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# Total phenolic and anthocyanin contents, as well as antioxidant activity, of black bean koji fermented by *Aspergillus awamori* under different culture conditions

I-Hsin Lee, Yu-Hsiang Hung, Cheng-Chun Chou *

*Graduate Institute of Food Science and Technology, National Taiwan University, 59, Lane 144, Keelung Rd., Sec. 4, Taipei, Taiwan*

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**Abstract**

Solid state fermentation was performed by growing *Aspergillus awamori*, a food grade fungus, on steamed black bean at 25, 30 or 35 °C for 3 days or at 30 °C for 0–5 days to prepare black bean kojis. It was found that fermentation for a period of 3 days at 30 °C yielded a koji that contained the highest amount of total phenolics and anthocyanins among the various kojis examined. Using this 3-day cultivation period, the 30 and 35 °C-koji exhibited the highest $Fe^{2+}$-chelating ability and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging effect, respectively. Results obtained with kojis prepared at 30 °C for 0–5 days revealed that total phenolic content increased as the fermentation period was increased and became highest on the 4th day and then declined, while the 3-day koji showed the highest anthocyanin content. Further extending the fermentation period did not change the anthocyanin content of the koji significantly ($p > 0.05$). Generally, the highest DPPH radical-scavenging effect and $Fe^{2+}$-chelating ability could be obtained with kojis fermented at 30 °C for 3–4 day. The DPPH radical-scavenging effect and the $Fe^{2+}$-chelating ability exhibited by these kojis were about 2.64–3.20- and 1.77–2.16-fold greater than those of the unfermented black bean, respectively.


*Keywords:* *Aspergillus awamori*; Black bean koji; Anthocyanin; Total phenolic compound; DPPH radical-scavenging effect; $Fe^{2+}$-chelating ability

## 1. Introduction

Oxygen free radicals and other reactive oxygen species can cause deterioration of biomolecules, such as membrane proteins, enzymes, lipids, and nucleic acids (Halliwell, Murcia, Chirco, & Aruoma, 1995), in addition to food deterioration (Duthie, 1993). The oxidative damage induced by these free radicals has been implicated in diseases such as atherosclerosis, cancer, emphysema, cirrhosis and arthritis (Jacob, 1994; Kehrer, 1993). On the other hand, epidemiological studies have shown that consumption of antioxidants and phytonutrient-containing foods may reduce this degenerative process (Halliwell, 1977; Rapisarda et al., 1999). Consequently, the intake of food-derived antioxidants in our daily diet is widely recommended as a strategy for reducing the oxidative damage caused by free radicals, thus yielding a beneficial effect on human health (Lin & Yen, 1999; Meydani, 1995).

Black bean [*Glycine max* (L.) Merr.] is a nutritionally rich food with a plentiful supply of protein and calories. It also contains vitamin E, isoflavones, saponins, carotenoids and anthocyanins, which have been reported to exert biological activity (Choung et al., 2001; Murakami, Asakwa, Terao, & Matsushita, 1984). In China, black bean koji was first prepared by growing fungi on a steamed black bean substrate. It was further processed to produce traditional fermented condiments, such as *In-yu* black sauce and *In-si* or *Ttou-si*, the dried by-product of the mash of black bean sauce (Su, 1980). Probably, due to the presence of abundant hydrolytic enzymes (Wang, Ellis, & Hesseltine, 1972), the dried koji powder is mixed with other ingredients to prepare healthy food. The beneficial effects

* Corresponding author. Tel.: +886 2 3366 4111; fax: +886 2 2362 0849.
*E-mail address:* fstcchou@ntu.edu.tw (C.-C. Chou).

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of black bean appeared in Ben-Tsao Gong-Mu, an ancient Chinese Botanical Encyclopedia written in the early 16th century (Li, 1990). Recently, black bean has been reported to reduce the incidence of DNA damage by cyclophosphamide (Riberio & Saloadori, 2003), to inhibit low-density lipoprotein oxidation (Takahashi et al., 2005), and to suppress the mutagenesis induced by various mutagens (Hung, 2006; Xochitl, Lourdes, & Guadalupe-Flavia, 2005). Furthermore, some have suggested that a nutritious weaning food can be developed by combining the *Rhizopus oligosporus*-fermented black bean with rice (Rodríguez-Bűrger, Mason, & Nielsen, 1998).

In our laboratory, we have conducted a series of studies on the solid fermentation of black bean with various fungi. We found that fermentation caused a marked increase in the content of aglycone (daidzein, glycitein and genistein), of the bioactive isoflavone, compared with the unfermented steamed black bean (Lee & Chou, 2006). Furthermore, the fungi-fermented black bean kojis were noted to show an increase in the content of total extractable phenolics and anthocyanins, as well as increased antioxidative activity. However, these effects varied with the starter organisms, with *Aspergillus awamori* exhibiting the most powerful enhancing effect (Lee, 2005). In this study, both antioxidative activity and the content of total phenolics and anthocyanins of the *A. awamori*-fermented black bean kojis, prepared under different fermentation temperatures and periods, were compared.

## 2. Materials and methods

### 2.1. Starter organism and black bean

*A. awamori*, obtained from Professor Yu, Graduate Institute of Food Science and Technology, National Taiwan University, was used as the starter organism to prepare black bean koji. Black beans were obtained from a local market.

### 2.2. Preparation of black bean koji

Solid state fermentation, as described in our previous paper (Lee & Chou, 2006), was performed to prepare kojis. Briefly, black beans were first washed, and soaked overnight at room temperature in distilled water that was six times the weight of the beans. After decanting the water, the black beans were steam-cooked in an autoclave (121 °C, 15 min). After cooling, the steamed black bean substrate (50 g) was inoculated with 1.0 ml of spore suspension (ca. $10^6$ $ml^{-1}$) of *A. awamori*. The inoculated black bean substrate, after a thorough mixing, was placed on a round screen (60-mesh) and then incubated for 3 days at 23, 30 or 35 °C and 95% RH. In addition, black bean kojis were also fermented at 30 °C for a period of 0–5 days. During the cultivation period, the black beans were stirred and mixed after 17 h and 25 h of cultivation to accelerate the release of fermentation heat.

### 2.3. Determination of mycelial propagation

The mycelial mass in the soybean koji was estimated by measuring the amount of glucosamine, as described by Desgranges, Vergoignan, Georges, and Durand (1991).

The glucosamine content of mycelia obtained from the culture of test organisms was first measured. The glucosamine content in soybean koji, due to mycelial propagation, was then obtained by subtracting glucosamine content in the unfermented soybean from that found in the black bean koji. The mycelial propagation of starter organism in the black bean kojis was then estimated by dividing the amount of glucosamine due to growth, by the glusosamine content in mycelia of test organisms.

### 2.4. Measurements of total phenolics and anthocyanins

Content of total phenolics was determined according to the method described by Quettier-Deleu et al. (2000) with minor modification. Essentially, an aliquot of 0.1 ml of methanol extract was added to 1.9 ml of deionized water and 1.0 ml of Folin-Ciocalteu phenol reagent (Sigma). After 8 min, 5.0 ml of 20% $Na_2CO_3$ were added and the mixture was heated in a boiling water bath for 1 min comparatively to gallic acid standard. Absorbance was measured at 750 nm after cooling in darkness and the results were expressed in mg of gallic acid/g dried koji.

The method described by Abdel-Aal and Hucl (1999) was followed to determine the content of total anthocyanin, which was expressed as cyanidin 3-glucoside equivalents (mg/g dried koji).

### 2.5. Determination of DPPH radical-scavenging effect

To determine the antioxidant activity, methanol extract of kojis and unfermented steamed bleak bean were prepared. Samples were first dried by a freeze-dryer (77500-00 M, Labconco Co., MO, USA) and homogenized. The ground powder of the samples was then extracted with methanol (1:10, w/v) by shaking at ca 25 °C for 24 h. After filtering through Whatman No.1 filter paper, the extract was vacuum- concentrated and freeze dried.

The method described by Lee et al. (2005) was used to assess the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma) free radical-scavenging activity of the methanol extract of steamed black bean and black bean kojis. Briefly, 75 μM DPPH solution in methanol was prepared and 2.5 ml of this solution were added to 0.5 ml test samples at different concentrations. After a 90 min incubation period at ambient temperature, the absorbance at 517 nm was measured. The scavenging percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging effect}, \% = [1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}] \times 100$$