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Cultivation Temperature and Length Affect the Antioxidant Activity and Total Phenolic Content of Soybean Koji Prepared with *Aspergillus awamori*

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ABSTRACT

In this study, soybean koji was first fermented with *Aspergillus awamori*, a starter organism commonly used for the fermentation of sake, at 25, 30 or 35°C for a period of 1~5 days. Antioxidant activity, including DPPH radical-scavenging effect and Fe^{2+}-chelating ability, and total phenolic content of the methanol extract of these fermented soybean koji as well as mycelial propagation of starter organism were compared. Results revealed that fermented for a same period of 3 days, the DPPH radical-scavenging effect exerted by the 30°C-koji extract was the highest showing 2.5~3.5 folds of that fermented at 25 or 35°C. The Fe^{2+}-chelating ability exerted by the 30°C-koji extract was also found to be 3.6~3.8 times higher than those fermented at other temperatures. Furthermore, it was also noted that increasing or decreasing cultivation time from 3 days resulted in a decreased antioxidant activity of the koji extract. Cultivation temperature and length were also observed to affect the mycelial propagation and the total phenolic content of the koji extract. Maximum mycelial propagation of *A. awamori* was achieved after cultivation at 30°C for 3 days. Finally, the highest phenolic content noted in 30°C-koji extract was closely correlated to the highest antioxidant activity observed.

Key words: soybean, soybean koji, *Aspergillus awamori*, antioxidanr activity, total phenolics, mycelial propagation

INTRODUCTION

A variety of oxygen free radicals, in addition to inducing lipid peroxidation that results in food deterioration, may also cause oxidative damage by oxidizing biomolecules and thus lead to cell death and tissue damage[1]. Antioxidants are commonly employed in the food industry to prevent lipid peroxidation. Besides, the intake of food-derived antioxidants is currently believed to reduce oxidative damage and to exert a beneficial effect on human health[2,3].

In oriental countries, filamentous fungi such as *Aspergillus* and *Rhizopus*, are commonly used as a starter to prepare traditional fermented food products. These starters are usually inoculated into the solid culture of steamed soybean, rice, barley, wheat and the mixture of wheat flour used to prepare koji[4]. These starter organisms usually possess high proteolytic, saccharolytic enzyme activity, and may also impart special flavors and colors to the fermented products. In addition, some of these starter organisms have also been reported to produce physiological substances associated with antioxidant, antibacterial, and anti-tumor activity as well as reducing hypertension effects[3,5,6]. Along with the enhanced antioxidant activity observed on tempeh, natto and rice koji[3,7,8], a comparison of the antioxidant activity of soybean koji fermented by five filamentous fungi has been conducted previously in our laboratory[9]. It was noted that the enhanced effect on antioxidant activity of the prepare koji varied with the starter organism employed. Among the five starter organisms tested, koji fermented with *A. awamori* exhibited the most markedly enhanced antioxidant activity. Despite the numerous reports related to the enhanced antioxidant activity of soybean after fermentation, investigation on the effects of fermentation parameters on the antioxidant activity of fermented soybean products is still rather limited. Therefore, this study was conducted to examine the effect of fermentation parameters: cultivation temperature and length on the antioxidant activity, and total phenolic content of koji fermented with *A. awamori*. In addition, mycelial propagation of test organism was also determined.

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MATERIALS AND METHODS

I. *Test Organism and Preparation of Inoculum*

A. awamori, one of the starter organisms commonly used for the preparation of sake, was used as the test organism in the present study. Previously, steamed soybean fermented with this starter organism has been found to exhibit the most markedly enhanced antioxidative and antimutagenic activity among the various starter organisms examined[9]. Before

the experiment, *A. awamori* was activated by two successive transfers to Potato dextrose agar (PDA, Difco, Detroit, MI, USA) slants and incubated at 30°C for 3 days. The activated culture was then inoculated into PDA and incubated at 30°C for 3 days. Spores of the test organism were harvested by flooding the surface of the agar with sterile distilled water containing 0.1% tween 80. The spore suspension was adjusted with sterile distilled water to a concentration of 10^6/mL and served as inoculation for the fermentation of soybean koji.

II. *Fermentation of Soybean Koji*

Whole soybeans were washed and then soaked in distilled water that was six times the soybeans weight at room temperature overnight. After decanting the water, soybeans were cooked in an autoclave (121°C, 15 min) and then cooled. Solid state fermentation to prepare koji was performed by evenly spraying 1.0 mL spore suspension into the steamed soybean substrate (50 g). After mixing thoroughly, the inoculated soybean substrate was placed on a round screen with 60 mesh. To examine the effect of cultivation temperature, the inoculated steamed soybean was incubated at 25, 30 or 35°C and 95% RH for 3 days. To investigate the effect of cultivation time, soybean inoculated with test organism was incubated at 30°C and 95% RH for a period of 1~5 days. During the cultivation period, the soybean was stirred and mixed after 17 and 25 hr of cultivation to accelerate the release of the fermentation heat.

III. *Preparation of Methanol Extracts of Koji Soybean*

After drying at 60°C for 24 hr, the prepared koji were pulverized to pass 30-mesh screen. Samples of the ground fermented soybean powder were extracted with methanol (1:10, w/v) by refluxing at 65°C for 3 hr. After filtering through Whatman No.1 filter paper, the extract was vacuum concentrated and dried by a freeze-dryer (77500-00 M, Labconco Co., MO, USA). Extract of soybean without fermentation was prepared as described above and served as a control.

IV. *Measurement of Antioxidant Activity*

In this study, antioxidant activity including DPPH radical-scavenging activity and Fe^{2+}-chelating ability of the soybean koji extracts were determined.

DPPH-radical-scavenging activity was determined according to the method of Shimada *et al.*[10]. Extract in 4.0 mL of methanol solution was added to methanolic solution of 10 mM DPPH (1.0 mL). The mixture was shaken and left standing at room temperature for 30 min, and the absorbance of the mixture at 517 nm was then measured spectrophotometrically. The inhibitory percentage of DPPH was calculated according to the following equation:

Scavenging effect (%) = [1–(absorbance$_{sample}$/absorbance$_{control}$)]×100%

The method described by Decker & Welch[11] was followed to determine Fe^{2+}-chelating ability of the koji extract. The Fe^{2+} level was monitored by measuring the formation of the ferrous ion-ferrozine complex. The fermented soybean 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 mixture was measured at 562 nm. A low absorbance indicates a strong Fe^{2+}-chelating ability. The ability to chelate the ferrous ion was calculated as follows:

Chelating effect (%) = [1–(absorbance$_{sample}$/absorbance$_{control}$)]×100%

V. *Measurement of Total Phenolic Content and Mycelial Propagation*

Total phenolic content, expressed as gallic acid equivalents, was determined according to that described by Quettier-Delau *et al.*[12] with minor modification. An aliquot of 0.5 mL methanol koji extract was added to 7.0 mL of deionized water and 5.0 mL of Folin-Ciocalteu phenol reagent (Sigma, St. Louis, MO, USA). After 3 min, 2.0 mL of 20% Na_2CO_3 was added and heated in a boiling water bath for 1 min corresponding to the gallic acid standard. Absorbance was measured at 750 nm after cooling in the dark.

The glucosamine content of the soybean koji was determined to estimate the mycelial mass as described by Desgranges *et al.*[13]. Content of glucosamine in the mycelia of *A. awamori* was first measured. Glucosamine content in fermented soybean due to mycelial propagation was obtained by subtracting glucosamine content in non-fermented soybean from that found in the fermented soybean. The mycelial propagation of test organism in the fermented soybean was then obtained by dividing the amount of glucosamine due to growth, with the glusosamine content in mycelia of test organisms.

VI. *Statistical Analysis*

The mean value and standard deviation was calculated from the data obtained from the three separate experiments. These data were compared using Duncan's multiple range test[14].

RESULTS

I. *Antioxidant Activity*

The dose response curves for the DPPH radical-scavenging activity and Fe^{2+}-iron chelating ability of the methanol extract of koji fermented at 25, 30 and 35°C are shown in Figures 1 and 2, respectively. Below the level of 20 and 5 mg/mL, respectively, the DPPH radical-scavenging

Table 1. IC_{50} of the extracts of soybean koji *A. awamori* at various temperatures for 3 days

	DPPH radical-scavenging	Fe^{2+}-chelating ability
Fermented temperatures (°C)	IC_{50} (mg/mL)* of koji extract	IC_{50} (mg/mL) of koji extract
Control	12.61 ± 0.7 a**	1.54 ± 0.12 a
25	6.27 ± 0.22 b	1.50 ± 0.24 a
30	2.40 ± 0.18 c	0.39 ± 0.02 b
35	7.43 ± 0.76 b	1.41 ± 0.31 a

*IC_{50}: the efficient concentration of antioxidant decreasing initial DPPH radical or Fe^{2+} concentration by 50%. IC_{50} was obtained by interpolation from linear regression analysis.

**IC_{50} values are given as mean ± S.D. (n = 3) and means with the same letters in the same column are not significantly different ($p > 0.05$).

effect (Figure 1) and Fe^{2+}-iron chelating activity (Figure 2) increased as the concentration of the methanol extract of the soybean koji was increased, regardless of fermentation temperature. No significant ($p > 0.05$) increase in these antioxidative activities was noted with further increases in dosage. In general, at the same dosage level, methanol extract of the soybean koji prepared at 30°C exhibited a higher DPPH scavenging activity and Fe^{2+}-iron chelating ability than did those prepared at other temperatures.

The half-inhibition concentration (IC_{50}) required to decrease initial DPPH concentration and to chelate Fe^{2+} ion by 50%, obtained by interpolation from linear regression analysis of data shown in Figures 1 and 2, is presented in Table 1. IC_{50} of the methanol extracts of the prepared koji for DPPH radical-scavenging effect ranged from 2.4 to 7.43 mg/mL, while Fe^{2+}-iron chelating ability ranged between 0.39 and 1.54 mg/mL, depending on the fermentation temperature. The extract of the 30°C-soybean koji, having the lowest IC_{50}, exhibited the highest DPPH radical-scavenging effect and Fe^{2+}-iron chelating ability among the various koji extracts examined.

The antioxidant activity of soybean koji obtained from various fermentation periods (1~5 days) at 30°C was further investigated and shown in Figures 3 and 4. Dose response studies also showed that before reaching a threshold level, there was a positively linear relationship between antioxidative activity and the concentrations of koji extract. Based on data shown in Figures 3 and 4, IC_{50} of the various koji extracts ranged from 2.40 to 9.33 mg/mL for DPPH radical-scavenging effect and from 0.39 to 2.40 mg/mL for Fe^{2+}-chelating ability, respectively (Table 2). Among the koji extracts tested, the 3 days-soybean koji extract showed the lowest IC_{50} for DPPH radical-scavenging effect and Fe^{2+}-chelating ability.

II. *Mycelial Propagation and Total Phenolic Content*

Figure 5 shows the mycelial propagation of *A. awamori* in soybean after 3 days of cultivation at 25, 30 and 35°C.

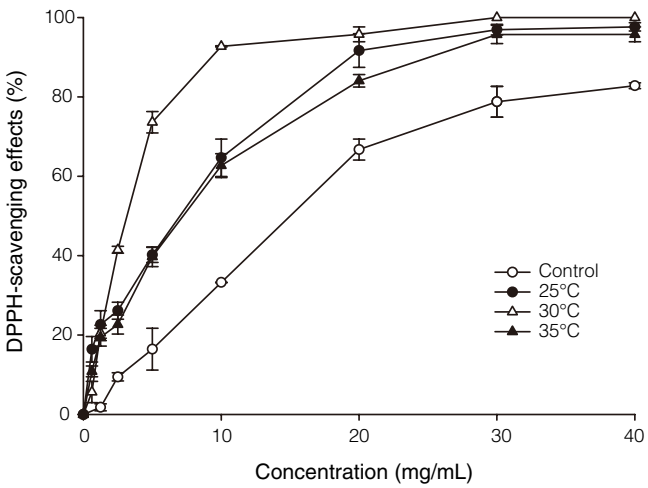

Figure 1. DPPH radical-scavenging effects of various extract of soybean koji fermented by *A. awamori* at different temperatures for 3 days. Each value represents means ± S.D. (n = 3).

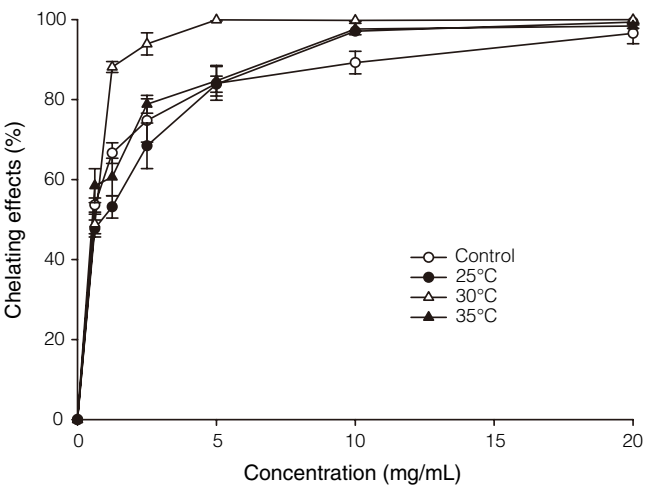

Figure 2. Fe^{2+}-chelating ability of various extracts of soybean koji fermented by *A. awamori* at different temperatures for 3 days. Each value represents means ± S.D. (n = 3).

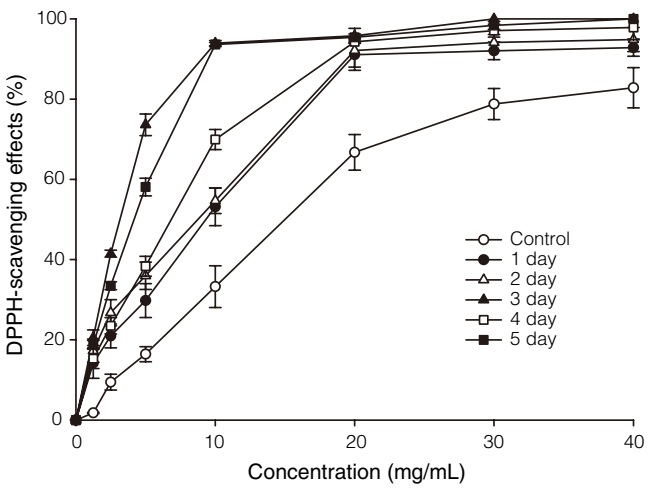

Figure 3. DPPH radical-scavenging effects of various extract of soybean koji fermented by *A. awamori* at 30°C for different periods. Each value represents means ± S.D. (n = 3).

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Among the various cultivation temperatures examined, mycelial propagation at 35 and 30°C showed no significant difference ($p > 0.05$) but was higher than that at 25°C. Further examination of the effect of cultivation period on the mycelial propagation of *A. awamori* at 30°C revealed that the maximum growth of test organism was achieved after 3 days of cultivation (Figure 6). Besides, after 3 days of cultivation, the appearance of abundant mycelia and some black conidia was particularly noteworthy. The surfaces of soybeans were covered entirely by the black conidia with extended cultivation to 4 or 5 days.

Figure 7 shows the total phenolic content of the methanol extract of the soybean koji prepared at different temperatures for 3 days. The extract of soybean without fermentation contained a lower total phenolic content of 14.81 mg gallic acid/g extract compared to that with fermentation (30.35~41.42 mg gallic acid/g extract). In addition, the highest total phenolic content was found in the extract of the 30°C-soybean koji. As shown in Figure 8, increasing the fermentation time from 1 to 3 days resulted in an increased of total phenolic content in the methanol extract of the soybean koji. On the other hand, the total phenolic content in the soybean koji extract reduced as the fermentation time was further extended from 3 days.

Table 2. IC_{50} of the extracts of *A. awamori*-koji prepared with different fermented periods

	DPPH radical-scavenging	Fe^{2+}-chelating ability
Fermented period (day)	IC_{50} (mg/mL)* of koji extract	IC_{50} (mg/mL) of koji extract
1	9.33 ± 0.65 a**	2.40 ± 0.29 a
2	7.74 ± 1.27 b	1.42 ± 0.10 b
3	2.40 ± 0.18 e	0.39 ± 0.02 d
4	4.70 ± 0.98 c	1.02 ± 0.12 c
5	3.94 ± 0.16 d	0.95 ± 0.12 c

*IC_{50}: the efficient concentration of antioxidant decreasing initial DPPH radical or Fe^{2+} concentration by 50%. IC_{50} was obtained by interpolation from linear regression analysis.

**IC_{50} values are given as mean ± S.D. (n = 3) and means with the same letters in the same column are not significantly different ($p > 0.05$).

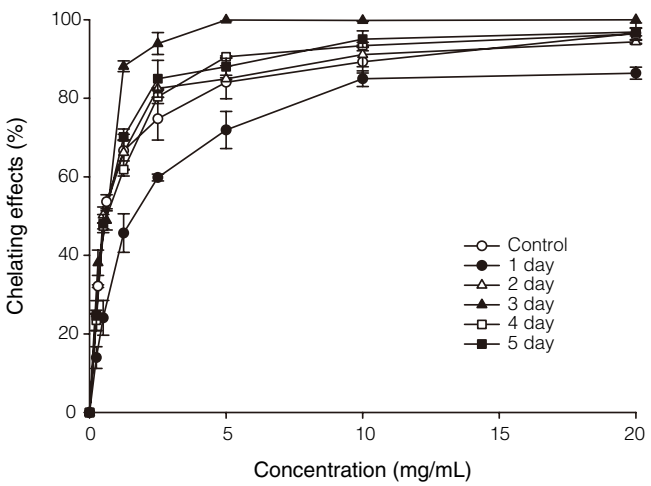

Figure 4. Fe^{2+}-chelating ability of various extracts of soybean koji fermented by *A. awamori* at 30°C for different periods. Each value represents means ± S.D. (n = 3).

DISCUSSION

Antioxidants have received considerable attention by the food industry, physicians, and consumers due to their ability to inhibit the deterioration of food resulting from lipid oxidation and to protect people from the free radical damage that may lead to many diseases including atherosclerosis,

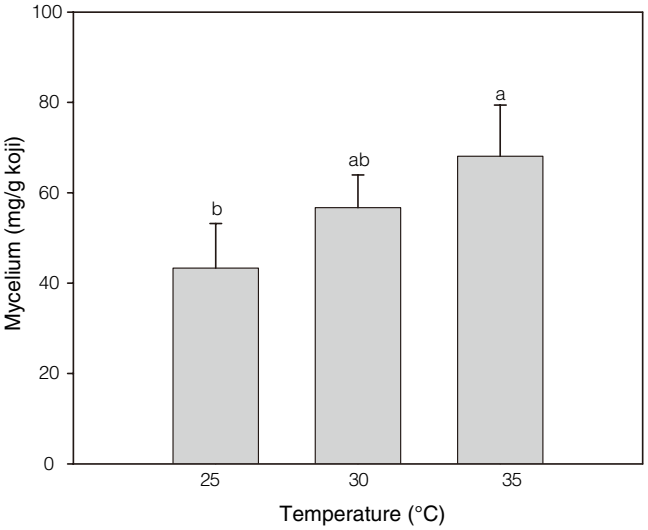

Figure 5. Mycelial propagation in various soybean koji fermented by *A. awamori* at various temperatures for 3 days. Each value represents mean ± S.D. (n = 3). Means (bar value) with the same letters are not significantly different ($p > 0.05$).

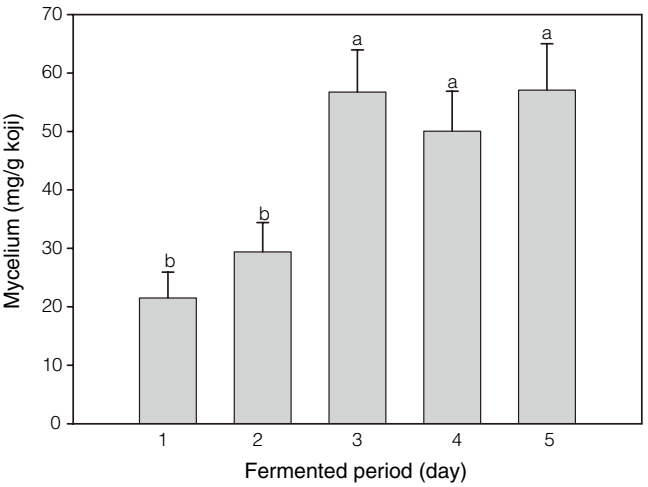

Figure 6. Mycelial propagation in various soybean koji fermented by *A. awamori* at 30°C for different periods. Each value represents mean ± S.D. (n = 3). Means (bar value) with the same letters are not significantly different ($p > 0.05$).

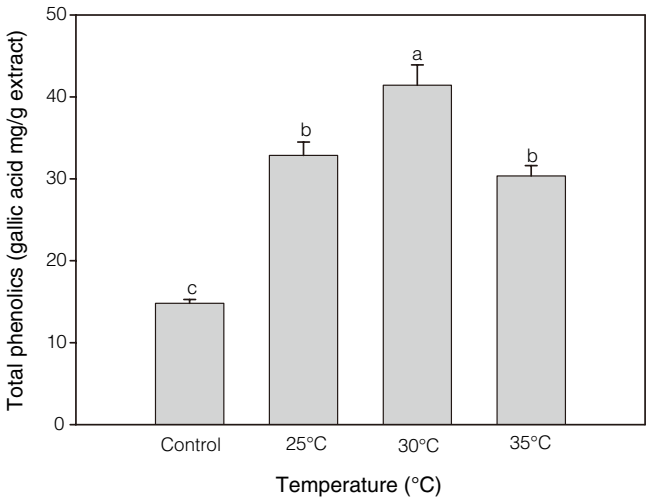

Figure 7. Total phenolic content of various soybean koji fermented by *A. awamori* at various temperatures for 3 days. Each value represents mean ± standard deviation (n = 3). Means (bar value) with the same letters are not significantly different ($p > 0.05$).

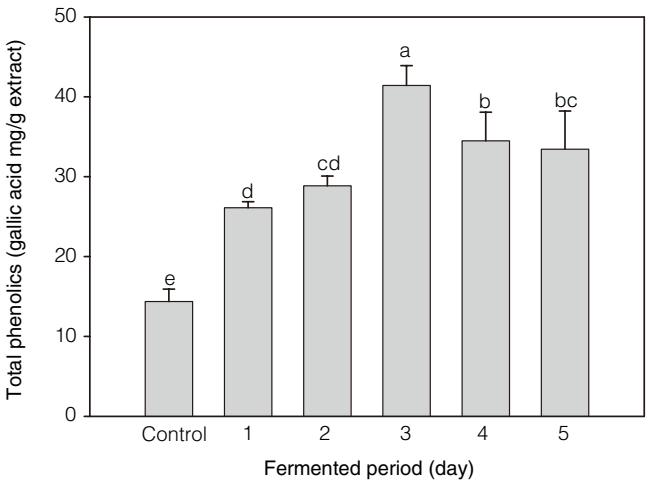

Figure 8. Total phenolic content of various soybean koji fermented by *A. awamori* at 30°C for different periods. Each value represents mean ± standard deviation (n = 3). Means (bar value) with the same letters are not significantly different ($p > 0.05$).

arthritis and cancer[1,15]. Health professionals have suggested that supplementing the diet with antioxidants is an effective way to reduce oxidative damage and thus has a corresponding beneficial effect on human health[2,16].

Enhanced antioxidative activity of soybean products such as miso, tempeh and natto obtained through fermentation with *A. oryzae*, *Rhizopus oligosporum* and *Bacillus natto*, respectively has been reported[7,8]. In our previous research we have observed that antioxidative activities, and the phenolic content of soybean, were enhanced after fermentation with various GRAS filamentous fungi[9]. Liberation of lipophilic aglycones of isoflavone glucosides such as daidzein and genestein by the catalytic action of β-glucosidase during fermentation was suggested to be one important factor contributing to the increased antioxidative activity of these fermented products. In contrast, Esakai *et al.*[17] have suggested that a significant increase in the formation of a water-soluble antioxidant, not the aglycones, leds to the enhanced antioxidative activity of natto.

In the present study, using *A. awamori* as the starter organism, we demonstrate that the cultivation temperature and length can influence the DPPH radical-scavenging effect and Fe^{2+}-chelating ability of the fermented soybean extract. As shown in Table 1, with a similar fermentation period of 3 days, extract of soybean koji fermented by *A. awamori* at 30°C exhibited the highest antioxidative activity tested. Based on the IC_{50}, the DPPH radical-scavenging effect exerted by this koji extract was about 2.5 and 3.5-fold that of the extract of 25°C- and 35°C-soybean koji, respectively. On the other hand, Fe^{2+}-iron chelating ability of the 30°C-koji extract was 3.6~3.8 times that of koji prepared at other cultivation temperature. In addition, the antioxidative activity was the highest with the extract of koji fermented by test organism at 30°C for 3 days (Table 2). Increasing or decreasing the fermentation length beyond 3 days resulted in a decreased antioxidative activity.

Cultivation temperature and length were also found to affect the phenolic content of the koji extract and the mycelial propagation of test organism. Mycelial propagation of *A. awamori* cultivated at 30°C was the greatest. Moreover, the total phenolic content of the soybean koji extract was higher when fermentation was conducted at 30°C instead of at 25 or 35°C. It reached its maximum after 3 days of fermentation at 30°C and decreased thereafter (Figure 8). This observation was different from the reports of McCue and Shetty[18] and Randhir *et al.*[19]. McCue and Shetty indicated that antioxidative activity (DPPH scavenging effect) in the ethanol extract of soybean fluctuated during the 10-day fermentation period with *R. oligosporus* while total phenolic content in the extract did not increase significantly until 4-days of cultivation, with the content level increasing only slightly thereafter. Randhir *et al.* conducted a 20-day solid fermentation of fava bean with *R. oligosporus* and observed that total phenolic level reduced during the first 8 days of fermentation then increased substantially thereafter. They also noted that the DPPH scavenging effect was the lowest on 8th day of fermentation. Differences in the test organism employed as the starter organism and the bean substrate may be the causes of these discrepancies.

Phenolics are reported to possess antioxidant properties[20,21]. We observed that extract of the soybean koji fermented by *A. awamori* at 30°C for 3 days contained the highest amount of phenolics and was closely correlated to the highest antioxidant activity examined. This observation further demonstrates that total phenolic content changes during the fermentation process, and knowing this enables one to derive the greatest amount of antioxidative activity from the *A. awamori*-fermented soybean.

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CONCLUSIONS

Developing food ingredients with enhanced antioxidative activity is desirable for both the food industries and consumers. This study shows that cultivation temperature and length significantly affect the antioxidative activity and phenolic content of the soybean koji extract. Cultivation of *A. awamori* in soybean at 30°C for a period of 3 days resulted in the highest antioxidative activity as exhibited by the prepared soybean koji. The significant impact of cultivation parameters observed in the present study provides useful information in using *A. awamori* to develop soy-based food ingredients.

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