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# Microbial Degradation of *n*-Eicosane by Filamentous Fungi

Tony HADIBARATA[1,2] and Sanro TACHIBANA[1]

[1]*Department of Applied Bioscience, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan*
[2]*Laboratory of Pulp, Paper and Environment, Department of Forest Product Technology, Mulawarman University, Kampus Gunung Kelua, Jl. Ki Hajar Dewantara No. 1, Samarinda 75119, Indonesia*

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**Abstract**—The degradation of *n*-eicosane by *Trichoderma* sp. S019, a fungus collected from petroleum-contaminated soil with extensive degradation capability on an agar medium containing *n*-eicosane, was demonstrated in liquid medium and in soil. The optimum carbon source on degradation (73%) was obtained when *Trichoderma* sp. S019 was incubated with glucose for 30 days while the optimum nitrogen source for degradation (63%) was obtained in culture with the addition of polypeptone. Furthermore, the degradation of *n*-eicosane was affected by the addition of carbon and nitrogen source. *n*-Eicosane was indeed degraded by *Trichoderma* sp. S019 because nonadecanoic acid considered to be the metabolite in the biodegradation of *n*-eicosane, were detected among the reaction products.

Keywords: *n*-eicosane, aliphatic hydrocarbon, filamentous fungus

## INTRODUCTION

The majority of molecules in petroleum oil spills and refined products are biodegradable, and they will finally fade away from the environment as they are utilized by microorganisms (Prince, 1993). These are usually considered to be weakly biodegradable compounds compared with most of the organic molecules concerned in the biological carbon cycle (Bertrand *et al*., 1989). Crude oil is mainly composed of hundreds of different hydrocarbon molecules, mainly alkanes from C1 to C40 straight chain, C6–C8 branched-chain, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen (Stafford *et al*., 1982).

Of the aliphatic hydrocarbons in petroleum hydrocarbon mixture, it is *n*-alkanes that are most quickly degraded in both laboratory cultures and in the environment. Most of the microorganisms convert *n*-alkanes to matching alkan-1-ol by means of a monooxygenase enzyme (Betts, 1993). The enzymatic actions of filamentous fungi are highly regulated by nutrients. Most detailed studies have been carried out mainly on several filamentous fungi such as *Trichoderma*

species. In this fungus, nitrogen, carbon and sulfur act as the limiting factors of the enzyme synthesis (Jeffries *et al*., 1981). In particular, manganese and nitrogen were found to have strong regulatory effects (Kirk and Farell, 1987).

Alkanes are normally degraded by terminal oxidation to alcohol and the fatty acids, with subsequent entry to the $\beta$-oxidation pathway. Sub terminal oxidation to secondary alcohol may also occur (Prince, 1993). Degradation mechanism of alkane was examined which showed that the chain lengths of alkane substrates had a pronounced effect on the total cellular fatty acid composition. An alkane with an odd number of C atoms yielded predominantly fatty acids with an even number of C atoms, and *vice versa*. Alkane was altered through an odd number carbon during its transformation to fatty acid (Aeckersberg *et al*., 1991).

In the natural environment, a spill containing only one fraction of oil such as aromatics or alkane compounds is unlikely (Atlas, 1981). However, the study on microbial breakdown of groups of pure *n*-alkanes is important from a scientific point of view since there is lack of information in this field, particularly in the area which deals with specific microorganisms and their relative effectiveness in the breakdown of long chain hydrocarbon (Fayad and Overton, 1995). In the present report, we describe the results of an investigation of the degradation of *n*-eicosane by *Trichoderma* sp. S019, a fungus collected from petroleum-contaminated soil and capable of degrading polycyclic aromatic hydrocarbons (PAHs). The catabolic pathway for *n*-eicosane was also examined.

## MATERIALS AND METHODS

### *Chemicals*

*n*-Eicosane and nonadecanic acid were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Malt extract and polypeptone were acquired from Difco (Detroit, USA). Thin layer chromatography (TLC) aluminium sheets (Silica gel 60 $F_{254}$, $20 \times 20$ cm) were obtained from Merck (Darmstadt, Germany). The silica gel used for column chromatography (Wakogel S-1) and all other chemicals were purchased from Wako Pure Chemical Industry Co. Ltd. (Osaka, Japan).

### *Fungi and medium*

The hydrocarbonoclastic fungus *Trichoderma* sp. S019 was originally isolated from soil. The fungus is able to grow on alkanes, particularly on *n*-eicosane as its sole source of carbon and energy. *Trichoderma* sp. S019 was isolated from soil at the Campus of the Faculty of Agriculture, Ehime University. A 5-mm disk of selected fungi was aseptically inoculated into a 100-ml Erlenmeyer flask containing 20 ml of mineral salt broth (MSB) containing (in g/l distilled water): glucose (10), $KH_2PO_4$ (2), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.1), Ammonium tartrate (0.2) and trace elements (10 ml) as described by Arora and Gill (2001). Each flask was incubated for 15 or 30 days at 25°C in the dark. After autoclaving for 20 minutes at 120°C, 1 mM of *n*-eisosane dissolved in 1 ml of dimethylformamyde containing

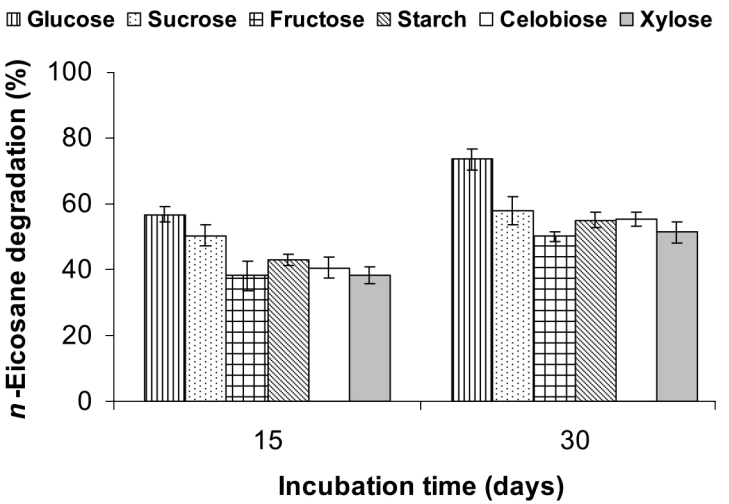


Fig. 1. Effect of carbon sources on degradation of *n*-eicosane in liquid medium by *Trichoderma* sp. S019.

100 $\mu$L of tween 80 was added to the flask. Control experiments were performed by incubating MSB medium with the test alkane without the inoculum. All assays were conducted in triplicate. Glucose, sucrose, fructose, starch, cellobiose, and xilose were used as carbon sources. Ammonium tartrate, ammonium nitrate, yeast extract and polypeptone were used as nitrogen sources. All media were sterilized by autoclaving at 120°C for 20 minutes.

*Preparation and identification of metabolites*

After seven days of growth, the cells were removed by centrifugation and the supernatants were extracted with three volumes of ethyl acetate. The aqueous fractions after extraction were acidified with concentrated hydrochloric acid to pH 2 and extracted again with three volumes of ethyl acetate. The organic extracts were dried over anhydrous sodium sulfate and the solvent was removed in vacuum at room temperature. *n*-Eicosane and their degradation products were separated by column chromatography using S-1 silica gel. 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). GC-MS was performed with a column 30 m in length and 0.25 mm in diameter, and a helium pressure of 100 kPa. The temperature was initially 80°C, held for 2 minutes, raised from 80°C to 200°C at 20°C/min, then to 260°C at 7.5°C/min, and held for 4 minutes. The flow rate was 1.5 ml/min, interface temperature was 260°C, and injection volume was 1 $\mu$l. Degree of degradation was determined by comparison of the remaining *n*-eicosane between control and samples. As the compound for authentication, the

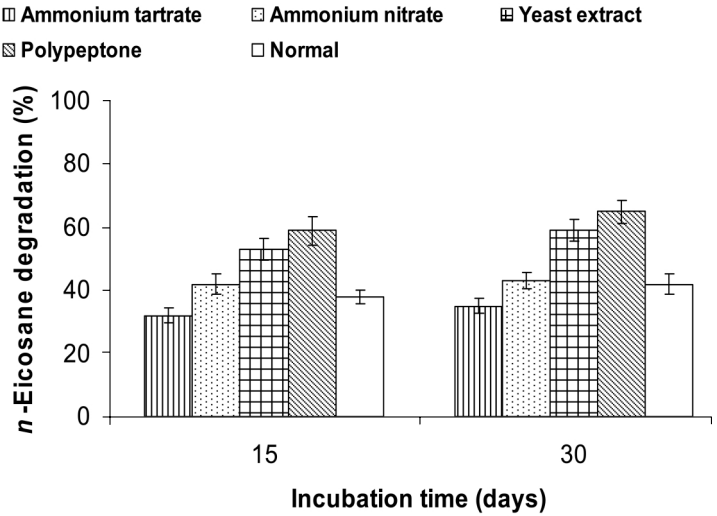


Fig. 2. Effect of nitrogen sources on degradation of *n*-eicosane in liquid medium by *Trichoderma* sp. S019.

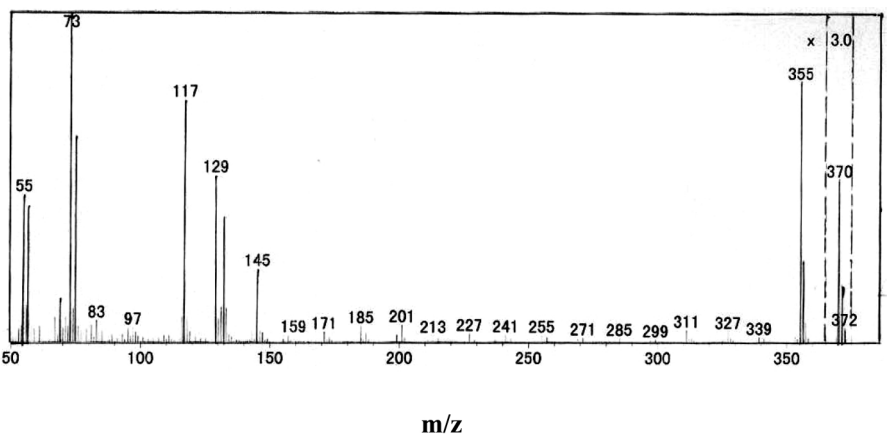


Fig. 3. Mass spectral profiles of nonadecanoic acid produced from *n*-eicosane by *Trichoderma* sp. S019.

hexadecanoid acid, could not be detected directly by GC/MS, an analytical derivatization procedure was used to detect these compounds with GC/MS; these compounds were subjected to trimethylsilylation (TMS). Similarly, extracts from *n*-eicosane-grown cultures were also derivatized and subjected to tests for the presence of these three compounds.

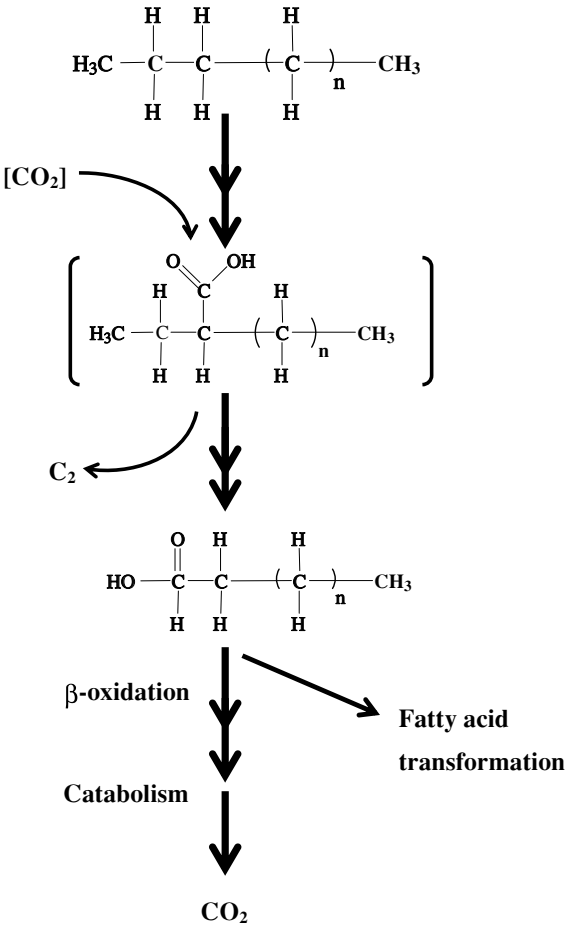


Fig. 4. A proposed pathway for the degradation of *n*-eicosane by *Trichoderma* sp. S019.

## RESULTS AND DISCUSSION

### *Degradation of n-eicosane by selected fungi*

Figure 1 shows the effect of different carbon sources on the degradation of *n*-eicosane in soil by *Trichoderma* sp. S019. The lowest degradation rate was observed with fructose (50%) after 30 days of incubation. Degradation of *n*-eicosane with glucose was the best, at 73%. Addition of ammonium tartrate, ammonium nitrate, yeast extract and polypeptone were performed in order to understand the effect of a nitrogen source on the degradation of the compound and the results are shown in Fig. 2. *Trichoderma* sp. S019 degraded 35, 43, 59, and 65% of *n*-eicosane with addition of ammonium tartrate, ammonium nitrate, yeast

extract and polypeptone in the cultures for 30 days. The highest rate of degradation was 65% when *Trichoderma* sp. S019 was incubated in the culture with polypeptone for 30 days. It has been reported that production of enzyme by fungi in the medium supplemented with nutrients was high (Asther *et al*., 1987). Since fructose is a poor carbon source compared to glucose, growth on glycerol leads to carbon limitation which affects the onset of secondary metabolism.

Al-Hadhrami *et al*. (1996) showed that addition of an alternative carbon and nutrient source, such as mollase, increased respiration and *n*-alkane degradation in synthetic seawater. We suggest that the addition of carbon and nitrogen sources such as glucose and polypeptone, increased the metabolic activity, and thereby stimulated the production of enzymes effective in alkane's degradation. This result is also similar to that of Khleifat (2006) who found that the rate of degradation of a substrate increased after addition of a carbon source to the medium. Carbon sources at 0.2%, except fructose, allowed phenol degradation to proceed faster at ranges between 1.4-fold and 3-fold higher than that of the control, and in all cases the growth of the microorganism was in accordance with the standard microbial batch growth culture.

*Identification of metabolites*

The cellular fatty acid composition of *Trichoderma* sp. S019 was clearly affected by the chain length of the alkane substrate. *Trichoderma* sp. S019, grown in MSB liquid medium with *n*-eicosane for 15 days was able to mineralize *n*-eicosane to form nonadecanoic acid (C-odd). Two peaks having a GC retention time at 8.9 minutes were detected. The GC retention time at 8.9 minutes, MS properties of the $M^+$ at *m/z* 370, and fragment ions at *m/z* 355 [$M^+$-15], corresponding to the respective sequential losses of methyl (-$CH_3$), as well as the expected fragment ions at 145, 117 and 73 [$(CH_3)_3Si$] were identical to those of authentic nonadecanoic acid (Fig. 3).

The filamentous fungi convert alkanes to fatty acids and incorporate them into cellular lipids, a metabolic process observed in many aerobic alkane-degrading organisms. The carbon numbers of fatty acids also correlate with those of the alkane substrate. A C-odd alkane yielded predominantly C-even fatty acids, and a C-even alkane yielded C-odd fatty acids. The initial attack on alkane involves a novel mechanism in which an odd-numbered carbon was added to or removed from the alkane chain (Aeckersberg *et al*., 1991; Doumenq *et al*., 2001). In this study, we confirmed that *n*-eicosane was oxidized to nonadecanoic acid by *Trichoderma* sp. S019. It was suggested that C-even alkanes are indeed transformed to C-odd fatty acids by *Trichoderma* sp. S019 (Fig. 4).

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T. Hadibarata (e-mail: thadibrata@fahutan.unmul.ac.id) and S. Tachibana