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Tempeh Antioxidant Activity using DPPH Method: Effects of Fermentation, Processing, and Microorganisms

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Abstract: Tempeh is the main type of traditional Indonesian food that is processed from soybeans fermented by *Rhizopus microsporus*. This study aims to measure the antioxidant activity of some kind tempeh using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Measurement of antioxidant activity toward tempeh took directly from the producer, tempeh produced on a laboratory scale using *Rhizopus spp*., *Bacillus spp*., and *Klebsiella sp*. K110, tempeh fried and steamed, and tempeh during the fermentation stage. The results showed that the fermentation process soybeans into soybean increase antioxidant activity. The antioxidant activity of tempeh from producers varies between approximately 52-70%. It is because of tempeh fermented at uncontrol conditions so that the microorganisms involved in the fermentation time is also uncontrol. Potential microorganisms vary in determining antioxidant activity. In the group of *Rhizopus spp.* (ATH 35, ATH 24, ATH 53), it showed that the highest antioxidant activity was found in ATH 35 (84%). In the group of *Bacillus spp*. the highest antioxidant activity produced by *B. megaterium* (76%) and higher than the *Klebsiella sp.* K110 (75%). Thus, the fermented soybeans into tempeh increase antioxidant activity. The existence of the antioxidant activity of tempeh was affected by strains of microorganisms involved during fermentation and processing time that will be consumed.
Keywords: Soybean; Tempeh; Microorganisms; Antioxidant activity; DPPH.

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1. Introduction

Currently, there is a growing interest to study antioxidative properties in many natural sources [1]. Free radicals are one of the causes of cancer and other causes of degenerative diseases [1]. These radicals cause oxidative damage by oxidizing biomolecules leading to tissue damage and cell death, such as cancer, cirrhosis and arthritis [2]. Antioxidants are known to protect cells by reducing the formation of free radicals, taking free radicals, converting existing free radicals into less dangerous molecules, and disrupting radical chained reactions [3].

The antioxidant content is found in fermented processed foods, such as tempeh. Tempeh is made from soybeans fermented with *Rhizopus oligosporus*. Antioxidants in tempeh include daidzein, genistein, glycitein, and factor 2 (6,7,4-trihydroxyisoflavones). Factor 2 produces biological activities such as antioxidants, antihemolysis, antibacterial, anti-diarrhea, and antitoxin [4]. In addition, tempeh also contains vitamin B12 [5] which functions to prevent anemia [6], increase the menstrual cycle of pre-menopausal women [7], also reduces risk such as heart disease and stroke, also digestive disorders [8]. The role of soybean processed products is very good for reducing the risk of cancer (breast and prostate) and osteoporosis [9].

One common method of determining the antioxidant content of the food sample is the DPPH (2,2-diphenyl-1-pikrilhidrazil) activity test. Antioxidants as proton donors to DPPH free radicals so that DPPH will be reduced to a stable molecule form, by indicating the color changes from purple to yellow, which can be measured its percentage of free radical capture at a wavelength of 517 nm [10].

This study aims to measure the antioxidant activity of several types of tempeh using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Measurements of antioxidant activity were carried out on tempeh taken directly from artisans; tempeh produced on a laboratory scale using *Rhizopus spp.*, *Bacillus spp*., *Klebsiella sp.* K110; fried and steamed tempeh; and tempeh during the fermentation process.

2. Materials and methods

2.1 Tempeh sample collections

Tempeh was obtained from 10 artisans in Jakarta (Indonesia) wrapped in plastic. Sources of all soybeans from KOPTI East Jakarta, the type of inoculum comes from the same source, namely inoculum with the trademark of

Raprima produced by PT. Aneka Fermentation, Bandung. The fermentation process of tempeh by all craftsmen is the same. All tempeh to be measured for antioxidant activity is frozen in freeze drier for 4x8 hours then were stored at 20°C for further analysis.

2.2 Tempeh fermented with many types of microorganisms

Tempeh samples were produced on a laboratory scale with 13 types of microorganisms obtained from the Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, as listed in Table 1. Tempeh samples were produced based on the method of Barus et al. (2008) by following the stage of tempeh production as depicted in Fig.1 [11].

Table 1. Tempeh fermented with *Rhizopus* spp., *Bacillus* spp., and *Klebsiella* sp.

Tempeh	Microorganism
ATH 35	*R. microsporus ATH 35*
ATH 24	*R. microsporus* ATH 24
ATH53	*R. delemar* ATH 53
T4B	*R. microsporus* ATH 35 + *B. pumillus* T4B
T3G	*R. microsporus* ATH 35 + *B. pumilus* T3G
B4C	*R. microsporus* ATH 35 + *B. pumilus* B4C
B1D	*R. microsporus* ATH 35 + *B. pumilus* B1D
L15	*R. microsporus* ATH 35 + *B. subtillis* L15
GR4	*R. microsporus* ATH 35 + *B. subtillis* GR4
E23	*R. microsporus* ATH 35 + *B. subtilis* E23
E22	*R. microsporus* ATH 35 + *B.amyloliquefaciens*E22
ER4	*R. microsporus* ATH 35 + *B. megaterium* ER4
K110	*R. microsporus* ATH 35 + *Klebsiella sp*. K110

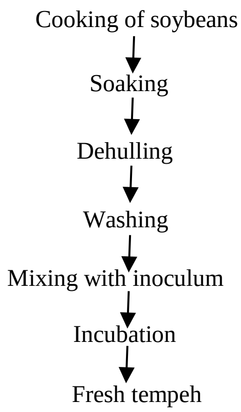

Fig. 1. Stage of tempeh produced on a laboratory scale with many types of microorganisms

2.3 Antioxidant activity measurement during tempeh making

Samples to measure the antioxidant activity of tempeh during the manufacturing process consisted of raw soybeans, boiled soybeans, soaked soybeans, and fresh tempeh which were ready to be consumed from 3 tempeh producers in Jakarta.

2.4 Preparation of cooking tempeh

Tempeh was obtained from a local tempeh producer in Jakarta, Indonesia. Tempeh samples were cut to size of 8 x 5 x 5 cm (±100 g). Tempeh pieces divided into two cooking methods include tempeh steamed at 70°C for 8 minutes and tempeh fried at 140°C for 4 minutes using cooking oil.

2.5 Sample extraction

Extraction was done using the method of Amarowicz *et al*. (2000) [12]. A total of 5 g of mashed samples were macerated with ethanol and methanol. The first step was to add 60 mL of the solvent in a closed erlenmeyer flask then shaken with a shaker overnight (20 hours) at room temperature. The second stage was added with the same solvent as much as 20 mL and shaken with a shaker for 1 hour. The third stage was added with the same solvent as much as 15 mL and shaken with a shaker for 30 minutes. After that, samples were filtered into a 100 mL volumetric flask and added the same solvent then homogenized. The solvents used for initial optimization were ethanol and methanol. The microorganisms listed in Table 1 were all isolated from tempeh and have been molecularly identified.

2.6 Antioxidant activity assay

The amount of 1 mL of filtrate was taken from the sample extract then put it in a test tube, added with 2 mL of DPPH 0.2 mM solution. Ethanol and methanol solvents were used as a negative control. After that, allowed to stand for 30 minutes at room temperature then the absorbance was measured with a spectrophotometer at a wavelength of 517 nm [10]. Antioxidant activity was expressed in % inhibition and calculated by the following formula:

$$\text{Inhibition activity (\%)} = \frac{\text{abs.control} - \text{abs.sample}}{\text{abs.control}} \times 100\% \quad (1)$$

3. Results and discussion

3.1 The difference in the antioxidant activity extracted using ethanol and methanol

In this study, two types of solvents were used to extract antioxidant from tempeh samples, namely ethanol and methanol. Fig. 2 showed that antioxidants extracted using methanol consistently produced higher antioxidant activity.

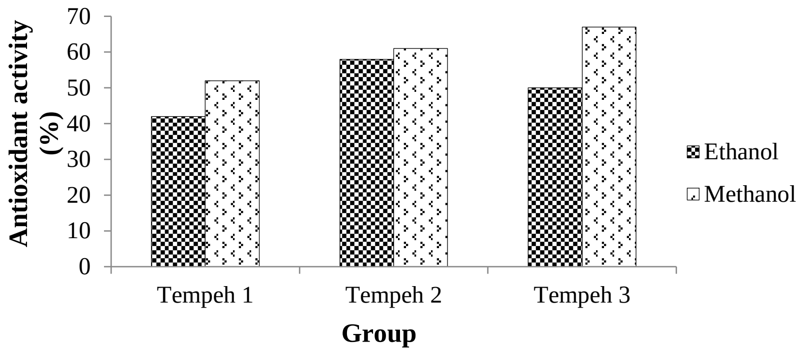

Fig. 2. The difference in the antioxidant activity was extracted using ethanol and methanol

3.2 Antioxidant activity during tempeh making

Table 2 shows the measurement of level antioxidant activity during the process of tempeh making, namely in soybeans, boiled soybeans, soaked soybeans for acidification, and fresh tempeh. Soybeans contain antioxidants around 43.04% -52.79%. The antioxidant activity of the soybeans decreases after boiling (28.68%-42.78%) and soaking (21.53%-23.44%). However, antioxidant activity was found to increase in tempeh (52.72%-67.61%).

Table 2. Measurement of level antioxidant activity during tempeh making

Location of producers	Source of antioxidants	Antioxidan activity (%)
Tebet, Jakarta	Soybeans	52.79 ± 0.6
	Boiled soybeans	28.68 ± 0.1
	Soaked soybeans	23.44 ± 0.3
	Fresh tempeh	56.27 ± 0.6
Pancoran, Jakarta	Soybeans	43.04 ± 0.7
	Boiled soybeans	42.78 ± 1.7
	Soaked soybeans	21.53 ±0.6
	Fresh tempeh	67.61 ± 0.6

3.3 Antioxidant activity of tempeh on several producers

Table 3 shows the level of antioxidant activity of tempeh taken from 10 tempeh producers. All producers make tempeh using the same method and the same type of soybeans. Levels of antioxidant activity appear to vary in tempeh from different producers. The highest antioxidant activity was found in tempeh producer (70%), and the lowest was found in tempeh producer 2,3,5.

Table 3. Antioxidant activity of tempeh on several producers in Indonesia

Source of tempeh	Antioxidan activity (%)
Tempeh producer 1	62
Tempeh producer 2	52
Tempeh producer 3	52
Tempeh producer 4	58
Tempeh producer 5	52
Tempeh producer 6	69
Tempeh producer 7	54
Tempeh producer 8	53
Tempeh producer 9	58
Tempeh producer 10	70

3.4 Antioxidant activity of tempeh prepared with various microorganisms

Table 4 shows the measurement of the level of antioxidant activity of tempeh prepared with various microorganisms. The types of microorganisms used to affect the antioxidant activity of tempeh. The highest antioxidant activity of tempeh was found in ATH 35 tempeh (84%) and lowest in T3G tempeh (50%).

Table 4. Antioxidant activity of tempeh prepared with various microorganisms

Microorganisms	Tempeh code	Antioxidant activity (%)
R. microsporus ATH 35	ATH 35	84
R. microsporus ATH 24	ATH 24	69
R. delemar ATH 53	ATH 53	53
R. microsporus ATH 35 + *B. pumillus* T4B	T4B	62
R. microsporus ATH 35 + *B. pumilus* T3G	T3G	50
R. microsporus ATH 35 + *B. pumilus* B4C	B4C	70
R. microsporus ATH 35 + *B. pumilus* B1D	B1D	63
R. microsporus ATH 35 + *B. subtillis* L15	L15	67
R. microsporus ATH 35 + *B. subtillis* GR4	GR4	69
R. microsporus ATH 35 + *B. subtilis* E23	E23	68
R. microsporus ATH 35 + *B.amyloliquefaciens* E22	E22	65
R. microsporus ATH 35 + *B. megaterium* ER4	ER4	76
R. microsporus ATH 35 + *Klebsiella sp*. K110	K110	75

Plants are good sources of natural antioxidants and can protect against harmful free radicals. Free radicals and reactive oxygen species are generated by exogenous chemicals and endogenous metabolic processes in the human body [13]. An antioxidant is any substance present in low concentrations compared to that of an oxidisable substrate significantly delays or inhibits the oxidation of that substrate [14]. Soybeans-based foods have received considerable attention of their potential role in reducing the formation and progression of certain types of chronic diseases such as Alzheimer’s disease, cardiovascular disease, osteoporosis and cancers [9]. Several anti-carcinogens have been studied in soybeans, especially isoflavones. Isoflavones are the predominant phenolics in soybean, and the glucoside form of isoflavones represents 99% of the total isoflavones in soybeans [15].

The presence of antioxidants in fermented soybeans has been widely reported (Wang et al. 2006; Iwai 2002; Morikawa 2014). In this study, we examined the antioxidant properties of tempeh. We used two types of solvents to extract antioxidants from tempeh: ethanol and methanol (Fig. 2). Ethanol and methanol are widely used as solvents in the extraction of antioxidants from plants [16-19]. Extraction using methanol is consistently higher so that methanol is used as a solvent for detecting antioxidants in tempeh extracts.

Soybeans are processed into various types of fermented foods in various countries, especially in Asia. In Indonesia, many soybeans are processed into tempeh. Tempeh is an indigenous Indonesian food fermentation and has become an important part of the Indonesian diet as a source of protein for hundreds of years. The tempeh making was done through several stages, such as boiling, soaking, and fermentation with *Rhizopus spp.* [11]. Table 2 shows the measurement of level antioxidant activity during the process of tempeh making. The measurement of antioxidant activity of soybeans dropped after boiling and dropped again when soaking. However, antioxidant activity was found to increase in tempeh.

The effect of processing on the antioxidant activity of plants has been widely reported. Turkmen et al. (2005) reported that total antioxidant activity increased or remained unchanged depending on the type of vegetable after cooking [20]. In this study, it was found that boiling soybeans reduced antioxidant activity. Boari et al. (2013) reported that boiling caused the highest losses of total antioxidant activity [21]. Stintzing et al. (2006) reported that isoflavones in aglycone forms are mostly degraded by environmental factors, particularly heat and acids [22]. Sebastian et al. (2008) also reported that post-fermentation sterilization of fermented soybean milk reducing the antioxidant activity [18]. This report is in line with our results, where the antioxidant activity of soybeans drops after boiling and soaking. The pH of soaked soybeans in making pH tempeh formed approximately pH 4 [11].

Table 3 shows that the antioxidant activity of tempeh was the highest compared to soybeans, boiled soybeans, and soaked soybeans. Wang et al. (2006) reported that the antioxidant activities of soymilk increased after fermentation using bifidobacteria and lactic acid bacteria [23]. Moktan et al. (2008) also reported that kinema extract was found to be a better free radical scavenger than the cooked non-fermented soybeans extract [19]. This report is in line with our results, where the fermentation process in the making of tempeh increases antioxidant activity. The increasing antioxidant activity may be due to the increasing of aglycone isoflavones from glucosides isoflavones during the fermentation process. The antioxidant may come from many compounds in products, including isoflavones. Isoflavones are known to protect cells from the damaging effects of free radicals [24]. Main isoflavones in soybean are in glucosides form. In fermented soybeans such as natto, miso, soy sauce, or fermented soymilk, isoflavone glucosides are hydrolyzed by α-glucosidases of microorganisms into isoflavone aglycone form. Wang et al. (2006) reported that the total glucosides decreased from 49.68 μg/mL to 14.49 μg/mL, while total aglycones increased from 21.91 μg/mL to 59.01 μg/ mL after soybean milk fermented by LAB and bifidobacteria [23]. The transformation isoflavones glucosides into isoflavones aglycone have also been reported by Chien et al. (2006) [25]. Isoflavone aglycones are absorbed faster and in higher amounts than isoflavone glucosides in humans' body [26].

Table 4 shows the difference in antioxidant activity of tempeh using several types of microorganisms. This study found that the highest antioxidant activity (84%) was found in tempeh with *R. microsporus* ATH 35 and the lowest antioxidant (50%) in tempeh with a mixture of *R. microsporus* ATH 35 + *B. pumilus* T3G. The differences antioxidant activity of aglycone isoflavones from glucosides isoflavones of this tempeh may be differences during the fermentation process. The potential of microorganisms in determining the antioxidant activity is different. The results of this study are in line with several other research results that have been reported. Lin et al. (2006) reported that high antioxidant activity was only found in several strains of lactic acid bacteria [27]. Zhang et al. (2017) reported that *Lactobacillus curvatus* SR6 had better DPPH scavenging activity (59.67% ± 6.68%) than *Lactobacillus paracasei* (SR10-1) [28]. The antioxidative activities of 27 *L. plantarum* strains were characterized, and their DPPH scavenging abilities ranged from about 11% to 43% [29].

4. Conclusions

This study found that fermentation of soybeans into tempeh increased antioxidant activity. The antioxidant activity of tempeh among artisans varies even though it has used the same raw material and fermentation process. The potential of microorganisms in determining antioxidant activity may vary where the antioxidant activity of steamed tempeh was higher than fried tempeh.

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