



Antioxidant Properties of Fermented Sesame Milk Using *Lactobacillus plantarum* Dad 13

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Abstract

The objectives of this research were to investigate the effect of sucrose addition on the growth of *L. plantarum* Dad 13 in sesame milk fermentation, β -glucosidase activity, sesaminol triglucoside concentration and antioxidant properties of fermented sesame milk. Sesame milk was extracted by blending 12% (w/v) sesame seed in water. Fermentation was done by inoculation of *L. plantarum* Dad 13 to sesame milk with and without addition of sucrose (2% and 4% (w/v)) and incubation at 37°C for 18 h. At the initial and end of fermentation, the viable cells, titratable acidity, pH, β -glucosidase activity, sesaminol triglucoside concentration, total phenolic content, and antioxidant activity were determined. The results showed that *L. plantarum* Dad 13 grew well in sesame milk with or without sucrose addition. Addition of sucrose didn't significantly affect the growth of bacteria, but increased production of acid. Fermentation of sesame milk without sucrose addition showed a significantly greater β -glucosidase activity than sesame milk with sucrose addition. Fermentation of sesame milk without sucrose addition reduced the concentration of sesaminol triglucoside, increased total phenolic content and DPPH radical scavenging activity of fermented sesame milk. The highest antioxidant properties obtained in the sesame milk fermented without addition of sucrose.

Keywords: Sesame milk fermentation, sesaminol triglucoside, antioxidant activities, β -glucosidase, lactic acid bacteria.

Introduction

Sesame seed (*Sesamum indicum*) is well-known as a source of high activity antioxidant with tocopherol and lignans as primary compounds¹. Tocopherol in sesame seed mainly consists of γ tokoferol (25 mg/100g), while α and δ tocopherol may be exist in a minor content. Lignans has higher antioxidant activity than tocopherol². Lignans in sesame seed are hydrophobic lignans and hydrophilic lignan glucosides. The major hydrophobic lignans in sesame seed are sesamin and sesamol which their concentration are 490.6 mg/g and 350 mg/g sesame oil respectively. The major hydrophilic lignan glucosides in sesame seed are sesaminol, sesamolol and pinoresinol glucoside. The total content of lignan glucoside in sesame seed is about 100-120 mg/100g, which sesaminol triglucoside is the most dominant lignan glucoside³. However, only small amount of antioxidant present in a free form and the rest of antioxidant compounds are attached to glucose in the form of lignan mono/di/tri-glucoside, which have no role or low antioxidant activity. In the intestinal track sesaminol glucoside can be hydrolyzed by β -glucosidase and produced an active aglycone after passing the digestive system⁴. Thus it could be that fermentation of sesame milk using β -glucosidase-producing lactic acid bacteria may break the glucoside bond, results in more active aglycone compounds and therefore increase antioxidant activity.

In Indonesia beside for sesame oil production, sesame seed is also used as ingredient for many traditional food products, i.e. snacks such as an *onde-onde*, cookies, and fermented food called *cabuk*. Like soybean and peanut, sesame seed can be processed to make sesame milk. Sesame milk contains sterol, lower chain saturated fatty acid, high protein and calcium content and no lactose¹. In addition sesame milk contains less stachyose and raffinose than soymilk thus less risk of flatulence. The processing of sesame seed itself as vegetable milk has been resulted in a positive sensory acceptance⁵. However, like other vegetable milks, sesame milk also presents an unfavored flavor. This unfavored flavor can be covered by the flavor produced by lactic acid fermentation⁶. Lactic acid fermentation of vegetable milk may offer another beneficial property. Fermentation of soymilk using lactic acid bacteria have been reported to enhance the antioxidant activities due to the formation of isoflavone aglycones^{7,8}. This phenomenon was associated with the activity of β -glucosidase produced by lactic acid bacteria. The growth and activity of lactic acid bacteria depend on the ability to use nutrient especially carbon source in the fermentation media. Therefore we investigated sesame milk fermentation with and without addition of sucrose, and their effect on antioxidant properties of the product.

The objectives of this research were to investigate the effect of sucrose addition on the growth of *L. plantarum* Dad 13 in sesame milk fermentation, β -glucosidase activity, sesaminol

triglycoside concentration and antioxidant properties of fermented sesame milk.

Material and Methods

The starter culture of *L. plantarum* Dad 13 was obtained from FNCC (Food and Nutrition Culture Collection), Centre for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta, Indonesia. The culture stock was kept in 10% glycerol and 10% skim milk with the ratio 1:1 (v/v). One milliliter culture in sterile 1.5 mL polyethylene screw cap tube was added with 1 mL glycerol-skim milk and stored at -20°C. The strain was activated by adding 1 mL of stock solution with 9 mL of 0.85% NaCl in water, vortex and then rejuvenated in 10 mL of MRS (De mann Rogosa Sharpe) broth (Oxoid) at 37°C for 18 h.

Preparation of sesame milk: Decorticated sesame seeds of Winas variety were obtained from Brajan Village, Prambanan District, Klaten Regency, Indonesia. Sesame milk was prepared by using 12 % (w/v) initial concentration of sesame seed⁵. It was blended for 10 minutes using Philips HR2071 blender. The resultant slurry was filtered using triple-layered cheesecloth to obtain sesame milk. The sesame milk was pasteurized at 75°C for 5 min.

Fermentation of sesame milk: Each of sesame milk added with addition of 2% and 4% sucrose was inoculated with 1% (v/v) of 18 h the single culture of *L. plantarum* Dad 13 in MRS broth. Fermented sesame milk without sucrose serves as a control. Sesame milk containing *L. plantarum* Dad 13 was incubated at 37°C for period of 18 h. The population of the lactic acid bacteria, titratable acidity, pH, β -glucosidase activity and sesaminol triglycoside concentration were determined in the initial and end of fermentation periods. Sesame milk fermentation was stored immediately at -20°C and then freeze dried using a Dynavac FD 300 freeze drier (Rowville, Vic., Australia) for the analysis of sesaminol triglycoside.

Analytical Methods: MRS broth (Oxoid) with 1 % CaCo₃ was used for the enumeration of lactic acid bacteria. One milliliter of appropriate serial dilutions of each sample was pour-plated on to MRS broth (Oxoid). After 48 h of incubation at 37°C, the colonies with the clear zone that appeared on the plates were counted and calculated as log CFU/ml. Titratable acidity was determined by titration of sample with 0.1 N NaOH solutions, and expressed as percent of lactic acid⁹. The pH value of the samples was measured using pH meter (Thermo scientific).

Beta glucosidase activity: Beta glucosidase enzyme had higher activity in *p*-nitrophenyl- β -D-glucoside substrat than others. Hydrolysis rate of *p*-nitrophenyl- β -D- glucopyranoside (*p*NPG) (Sigma Chemical Co., St. Louis, Mo., USA) was used to determine β -glucosidase activity. The procedure was not different with other authors¹⁰. Ten ml of each aliquot was added to one milliliter of 5 mM *p*NPG which prepared to 100 mM

sodium phosphate buffer (pH 7.0) and incubated at 37°C for 30 min. To stop the reaction, 500 μ l of 1 M cold sodium carbonate (4°C) was added. The aliquots were placed in a 1.5 ml eppendorf centrifuge tubes then followed by centrifugation (14,000 g for 30 min). Spectrophotometer UV-Vis (Shimadzu, UV-1656 PC) at λ 420 nm was used to measure the amount of *p*-nitrophenol that released. The amount of β -glucosidase that releases 1.0 nmol of *p*-nitrophenol from the substrate *p*-NPG per milliliter per min, under assay conditions was named as one unit of the enzyme activity. *p*-nitrophenol was used as standard in the enzyme assay.

Preparation of crude extract of sesame milk and fermented sesame milk: One milliliter of sesame milk or fermented sesame milk was transferred into flasks and 5 mL of methanol 70% were added into the solution. The flasks were then placed in shaker (SIBATA, SU-2TH) (120 rpm) for 1 h in room temperature, followed by maceration at 4°C for 24 h. The crude extracts were obtained by centrifugation (eppendorf centrifuge 5417 R Hamburg German) at 4000 g 4°C for 10 min, filtration through a Whatman paper no 42. The supernatant (S1) was stored at 20 °C, the natant was transferred into flasks and 5 ml of methanol 70% were added into solution. The flasks were then placed in shaker (SIBATA, SU-2TH) (120 rpm) for 1 h in room temperature, followed by maceration at 4°C for 24 h. The crude extracts were obtained by centrifugation (eppendorf centrifuge 5417 R Hamburg German) at 4000 g 4°C for 10 min, filtration through a Whatman paper no 42. The supernatant (S2) and supernatant (S1) was mixtured and stored at -20°C until analysis for determination of DPPH radical scavenging activity and total phenolic content¹¹.

Determiation of DPPH (2,2-diphenyl-1-picrylhydrazil) radical scavenging activity: DPPH solution in methanol (0.06 mM) was prepared, and then 3 ml DPPH solution was mixed with 1 ml crude extract of sesame milk and sesame milk fermentation. The mixtures were incubated for 1 h in the dark room¹². Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) was used to measure the absorbance at λ 516 nm. Increasing of DPPH radical scavenging activity was shown by decrease of the DPPH solution absorbance. The equation to calculate antioxidant activity was given as percent DPPH radical scavenging activity.

$$\text{Radical scavenging activity (\%)} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100\%$$

The control contained 3 ml of 0.06 mM DPPH solution and 1 ml of methanol. Ascorbic acid was used as positive controls. Data were reported as means \pm SD for three replications.

Analysis of total phenolic content: The crude extract (2 ml) was placed in a tube, and 1 ml Folin-Ciocalteu reagents was added, mixed and allows standing for 1 min. Then 4 ml of 15%

of sodium carbonate (Na₂CO₃) solution were added, mixed and placed in a dark room for 2 h at room temperature¹³. Absorbance of the resulting blue complex was then measured at 760 nm using a shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Methanol was used as the blank and gallic acid used as standard. The used of gallic acid based on its stability and purity. The results were expressed as mg gallic acid equivalents (GAE)/g of dry sesame seed. Data were reported as means ± SD for three replications.

Analysis of sesaminol triglucoside: The change of sesaminol triglucoside was considered as the cause of antioxidant properties change during fermentation. The research procedure was not different with other author but with little modification¹⁴. Naringenin was used for internal standard. Glass tube (35 ml) was containing 0.01 g freeze-dried sesame milk samples in triplicate and extracted for 5 h with 8.25 ml of 85% ethanol containing 100 µg/mL of naringenin. Then, the extraction was continued whole night after adjusting the ethanol concentration up to 70% by putting 1.75 mL of distilled water to the extraction tubes. Soon after that, the tubes were centrifuged for 10 minutes at 2000 rpm and the supernatants were filtered 0.45 µm membrane sartolon polyamide.

HPLC (Waters), Binary 1525 EF model pump, with a photodiode array detector (PDA) 2996 which also provided the UV spectra of the peaks and an Phenomenex® Luna C 18 (250 x 4.60 mm, 5 µm), Alltech Co., Waukegan Road, Deerfield, IL), was used to analyze the sample. To get sesaminol triglucoside, eluents of 0.01 M Phosphat buffer (pH 2.8) containing 5 % acetonitrile (A) and acetonitrile (B) being used. The elution conditions were 0-5 min (15%B), 30 min (30%B), and 40-50 (70% B) and the flow rate was 1.0 mL/min¹⁴.

The concentration of sesaminol triglucoside was counted from the peak area at 290 nm against the internal standard (naringenin). The relative response factors (RRFs) need to calculate with the internal standard reference which includes to sesaminol triglucoside and naringenin with 8 different concentrations were analyzed by HPLC. The peak areas were used to draw the calibration curve for sesaminol and naringenin. The respond factor for sesaminol relative to naringenin was calculated as 0.0008 and was used to count the sesaminol triglucoside concentration in freeze dried sesame milk fermented¹⁴.

Statistical analysis: The experiment design used ccompletely randomized design in triplicate. Results were presented as means value ± standard deviation. Statistical analysis between experimental results based on one-way ANOVA; pair-comparison of treatment means was achieved by Duncan's procedure at P<0.05 using statistical software SPSS 17 for Windows.

Results and Discussion

The effect of sucrose addition on the fermented sesame milk: Lactic acid bacteria utilized carbon source for supporting growth and metabolic activity. Table-1 showed the growth of lactic acid bacteria in sesame milk without addition of sucrose was relatively similar to that of with addition of 2% and 4% (w/v) sucrose. This result showed that *L. plantarum* Dad 13 grew well in sesame milk fermentation with and without addition of sucrose. It means that carbon source in sesame milk was enough for *L. plantarum* Dad 13 growth, so addition of sucrose in sesame milk didn't give any effect for its growth. Sesame seed contained glucose (3.63%), fructose (3.43%), raffinose (0.59%), galactose (0.40%), stachyose (0.38%) and sucrose (0.17%)¹⁵. *Lactobacillus plantarum* can metabolized raffinose, fructose, galactose and sucrose to simple saccharide¹⁶. It means that *L. plantarum* Dad 13 can use carbon source in sesame seed for the growth. This result is in agreement with result reported by other researcher that *L. plantarum* Dad 13 could grow well in peanut milk without addition of sucrose. Peanut milk ccontained glucose (0.05%), sucrose (0.63%), and pentosan (0.20%). It means that the carbon source in peanut milk enough for the growth. So that, peanut milk ffermentation with the addition of 2% sucrose did not make any different number of viable cells¹⁷.

The result showed that the addition of sucrose increased significantly the production of acid and lowered the pH value (table-1). This result is in agreement with result reported by other researcher that the addition of sucrose in fermentation of peanut milk using *L. plantarum* Dad 13 increased production of acid and lowered pH, but didn't effect of cell growth¹⁷. It means that fermentable sugars content in sesame milk was enough for bacteria growth but not for metabolic activity. Addition of sucrose increased the metabolic activity of bacteria, as indicated by higher acid production and lower pH value.

Table-1
Viable cell, titratable acidity, and pH of fermented sesame milk with and without addition of sucrose

Sucrose addition (%) w/v	Viable cells (log CFU/mL)		Titratable acidity (%)*		pH value	
	Initial	18h	Initial	18h	Initial	18h
0	7.39 ^a	8.88 ^a	0.06 ^a	0.24 ^a	6.03 ^a	4.50 ^a
2	7.41 ^a	9.02 ^a	0.06 ^a	0.63 ^b	6.03 ^a	4.13 ^b
4	7.45 ^a	9.03 ^a	0.06 ^a	0.63 ^b	6.03 ^a	4.00 ^b

* Fermented of sesame milk was carried out using *L. plantarum* Dad 13 at 37°C, 18 h., **Titratable acidity expressed as percent of lactic acid, ***Values in the same column with different superscripts are significantly different (P<0.05)

Beta glucosidase activity of fermented sesame milk: Beta glucosidase activity in fermented sesame milk without addition of sucrose was (70.3±0.023 mU/mL fermented sesame milk). Addition of sucrose tend to decrease the activity of β-glucosidase in the fermented sesame milk (figure-1). Addition of sucrose provided more fermentable sugars in the sesame milk. β-glucosidase is enzyme which involved in hydrolysis of β-1,2-glycosidic bond between sesaminol aglycone and glucose. β-glucosidase is inducible enzyme, so it will be synthesized in the present of its inducer. Cellobiose is an inducer for the activity of β-glucosidase, enabling a higher β-glucosidase activity when added to the fermentation media. Other researches reported that an elevated β-glucosidase activity in fermentation of soymilk using *W. cibaria* 37 when induced by cellobiose¹⁸. During fermentation of sesame milk without addition of sugar, bacteria used fermentable sugars from sesame milk. It would be when the fermentable sugars were limited *L. plantarum* Dad 13 produced β-glucosidase to hydrolize sesaminol triglycoside in order to get glucose. In the case of fermentation of sesame milk with addition of sucrose, the media provided enough carbon source of its growth and metabolic activity. Therefore *L. plantarum* Dad 13 did not synthesize β-glucosidase as high at the one without addition of sucrose.

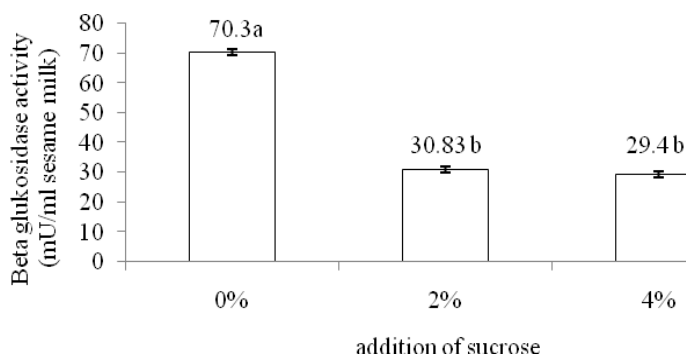


Figure-1

Beta glucosidase activity of fermented sesame milk with and without addition of sucrose. Fermented of sesame milk was carried out using *L. plantarum* Dad 13 at 37°C, 18 h. Values with different superscripts are significantly different (P<0.05)

Sesaminol triglycoside concentration, total phenolic content and antioxidant activity: The concentration of sesaminol triglycoside decreased during fermentation of sesame milk (table-2). The higher the concentration of sucrose added, the higher the concentration of sesaminol triglycoside in the end of fermented sesame milk. It was a correlation between the sesaminol triglycoside content and β-glucosidase activity (figure-1). The limited of fermentable sugars available in sesame milk without addition of sucrose tend to the higher activity of β-glucosidase to hydrolize sesaminol triglycosides into sesaminol aglycone and glucose, thus lower sesaminol triglycoside concentration. The glucose was consumed by *L. plantarum* Dad 13 for metabolic activity. The sesaminol

aglycone contain more reactive phenolic compound result in higher total phenolic content and antioxidant activity (figure 2 and 3). This in line with other researches that enzymatic hydrolysis of ethanolic sesame cake with β-glucosidase resulted in sesaminol aglycone in sesame seed².

Table-2
Sesaminol triglycoside concentration of fermented sesame milk with and without addition of sucrose

Sucrose addition (% w/v)	Sesaminol triglycoside concentration (mg/100 ml of sesame milk)	
	Initial fermentation	End of fermentation
0	11.72	6.05
2	11.72	6.84
4	11.72	7.93

* Fermented of sesame milk was carried out using *L. plantarum* Dad 13 at 37°C, 18 h.

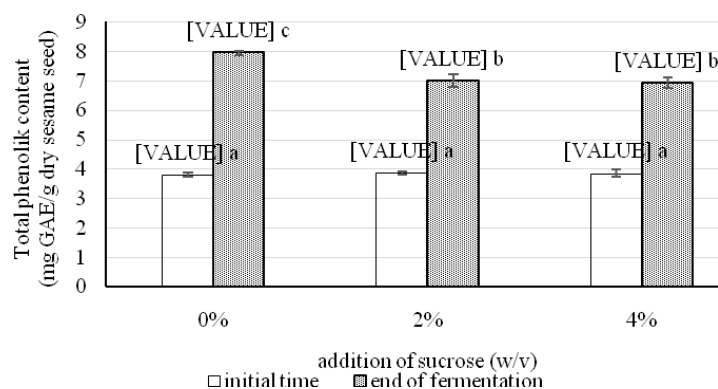


Figure-2

Total phenolic content of fermented sesame milk with and without addition of sucrose. Fermented of sesame milk using *L. plantarum* Dad 13 at 37°C, 18 h. Values with different superscripts are significantly different (P<0.05)

Fermentation of sesame milk by *L. plantarum* Dad 13 (37°C, 18 h) resulted in a significant increase of total phenolic content (figure-2). In this research, The highest total phenolic content was in sesame milk without addition of sucrose. The bacteria synthesized more β-glucosidase in sesame milk without addition of sucrose than in sesame milk with addition of sucrose for hydrolize of sesaminol triglycoside in order to obtain glucose as carbon source. Thus, sesaminol triglycoside concentration was lower, sesaminol aglycone was bigger than fermented sesame milk with addition of sucrose.

The free sesaminol aglycone exhibited a higher reactivity in scavenging radicals, resulted in a higher antioxidant activity. Fermentation of sesame milk by *L. plantarum* Dad 13 (37°C, 18 h) resulted in a significant increase of radical scavenging activity. The highest antioxidant activity was in sesame milk without addition of sucrose (figure 3). During fermentation,

hydrolysis of sesaminol triglucoside into aglycone was occur due to the β -glucoside activity production by the lactic acid bacteria. Lactic acid fermentation in sesame milk might increase the antioxidant activity by releasing various antioxidative component which initially present in an inactive form¹⁹. For the requirement of carbon source, the bacteria synthesize more β -glucosidase to obtain the glucose moiety in the sesaminol glucoside complex. The free sesaminol aglycon exhibit a higher reactivity in scavenging radicals, resulted in a higher antioxidant activity. This result was in agreement with other researches that pointed a significant increase of total phenolic content followed by an increase of antioxidant activity²⁰ such as in fermentation of buckwheat, barley, wheat dan rye mixture by *Lactobacillus rhamnosus* dan *Saccharomyces cerevisiae* separately²¹.

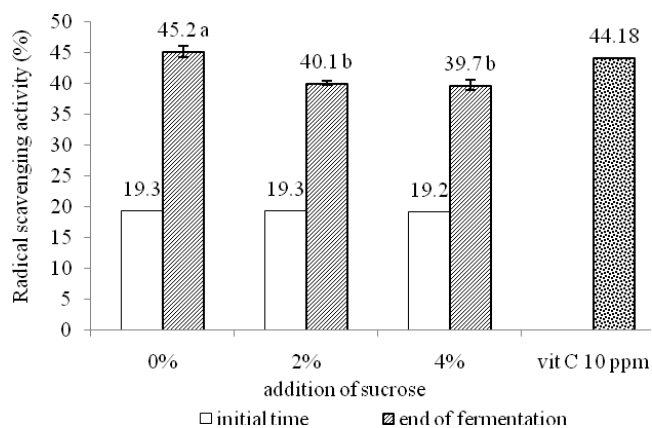


Figure-3

DPPH-scavenging activities of fermented sesame milk with and without addition of sucrose. Fermented of sesame milk using *L. plantarum* Dad 13 at 37° C, 18 h. Values with different superscripts are significantly different (P<0.05)

Conclusion

Fermentation of sesame milk using *L. plantarum* Dad 13 increased the antioxidant activity. The addition of sucrose (2% and 4% w/v) in sesame milk fermentation did not effect the growth of *L. plantarum* Dad 13, but increased the acid production. The addition of sucrose in sesame milk fermentation decreased β -glucoside activity resulted in lower antioxidant activity. *Lactobacillus plantarum* Dad 13 could grow using fermentable sugars present in sesame milk. Without addition of sucrose in sesame milk fermentation resulted in the higher β -glucoside activity and antioxidant properties than addition of sucrose in sesame milk fermentation.

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