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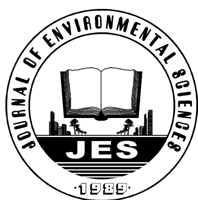

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Identification of naphthalene metabolism by white rot fungus *Armillaria* sp. F022

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Abstract

Armillaria sp. F022, a white rot fungus isolated from tropical rain forest (Samarinda, Indonesia) was used to biodegrade naphthalene in cultured medium. Transformation of naphthalene by *Armillaria* sp. F022 which is able to use naphthalene, a two ring-polycyclic aromatic hydrocarbon (PAH) as a source of carbon and energy was investigated. The metabolic pathway was elucidated by identifying metabolites, biotransformation studies and monitoring enzyme activities in cell-free extracts. The identification of metabolites suggests that *Armillaria* sp. F022 initiates its attack on naphthalene by dioxygenation at its C-1 and C-4 positions to give 1,4-naphthoquinone. The intermediate 2-hydroxybenzaldehyde and salicylic acid, and the characteristic of the *meta*-cleavage of the resulting diol were identified in the long-term incubation. A part from typical metabolites of naphthalene degradation known from mesophiles, benzoic acid was identified as the next intermediate for the naphthalene pathway of this *Armillaria* sp. F022. Neither phthalic acid, catechol and *cis,cis*-muconic acid metabolites were detected in culture extracts. Several enzymes (manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase) produced by *Armillaria* sp. F022 were detected during the incubation.

Key words: *Armillaria* sp. F022; dioxygenase enzymes; ligninolytic enzymes; naphthalene metabolites; 1,4-naphthaquinone

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Introduction

Polycyclic aromatic hydrocarbons (PAHs), has been recognized institutionally by the Unites States Environmental Protection Agency (US EPA) which has proposed 16 PAH as priority pollutants including naphthalene. Besides being toxic, carcinogenic and mutagenic compounds, the semi-volatile properties of some PAH make them highly mobile throughout the environment (Samanta et al., 2002). In addition, PAH have a high trophic transfer and biomagnification within the ecosystems due to the lipophilic nature and the low water solubility that decreases with molecular weight. Consequently, the environment has become highly polluted with different PAHs. Removal of PAHs from the environment is a necessity for ensuring human health (Long et al., 2008). Many strategies have been developed to remove PAHs from the environment, including volatilization, photo-oxidation, chemical oxidation, adsorption, and biodegradation (Rivas, 2006; Semple et al., 2007). Of these, biodegradation techniques have received considerable attention, particularly in the context of cleaning up contaminated sites, due to their comparatively low cost (Bamforth and Singleton, 2005).

Naphthalene is a two-ring PAH that is of environmental concern due to its carcinogenicity and persistent organic pollutant properties (Santos et al., 2008). Naphthalene degradation has been extensively studied in many microorganism especially bacteria (Zeinali et al., 2008; Abou Seoud and Maachi, 2003). However, degradation pathways for naphthalene's catabolism by *Armillaria* species are not well explored. Under mesophilic conditions, white rot fungi species metabolize naphthalene at different sites of the molecule, presumably via ligninolytic enzymes and dioxygenase on the aromatic nucleus.

Armillaria sp. F022 was originally isolated in our laboratory after decolorizing azo, anthraquinone and triphenylmethaneas as the source of carbon and energy (Hadibarata et al., 2011). Temperature growth range for this strain was 30–55°C with the maximum growth rate at 35°C. The current study represents an effort to characterize the structures of naphthalene metabolites produced by this *Armillaria* species under thermophilic condition. The ring-cleavage pathway of naphthalene transformation was studied with identification of key metabolites using thin layer chromatography (TLC), UV-Vis spectrophotometer, gas chromatography mass spectrometry (GC-MS), and enzymatic assay analyses.

1 Materials and methods

1.1 Microorganism and growth conditions

Armillaria sp. F022 isolated from a tropical rain forest

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in Samarinda, East Kalimantan, Indonesia was used for experimentation. The macroscopic morphological characteristics and the phylogenetic position of this fungus were described in previous research (Hadibarata et al., 2011). The fungus 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 dish at 4°C prior to use. *Armillaria* sp. F022 was selected based on its ability to grow on a solid agar medium containing 20 mL of malt extract agar with the addition of naphthalene dissolved in dimethylformamyde (DMF) and Tween 80 (1%) and addition of 300 mg/L benomill to inhibit bacterial growth, then incubated at room temperature for two weeks and observed daily. A single colony of naphthalene-degrading fungus was transferred to a mineral liquid medium containing naphthalene. The constituent of modified mineral liquid medium was described previously (Hadibarata et al., 2007). The fungal inoculum was prepared by growing fungus on malt extract agar plates at 25°C for 7 days. The inoculum was added to a flask containing the mineral salt broth medium. The cultures were kept shaking (120 r/min) at 35°C for 7 and 14 days. Also, naphthalene-grown cells were washed twice in mineral liquid medium and used as inocula for the metabolite degradation studies. Control experiments were performed with autoclaved *Armillaria* sp. F022 cells.

1.2 Chemicals

Organic solvents were obtained from Qrec (New Zealand). Naphthalene and reference compounds (≥ 98% pure) for the identification of metabolites were purchased from Sigma-Aldrich (Detroit, USA). Malt extract and polypeptone were purchased from Difco (Detroit, USA). Thin layer chromatography (TLC) aluminium sheets (Silica gel 60 F_{254}, 20 cm × 20 cm) were obtained from Merck (Darmstadt, Germany).

1.3 Extraction and analysis of metabolites

After growth on naphthalene, contents of the flasks was blended with ethyl acetate and acidified with 1 mol/L HCl. The filtrate (liquid medium) and residue (fungal body) were separated by filtration and the supernatants were extracted with three equal volumes of ethyl acetate. The residual extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure at room temperature using a vacuum concentrator. The products of naphthalene's degradation in culture extracts were analyzed by TLC on silica gel 60 F_{254} (20 × 20 cm, thickness 0.25 mm) using hexane:chloroform (30:10, V/V) as the solvent system for short-term incubation, and dichloromethane:ethyl acetate (10:30, V/V) as the solvent system for long-term incubation. The locations of the compounds on the TLC plates were detected by the use of UV light. The R_f values of the detected spots were compared with those of authentic compounds known or suspected to be metabolites of naphthalene's degradation. Naphthalene and its metabolites were detected under UV illumination at 254 nm. UV-Vis absorption spectra were recorded on a UV-Vis spectrophotometer (Shimadzu 1600, Japan).

Derivatization of carboxylic acids and aromatic hydroxyl groups for GC-MS analysis was performed with a mixture of N,O-bis-trimethylsilyl acetamide, pyridine, and trimethylchlorosilane (40:40:20, $V/V/V$) at 80°C for 10 min. A portion of the resulting products was injected into a TC-1 capillary column (30 m × 0.25 mm) ID 0.25 μm using a gradient of 60°C for 2 min, raised to 150°C at 15°C/min, then raised to 300°C at 25°C/min, and maintained at 300°C for 6 min. Injector and interface temperatures were 260°C (Hadibarata and Tachibana, 2010). In order to confirm the metabolites of naphthalene's degradation and to determine the degradation pathway, a GC-MS (Shimadzu QP-5050, Japan), was used in this experiment. The conditions for GC-MS consisted of the use of a detector at 1.3 eV, scan intervals of 1 sec, and a mass range of 50–500. Mass profiles were also compared with spectra of the authentic standards which were analyzed similarly. Mass spectra of individual total ion peaks were identified by comparison with authentic standards and the Wiley 275L mass spectra data base. The metabolites were identified by comparing their TLC analyses, UV absorption spectra, retention times, and mass spectra with those of the corresponding authentic standards.

1.4 Preparation of cell-free extract and enzyme assays

Naphthalene-grown cultures were harvested at different time intervals incubation (7 and 15 days) and centrifuged (10,000 r/min, 15 min) at room temperature and rapidly washed three times with 50 mmol/L phosphate buffer (pH 7). Cells were disintegrated with a probe-type sonic oscillator for 10 min. Whole cells and large debris were removed from the extract by 20-min centrifugation (15,000 r/min, at 4°C) and the supernatant was used immediately for enzymatic experiments. The supernatant was separated into two parts: one for assay of phenoloxidases activity, the other for detection of dioxygenases activity. Manganese peroxidase activity was assayed using 50 mmol/L malonate buffer and dimethoxyphenol in 20 mmol/L $MnSO_4$ (Wariishi et al., 1992). Laccase activity was assayed using syringaldazine in 100 mmol/L sodium acetate buffer (Tien and Kirk, 1984). Lignin peroxidase activity was determined using veratryl alcohol as a substrate (Kuwabara et al., 1984). 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 U/L.

2 Results and discussion

2.1 Enzymatic activity of selected fungi

Several enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), laccase, 1,2-dioxygenase and 2,3-dioxygenase were detected in the culture produced by *Armillaria* sp. F022. The highest levels of enzyme ac-

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tivity were shown by 1,2-dioxygenase (103.4 U/L) after 14 days incubation. A few activity was shown by MnP, LiP and 2,3-dioxigenase at the end of incubation (Fig. 1). 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 (Mester and Tien, 2000). The oxidation of both phenolic and non-phenolic units was catalized by lignin peroxidase catalyzes through a mechanism of one electron oxidation, resulting in the phenoxy radicals formation and cation radicals. Depending on the substrate these radicals may couple and partly polymerize at least *in vitro*. Laccases frequently demonstrate high oxidative selectivity in an aqueous solution and provide a unique green chemistry solution for a variety of oxidations. These properties make laccase attractive biocatalysts in organic synthetic chemistry. In similarity with laccase, MnP oxidizes some phenolic compounds to give phenoxy radicals and quinones, which may undergo coupling and condensation (Ander et al., 1990; Witayakran et al., 2007). Dioxygenase is the enzyme in bacteria that degrades PAHs (Pinyakong et al., 2000). In this research, crude enzyme preparation on the involvement of any ligninolytic and dioxygenase enzymes using naphthalene showed the presence of extracellular ligninolytic enzyme activity, which may be responsible for the production of quinone in the degradation process.

2.2 Identification of metabolites

The TLC and UV-Vis spectrophotometric analyses were initially performed to indicate the presence of different intermediates in the degradative pathway by combining short- and long-term incubation extracts of the naphthalene-grown culture. GC-MS analysis of the extract from naphthalene incubation revealed the presence of four metabolites using extracts of short- and long-term incubations separately (Table 1). Analysis of TLC which was obtained using the ethyl acetate extractable metabolites of short term incubation of naphthalene formed by *Armillaria* sp. F022 showed the presence of one metabolite (Fig. 2a). One metabolite (I) having an R_f value of 0.65, gave an UV spectrum with λ_{max} of 437, 463 and 482 nm, similar to that of 1,4-naphthaquinone. The spectrum of compound I (m/z 208, M^+), which had a retention time of 8.6 min, is shown in Fig. 3a. The GC retention time, MS properties of the M^+ at m/z 158, and the significant fragment ions at m/z 102 and 130 (M^+28), corresponding to the respective sequential losses of –CO, were identical to authentic 1,4-naphthaquinone.

Armillaria sp. F022 degraded naphthalene via 1,4-dihydroxynaphthalene to 1,4-naphthaquinone in short term incubation time. Unfortunately, 1,4-dihydroxynaphthalene was not confirmed in our culture extract. However, when a long-term extract was analyzed, other peaks from the short-term extract was decreased and new peaks appeared, the retention time and fragmentation pattern of compound II, III and IV matching those of authentic 2-hydroxybenzaldehyde, salicylic acid, and benzoic acid, respectively (Fig. 2b). Few naphthalene peaks was still apparent on chromatogram, probably because naphthalene was not totally degraded after long-term incubation.

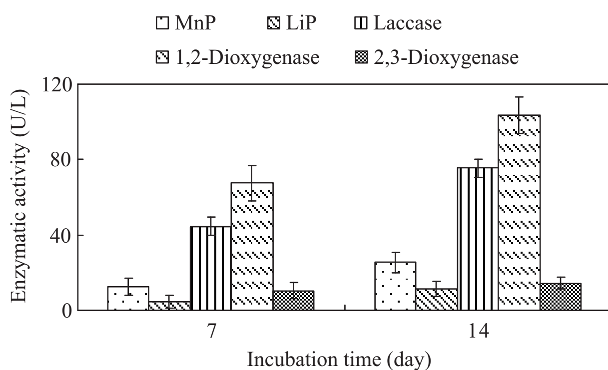

Fig. 1 Change in enzymatic activity during incubation of *Armillaria* sp. F022.

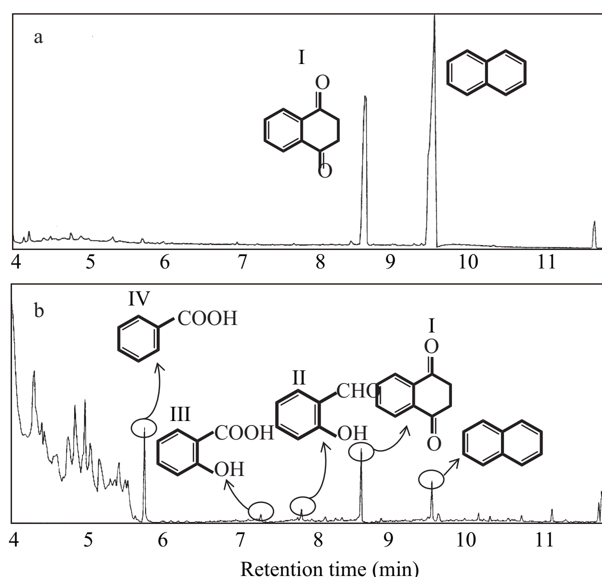

Fig. 2 GC chromatogram of naphthalene metabolisms after short-term incubation (a), and long-term incubation (b).

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

Metabolite	Retention time (min)	m/z of fragment ions (% relative abundance)	Possible structure
I	8.6	158 (100, M^+), 50 (44), 76 (84), 102 (96), 104 (77), 130 (51),159 (11)	1,4-Naphthaquinone (confirmed with standard)
II	7.7	194 (7, M^+), 73 (100), 79 (86), 93(16), 121 (24), 145(27), 178 (12),179 (32), 195 (5)	2-Hydroxybenzaldehyde-TMS derivative (confirmed with a standard)
III	7.3	267 (94, M^+-15), 73 (100), 74 (22), 75 (19), 91 (16), 135 (18), 147(23), 149 (19), 209 (15), 268 (38)	Salicylic acid-TMS derivative (confirmed with standard)
IV	5.8	194 (8, M^+), 73 (9), 77 (72), 105 (96), 135 (52), 179 (100), 180 (14)	Benzoic acid-TMS derivative (confirmed with standard)

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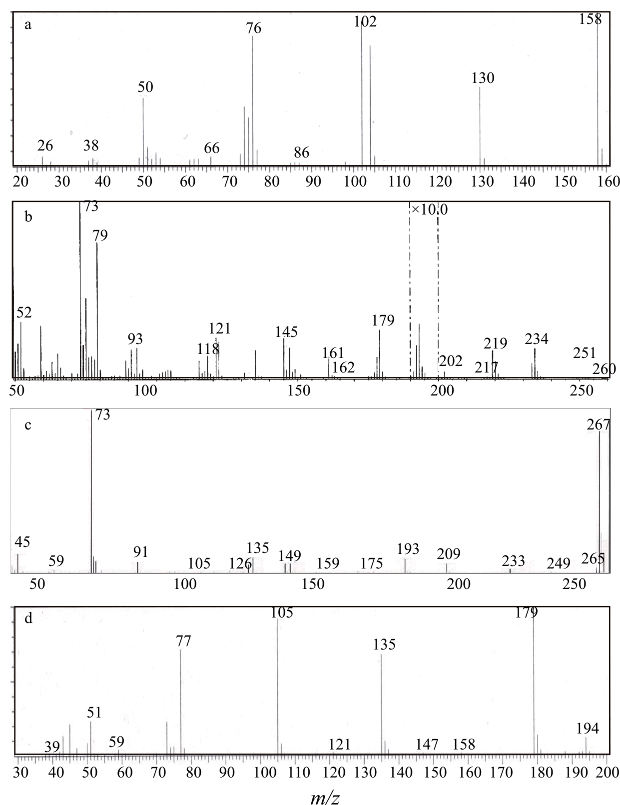

Fig. 3 Mass spectral profiles of naphthalene metabolites. (a) 1,4-naphthaquionone; (b) 2-hydroxybenzaldehyde-TMS derivative; (c) salicylic acid-TMS derivative; (d) benzoic acid-TMS derivative.

TLC of the ethylacetate-extractable metabolites of long term incubation of naphthalene showed one metabolite(II) having an R_f value of 0.4. This metabolite had the same R_f value and UV characteristics (λ_{max} 85 nm and 109 nm) as the authentic 2-hydroxybenzaldehyde standard. The retention time peak at 7.7 min refers to authentic 2-hydroxybenzaldehyde (Fig. 2b). MS analysis of the 2-hydroxybenzaldehyde produced from naphthaquinone gave an apparent molecular ion [M^+] at m/z 194 for TMS-derivatives and apparent losses of [M^+-15] at m/z 179 corresponding to the respective sequential losses of methyl ($-CH_3$), and [M^+-16] at m/z 178 corresponding to the respective sequential losses of oxygen (O) as well as the expected fragment ions at m/z 179 and 73 (Fig. 3b). Based on data obtained above, the compound should be 2-hydroxybenzaldehyde.

TLC of the ethylacetate-extractable metabolites of naphthalene showed one metabolite (III) having an R_f value of 0.28. This metabolite had the same R_f value and UV characteristics (λ_{max} 211, 236 and 305) as the authentic salicylic acid standard. The mass spectrum of compound III (Fig. 3c), eluting at retention time 7.3 min (Fig. 2b), had a molecular ion at m/z 282 and fragmentation ions at m/z 267 (M^+15), sequential loss of methyl ($-CH_3$), and 193 (M^+89), sequential loss of $-OSi(CH_3)_3$, as well as the expected fragment ions at 147, 209, and 73 [$(CH_3)_3Si$].

The last metabolite (IV) with an R_f value of 0.63, gave an UV spectrum with λ_{max} of 220 and 273 nm, similar to that of the authentic benzoic acid standard. Mas spectrum of compound IV (Fig. 3d) with a GC retention time of 5.8 min (Fig. 2b) indicated a TMS compound. MS of the benzoic acid produced from naphthalene gave an apparent molecular ion [M^+] at m/z 194 for TMS-derivatives and significant fragment ions at m/z 179 (M^+15), sequential loss of methyl ($-CH_3$), and as well as the expected fragment ions at 135, 105 and 73 [$(CH_3)_3Si$].

The degradation of naphthalene was a complicated process because many enzymes and different catabolic pathways are involved and many intermediates were produced and consumed when the degradation proceeds. The pathway of naphthalene's degradation by *Armillaria* sp. F022 is proposed in Fig. 4. *Armillaria* sp. F022 grows in mineral liquid medium with naphthalene as a source of carbon and energy. Analysis of the culture extracts showed that *Armillaria* sp. F022 did not accumulate large quantities of aromatic metabolites during the cultivation period. Low levels of metabolite production by some PAH-degrading fungi may be related to physical interaction between the cell and the hydrophobic substrates (Guerin and Jones, 1988). Based on the identification of various metabolites produced during the initial ring oxidation and ring cleavage processes, the metabolism of naphthalene by *Armillaria* sp. F022 was explored.

It is possible that a fungal culture could utilize the dioxygenase system to transform naphthalene to *cis*-naphthalene or *trans*-naphthalene dihydrodiol, and further to dihydroxy naphthalene, respectively. However, only 1,4-naphthoquinone was detected in the present study, suggesting that the fungus utilized the dioxygenase system to transform naphthalene. These results showed that 1,4-oxidation and ring cleavage to give 1,4-naphthaquinone is the major fate of naphthalene in ligninolytic *Armillaria* sp. F022. Naphthalene's metabolism in ligninolytic *Armillaria* sp. F022 differs from the pathway employed by most bacteria, which cleave naphthalene between C1 and C2 to produce 1,2-dihydro-1,2-dihydroxynaphthalene and 1,2 dihydroxynaphthalene (Zeinali et al., 2008; Lin et al., 2010).

Benzoic acid is proposed as the decarboxylation product of 1,2-hydroxybenzaldehyde and salycilic acid. Benzoic acid can be further metabolised into carbon dioxide and water. This is in contrast to metabolism to the metabolism of naphthalene in well studied bacteria, which employs the common route for naphthalene degradation via phthalic

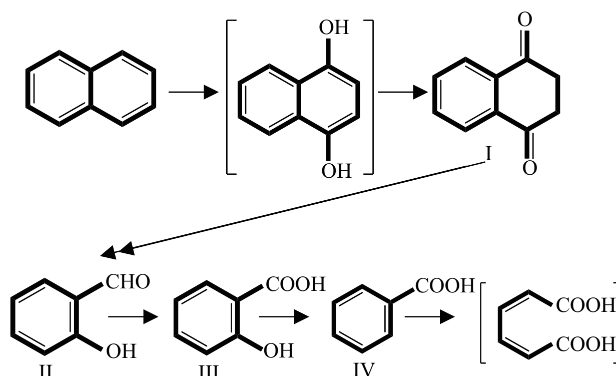

Fig. 4 Proposed pathway for the degradation of naphthalene by *Armillaria* sp. F022. The metabolites in brackets have not been identified in our culture extracts.

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acid (Lin et al., 2010). In our study, phthalic acid was not identified in the culture extract and growth on phthalic acid was also negative. Furthermore, whereas in bacteria, phthalic acid acts as inducer for naphthalene degradation pathway, this was not observed in white rot fungus *Armillaria* sp. F022. Also in current study, catechol and *cis,cis*-muconic acid were not detected in culture extract and spectrophotometrically indicating β-ketoadipate pathways is not playing an important role during naphthalene metabolism.

3 Conclusions

Armillaria sp. F022, a white rot fungus collected from tropical rain forest produced several enzymes (manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase) that are playing an important role in metabolism of naphthalene. Analysis of culture liquids showed that *Armillaria* sp. F022 grown on naphthalene did not accumulate any intermediates throughout the cultivation period and all detected metabolites except benzoic acid were present in slight concentrations. 1,4-Naphthoquinone, 2-hydroxybenzaldehyde and salicylic acid ad benzoic acid were detected as metabolic products of naphthalene degradation by *Armillaria* sp. F022. Naphthalene intermediates and the products of their autoxidation accumulating in the environment can be toxic to the degraders themselves and to the environmental microflora in general. This should be taken into account when choosing efficient PAH degraders for bioremediation. Capability of *Armillaria* sp. F022 to degrade PAH at high temperature efficiently may make it an ideal candidate to threat oil-contaminated soils. However, incomplete naphthalene degradation may still have threat to ecosystem or even worse the metabolic products would be more toxic than the parent compound.

Acknowledgments

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