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# Identification of Metabolic Intermediates in Microbial Degradation of Chrysene by *Armillaria sp*. F022

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## *Abstract*

To degrade chrysene, a polycyclic aromatic hydrocarbon (PAH), *Armillaria* sp. F022, a fungus collected from a soil, was used. Maximal degradation (77%) was obtained when *Armillaria* sp. F022 was incubated in cultures agitated at 120 rpm for 30 days, as compared to just 41% degradation in stationary culture. Furthermore, the degradation of chrysene was affected by the addition of surfactants. The mechanism of degradation was determined through identification of the intermediates. Several enzymes (manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase) produced by *Armillaria* sp. F022 were detected in the culture. The highest level of activity was shown by 1,2-dioxygenase after 20 days (143.6 U $l^{-1}$). These ligninolytic and dioxygenase enzymes played an important role in the oxidation of chrysene. Chrysene was indeed degraded by *Armillaria* sp. F022 through several intermediates, chrysenequinone, 2-((1*E*,3*E*)-4-carboxy-3-hydroxybuta-1,3-dien-1-yl)-1-naphthoic acid , 1-hydroxy-2-naphthoic acid, and gentisic acid.

**Keywords :** Biodegradation, Chrysene, Metabolites, *Armillaria* sp. F022

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment and their fates in nature are of great environmental concern due to their potential toxicity, mutagenicity, and carcinogenicity (Keith and Telliard, 1979). They are hydrophobic and readily adsorbed onto particulate matter, thus, coastal and marine sediments become the ultimate sinks for PAHs. Although PAHs may undergo volatilization, photolysis, bioaccumulation, and adsorption, microbial degradation is the main process affecting PAH persistence in nature (Cerniglia, 1993; Yuan *et al*., 2000). Recently, biodegradation, which is expected to be an efficient alternative method to other degradation processes such as physical or chemical ones, has been developed as a environment cleanup technique (Potin *et al*. , 2004; Antizar-Ladislao *et al*., 2004).

Chrysene is a polycyclic aromatic hydrocarbons (PAHs) with the molecular formula $C_{18}H_{12}$ that consists of four fused benzene rings. It is a natural constituent of coal tar, from which it was first isolated and characterized. It is also found in creosote, a chemical used to preserve wood. Chrysene is formed in small amounts during the burning or distillation of coal, crude oil, and plant material (Harvey, 1991). Larger PAHs have also been used as models to determine factors that affect the bioavailability, biodegradation potential, and rate of microbial degradation

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of PAHs in the environment (Kanaly and Harayama, 2000; Sutherland *et al.*, 1995). The metabolism of more complex PAHs with four or more rings has been less extensively studied when they are used as a sole carbon source. However, the very low solubility of complex PAHs, in fact, strongly reduces their bioavailability and makes microbial growth and biodegradation difficult. (Boldrin *et al.*, 1993).

Numerous investigators have attempted to measure microbial transformation rates of PAH, particularly as a component fraction of petroleum. Microbial communities could have considerable potential to remedy oil-contaminated sediment and remove PAHs from aqueous solution (Ramsay *et al.*, 2000; Tam *et al.*, 2002). White rot fungi (WRF) play an important role in the degradation of many chemicals, including aromatic hydrocarbons. Fungal oxidation of aromatic hydrocarbons results in the production of metabolites with higher aqueous solubility and generally less biological reactivity than the parent compounds. White rot fungi possess a number of advantages not associated with other bioremediation systems. The key components of their degrading system are extracellular so the fungi can degrade compounds that are not easily taken up by the cell such as lignin and many hazardous environmental pollutants (Martens and Zadrazil, 1998; Barr and Aust, 1994).

The present study therefore aims to examine the capability to degrade chrysene of *Armillaria* sp. F022, to investigate the metabolites produced during the degradation process, and to investigate the enzymes which play an important role in the degradation.

## Materials and Methods

### *Chemicals*

Chrysene used in this study was purchased from Alfa Aesar. 1- protocatechuic acid and catechol were obtained from Tokyo Chemical Industry Co. Ltd.. Malt extract and polypeptone were purchased from Difco. Thin layer chromatography (TLC) aluminium sheets (Silica gel 60 F254, 20x20cm) were obtained from Merk. Salicylic acid, gentisic acid, the silica gel used for column chromatography (wakogel S-1), and all other chemicals were purchased from Wako Pure Chemical Industry Co. Ltd. at the highest purity available.

### *Fungal culture*

*Armillaria* sp. F022 isolated from a soil in Samarinda, Indonesia, was used for experimentation. The strain was maintained on malt extract agar (2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) polypeptone, and 1.5% (w/v) agar) in a plastic Petri disk at 4°C prior to use. Agar plates were incubated at 25°C for up to 21 days. Chrysene-degrading cultures were identified by a distinct chrysene-clear zone surrounding individual colonies. A single colony of chrysene-degrading fungus was transferred to mineral salt broth medium containing chrysene. The fungus used in the present research was capable of utilizing chrysene as a sole carbon source as determined using mineral salt broth (MSB) medium containing (in g/l distilled water): glucose (10), $KH_2PO_4$ (2), $MgSO_4.7H_2O$ (0.5), $CaCl_2.2H_2O$ (0.1), ammonium tartrate (0.2), and trace elements (10 ml). The pH of the medium was adjusted to 5.7. The fungal inoculum was prepared by growing each fungus on malt extract agar plates at 25°C for 7 days. The inoculum was added to a flask containing mineral salt broth medium. Flasks were shaken at 120 rpm for 3 days at 25°C, and filtered through filter paper under sterile conditions. Mycelia were then added to each vial.

### *Experimental design*

Experiments were performed in 100-ml Erlenmeyer flasks containing 20 ml of liquid medium plus 1 mM chrysene dissolved in dimethylformamide (DMF) to 1 ml. In addition, as the strains have different growth rates, the period of incubation was varied from 5 to 7 days in order to obtain a similar radial growth and to minimize variation in the starting inoculums. Mycelial plugs of a selected fungus were cut from the outer edge of an actively growing culture on an inoculum plate. Three 5 mm disks obtained by punching out with a cork-borer from the outer edge of an actively growing culture of a particular fungus were inoculated into a flask containing 20 ml of liquid medium supplemented with 1 mM of substrates. The flasks were incubated at 25°C. Growth and substrate consumption were determined at 7-day intervals. One set of inoculated flasks was incubated stationary. The effect of varying the surfactant on chrysene's degradation was studied using nonionic tween 80 and anionic perfluorononanoic acid (PFNA). Agitation at 120 rpm was conducted to enhance the degradation of chrysene in the liquid medium. All media were sterilized by autoclaving at 120ºC for 20 min. Control experiments were performed by incubating chrysene in autoclaved cultures (121°C for 20 min) and by incubating MSB medium with chrysene without a inoculum. All assays were conducted in duplicate. Before the incubation, a flask of each treatment was selected for immediate extraction. All remaining flasks were incubated for 15 and 30 days. The culture broth was blended with ethyl acetate to extract the aromatic hydrocarbon and metabolites from the mycelia.

### *Analytical procedures*

After incubation, culture broth was mixed with ethyl acetate and acidified with 1N HCl. The filtrate (liquid media) and residual (fungus body), which are separated by filtration, was extracted with ethyl acetate respectively. Each combined extract and purified by column chromatography using dichloromethane (150 ml). With this method, all substrates initially present in the liquid medium were recovered. The extracts were concentrated and analyzed by gas chromatography-mass spectrometry (GC-MS Shimadzu QP-5050). The amount of substrate was determined using 4-chlorobiphenyl as an internal standard. GCMS was performed with the following conditions: column TC-1; 30 m in length and 0.25 mm in diameter, helium pressure 100 kPa. The temperature program was started at 80°C, held for 2 min, raised from 80°C to 200°C at 20°C min$^{-1}$, then to 260°C at 7.5°C min$^{-1}$, then held for 4 min. The flow rate was 1.5 ml min$^{-1}$, interface temperature was 260°C, and injection volume was 1 μl. The degree of degradation was determined by comparing the amount of chrysene remaining between the control and samples.

### *Enzyme assays*

The production of extracellular enzymes was investigated in the medium mineral salts broth. After homogenization at 10.000 rpm, the enzymatic activity in the crude supernatant was determined using UV-Vis spectrophotometer. All activities were expressed in U, defined as the amount of enzyme required to oxidize one μmol substrate in one min. Manganese peroxidase activity was assayed using malonate buffer 50mM and dimethoxyphenol in 20mM $MnSO_4$ (Wariishi *et al.,* 1992). One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of dimethoxyphenol per min and activities were expressed in Ul$^{-1}$. Laccase activity was assayed using syringaldazine in 100 mM sodium acetate buffer. The enzymatic reaction was carried out at room temperature and one unit of activity was defined as the amount of enzyme oxidizing 1 μmol of substrate in 1 min. Lignin peroxidase activity was determined using veratryl alcohol as a substrate (Kuwabara *et al.*, 1984). One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of veratryl alcohol per min and the activity was reported as Ul$^{-1}$. 1,2-Dioxygenase and 2,3-dioxygenase were measured by a modified previous method (Nakazawa and Nakazawa, 1970). 1,2-Dioxygenase and 2,3-dioxygenase activities were assayed using catechol as a substrate. One unit of activity was defined as the amount of enzyme that oxidized 1 µmol of substrate per min and the activity was expressed in $Ul^{-1}$.

### *Detection of metabolites*

MSB medium was prepared as described above. After inoculation of the medium with *Armillaria* sp. F022, the culture was pre-incubated by standing for 7 days at 25°C in the dark. Chrysene dissolved in 100 µl of dimethylformamide (DMF) and 10 µl of tween 80 (1% solution) were added to each culture medium as described above. The incubation was conducted for 7-30 days at 25ºC in the dark. The extracts were purified using silica gel column chromatography by successive elution with several solvent combinations. After the vacuum drying of each eluate (100 µl) in a vial, N, O-bis-trimethylsilyl acetamide (40 µl), pyridine (40 µl), and trimethylcholorilane (20 µl) were added. Trimethylsilylation of the eluate was conducted for 10 min at 80ºC without contact with moisture. The trimethylsilyl (TMS) derivatives of the extract were analyzed by gas chromatography (GC) using a Shimadzu GC-17 as described above.

## Results and Discussion

### *Investigation of degradation of chrysene by Armillaria sp. F022*

The rate of degradation was above 40% for 30 days of incubation with *Armillaria* sp. F022. *Armillaria* sp. F022 degraded 41% of chrysene at 1 mM in 30 days. It was observed that by 30 days in the stationary cultures, *Armillaria* sp. F022 formed filamentous mats at the surface of the growth medium, while in the set incubated with agitation, uniform pellets were formed. From Figure 1, it could be seen that 77% degradation was achieved in 120 rpm agitated cultures in 30 days, as compared to 41% degradation in stationary cultures.

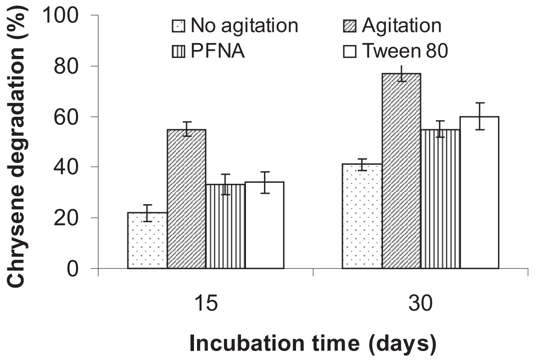

Figure 1. Effect of agitation and kind of surfactants on degradation of chrysene by *Armillaria* sp. F022.

Efficiency improvement could be due to degradation of fungal physiological conditions as pellets and increase the mass transfer between cells and medium. Biodegradation by white rot fungi have been associated with the activity of extracellular oxidative enzymes such as laccase. Some references indicate that laccase production was the largest in the culture of restlessness and therefore the maximum degradation was achieved in agitated culture. Oxygen concentration was directly dependent on the rate of air flow. Stirring increases the contact between the reagents (substrate, oxygen, and biomass), thus increasing the mass transfer and, consequently, the rate of biodegradation Listen Read phonetically (Collina *et al.*, 2005).

In stationary culture, forming a mat on the surface limit the transfer of oxygen to the cells below the surface and in the media which resulted in limited oxygen, which inhibits oxidative enzymes and prevent degradation. Figure 1 also shows the effect of surfactants on the degradation of chrysene. The highest degradation levels was obtained with nonionic surfactant tween 80 (60%) compared with anionic PFNA (55%) after 30 days incubation. When culture comes equipped with 80 tween, the rate of decline of about 2-fold higher than that obtained in control cultures (without tween 80). In addition, the level of enzyme activity persists throughout the culture, which may correlate with greater stability of isoenzyme produced. One benefit of using a surfactant such as tween 80 was a better dissolution of the very hydrophobic substrate (Kapich *et al.* 1999). On the other hand, tween 80 was proved to promote better absorption and release of compounds from cells through modification of plasma membrane permeability. In addition, tween 80 increases the solubility of petroleum components such as chrysene, or reduce interfacial tension to increase the mobility of petroleum. General desirable properties including increased solubility, decreased surface tension, critical micelle concentration, wet-ability, and foaming capacity (Asther *et al.*, 1987).

### *Investigation of enzyme activity of selected fungi in the liquid medium*

Several enzymes (manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase) were detected in the culture produced by *Armillaria* sp. F022. The levels of MnP and LP activity were highest after 15 days of cultivation (54.3 and 40.3 Ul$^{-1}$) while 1,2- and 2,3-dioxygenase showed the highest level after 20 days (143.6 and 34.3 Ul$^{-1}$). *Armillaria* sp. F022 showed the greatest laccase production after 25 days (92.4 Ul$^{-1}$) (Figure 2). Those ligninolytic and dioxygenase enzymes play an important role in the oxidization of various environmental pollutants such as chlorophenol, aromatic dyes, and polycyclic aromatic hydrocarbons including chrysene. LP is able to oxidize various aromatic compounds, while MnP oxidizes almost exclusively Mn (II) to Mn(III), which then degrades phenolic compounds (Mester and Tien, 2000). Laccase is a copper-containing oxidase that reduces molecular oxygen to water and oxidizes phenolic compounds. In most species, peroxidase and laccases presented as several isoenzymes. Both types of ligninolytic enzymes are glycosylation, which may increase their stability (Nie *et al.*, 1999). Previous studies showed that the dioxygenases were used to degrade polycyclic aromatic hydrocarbons by a strain. Thus dioxygenase initially attacked the aromatic compound at both the 1,2-position and the 2,3-position (Pinyakong *et al.*, 2000).

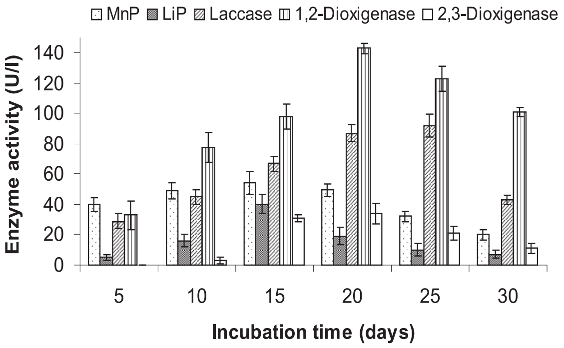

Figure 2. Enzyme activity during incubation of *Armillaria* sp. F022.

### *Identification of metabolites*

Four metabolites were detected during the degradation of chrysene by *Armillaria* sp. F022 (Table 1). The identity of four of the metabolites was confirmed using authentic standards. Other compounds are chrysenequinone based oxygenase reaction may start with chrysene. The other compound was chrysenequinone based on the possible initial oxygenase reaction with chrysene. By comparing the GC elution and DI profile of I, II, III and IV, with standards or synthesized compound, the identity of these compounds could be confirmed. Compound I (m/z, 258) was possibly chrysenequinone as reflected by a major peak at 3.3 min, its mass spectrum, and the presence of dihydroxy chrysene, a dehydrogenation product from dihydrodiol. The MS properties of the M$^+$ at m/z 258, and fragment ion at m/z 230 [M$^+$-28], corresponding to the respective sequential losses of -CO, were identical to those of chrysenequinone. *Armillaria* sp. F022, grown in MSB with chrysene, was able to mineralize chrysene to compound II.

The DI properties of the M$^+$ at m/ 284, and fragment ions at m/z 256 [M$^+$-28],

Table 1 Mass spectra analysis of the principal metabolites detected during the degradation of chrysene by *Armillaria* sp. F022.

| Metabolites | m/z of fragment ions (% relative abundance) | Possible structures |
|---|---|---|
| I | 258 (15, $M^+$), 230 (100), 231(26), 202 (25), 200 (12), 201 (11), 228 (6) | Chrysenequinone (confirmed with a synthesized compound) |
| II | 188 (50, $M^+$), 170 (100), 114 (61), 115 (53), 77 (29) | 1-Hydroxy-2-naphthoic acid (confirmed with a standard) |
| III | 256 (100, $M^+$), 129 (44), 213 (34), 185 (20), 227 (18), 282 (13) | 2-((1*E*,3*E*)-4-carboxy-3-hydroxybuta-1,3-dien-1-yl)-1-naphthoic acid (confirm with $^1$H and $^{13}$C NMR) |
| IV | 154 (100, $M^+$), 136 (77), 108 (25), 137 (24) | Gentisic acid (confirmed with a standard) |

corresponding to the respective sequential losses of –CO. In addition to GC-MS results, 1H and 13C NMR spectra (data not shown), revealed that metabolite II was 2-((1*E*,3*E*)-4-carboxy-3-hydroxybuta-1,3-dien-1-yl)-1-naphthoic acid. An analysis of the ethyl acetate-extractable metabolites was conducted using MS, under normal conditions. A major peak at 2.2 min, which represented all of the metabolites, was identified as 1-hydroxy-2-naphthoic acid. The MS properties of $M^+$ at m/z 188, and fragment ions at m/z 170 [$M^+$-18], that compound III were identical to that of authentic 1-hydroxy-2-naphthoic acid. Compound IV (m/z, 154) was possibly gentisic acid as reflected by mass spectrum. Mass spectrum properties of the $M^+$ at m/z 154, and fragment ions at m/z 136 [$M^+$-18], were identical to those of authentic gentisic acid.

Many PAHs contain a "bay region" and a K-region". The bay- and K-regions, which can be formed metabolically, are highly reactive both chemically and biologically. As chrysene contains bay- and K- regions, it is also used as a model substrate for studies on the metabolism of bay-region- and K-region-containing carcinogenic PAHs such as benzo(a)pyrene and benzo(a)anthracene (Fawell and Hunt, 1988; Gibson and Subramanian, 1984). Based on the identification of various metabolites produced during the initial ring oxidation and ring cleavage processes, the metabolism of chrysene by *Polyporus* sp. S133, a fungus screened from nature, was successfully explored. The pathways for chrysene's degradation were proposed based on the identification of various metabolites (Figure 3).

It is possible that a fungal culture could utilize the dioxygenase system to transform chrysene to *cis*-chrysene or *trans*-chrysene dihydrodiol, and further to dihydroxy chrysene, respectively. However, only chrysenequinone was detected in the present study, suggesting that the fungus utilized the dioxygenase system to transform chrysene. Chrysenequinone was further degraded to 2-((1*E*,3*E*)-4-carboxy-3-hydroxybuta-1,3-dien-1-yl)-1-naphthoic acid. *Armillaria* sp. F022 can degrade chrysenequinone through a highly complex initial metabolic pathway but this pathway converged into 1-hydroxy-2-naphthoic acid. This reaction is presumably catalyzed by salicylate hydroxylase or equivalent enzymes (Nie *et al.*, 1999; Balashova *et al.* 2001). 1-Hydroxy-

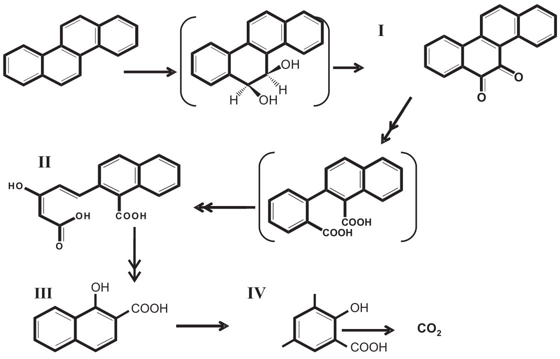

Figure 3. A pathway for the degradation of chrysene by *Armillaria* sp. F022

2-naphthoic acid can be further degraded to gentisic acid. Gentisic acid undergoes ring fusion to form tricaboxylic acid-cycle intermediates (Gibson and Subramanian, 1984; Houghton and Shanley, 1994).

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