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Detection of Oleic Acid Biodegradation by Fungi

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Abstract To investigate oleic acid biodegradation, 47 fungal strains were tested with modified Czapek Dox broth media containing oleic acid, and their biodegradative activities were assayed by measuring the release of [^{14}C]CO_2 from the ^{14}C-labeled oleic acid. After 72 h of cultivation, *Aspergillus flavus*, *Aspergillus ochraceus*, and *Alternaria* species metabolized approximately 25% to 35% of the supplied oleic acid. The relationship between the fungal degradation of oleic acid and the fungal growth was also examined using 7 strains of *Aspergillus niger*. *A. niger* YMC 0100 and YMC 0322 degraded about 26% of the oleic acid after 72 h, while their germination ratios were more than 30%.

Key words: Oleic acid, biodegradation, fungi, modified Czapek Dox Broth media, *Aspergillus niger*, germination

Molokwu and Okpokwasili [11] recently reported on the ability of microorganisms (bacteria, moulds, and yeast) to use organic compounds found in vegetable oils as a source of cellular carbon and energy. In their study, the growth of pure cultures of certain mould species (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp.) and a mixed yeast culture (*Saccharomyces* sp., *Candida* sp., and *Hansenula* sp.) were monitored in the presence of vegetable oils containing lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, and linoleic acid as substrates, and the biodegradabilities of the six vegetable oils by the fungi were shown by gas chromatograms of the fatty acid components of the vegetable oil samples. Sowunmi [15] also reported an increase in the mycelial dry weight of *A. niger* and *A. flavus* growing on palm oil including oleic acid. Evidences, which show that *Aspergillus nidulans* is able to grow on oleic acid as the sole carbon source [5] and that two enzyme activities, acyl-CoA oxidase and acyl-CoA dehydrogenase, are involved in the first step of oleate-induced β-oxidation [18], have also been demonstrated. Evers *et al.* [7] and Karpichev *et al.* [9] showed the influence of specific growth conditions on the induction of β-oxidation enzymes and rate of microbody (peroxisome) proliferation in *Saccharomyces cerevisiae* by oleic acid. However, in spite of the wide range of studies on the β-oxidation of fatty acids, most investigations have focused on the peroxisomal enzyme-dependent β-oxidation machinery in yeast species such as *Saccharomyces* or *Candida* [6, 10]. Moreover, comparisons of fungal oxidative capacities on the basis of enzyme production are considerably time consuming, especially if the fungi must be obtained in a pure culture and the enzymes must be isolated for a direct assay. As such, the current study was intended to evaluate the potential for oleic acid biodegradation by individual fungal strains isolated from indoor dust and air and examine the relationship between the fungal degradation of oleic acid and the fungal growth.

A total of 47 fungal strains were used which were isolated from a housing environment, such as indoor dust and air. All the strains were maintained on potato-dextrose agar (Difco, Detroit, MI, U.S.A.) slants that contained 30 μg/l chloramphenicol (Wako Pure Chemical, Tokyo, Japan) to suppress any bacterial growth [12, 16]. Conidia or mycelia were produced in bulk by growth on the same medium at 25°C for 7–10 days in the dark.

Using a similar method as previously described [12, 13], modified Czapek Dox broth (CzDB) media (CzDB consists of $NaNO_3$, 2.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01 in grams per liter of distilled H_2O, pH 7.0) were prepared. The [1-^{14}C]oleic acid (specific activity, 51.0 mCi/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, Mass, U.S.A.) and had a purity of greater than 98%. ^{14}C-labeled oleic acid and nonlabeled oleic acid (Kanto Chemical, Tokyo, Japan) were added to the CzDB media at a final concentration of 0.05% (v/v)

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and 0.5% (v/v), respectively, as the sole source of carbon. Approximately 55,000 dpm of the ^{14}C-labeled substrate was finally included in the medium. For the fungal spore suspension [17], a sterilized Tween® 80 (polysorbate 80, Difco) solution was also added to the screening medium at 0.02% (v/v) of the final concentration. Although Tween® 80 is not an inert surfactant, but rather an oleic acid ester that may be used by some fungi for growth [14], such a possibility was excluded in the current study.

As described in a previous study by the current authors [13], biodegradation was defined as the evolution of [^{14}C]CO_2, as determined by a liquid scintillation counter (LSC, 1450 MicroBeta TRILUX; Wallac, Turku, Finland). Briefly, the fungal strains were suspended in the CzDB screening media, and the suspensions of 1.0×10^6 fungal spores were then inoculated into each well of a 24-well plate (Corning Costar Corporation, Cambridge, Mass, U.S.A.) in duplicate. The well plate was covered with a sheet of Parafilm®, in which 24 holes had been punched with a ticket punch so that the holes were centered over each well. The Parafilm® was firmly pressed against the rims of the wells to form a shallow depression. A paper disk was then placed over each hole and moistened with 500 µl of 1 N NaOH solution. The plate was covered with another sheet of Parafilm®, followed by a rubber sheet of the same size. The entire apparatus was then finally secured together with several rubber bands and incubated at 25°C in the dark for 12, 24, 36, 48, and 72 h. After incubation, the paper disks were transferred into scintillation vials containing 1 ml of liquid scintillation cocktail (Ultima Gold™, A Packard Bioscience Company, Meriden, CT, U.S.A.), and the dpm value of the adsorbed [^{14}C]CO_2 was then counted using an LSC. The percentage of biodegradation was determined as the percentage of the dpm value in the evolved [^{14}C]CO_2 to the 55,000 dpm of the initially supplied [1-^{14}C]oleic acid. Negative (blank) controls were defined as the wells without any fungal cell inoculation.

The term spore germination was here intended to include both germinated spores after swelling and elongated spores [2, 3]. In the experiments for detecting fungal growth, CzDB media containing only 0.5% (v/v) nonradioactive oleic acid were prepared. Among the 47 fungal strains tested in the above biodegradation assay, 7 strains of *A. niger* were used with the following strain numbers: YMC (Yonsei Medical Center) 0058, YMC 0061, YMC 0062, YMC 0100, YMC 0160, YMC 0244, and YMC 0322. In brief, the fungal spore suspensions (1.0×10^6 cells) were inoculated into each well of a 12-well plate and incubated at 25°C in the dark. After 12, 24, 36, 48, and 72 h of incubation, the number of germinated spores was counted using a hemacytometer under a microscope.

The 47 fungal strains used in the current study exhibited variable metabolic capacities to decompose the substrate supplied, showing a remarkable variation in their ability to evolve [^{14}C]CO_2 from [1-^{14}C]oleic acid. The oleic acid degradation by 10 strains of *Aspergillus* species grown in the modified CzDB medium containing oleic acid as the sole carbon source is described in Table 1. The biodegradation efficiencies of the two *A. ochraceus* strains (YMC 0144 and 0245) noticeably increased after 48 h, reaching 31% and 21% after 72 h. *Aspergillus flavus* YMC

Table 1. Oleic acid degradation by 40 fungal strains grown in oleic acid-CzDB medium.

Fungus	YMC[a] #	Oleic acid degradation			
		24 h	36 h	48 h	72 h
Aspergillus flavus	0065	–[b]	–	+++	++++
	0269	–	–	++	++++
A. fumigatus	0064	–	–	++	+++
	0270	–	–	++	++
A. oryzae	0063	–	–	+	+++
	0271	–	–	+	++
A. ochraceus	0144	–	+	++++	++++
	0245	–	+	++++	+++
A. versicolor	0049	–	–	+	++
	0067	–	–	+	++
Candida albicans	0078	–	–	+	+
Saccharomyces cerevisiae	0079	+	+++	++++	+++
Rhodotorula rubra	0080	+	++	+++	++
Alternaria sp.	0136	–	–	+	++++
A. alternata	0150	–	–	+	+++
Fusarium solani	0169	–	+	++	+++
F. oxysporum	0218	–	–	++	++
F. camptoceras	0212	–	–	+	+
F. graminearum	0215	–	+	++	+
Trichoderma viride	0082	–	–	++	++
	0238	–	+	++	+++
Chaetomium sp.	0099	–	+	++	+++
	0129	–	+	++	+++
Penicillium citrinum	0016	–	–	+	++
P. viridicatum	0018	–	–	–	++
P. commune	0020	–	–	–	++
P. chrysogenum	0023	–	–	+	++
P. citrero-viride	0031	–	–	+	++
P. frequentans	0033	–	–	+	++
P. expansum	0035	–	–	–	++
P. funuculosum	0040	–	–	+	+
P. italicum	0041	–	–	+	+
Cladosporium herbarum	0162	–	–	–	+
C. cladosporioides	0077	–	–	–	+
	0253	–	–	–	+
C. macrocarpum	0167	–	–	–	++
C. sphaerospermum	0076	–	–	–	+
	0241	–	++	++	++
Aureobasidium pullulans	0060	–	–	+	+
	0074	–	–	+	++

[a]YMC is an abbreviation for Yonsei Medical Center.
[b]–, less than 5% biodegradation; +, 5% to 10%; ++, 10% to 15%; +++, 15% to 20%; ++++, more than 20%.

0269 showed the highest degradation efficiencies (34%) of oleic acid after 72 h, while *A. fumigatus*, *A. oryzae*, and *A. versicolor* showed 10% to 17% biodegradation.

As shown in Table 1, there was a considerable difference in the oxidative abilities among the 3 yeast-like strains grown in the oleic acid-CzDB medium. *Saccharomyces cerevisiae* and *Rhodotorula rubra* showed significant oleic acid biodegradation (24% and 16%) after 48 h, while the degradation by *Candida albicans* was less than 8% even after 72 h of incubation. In contrast, the 2 species of *Alternaria* (YMC 0136 and YMC 0150) degraded only a little amount of oleic acid up to 48 h, however, substantial oleic acid biodegradation (25% and 16%) was found after 72 h. The 2 species of *Chaetomium* (YMC 0099 and YMC 0129) showed about 17% degradation of oleic acid, while the oleic acid oxidation occurred by only *F. solani* among the 4 species of *Fusarium* and became greater than 15% after 72 h. The 2 strains of *Trichoderma viride* (YMC 0082 and YMC 0238) and 7 species of *Penicillium*, except for *P. funiculosum* and *P. italicum*, showed 10% to 15% biodegradation, whereas the biodegradation efficiencies by the other 10 strains, including the 2 strains of *Cladosporium cladosporioides* and *Cladosporium herbarum*, were 13% or below.

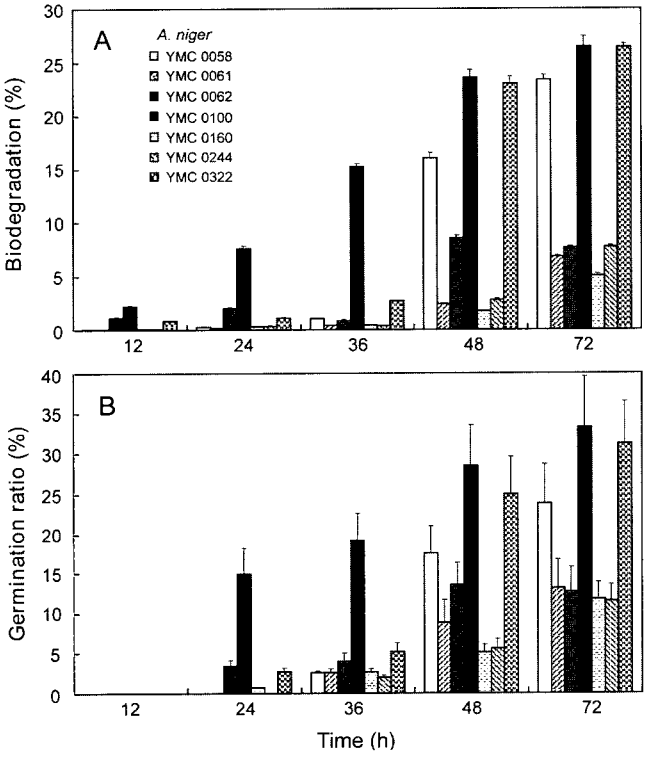

Fig. 1. Fungal degradation of oleic acid and fungal growth on oleic acid.

(A) Biodegradation of oleic acid by 7 strains of *A. niger* grown in the oleic acid-CzDB medium over 12, 24, 36, 48, and 72 h, (B) Germination ratios of 7 strains of *A. niger*. Experimental results were expressed as the mean±standard deviation.

Baltazar *et al.* [4] earlier demonstrated that certain strains of *Aspergillus niger* could grow on glyceryl trioleate and measured the activities of β-oxidation enzymes in the extracts of glucose- and triolein-grown cells of *A. niger*. As shown in Fig. 1A, the oleic acid degradation by *A. niger* YMC 0100 approached a 15% degradation after incubation for 36 h, while only a little amount of oleic acid was degraded by *A. niger* YMC 0061, YMC 0062, YMC 0160, and YMC 0244. The biodegradation efficiencies of *A. niger* YMC 0058 and YMC 0322 significantly increased after 48 h, reaching 16 and 10 times greater than after 36 h, respectively. After incubation for 72 h, *A. niger* YMC 0100 and YMC 0322 showed significant oleic acid biodegradation (26%, both). The oleic acid degradation by *A. niger* YMC 0058 reached 23% after 72 h, while the other 4 strains of *A. niger* showed 5% to 7% biodegradation.

The possible relationship between the fungal degradation of oleic acid and the fungal growth on oleic acid was examined using 7 strains of *A. niger*. When grown in the CzDB media supplemented with oleic acid, all the strains germinated after 36 h (Fig. 1B). In the case of *A. niger* YMC 0100, the relative ratio of germinated spores approached approximately 15% after 24 h and reached more than 33% after 72 h. The relative ratios of germination in *A. niger* YMC 0058 and YMC 0322 significantly increased after 48 h, and were about 7 and 5 times greater than after 36 h, respectively. After 72 h, *A. niger* YMC 0058 and YMC 0322 showed significant spore germination (24% and 31%), whereas the spores of the other 4 strains showed only 11% to 13% germination.

A study to detect fungal growth was conducted to correlate biochemical events such as fatty acid oxidation with morphological events of the spore germination. The fungal mycelia were clearly observed to grow in the oleic acid supplementation as a carbon source, whereas all the *A. niger* strains showed poor or little growth in the CzDB media without oleic acid (data not shown). This observation confirmed the involvement of the fungal metabolic capacity and spore germination in the biodegradation of oleic acids. While several reports described fatty acid oxidation, no previous study on the correlation of fungal metabolism with spore germination has been found. The fact that the germination of conidiospores of *A. niger* was stimulated by the addition of certain carbohydrates (glucose, sucrose, and fructose) and nitrogenous compounds (glutamic acid and valine) to the growth medium has earlier been shown [1]. When these sugars or nitrogenous compounds were added for the spore germination of the fungus, germination started within 6 or 9 h of incubation. Moreover, Hagerman *et al.* [8] found that the production of enzymes, such as protease, cellulase, or pectinase, was related to the extent of fungal spore germination.

The current study demonstrated that the detection of oleic acid degradation was relatively simple and effective

when a well plate equipped with filter papers as CO_2 traps was used. Furthermore, the direct screening using a 24-well plate with oleic acid-CzDB media gave acceptable results in terms of detection and comparison of oleic acid biodegradation by fungi. The oleic acid-CzDB media used in the present study made it possible for rapid examination of a particular fungal biodegradative capability of oleic acid, and facilitated the identification of fungi that evolved carbon dioxide from the supplied oleic acids. Accordingly, the screening method and media used in the current study could also be used in surveys of many fungal strains and several fatty acids to evaluate their biodegradation potential.

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REFERENCES

1. Abdel-Rahim, A. M. and H. A. Arbab. 1985. Nutrient requirements in germination of conidiospores of *Aspergillus niger* V. Tieghen. *Mycopathologia* **92:** 111–113.
2. Aderiye, B. I., S. K. Ogundana, S. A. Adesanya, and M. F. Roberts. 1989. The effect of β-sitosterol on spore germination and germ-tube elongation of *Aspergillus niger* and *Botryodiplodia theobromae*. *Int. J. Food Microbiol.* **8:** 73–78.
3. Anderson, J. G. and J. E. Smith. 1972. The effects of elevated temperatures on spore swelling and germination in *Aspergillus niger*. *Can. J. Microbiol.* **18:** 289–297.
4. Baltazar, M. F., F. M. Dickinson, and C. Ratledge. 1999. Oxidation of medium-chain acyl-CoA esters by extracts of *Aspergillus niger*: Enzymology and characterization of intermediates by HPLC. *Microbiology* **145:** 271–278.
5. De Lucas, J. R., S. Valenciano, A. I. Dominguez, G. Turner, and F. Laborda. 1997. Characterization of oleate-nonutilizing mutants of *Aspergillus nidulans* isolated by the 3-amino-1,2,4-triazole positive selection method. *Arch. Microbiol.* **168:** 504–512.
6. el-Sharkawy, S. H., W. Yang, L. Dostal, and J. P. Rosazza. 1992. Microbial oxidation of oleic acid. *Appl. Environ. Microbiol.* **58:** 2116–2122.
7. Evers, M. E., J. Hohfeld, W. H. Kunau, W. Harder, and M. Veenhuis. 1991. Physiological studies on the utilization of oleic acid by *Saccharomyces cerevisiae* in relation to microbody development. *FEMS Microbiol. Lett.* **69:** 73–78.
8. Hagerman, A. E., D. M. Blau, and A. L. McClure. 1985. Plate assay for determining the time of production of protease, cellulase, and pectinases by germinating fungal spores. *Anal. Biochem.* **151:** 334–342.
9. Karpichev, I. V., Y. Luo, R. C. Marians, and G. M. Small. 1997. A complex containing two transcription factors regulates peroxisome proliferation and the coordinate induction of β-oxidation enzymes in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **17:** 69–80.
10. Kunau, W. H. and A. Hartig. 1992. Peroxisome biogenesis in *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* **62:** 63–78.
11. Molokwu, C. N. and G. C. Okpokwasili. 1997. Biodeterioration potentials of fungal isolates from vegetable oils. *Int. J. Food Sci.* **48:** 251–255.
12. Park, J.-C., D.-W. Han, Y.-S. Hwang, H.-J. Lee, K. Takatori, and H. Suh. 1999. Determination of a favorable medium for detection of fungal extracellular protease. *Biocontrol Sci.* **4:** 91–95.
13. Park, J.-C., D.-W. Han, B. J. Park, D. H. Lee, K. Takatori, and H. Suh. 2001. Effective screening medium for the biodegradation of oleic acid by *Aspergillus niger*. *Biocontrol Sci.* **6:** 37–41.
14. Rao, K. K. and S. Rao. 1975. Effect of tweens on the production of ergot alkaloids by *Aspergillus fumigatus*. *Folia Microbiol.* **20:** 418–422.
15. Sowunmi, O. 1981. The effects of *Aspergillus niger* (van Teigh) and *Aspergillus flavus* (Link ex fr.) growing on palm oil. *Nig. Stored Prod. Res. Inst. Tech. Rep.* pp. 87–91.
16. Tsao, P. H. 1970. Selective media for isolation of pathogenic fungi. *Annu. Rev. Phytopathol.* **8:** 157–186.
17. Umesaki, Y., Y. Kawai, and M. Mutai. 1977. Effect of Tween 80 on glucosyltransferase production in *Streptococcus mutans*. *Appl. Environ. Microbiol.* **34:** 115–119.
18. Valenciano, S., J. R. De Lucas, A. Pedregosa, I. F. Monistrol, and F. Laborda. 1996. Induction of β-oxidation enzymes and microbody proliferation in *Aspergillus nidulans*. *Arch. Microbiol.* **166:** 336–341.