.23
COR	300	0.00014	>350.0	525.0	6.75	7.57

[a]Bogan and Lamar, 1995; Dabestan, 1999 [b]Octanol-water partition coefficient (concentration in the octanol-to-aqueous phase) [c]Cavalieri et al., 1983. The ionization potentials of these PAHs were determined from the absorption maximum of the charge-transfer complex of each hydrocarbon and choranil in chloroform

In this study, we examined the uptake of these HMW PAHs by *P. chrysosporium* cells through direct observation of Sudan III stained cells using fluorescence microscopy. In addition, the metabolism of these HMW PAHs by this fungus was investigated.

2. Materials and Methods

2.1. Chemicals

Anthracene, triphenylene, benzo(a)pyrene and coronene were purchased from Wako pure chemicals (Osaka, Japan). Sudan III was supplied from Kasayama. All other chemicals were reagent grade. Deionized water was obtained with a Milli Q System (Millipore).

2.2. Metabolism of PAHs by the Fungus *P. chrysosporium*

P. chrysosporium (ATCC 34541) was grown from conidia inocula at 37℃ in a stationary culture under air (10 mL of medium in a 100-mL Erlenmeyer flask). The medium used in this study was as previously described [31] with 1% glucose and 1.2 mM ammonium tartrate at pH 4.5.

After a 4-day pre-incubation, substrate was added to the cultures to become final concentration of 25 μM. Coronene in dimethyl formamide (2.5 mM), triphenylene and benzo(a)pyrene in acetonitrile (2.5 mM) were utilized substrates-stock solution. Anthracene (2.5 mM) was used for co-metabolic studies with coronene. NaN_3 (1 M) treated cultures were used as controls in the biodegradation studies. To detect abiotic degradation of substrates, blanks without fungi were prepared and processed analogously. Cultures were incubated at 37℃ without agitation under 100% oxygen for periods ranging from 20-30 days. Periodically harvested cultures and controls were homogenized in triplicates with 20 ml acetonitrile, centrifuged at 25,000 x g for 10 min at 4℃ to separate the mycelium from the aqueous fraction and filtered with a membrane (0.45 μm). The aqueous fraction obtained was analyzed by HPLC.

2.3. High Performance Liquid Chromatography Analysis

HPLC analysis of HMW PAH metabolism was done with a Shimadzu STR ODS-II column (4.6 by 150 mm I.D.) Detection of triphenylene and benzo(a)pyrene metabolites was achieved at 257 and 254 nm respectively with a linear gradient from 20% acetonitrile in water, isocratic for 5 mins, to a 100% acetonitrile (21-31 min) at a flow rate of 1.0 ml/min. Coronene samples were detected at 304 nm with a linear gradient from 50% acetonitrile in water, isocratic for 5 mins, to a 100% acetonitrile (21-31 min) at a flow rate of 1.0 ml/min.

2.4. Fluorescence Observations

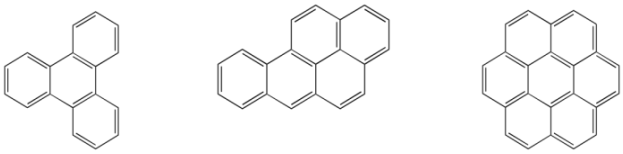
Cultures were grown at 37℃ without agitation under 100% oxygen for five days after substrate addition. Fungal samples were collected from liquid cultures containing no PAH (control sample) or one of the different PAHs used. Observations were conducted with a fluorescent microscope (Leica). PAHs fluorescence wavelengths range from 210 -380 nm.

2.5. Sudan III Staining Protocol

Stock solution of Sudan III (0.2% w/v) was prepared using 70% (v/v) ethanol and warmed in a water bath. The solution was then cooled down to room temperature, filtered and kept in dark at room temperature. Fungal cells were washed thrice with distilled water, and twice with acetone to remove PAH residues. The cells were immersed in the working solution of Sudan III in the dark for 20 min. Hereafter sections were washed with 3 exchanges of deionized water for 30s each. Cells were mounted for observation in an aqueous medium and or glycerin [21].

3. Results and Discussion

3.1. Uptake of Polycyclic Aromatic Hydrocarbons by *P. chrysosporium*

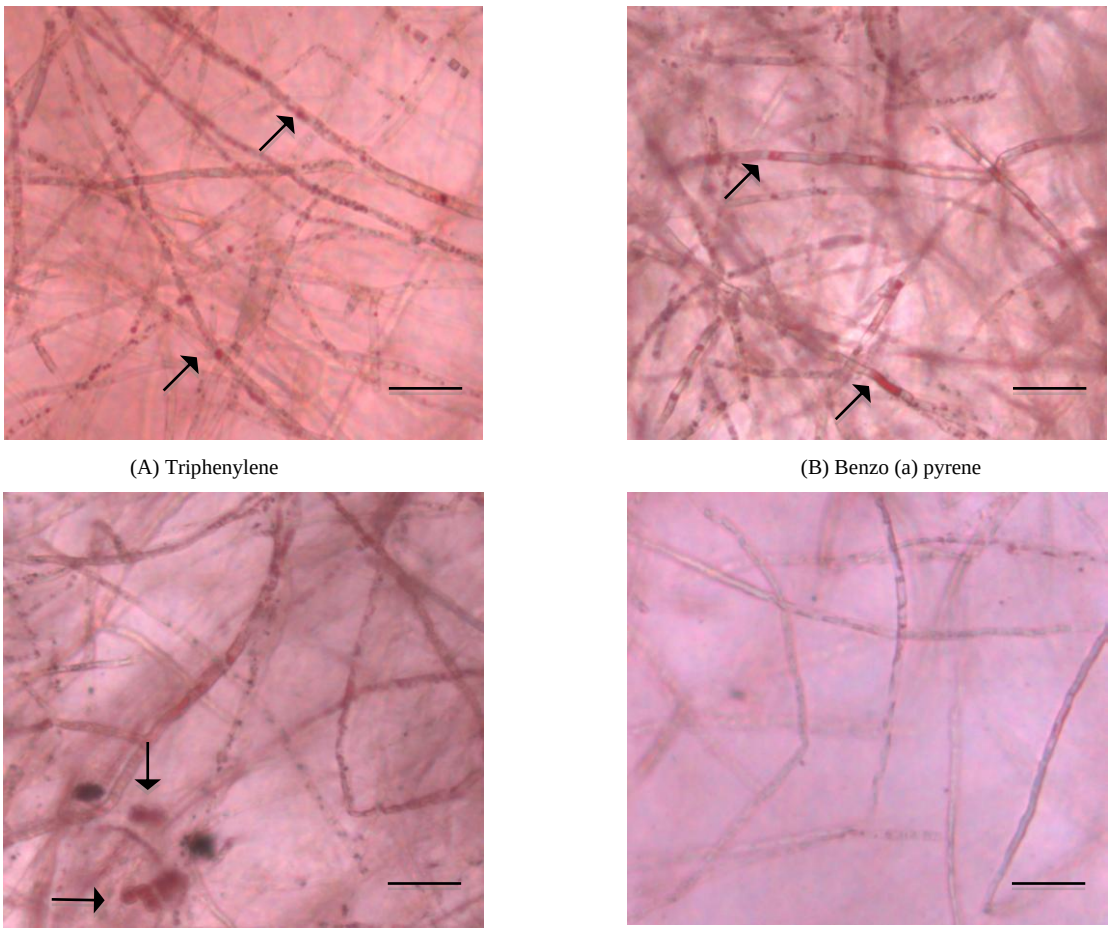


Triphenylene **Benzo (a) pyrene** **Coronene**

Figure 2. Chemical structures of HMW PAHs used in this study

PAHs are lipophilic molecules that are easily incorporated into lipid vesicles for transport and storage ([43, 54]). PAH containing elements in these lipid vesicles can be visualized with Sudan III and other dyes to stain lipid droplets for fluorescence microscopy. In this study, three recalcitrant HMW PAHs (Figure 2) were separately added to cultures of *P. chrysosporium* after a 4-day pre-incubation. Their uptake by the fungus during its cultivation was investigated using fluorescence microscopy. The results obtained were compared to those analogously done in the absence of PAHs.

Figure 3 depicts light microscopic images of fungal cells. Fungal cells incubated with PAH showed small inclusions colored rose-red (Figs. 3A, 3B and 3C) whereas rose-red inclusions were not seen in fungal cell hyphae grown in the absence of PAHs (Figs. 3D). These small inclusions are Sudan III stained lipid vesicles containing PAHs. According to previous literature ([22, 51, 54]), the lipid vesicles in hyphae were also the sites to accumulate PAHs. The absence of these lipid vesicles in fungal cell hyphae grown in the absence of PAHs (Figs. 3D) indicates that these are produced in response to the presence of PAHs in culture. Thus, it is reasonable to speculate that the small inclusions colored rose-red are used as vehicles of transport and storage of polycyclic aromatic hydrocarbons.



(A) Triphenylene (B) Benzo (a) pyrene

(C) Coronene (D) Fungus

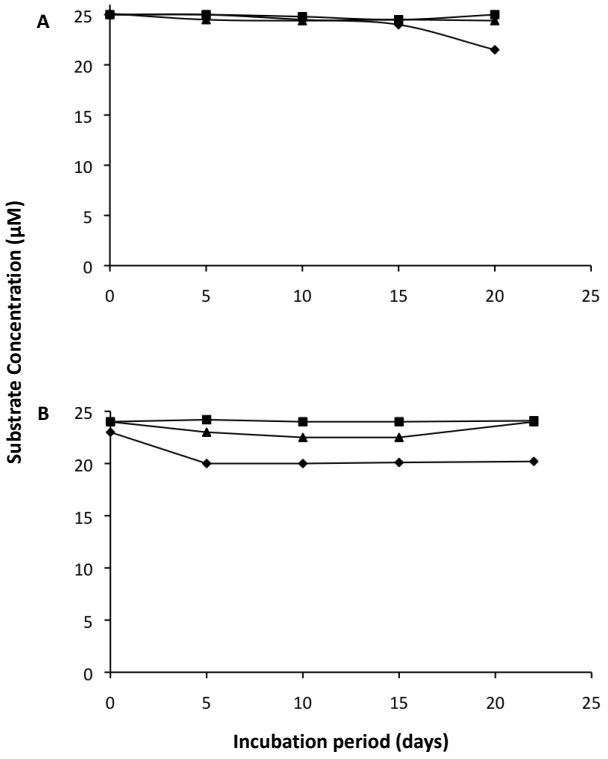
Figure 3. Light microscopic observation of the intracellular elements in *P. chrysosporium* grown in the presence of (A) - (C) and (D) in the absence of PAHs. The PAH containing lipid vesicles are stained red in the hyphae (20X objective lens): bright field images. Bar = 5 μm

This is in agreement with the report of [13], who found that the active microorganisms can be viewed both as a biosorbent that retains organic pollutants as well as a bioreactor that degrades them. A similar observation was reported using *Fusarium solani* that could store PAHs intracellularly in lipid vesicles [54]. It was also shown that the intracellular storage of the PAHs was not restricted to the imperfect fungus *F. solani*, but could be found in numerous other fungi belonging to different genera including *P. chrysosporium P11* [54]. It has been reported that the transport of PAHs inside fungal cells can be limited by the size and solubility of these compounds [11]. However, we revealed that the HMW PAHs used in this study were accumulated in hyphae of *P. chrysosporium* fungal cells, indicating that PAH transport into fungal cells is not impacted only by the size and solubility of the PAH. This is also in agreement with the results reported by [19] where an isolate of the fungus *F. solani* incorporated more BaP into cells than phenanthrene (PHE), despite the 400-fold higher aqueous solubility of PHE compared with BaP.

3.2. Metabolism of HMW PAHs by the White-Rot Fungus *P. chrysosporium*

In 2004, [55] demonstrated that fluorescent PAHs concentrate in the lipid droplets of fungi, which sequester these noxious compounds and perhaps metabolize them to less toxic derivatives. After evaluating the uptake of HMW PAHs, a further study was conducted to evaluate the potential ability of fungal degradation of these high molecular weight PAHs (shown in figure 2). The fungus *P. chrysosporium* degraded Benzo (a) pyrene significantly the results of which have been published [15]. In contrast, the fungus *P. chrysosporium* showed poor degradation of both triphenylene and coronene (figure 4). Cometabolism of coronene with anthracene, a relatively simple PAH can have multimechanistic effects on coronene metabolism, such as inhibition or induction [59]. In this study, the presence of anthracene in fungal cultures slightly improved the degradation of coronene.

Intracellular cytochrome P450 monooxygenase is believed to initially metabolize PAH molecules, having an IP of 8.03 eV ([2, 3, 34, 50]) or higher. The IPs of both triphenylene and coronene suggest that these PAHs would be ideal substrates for the intracellular cytochrome P450 monooxygenase and not the ligninolytic enzyme system ([2, 3, 38]). Poor degradation of both coronene and triphenylene could be as a result of their aromaticity that confers electrochemical stability to these compounds. Triphenylene is highly symmetrical and much more resonance stable than its isomers chrysene, benz(a)anthracene, benzo(c)phenanthrene and naphthacene (tetracene). On the other hand, coronene is a highly condensed PAH with seven benzene rings fused together. These attributes hinder the removal of electrons to participate in reactions during a cytochrome P450 monooxygenase activity. This result is in agreement with that reported by [54] that showed that *P. chrysosporium* was a poor degrader of the compound coronene. Of the PAHs used in this study, B(a)P has an IP of 7.23 making it amenable to biodegradation by both the ligninolytic and cytochrome P450 monooxygenase fungal systems. Benzo (a) pyrene can easily form radical cations by the removal of one electron ([10, 42]), the ease of formation of which depends on the IP of the PAH.



KEY: ◆ Fungal culture in limited nitrogen, ■ Abiotic control not inoculated with fungus and ▲ fungal culture treated with Sodium azide. Each data point is a mean value of three replicates.

Figure 4. Time course of (A) Triphenylene and (B) Coronene metabolism by the white rot fungus *P. chrysosporium* ATCC 34541

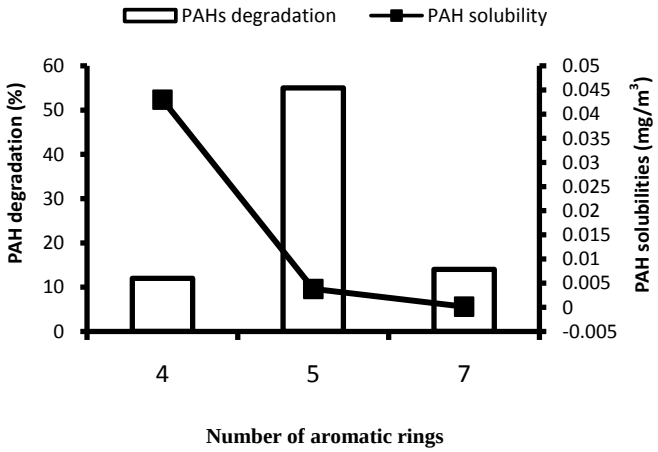


Figure 5. Correlation between the number of aromatic rings of PAHs, their % degradation and solubilities. 4, Triphenylene; 5, Benzo (a) pyrene; 7, Coronene

Correlation between the number of aromatic rings of the PAHs, their percentage degradation and solubilities was analyzed. Triphenylene has the least number of aromatic rings, is the most soluble of the HMW PAHs used in this study but showed the least percentage degradation (Figure 5) by the fungus *P. chrysosporium*. This suggests that no correlation exists between the number of aromatic rings of the PAHs, their solubilities and the extent of degradation. This is in contrast to results described by [12] which showed that degradation was inversely proportional to the number of rings in the PAH molecule.

More attention should be given to studies on the fate of PAH metabolites outside and inside of fungal cell. The result obtained here may be used as a template in future studies to design more improved and useful systems for the study of cytochrome P450s and other intracellular enzymes.

4. Conclusions

This study is one of the few concerned with the correlation between substrate uptake and metabolism. *P. chrysosporium* cells grown in the presence of triphenylene, benzo (a) pyrene, and coronene accumulated these PAHs and showed small intensely stained rose-red inclusions that were not observed in fungal cell hyphae lacking the PAHs. This fungus degraded Benzo (a) pyrene significantly but showed poor degradation of Triphenylene and Coronene. Therefore, evaluating transport of these recalcitrant HMW PAH compounds in the cells of *P. chrysosporium* help to discern lack of attack due to enzyme specificity or due to insufficient enzyme contact. This effort is of both a practical and theoretical interest. Effort to engineer modified enzymes or pathways ([14, 29]) for degradation of recalcitrant compounds would be wasted if the recalcitrance were essentially due to a transport problem.

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