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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:

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

2.6. Determination of Fe^{2+}-chelating ability

Determination of Fe^{2+}-chelating ability of the black bean and black bean koji extract was performed according to the method described by Dinis, Madeira, and Almeida (1994). The Fe^{2+} level was monitored by measuring the formation of the ferrous ion-ferrozine complex. The methanol extract (1.0 ml) was mixed with methanol (3.7 ml), 2 mM $FeCl_2$ (0.1 ml) and 5 mM ferrozine (0.2 ml) and the mixture was shaken and left at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. A lower absorbance indicates a stronger Fe^{2+}-chelating ability. The ability to chelate the ferrous ion was calculated as follows:

$$\text{Chelating effect } (\%) = [1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}] \times 100\%$$

2.7. Statistical analysis

The mean value and standard deviation were calculated from the data obtained with three separate experiments. These data were compared using Duncan's multiple range test (SAS, 2001).

3. Results and discussion

3.1. Mycelial propagation and contents of total phenolics and anthocyanins

Fermenting black beans at 30 °C for 3 days, yielded various amounts of mycelial propagation and an increased total phenolic and anthocyanin content, depending on starter organisms, as has been observed (Lee, 2005). Most phenolics that are found in plants exist conjugated to sugars (primarily glucose) as glycosides (Vattem & Shetty, 2002). β-Glucosidase is capable of hydrolyzing phenolic phucosides and releasing extractable free phenolics such as aglycones. Therefore, the increased content of phenolic compounds and anthocyanins was attributed to the catalytic action of β-glucosidase produced by the starter organism (Lee & Chou, 2006; Rodríguez-Bűrger et al., 1998). Phenolics have been reported to possess antioxidant properties (McCue & Shetty, 2003; Shahidi, Janitha, & Wanasundara, 1992). In addition, Kathkonen and Heinonen (2003) also demonstrated that anthocyanins were capable of scavenging free radicals.

Table 1 shows the mycelial propagation of *A. awamori*, contents of total phenolics and anthocyanins in the unfermented steamed black and black bean kojis after 3 days of fermentation at 25, 30 and 35 °C. It was noted, that at 30 °C, *A. awamori* in black bean exhibited the highest mycelial propagation, a level which is significantly higher ($p < 0.05$) than that observed at 35 °C. In contrast, mycelial propagation of test organism after 3 days of cultivation at 25 and 35 °C showed no significant difference ($p > 0.05$). Black bean koji fermented at 35 °C for 3 days yielded a lower content of total extractable phenolic compounds when compared with that of the unfermented steamed black bean. A significantly higher ($p < 0.05$) content of total phenolics was observed in kojis prepared at 30 or 25 °C than in the control, with the 30 °C-prepared koji showing the highest total phenolic content. Regardless of fermentation temperature, the content of anthocyanins detected in the prepared kojis, ranging from 1.1 to 1.4 mg of cyanidin 3-glucoside/g dried koji, were all significantly higher ($p < 0.05$) than that of the unfermented black bean (control).

Table 1
Mycelial propagation of *A. awamori* and contents of total phenics and anthocyanins in black bean kojis prepared at various cultivation temperatures for 3 days

Fermentation temperature (°C)	Mycelial propagation (mg/g koji)	Total phenolic content (mg gallic acid/g dried koji)	Total anthocyanin content (mg cyanidin 3-glucoside/g dried koji)
Control	0.0 ± 0.0d[A]	16.6 ± 1.1c	0.8 ± 0.0c
25	54.6 ± 2.4ab	19.0 ± 0.6b	1.1 ± 0.1b
30	57.5 ± 2.4a	27.2 ± 0.2a	1.4 ± 0.2a
35	48.2 ± 5.7b	14.1 ± 0.5d	1.3 ± 0.1a

[A] Values are presented as means ± SD, and means in the same row with different letters are significantly different by Duncan's multiple range test ($p < 0.05$).

To examine the effect of cultivation period on mycelial propagation, further experiments were conducted to prepare various kojis by fermenting black bean with *A. awamori* at 30 °C for a period of 1–5 days. During the fermentation period, visual observation revealed that mycelia appeared on the surface of black bean after 1 day of fermentation, while spores of test organism, black in colour, started to appear in the 3-day koji. The appearance of spores became abundant in kojis prepared with an extended cultivation period. As shown in Table 2, mycelial propagation of *A. awamori* in black bean koji increased with increased cultivation period under the test conditions. Mycelial propagation of *A. awamori* was the highest in the 5-day black bean koji among the various kojis examined.

Table 2
Mycelial propagation of *A. awamori* and content of total phenics anthocyanins in black bean kojis prepared at 30 °C for various periods

Cultivation periods (day)	Mycelial propagation (mg/g koji)	Total phenolic content (mg gallic acid/g dried koji)	Total anthocyanin content (mg cyanidin 3-glucoside/g dried koji)
0	0.0 ± 0.0f[A]	16.6 ± 1.1d	0.8 ± 0.0c
1	19.7 ± 1.8e	16.9 ± 0.2d	0.8 ± 0.0bc
2	30.2 ± 2.4d	20.4 ± 2.0c	1.0 ± 0.0b
3	57.5 ± 2.6c	27.2 ± 0.9b	1.4 ± 0.2a
4	77.6 ± 1.5b	30.1 ± 0.1a	1.4 ± 0.1a
5	82.2 ± 2.0a	28.7 ± 0.9a	1.3 ± 0.1a

[A] Values are presented as means ± SD, and means in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

Extending the fermentation period from 1 to 4 days at 30 °C was noted to result in an increased total phenolics content in the prepared black bean kojis, while extending the fermentation period to 5 days did not further increase the total phenolic content in the koji. As shown in Table 2, fermentation with *A. awamori*, regardless of fermentation length, resulted in increased anthocyanin content in the prepared black bean koji. In comparison, among the various fermentation periods examined, the kojis prepared at 30 °C for 3–5 days contained the highest amounts of anythocyanins.

Previous workers have investigated the effect of fermentation length on the total phenolic content in some bean substrates (McCue & Shetty, 2003; Randhir, Vattem, & Shetty, 2004). Randhir et al. (2004) conducted a 20-day solid fermentation of fava bean with *R. oligosporus* and found that total phenolic level was reduced during the first 8 days of fermentation but increased substantially thereafter. McCue and Shetty (2003) reported that the total phenolic content in the extract of soybean did not increase until 4 days of cultivation. Trends in the variation of total phenolic content in black bean observed in the present study appear not to be consistent with those reported by McCue and Shetty (2003) and Randhir et al. (2004). These discrepancies may be attributed to differences in the starter organism and the bean substrate studied.

3.2. Antioxidant activity

It was found that the content of methanol extract in the prepared kojis, fermented for 3 days at 25–35 °C, varied with the fermentation temperatures. Methanol extract contents of 8.75%, 9.21% and 14.4%, respectively, were noted with kojis prepared at 25, 30 and 35 °C. On the other hand, the unfermented steamed black bean had a methanol extract content of 9.64%.

Based on free radical chain-breaking, metal-chelating and singlet oxygen-quenching activities, various methods have been suggested to evaluate the antioxidant activity of a sample (Amarowicz, Naczk, & Shahidi, 2000). One important mechanism of antioxidation involves the scavenging of proton radicals. DPPH$^{\cdot}$, having a proton free radical, was used to determine the proton-scavenging activity of the various koji extracts. Additionally, the chelating ability toward Fe^{2+}-ion was investigated, since transition metal ions can initiate lipid peroxidation and start a chain reaction, which leads to the deterioration of flavour and taste in food (Gordon, 1990). Besides, It has also been proposed that the catalysis of metal ions might correlate with cancer and arthritis (Halliwell et al., 1995).

Figs. 1 and 2, respectively, show the dose-response curves for DPPH radical-scavenging activity and Fe^{2+}-ion chelating ability of the methanol extract of black bean koji fermented at 25, 30 and 35 °C for 3 days. In general, it was noted that the DPPH radical-scavenging activity (Fig. 1) and Fe^{2+}-ion chelating ability (Fig. 2) increased as the dosage of the methanol extract of koji, regardless

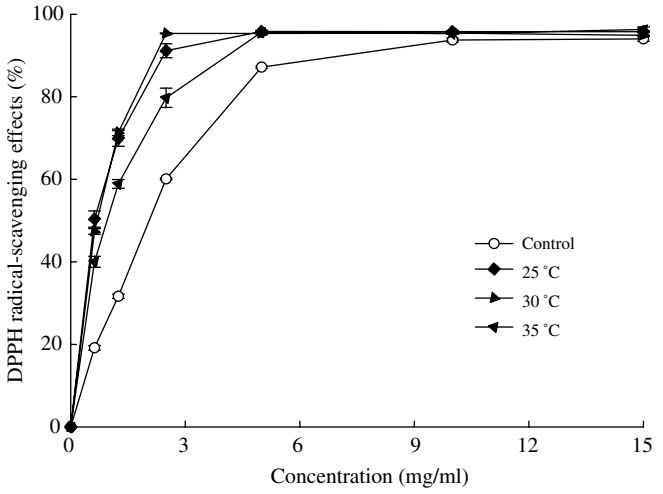

Fig. 1. DPPH radical-scavenging effects of the methanol extracts of black soybean koji prepared with *A. awamori* at different temperatures. Values are presented as means ± SD ($n = 3$).

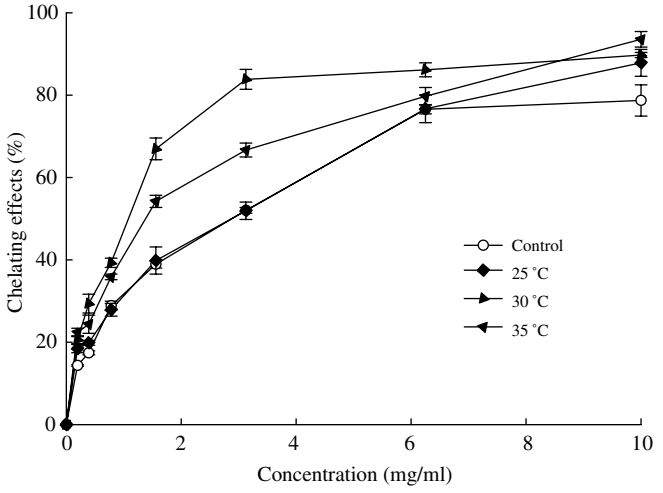

Fig. 2. Fe^{2+}-chelating ability of the methanol extracts of black soybean koji prepared with *A. awamori* at different temperatures. Values are presented as means ± SD ($n = 3$).

of fermentation temperature, increased. However, beyond a certain threshold, this activity levelled off, even with further increases in dosage. Nevertheless, the methanol extract of black bean koji, fermented at 30 °C, exhibited a relatively higher DPPH radical-scavenging activity and Fe^{2+}-iron chelating ability than did those kojis prepared at 20 or 35 °C or the unfermented steamed black bean (control). Table 3 shows the half-inhibition concentration (IC_{50}), the efficient concentration required to decrease initial DPPH$^{\cdot}$ concentration or to chelate Fe^{2+} ion by 50% of various methanol extracts, obtained by interpolation from linear regression analysis of data shown in Figs. 1 and 2, respectively. It was found that the IC_{50} of the methanol extract of kojis, depending on fermentation temperature, ranged between 0.69 and 1.01 mg/ml for DPPH radical-scavenging effect, and ranged between 1.19 and 2.74 mg/ml for Fe^{2+}-

chelating activity. In contrast, the methanol extract of the unfermented steamed black bean showed IC_{50} values of 1.95 and 2.68 mg/ml for DPPH radical-scavenging and Fe^{2+} ion chelating activity, respectively.

The IC_{50} values of the prepared black bean koji extracts, except the 25 °C-koji extract for Fe^{2+}-ion chelating ability, were significantly lower ($p < 0.05$) than that of the unfermented black bean extract for the respective antioxidant activity (Table 3). Further, considering the variation in the methanol extract of black bean and black bean kojis tested, the relative scavenging effect exhibited by black bean samples, taking into account the extract content, were calculated and are shown in Table 3. The relative antioxidative effect of the samples was obtained by dividing the methanol extract content of black bean or koji by the IC_{50} of the respective extract, and then comparing the result with the antioxidative activity of the unfermented steamed black bean. As shown in Table 3, all the prepared kojis, regardless of fermentation temperature, exhibited a relative higher DPPH radical-scavenging effect of 2.57–2.89 when compared to that of the unfermented black bean, which was assigned a value of 1.0. The highest DPPH radical-scavenging effect and Fe^{2+}-chelating ability were found with the 35 °C- and 30 °C-kojis, respectively. They were ca. 2.89- and 2.16-fold that of the unfermented black bean, respectively.

Based on the dose response curves of the methanol extract of kojis prepared at 30 °C for 0–5 days (data not shown) and methanol extract content, ranging from 8.96% to 12.0%, the IC_{50} values of the koji extracts and the relative antioxidative effect of the prepared kojis for DPPH radical-scavenging and Fe^{2+}-chelating ability are summarized in Table 4. It was noted that the DPPH radical-scavenging and Fe^{2+}-chelating ability of the *A. awamori*-kojis varied with fermentation length. Generally, the 3–4 day koji showed a relative high antioxidative activity. 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 that of the unfermented black bean, respectively.

The observed effect of fermentation length on the antioxidative activity of fermented black bean has been reported previously (McCue & Shetty, 2003; Randhir et al., 2004). McCue and Shetty (2003) observed that the DPPH·-scavenging effect in the ethanol extract of soybean fluctuated during a 10-day fermentation period while Randhir et al. (2004) reported that the DPPH·-scavenging effect of fava bean decreased from the start of fermentation

Table 3
Half-efficiency concentrations (IC_{50}) of the methanol extracts with DPPH radical-scavenging and Fe^{2+}-chelating ability and the relative antioxidative activities of black soybean kojis prepared with *A. awamori* at different temperatures

Temperature (°C)	DPPH radical-scavenging		Fe^{2+}-chelating ability	
	IC_{50} (mg/ml)[a] of koji extract	Relative effects of koji[c]	IC_{50} (mg/ml) of koji extract	Relative effects of koji
Control	1.95 ± 0.01A[b]	1.00C	2.68 ± 0.09A	1.00C
25	0.69 ± 0.04C	2.57B	2.74 ± 0.12A	0.89D
30	0.70 ± 0.01C	2.64B	1.19 ± 0.03C	2.16A
35	1.01 ± 0.02B	2.89A	2.17 ± 0.11B	1.85B

[a] IC_{50} is the efficient concentration of the test samples that decreases 50% of initial DPPH radical or Fe^{2+} concentration. IC_{50} was obtained by interpolation from linear regression analysis.
[b] Values are given as means ± SD ($n = 3$), and the means with different letters in the same column are significantly different ($p < 0.05$).
[c] Antioxidative activity of black soybean koji was obtained by dividing extraction rate by IC_{50} value. The relative antioxidative activity of control (non-fermented black soybean) is regarded as 1.00, and the test groups are expressed as values relative to the control.

Table 4
Half-efficiency concentrations (IC_{50}) of the methanol extracts with DPPH radical-scavenging and Fe^{2+}-chelating ability and the relative antioxidative activities of black soybean koji prepared with *A. awamori* during fermentation

Fermentation period (day)	DPPH radical-scavenging		Fe^{2+}-chelating ability	
	IC_{50} (mg/ml)[a] of koji extract	Relative effects of koji[c]	IC_{50} (mg/ml) of koji extract	Relative effects of koji
0	1.95 ± 0.01B[b]	1.00E	2.68 ± 0.09A	1.00E
1	2.24 ± 0.01A	0.88F	1.96 ± 0.05D	1.39C
2	1.33 ± 0.02C	1.32D	2.12 ± 0.06C	1.14D
3	0.70 ± 0.01E	2.64C	1.19 ± 0.03F	2.16A
4	0.69 ± 0.03E	3.20A	1.73 ± 0.10E	1.77B
5	0.80 ± 0.03D	3.05B	2.37 ± 0.06B	1.41C

[a] IC_{50} is the efficient concentration of the test samples that decreases 50% initial DPPH radical or Fe^{2+} concentration. IC_{50} was obtained by interpolation from linear regression analysis.
[b] Values are given as means ± SD ($n = 3$), and the means with different letters in the same column are significantly different ($p < 0.05$).
[c] Antioxidative activity of black soybean koji was obtained by dividing extraction rate by IC_{50} value. The relative antioxidative activity of control (non-fermented black soybean) is regarded as 1.00, and the test groups are expressed as values relative to the control.

to the lowest level on the 8th day then increased during 20 days of fermentation with *R. oligosporus*.

Phenolic compounds are frequently reported to covary with antioxidative activity (McCue & Shetty, 2003; Shahidi et al., 1992). However, total phenolic content and the extent of antioxidative activity of the prepared koji were found not to be strongly correlated. For example, the total phenolic content was less in the 35 °C-koji than in the 30 °C-koji (Table 1), with the former showing a significantly higher ($p < 0.05$) DPPH radical-scavenging effect and a lower Fe^{2+}-chelating ability than the latter (Table 3). Therefore, in addition to the amount of phenolics, the kind of phenolic compound present and other metabolites formed during the fermentation process may all affect the antioxidative activity of the black bean koji observed. These active components, responsible for the antioxidative activity observed, merit further investigation, which is now being conducted in our laboratory.

4. Conclusion

This study demonstrated that cultivation temperature and length significantly affected the enhancement of antioxidative activity of the black bean fermented by *A. awamori*. Generally, black beans, fermented at 30 °C for a period of 3–4 days, resulted in a product with higher DPPH radical-scavenging effect and Fe^{2+}-chelating ability. When solid fermentation of black bean with *A. awamori* is adapted for the development of food ingredients possessing enhanced antioxidative activity, these fermentation parameters should be recognized, so that the incorporation of the fermented black bean ingredient into healthy and nutritious food formulas may exert the most beneficial effect.

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