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## Screening of Antioxidant-Producing Fungi in *Aspergillus niger* Group for Liquid- and Solid-State Fermentation

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### Abstract

Filamentous fungi belonging to the *Aspergillus niger* group were screened for antioxidative activity to linoleic acid. Strains were inoculated in MYPG liquid media and seven kinds of cereals or their refuses, incubated at 30°C and then extracted with ethyl acetate. Most of the strains tested in the *A. niger* group had antioxidative activities in their ethyl acetate extracts. *A. niger* IFO 31125 had the highest activity in the MYPG medium. Fermentation with *A. niger* IFO 31125, AHU 7294, A-12 using rice bran and wheat bran as substrates gave higher antioxidative activities.

### Introduction

It has been known that some microorganisms have antioxidative effects.<sup>1,2)</sup> Antioxidative substances produced by *Penicillium* or *Aspergillus* species had been studied.<sup>3-7)</sup> Kawai et al.<sup>8)</sup> had screened for antioxidant-producing fungi and found that *A. niger* var. *niger* A-12 (*A. niger* A-12) had a higher antioxidative activity. An antioxidative substance from *A. niger* A-12 was isolated.<sup>9)</sup>

In relation with solid-state fermentation, Kato et al.<sup>10)</sup> and Rashid et al.<sup>11)</sup> applied a combination of *A. oryzae*, *A. sojae* K and *Saccharomyces cerevisiae* IFO 2114 to fermentation of fish meal and observed an inhibition of lipid oxidation during the fermentation process. They found an antioxidative peptide from *A. sojae* K.<sup>12)</sup> Hossain et al.<sup>13)</sup> also reported the lipid decomposition and decrease of oxidized lipid in mackerel meal by fermentation with *A. terreus*. Matsuo<sup>14,15)</sup> observed that the oxidation of vegetable oil was inhibited by an addition of soybean-curd refuse fermented with *A. oryzae* or *Rhizopus oligosporus*. During the processing of boiled, somke-dried and molded skipjack *Katsubushi*, molding is an important process for decrease in lipid content, antioxidation of lipid and expression of flavor.

The objectives of this work were to search for strains producing an antioxidative substance in the *A. niger* group and to obtain fundamental findings of the fungal applications for effective utilization of cereals or their refuses.

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## Materials and Methods

### *Fungal strains*

Fungi belonging to the *A. niger* group based on the method of Raper and Fennel,<sup>16)</sup> *A. awamori* AHU 7011, AHU 7098, IFO 4033, *A. carbonarius* AHU 7020, *A. ficuum* AHU 7015, *A. foetidus* AHU 7008, *A. japonicus* AHU 7205, *A. niger* AHU7217, AHU7294, AHU 7362, AHU 7409, IFO 4416, IFO 31125, IFO 31628, *A. usamii* IFO 8877 and *A. yezoensis* AHU 7196 were used. Also, three strains, A-7, A-8, A-12 of *A. niger* group which were isolated from foods in our laboratory were used. *A. fumigatus* IFO 4057, *A. giganteus* AHU 7339, *A. oryzae* IFO 4202, *A. sojae* AHU 7181, *A. terreus* IFO 30536 and *R. oligosporus* IFO 8631 were used as strains of the different groups from *A. niger*. These fungal strains were cultured on Poteto Dextrose Agar (PDA, Nissui) slants at 25°C.

### *Media and cultivation*

A loopful of spore of fungi pre-cultured on PDA at 30°C for 10 days was inoculated to 100 ml of MYPG medium composed of 0.3% malt extract (Difco), 0.3% yeast extract-S (Nippon Seiyaku), 0.5% polypeptone (Nippon Seiyaku) and 1.0% glucose, in a 200 ml Erlenmeyer flask and statically cultured at 30°C for 14 days.

As substrates for solid-state fermentation, soybean (Hokuren), powdered soybean-curd refuse (Misuzu Tofu), polished rice (Hakodate Beikoku), rice bran (Hakodate Beikoku), wheat bran (Nisshin Seifun), rapeseed-oil refuse (Honen Seiyu) and barley (Hamada Seibaku) were used. The polished rice, barley and soybean were ground with a National MK-5 mill to crude particles. Water was added to 10 g of solid to the following moisture content in a 1,000 ml Erlenmeyer flask : soybean, 60% ; soybean-curd refuse, 60% ; polished rice, 40% ; rice bran, 40% ; wheat bran, 50% ; rapeseed-oil refuse, 50% ; barley, 50%. Then they were autoclaved at 121°C for 15 min, inoculated with a loopful of the fungal spores and incubated at 30°C, RH 95% for 8 days, and were stirred up every 24 hours.

### *Extraction*

As for liquid-state fermentation, mycelial mat was homogenized with 10 volumes of ethyl acetate and filtered under reduced pressure. The filtrate was mixed with culture filtrate, shaken and left to stand over night in a separatory funnel. The ethyl acetate layer was dried with anhydrous sodium sulfate and evaporated using a rotary evaporator to remove the solvent *in vacuo*. For solid-state fermentation, solid was blended with anhydrous sodium sulfate to dehydrate and extraction was done with ethyl acetate. The extract was concentrated as above.

### *Autoxidation test*

Using peroxide-free linoleic acid as a substrate, an autoxidation test was carried out to determine an antioxidative activity of fungal extracts as described in a previous paper.<sup>8)</sup> Fungal extracts were added at the rate of 1,000 ppm to linoleic acid. The absorption of oxygen by the mixture was monitored during the 40°C-storage. The elongation time of the induction period for linoleic acid autoxidation was defined as the antioxidative activity.

*Thin layer chromatography (TLC)*

TLC was carried out using a Merck silica gel 60 F<sub>254</sub> plate 0.25 mm thick. The solvent system used was chloroform-methanol (88 : 12, v/v). After development, the components were detected under 254 nm UV. Also, antioxidative components were detected with carotenoid-UV irradiation method. 0.2% cantaxanthin (Extrasynthese) in chloroform-methanol (2 : 1, v/v) was sprayed on the TLC plate. Then antioxidative components could retard fading of carotenoid color under UV<sub>254</sub>.

*High performance liquid chromatography (HPLC)*

HPLC was done with a Jasco 880-PU pump with a Merck LiChrospher 100 RP-18 (e) column (4×250 mm) and the elution was done with 50% methanol-5% acetic acid for initial 25 min followed by a linear gradient of 50% to 95% methanol-5% acetic acid for 30 min with 0.7 ml/min of flow rate. The chromatograms were recorded by monitoring the absorbance at 254 nm using a Jasco Uvidec-100-V and processed using a Hitachi D-2500 integrator.

**Results and Discussion**

Antioxidative activities of ethyl acetate extracts from cultures in MYPG media are shown in Fig. 1. The extracts from *A. foetidus* AHU 7008, *A. niger* AHU 7362, IFO 31125, A-12, *A. usamii* IFO 8877 and *A. terreus* IFO 30536 had relatively higher antioxidative activities. The activity of *A. niger* IFO 31125 was especially the highest.

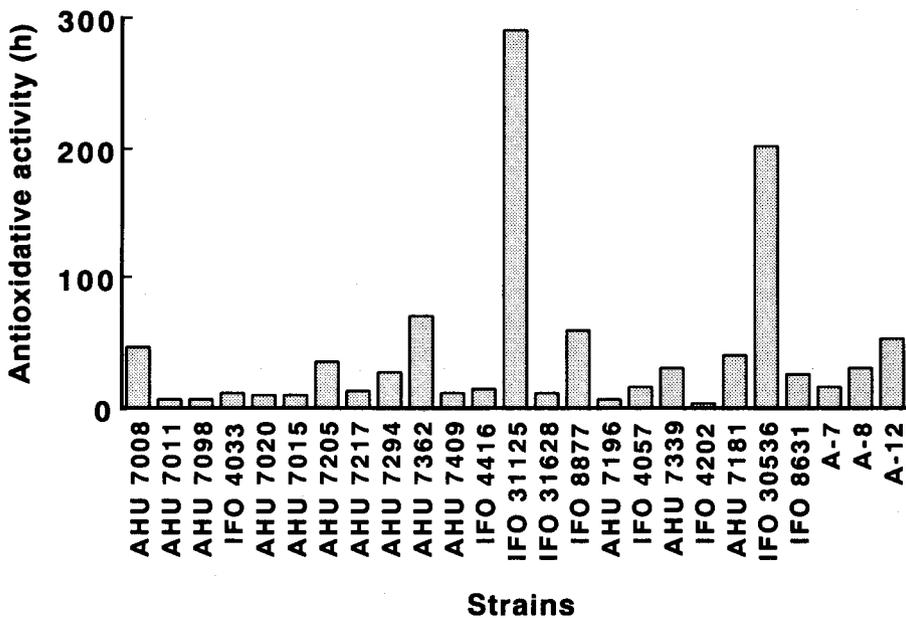


Fig. 1. Antioxidative activity of ethyl acetate extracts from fungi mainly belonging to the *A. niger* group cultured in MYPG media.

Oeda et al.<sup>9)</sup> reported that *A. niger* A-12 produced an antioxidative and synergistic compound (J) with tocopherols. On the basis of the HPLC pattern and UV-VIS absorption spectra, it was suggested that the strains AHU 7008, AHU 7362, IFO 31125 and IFO 8877 belonging to the *A. niger* group might produce the

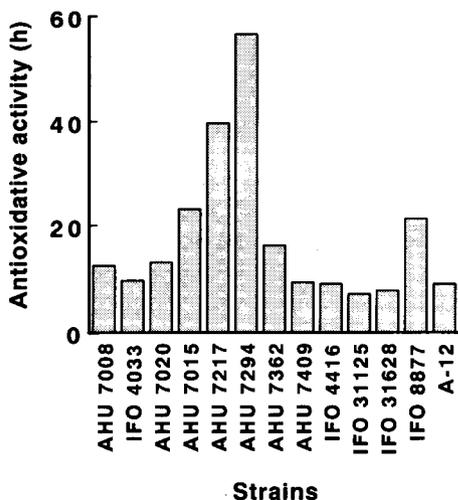


Fig. 2. Antioxidative activity of ethyl acetate extracts from soybean-curd refuse fermented with fungi belonging to the *A. niger* group.

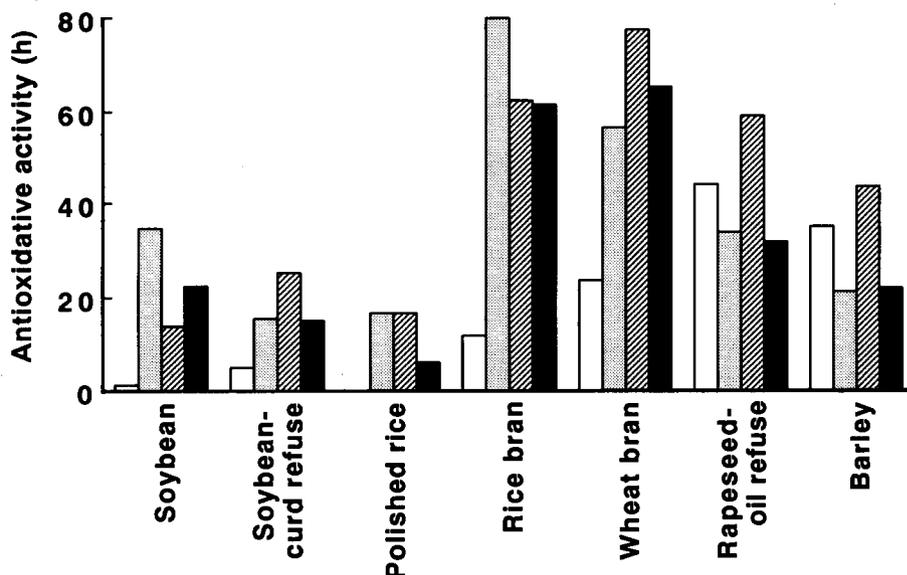


Fig. 3. Antioxidative activity of ethyl acetate extract from various substrates fermented with *A. niger*.

Columns: , unfermented substrate; , *A. niger* AHU 7294; , *A. niger* IFO 31125; , *A. niger* A-12.

antioxidative compound (J) or its homologues.

Antioxidative activity of 12 strains belonging to the *A. niger* group were investigated when they were inoculated in soybean-curd refuse and incubated at 30°C, RH 95% for 8 days. For all of the strains tested, the ethyl acetate extracts from the fermentations were more or less antioxidative as shown in Fig. 2. The fermentation with *A. niger* AHU 7294 had the highest effect.

Cereals, beans and their non-useful parts as fermentation substrates were inoculated with *A. niger* IFO 31125, AHU 7294 and A-12 and the fermentations were extracted with ethyl acetate. The antioxidative activities of the extracts are shown in Fig. 3.

The antioxidative effect was observed for all fermentations with the three strains tested. Antioxidative activities of extracts from fermentations of soybean, soybean-curd refuse, polished rice, rice bran and wheat bran were obviously greater than those of the unfermented matters. When rice bran and wheat bran were used as substrates, the activities were considerably higher. Antioxidative components corresponding to tocopherols and the compound (J) or its homologues were detected by TLC for the extracts from fermentations with *A. niger* IFO 31125 of rice bran, wheat bran and rapeseed-oil refuse. Therefore, the antioxidative effect of solid-state fermentations with the *A. niger* group might be somewhat induced by synergism of the compound (J) and tocopherols.

Kato et al.<sup>1)</sup> reported that many of fungi and yeast had antioxidative activity when they were incubated in a medium including fish oil or fish waste. This was especially true with *A. sojae*, *A. oryzae*, *Mucor javanicus* and *S. cerevisiae* which had a relatively great effect. They found *A. niger* also had some antioxidation effects. Hossain et al.<sup>13)</sup> reported that mackerel waste fermented with *A. terreus*, *A. oryzae*, *A. flavus* and *P. citrinum* inhibited autoxidation of fish lipid.

Tempeh, soybean fermented with *R. oligosporus*, has been known to have an antioxidative activity. György et al.<sup>17)</sup> had detected isoflavonoid compounds possessing antioxidative activity from tempeh: daidzein, genistein and 6, 7, 4'-trihydroxyisoflavone. These isoflavonoids were thought to be derived from isoflavone-glucosides such as daidzin and genistin in original soybean. The antioxidative effect of soybean-curd refuse fermented with *A. oryzae* and *R. oligosporus*<sup>14,15)</sup> may be induced by a similar mechanism to tempeh.

Thus, the antioxidative effect of fermentations with *A. niger* might be induced by antioxidative substances produced or derived from precursors in the substrates by the fungi. Further studies are needed on the conditions for fermentation and extraction and the structure of antioxidants produced.

### Acknowledgment

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