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Note

Effective Screening Medium for the Biodegradation of Oleic Acid by *Aspergillus niger*

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To investigate oleic acid biodegradation, 7 strains of *Aspergillus niger* were tested with 3 different types of Czapex-Dox broth (CzDB) medium containing oleic acid, and their metabolic abilities to decompose the fatty acid into carbon dioxide and water were compared. When the fungal strains were grown in the CzDB media with both ^{14}C-labeled and non-labeled oleic acid, *A. niger* YMC 0100 and YMC 0322 oxidized more than 58% of the supplied substrate within 72 h. The addition of saccharose as an additional carbon source substantially reduced the biodegradation of oleic acid to the point that all the strains showed less than 4% degradation.

Key words : Oleic acid/Biodegradation/*Aspergillus niger*/Czapek Dox broth medium/Saccharose.

There is considerable interest in utilizing abundantly available natural resources such as corn oil as renewable feedstock in the preparation of useful chemicals. Plant oils are also rich sources of materials, including mixtures of glycerides, fatty acids, glycerol, tocopherols, and various sterols, for use as potential chemical feedstock (Eckey and Miller, 1954). In recent years, microbiological transformations of corn oil and compounds that are readily derived from this renewable lipid feedstock have been investigated (Koritala et al., 1989; el-Sharkawy et al., 1992). Oleic acid (cis-Δ^9-octadecenoic acid) is a major component of corn oil, representing nearly 30 to 40% of the fatty acids present in triglyceride mixtures (Eckey and Miller, 1954). As a useful carbon source, oleic acid has been envisioned as a substrate capable of conversion to a variety of potentially valuable derivatives by both chemical and microbiological means. It is well understood that *Aspergillus nidulans* is able to grow using oleic acids as the sole carbon source and growth using oleic acid is associated with the development of many microbodies (peroxisomes) that are scattered throughout the cytoplasm of the cells (Valenciano et al., 1996 and 1998). In spite of a very broad range of studies on the microbial degradation of fatty acids, most investigations have not focused on a single microorganism that grows in the normal environment, but on undefined mixed microbial cultures. This study is intended to detect and compare the potential for oleic acid oxidation by *Aspergillus niger* grown in Czapek Dox broth (CzDB) medium containing oleic acid and to examine whether the use of saccharose as another carbon source has effects on the fungal degradation of oleic acid or not.

Seven strains of *A. niger* used in this study had been isolated from indoor dust and air and kept as stock cultures in our laboratory. They were all non-pathogenic, and their strain numbers were as follows: YMC (Yonsei Medical Center) 0058, YMC 0061, YMC 0062, YMC 0100, YMC 0160, YMC 0244, and YMC 0322, which were identified by their mycological characteristics (Raper and Fennell, 1965). All the strains were maintained on potato-dextrose agar (Difco, Detroit, U.S.A.) slants supplemented with 30 μg/l chloramphenicol (Wako Pure Chemical, Tokyo,

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Japan) in order to suppress bacterial growth (Tsao, 1970). Conidia or mycelia were produced in bulk by growth on the same medium at 25℃ for 7-10 days in the dark.

By a previously described method (Lee et al., 1996; Park et al., 1999), 3 different types of CzDB medium (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. All reagents of the highest grade available were purchased from Sigma (St. Louis, Mo., USA). [1-^{14}C]oleic acid (specific activity, 51.0 mCi/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, U.S.A.) and had a purity of greater than 98%. In one type of medium (Type A medium), only ^{14}C-labeled oleic acid was added to each liquid medium at a final concentration of 0.05% (v/v), as the sole source of carbon. Another type of medium (Type B medium) contained non-labeled oleic acid (Kanto Chemical, Tokyo, Japan) at 0.5% (v/v) of the final concentration in addition to the radiochemical. The third type of medium (Type C medium) was composed of Type B medium supplemented with 0.25% (w/v) saccharose (Difco) as an additional carbon source. Approximately 110,000 dpm of the ^{14}C-labeled substrate was finally contained in each medium. For the fungal spore suspension, a sterilized Tween 80 (polysorbate 80, Difco) solution was also added to all types of the screening medium at 0.02% (v/v) of the final concentration (Tomita et al., 1998; Umezaki et al., 1977). Although it is known that Tween 80 is not an inert surfactant but an oleic acid ester that may be used by some fungi for growth (Rao and Rao, 1975), such possibility is excluded here.

Biodegradation was defined as evolution of [^{14}C]CO_2 as determined by a liquid scintillation counter (LSC, 1450 MicroBeta TRILUX; Wallac, Turku, Finland). A schematic diagram for the biodegradation experiments is shown in Fig. 1. Fungal strains were respectively suspended in the 3 types of the CzDB screening media, and the suspensions of 1.0×10^6 fungal spores were inoculated into each well of a 24-well plate (Corning Costar Corporation, Cambridge, U.S.A.) in duplicate. Each well plate was covered with a sheet of Parafilm, in which 24 holes had been punched with a ticket punch, and the Parafilm was tightly pressed against the rims of the wells to form a shallow depression. Carbon dioxide traps were established by placing paper disks presoaked with 0.5 ml of a 1 N NaOH solution on the center of the holes. The plates were covered with another sheet of Parafilm, followed by a rubber sheet of the same size. The entire apparatus was finally secured together with several rubber bands and then incubated at 25℃ in the dark for 12, 24, 36, 48, and 72h. After incubation, the paper disks were transferred to scintillation vials containing 1 ml of a liquid scintillation cocktail (Ultima Gold™, A Packard Bioscience Co., Meriden, USA), and the dpm value of adsorbed [^{14}C]CO_2 was determined with the LSC. The percentage of biodegradation was determined as the percentage of dpm value in the evolved [^{14}C]CO_2 to the 110,000 dpm of the initially supplied [1-^{14}C]oleic acid. Negative controls (blank) were considered as the wells without the fungal spore inoculation.

A study to detect oleic acid degradation by 7 strains of *A. niger* was examined to correlate biochemical events such as fatty acid oxidation with the physical events of CO_2 evolution. Each of the *A. niger* strains tested in this study maintained the individual capability for oleic acid biodegradation and revealed marked variation in their abilities to bring about the evolution of [^{14}C]CO_2 according to the 3 types of oleic acid-CzDB medium. Molokwu and Okpokwasili (1997) have reported the ability of microorganisms (bacteria, mold and yeast) to use organic compounds found in vegetable oils as sources of cell carbon and energy and investigated the biodeterioration potentials of

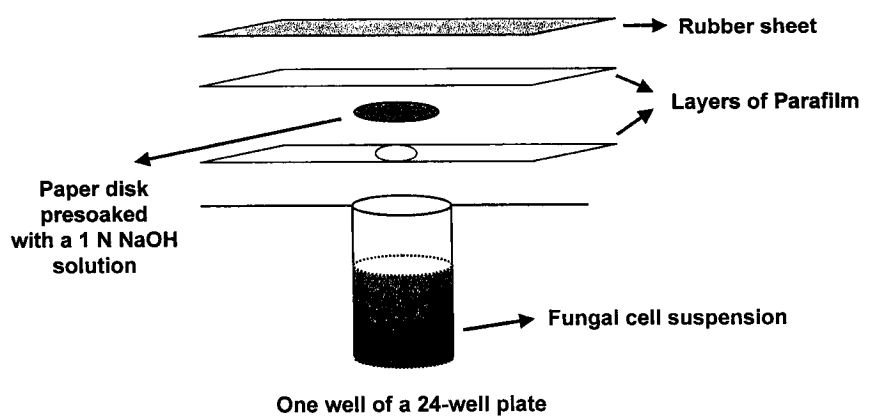

FIG. 1. A schematic diagram for the biodegradation experiments.

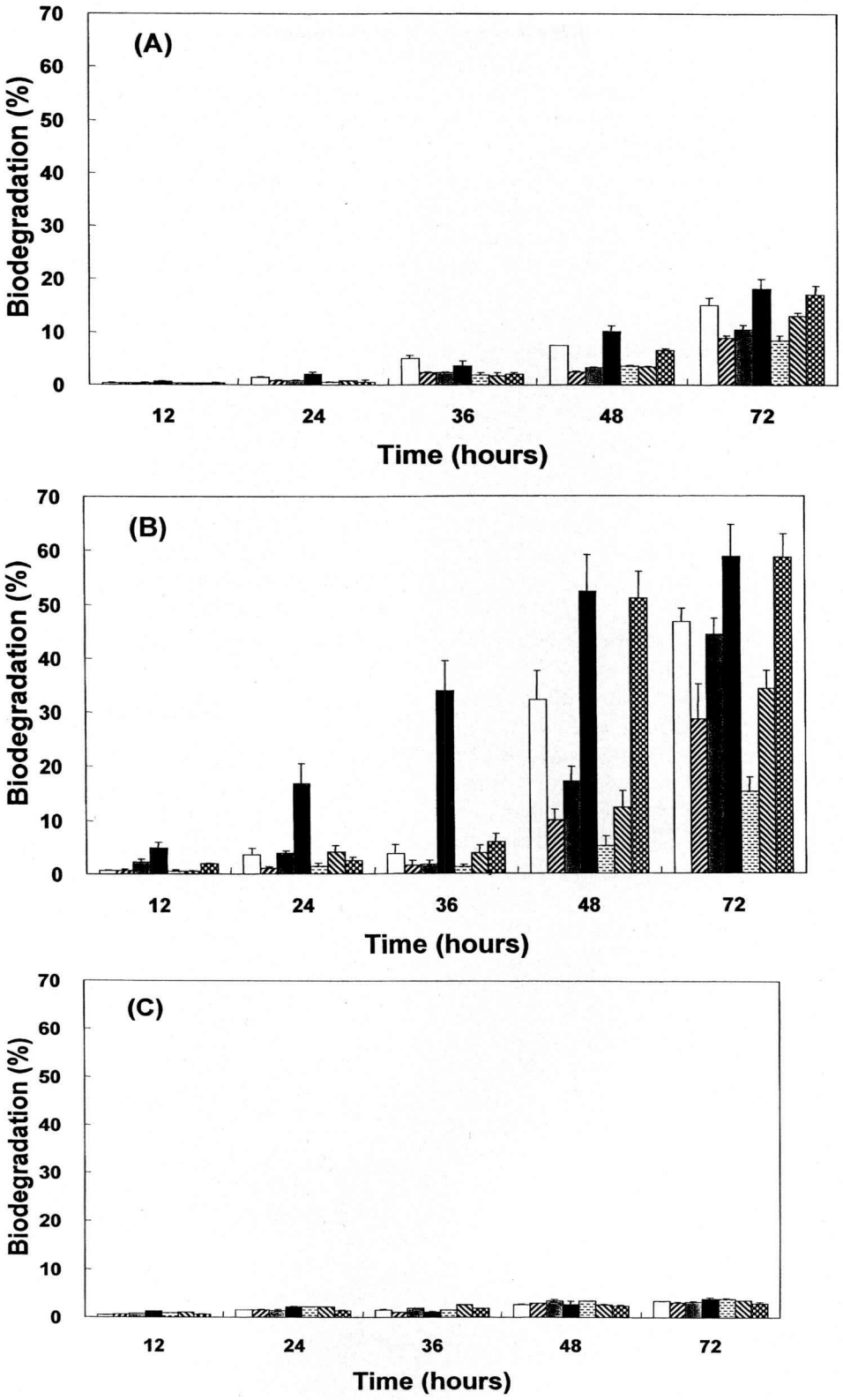

FIG. 2. Biodegradation of oleic acid by 7 strains of *A. niger* grown in 3 different types of oleic acid-CzDB medium for 12, 24, 36, 48, 72 h. (A) Type A medium: containing only 0.05% (v/v) of ^{14}C-labeled oleic acid; (B) Type B medium: containing both 0.05% (v/v) of ^{14}C-labeled and 0.5% (v/v) of non-labeled oleic acids; (C) Type C medium: containing 0.05% (v/v) of ^{14}C-labeled oleic acid, 0.5% (v/v) of non-labeled oleic acid, and 0.25% (w/v) of saccharose. Symbols: □, *A. niger* YMC0058; ▨, YMC 0061; ▩, YMC 0062; ■, YMC 0100; ▤, YMC 0160; ▧, YMC 0244; ▦, YMC 0322.

fungi isolated from vegetable oils. As shown in Fig. 2A, the carbon dioxide production by the *A. niger* strains grown in Type A medium slightly increased after 48h of incubation. Oleic acid degradation by *A. niger* YMC 0100 approached about 10% degradation after 48h, and this strain showed considerable oleic acid biodegradation (18%) after 72h. *Aspergillus niger* YMC 0058 and YMC 0322 also oxidized more than 15% of oleic acid after 72h of incubation, whereas the other 4 strains showed 8% to 13% degradation.

In the case of Type B medium (Fig. 2B), oleic acid biodegradation was substantially induced after 36 or 48h of incubation. Especially, $[^{14}C]CO_2$ evolution by *A. niger* YMC 0100 approached a rate greater than 16% already after 24h, and the degradation rate was continuously increased up to 58% until 72h. The biodegradation rate of *A. niger* YMC 0322 was noticeably increased at 48h, so that the rate after 72h was approximately 10 times greater than after 36 h. After incubation of 72 h, *A. niger* YMC 0058 and YMC 0062 showed significant oleic acid biodegradation (46 and 44%, respectively). *Aspergillus niger* YMC 0061 and YMC 0244 showed 28 to 34% biodegradation after 72h, while *A. niger* YMC 0160 degraded 15% of the oleic acid. These data suggest that addition of sufficient oleic acid to the media may enhance the levels of oleic acid oxidation by *A. niger* strains. In relation to this finding, it has been shown that some strains of *A. niger* could grow using glyceryl trioleate, and the activities of β-oxidation enzymes in the extracts of triolein-grown cells of *A. niger* were much more highly induced than in the glucose-grown cells (Baltazar et al., 1999). Moreover, De Lucas et al. (1997) demonstrated that the ultraviolet-irradiated conidia of *A. nidulans* were unable to grow on media with carbon sources requiring functional peroxisomes (oleate, butyrate, acetate, or ethanol), but grew well on media with carbon sources supposedly not requiring such organellae (glucose, glycerol, L-glutamate, or L-proline).

The effect of saccharose as an additional source of carbon on the biodegradation of oleic acid was also examined for the *A. niger* strains. When the fungal strains were grown in Type C medium (Fig. 2C), oleic acid was hardly degraded by all the organisms and $[^{14}C]CO_2$ production reached less than 4% even at 72 h after incubation. As previously described by Baumgartner et al. (1999), the use of glucose as the preferred carbon source has been shown to antagonize fatty acid induction of the peroxisomal β-oxidation machinery in *Saccharomyces cerevisiae*. It has been also reported that the activities of all individual β-oxidation enzymes in a particulate fraction from *Neurospora crassa* were enhanced in cells after a shift from a sucrose to an acetate medium, and the induction was even more pronounced in transfer to a medium containing oleate as the sole carbon and energy sources (Kionka and Kunau, 1985). These investigations were consistent with our results, showing that the tested fungal strains preferred saccharose to oleic acid as the carbon source.

From the present results, it would seem that *A. niger* strains can oxidize oleic acid only when sufficient amounts of oleic acids are supplied to the growth media, and saccharose as another carbon source has been shown to be ineffective in bringing about $[^{14}C]CO_2$ evolution from ^{14}C-labeled oleic acid. In conclusion, Type A media with both ^{14}C-labeled and non-labeled oleic acid gave significantly acceptable results in terms of detecting oleic acid biodegradation, and the use of the media provided a rather simple and rapid method to screen fungi able to produce CO_2 from the supplied substrate. The media described here would also be applicable to ecological studies in which biodegradative capabilities of fungi could be compared with those of other microorganisms.

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