Bull. Fac. Fish. Hokkaido Univ.
**44**(3), 141-146. 1993.

# Screening and Identification of Antioxidant-Producing Strains in Food-Borne Fungi

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## Abstract

The antioxidant-producing activity of 45 fungal strains isolated from various food samples were investigated. Ethyl acetate extracts from cultures of strains fermented in MYPG medium were added to peroxide-free linoleic acid at the rate of 1,000 ppm and stored at 40°C. The oxygen absorption of the extract-linoleic acid mixture was monitored during the storage.

Most of tested strains had antioxidative activity, especially the strain A-12 possessed the highest activity corresponding to 0.6 times of 200 ppm $\alpha$-tocopherol.

The strain A-12 was identified as *Aspergillus niger* var. *niger* belonging to the section *Nigri*, subgenus *Circumdati*, in genus *Aspergillus*. This species is identical to *Aspergillus niger* belonging to the *Aspergillus niger* group.

## Introduction

For preservation of fatty foods, the inhibition of oxidation of lipids is very important. One effective antioxidation method is the use of antioxidants. The antioxidative effect of tocopherols, which have been extensively used as natural antioxidants, is not superior to artificial antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Consequently, tocopherols have usually been used with synergists. Presently, the development of an effective natural antioxidant or synergist is highly anticipated.

Some filamentous fungi and yeasts have been found to have an antioxidative effect.[1] For example, antioxidants produced by *Penicillium janthinellum*[2], *P. commune*[3], *P. herquei*[4], *Eurotium chevalieri*[5] and *Aspergillus niger*[6] have been studied. In regards to feed and food, Hossain et al.[7] observed the decomposition of lipids and a decrease in oxidized lipids of mackerel meal when fermentation with *A. terreus* was induced. Matsuo[8,9] also reported the inhibition of autoxidation of vegetable oil mixed with bean-curd refuse, *Okara*, fermented with *A. oryzae* and *Rhizopus oligosporus*.

In this paper, the potential for producing effective antioxidants of food-borne filamentous fungi was investigated and an antioxidant-producing fungal strain was identified to species.

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## Materials and methods

*Isolation of filamentous fungi*

For 63 kinds of food or related materials, 1 g of aseptically well-chopped sample was suspended in 10 ml of physiological saline.

This suspension (0.1 ml) was spread on a Potato dextrose agar (PDA, Nissui) plate, and incubated at 25°C. The developed colonies were then picked up and isolated as pure cultures. These fungal strains were maintained on PDA slants at 25°C.

*Liquid cultivation*

A loop-full of fungal strain which was pre-cultured on PDA at 30°C for 14 days was inoculated to 100 ml of MYPG medium composed of 0.3% of malt extract (Difco), 0.3% of yeast extract-S (Nippon Seiyaku), 0.5% of polypeptone (Nippon Seiyaku), and 1.0% of glucose, in a 200 ml Erlenmeyer flask and stationarily cultured at 30°C for 14 days.

*Extraction with ethyl acetate*

After the cultivation, the mycelial mat was separated from the culture filtrate using a Toyo GA-200 glass fiber filter paper. The mycelial mat was homogenized with 10 volumes of ethyl acetate and filtered through two layers of Toyo No. 5A filter paper under reduced pressure. This extraction procedure was repeated 3 times. The filtrate was combined with the culture filtrate, arranging equal ratio of ethyl acetate and aqueous solution. The mixture was shaken and then left to stand overnight in a separatory funnel. This partition was repeated once more. The ethyl acetate layer obtained was then dried with anhydrous sodium sulfate and evaporated using a rotary evaporator to remove the solvent *in vacuo*. The MYPG medium without the inoculation of fungi was also processed as above.

*Autoxidation test*

The antioxidative activity of these extracts was measured by the oxygen absorption method with gas liquid chromatography (GLC). Linoleic acid (extra-pure grade, Wako Pure Chemical) was used as a substrate for the autoxidation test which was chromatographed with a Wakogel C-300 column to remove the peroxide in it before use. The peroxide-free linoleic acid was eluted with hexane-diethylether (92 : 8). The linoleic acid and the fungal extract were placed in a flat-bottomed test tube (30 × 120 mm). Then the test tube was tightly closed with a rubber stopper and stored at 40°C. The absorption of oxygen by the linoleic acid-extract mixture, i.e., the decrease in oxygen in the head-space during the storage was monitored using a Hitachi 263-30 gas chromatograph with a WG-100 column (1/4″ × 1.8 m, GL Sciences). The elongation time of the induction period for linoleic acid autoxidation was defined as the antioxidative activity. The conditions of the GLC were as follows: temperature of injector, column, and detector, 50°C; detection, TCD; bridge current, 100 mA; carrier gas, He, 40 ml/min; sample size, 75 $\mu$l. The chromatograms were processed using a Hitachi D-2500 integrator.

*Identification of fungi*

The identification of fungi was carried out based on the discriptions of Udagawa et al.[10], Samson and Hoekstra[11], Raper and Fennell[12], and Klich and Pitt[13].

The media and incubation conditions used for the identification key of Klich and Pitt[13] are: Czapek yeast-extract agar (CYA) with incubation at 25°C and 37°C; Malt-extract agar (MEA) incubated at 25°C; and Czapek yeast agar with 20% sucrose (CY20S) incubated at 25°C. Each plate was inoculated at three points and all plates were observed after 7 days incubation. Isolates were examined both macroscopically and microscopically staining by fuchsin-lacto-phenol (0.1% acid fuchsin dissolved in lacto-phenol). Simultaneously, the slide-culture method was used to observe the morphological structures of isolates.

For the identification key of Raper and Fennell[12], Czapek's solution agar (CZA) was used with incubation at 25°C for 10 days.

## Results and Discussion

*Antioxidative activity of extracts from isolated fungi*

From the 63 sources, 117 strains of fungi could be isolated. Most of the isolated strains belonged to the genus *Aspergillus* or *Penicillium* which are widely distribut-

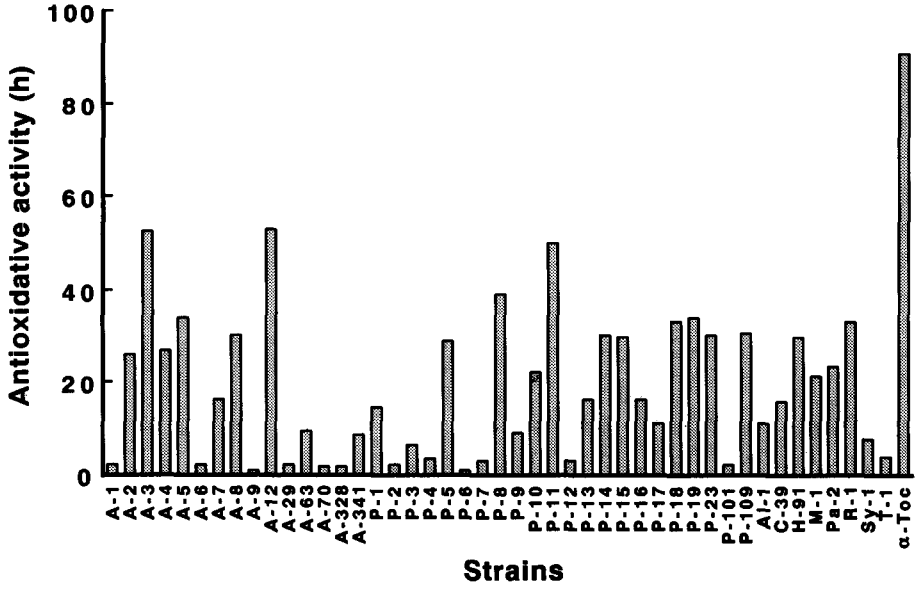


Fig. 1. Antioxidative activity of ethyl acetate extract from food-borne fungal isolates.
Extracts were added at the rate of 1,000 ppm and $\alpha$-tocopherol (Toc) was 200 ppm to linoleic acid.
Strains expressed by A-, P-, Al-, C-, H-, M-, Pa-, R-, Sy-, and T- refer to *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Cladosporium* sp., *Helminthosporium* sp., *Mucor* sp., *Paecilomyces* sp., *Rhizopus* sp., *Syncephalastrum* sp., and *Trichothecium* sp., respectively.

ed in nature : *Aspergillus* spp. and *Penicillium* spp. comprised 43% and 19% of the isolated strains, respectively. In addition to these two genera, the following genera were isolated ; *Alternaria*, *Cladosporium*, *Helminthosporium*, *Mucor*, *Paecilomyces*, *Rhizopus*, *Syncephalastrum*, *Trichothecium*, etc.

Antioxidative activity of extracts from the cultures of 45 selected strains are as shown in Fig. 1. Extracts from most of the subjected strains expressed a potential antioxidative effect. Especially, strain A-12 isolated from dried mushrooms, *Hoshi-shiitake*, had the highest antioxidative activity of its extract, followed by A-3 from almond and P-11 from smoked salmon. The activity of 1,000 ppm of the A-12 extract corresponded to 0.6 times that of 200 ppm of DL-$\alpha$-tocopherol (97.7%, Eisai). In the case of the extract of MYPG medium without inoculation of fungi no antioxidative activity was observed.

*Identification of the strain A-12*

The characteristics of strain A-12, having the highest antioxidative activity are macroscopically and microscopically as shown in Fig. 2.

The strain A-12 had swollen vesicles, phialides, metulae, conidia, and conidiophores of an unbranched stipe with a conidial head and foot cell. Therefore, the

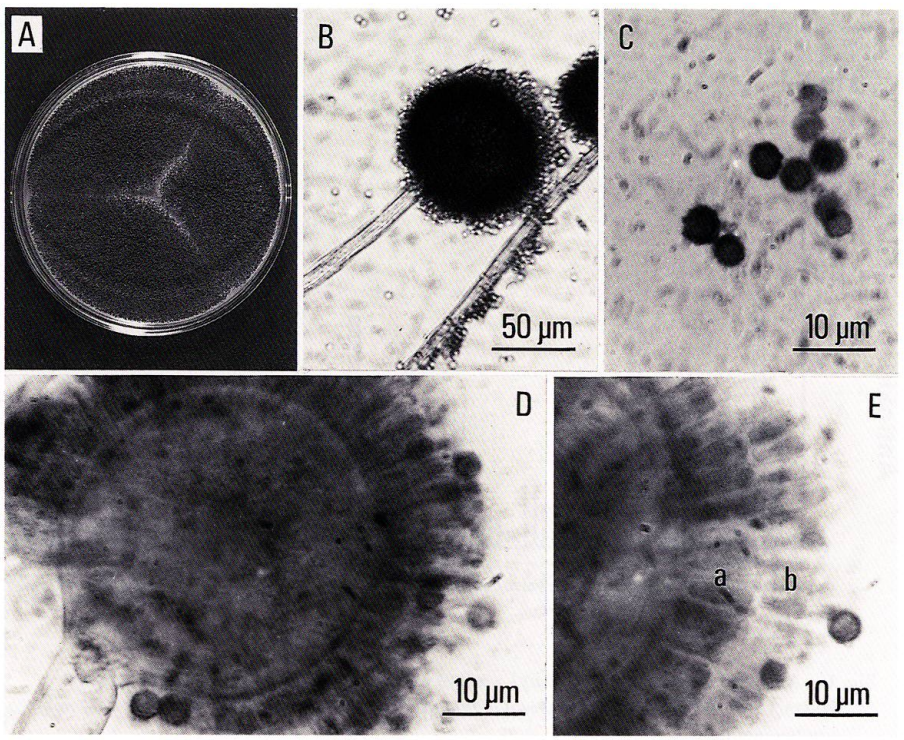


Fig. 2. Morphological structures of the strain A-12 cultured on CYA for 7 days at 25°C.
A, colonies on CYA ; B, conidial heads ; C, conidia ; D, aspergillum (vesicle) ; E, metulae (a) and phialides (b).

strain was obviously found to belong to the genus *Aspergillus*.

Colony diameter on CYA at 25°C was 67->70 mm; conidial areas were black to very dark brown and densely packed; hyphae were inconspicuous and white. Colony diameter on CY20S was 68->70 mm and the colonies were similar to those on CYA in appearance. Colony diameter on MEA was 48-55 mm and its conidial areas were black; mycelium was white. Colony diameter on CYA at 37°C was 67->70 mm.

Conidial heads were radiate; stipes>1,000×10-11 $\mu$m; walls thick, smooth; vesicles 38-52 $\mu$m wide, nearly spherical. Aspergilla were biseriate: metulae covering virtually the entire surface of the vesicle, measuring 4.5-13×3.5-4.5 $\mu$m; phialides 4.5-8.0×2.0-3.5 $\mu$m. Conidia were globose, 3.0-4.5 $\mu$m in diameter, and the surface was very rough with irregular warts.

From the above macroscopic and microscopic characteristics, using the identification key of Klich and Pitt[13] based on the new taxonomy of Gams et al.[14], it was considered that the strain A-12 could be identified as *Aspergillus niger* var. *niger*, belonging to section *Nigri*, subgenus *Circumdati*, in a genus *Aspergillus*.

After 10 days of incubation at 25°C on CZA, colonies were 28-30 mm in diameter and black; vesicles were spherical. Aspergilla were biseriate. Conidia were 2.8-3.8 $\mu$m in diameter and the surface was very roughened. These characteristics also suggested that the strain was *Aspergillus niger* belonging to *Aspergillus niger* group based on the identification key of Raper and Fennell[12].

This species is commonly isolated from soils, plant litter, plant rhizospheres, seeds, dried fruits and nuts, and is considered to be economically and industrially important[13].

Zaika and Smith[6] had reported that *Aspergillus niger* produced a antioxidative substance. However, they could not successfully isolate and/or determine its chemical structure. Therefore, the need arised to isolate the antioxidant from this *Aspergillus niger* var. *niger* A-12.

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