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Research Article

Mixed culture fermentation with *Rhizopus oligosporus* and *Micrococcus luteus* to enhance isoflavone aglycone, factor 2 production and antioxidant activity of soybean

Putu Virgina Partha Devanthi and Pingkan Aditiawati

School of Life Sciences and Technology, Bandung Institute of Technology, Jl. Ganeca 10, Bandung, Indonesia.

Email: putuvirgina@gmail.com

Abstract

In this study, mixed culture fermentation of soybean under solid-state fermentation using *R. oligosporus* and *M. luteus* was performed. *M. luteus* was inoculated at different times of fermentation process, together with *R. oligosporus* at the beginning and after 6 h, 12 h, 24 h and 36 h fungal fermentation. The methanolic extracts of soybeans were analyzed using HPLC and subjected to free radical scavenging activity measurement using DPPH method. The results of HPLC analysis showed that inoculating *M. luteus* after 24 h fungal fermentation increased total isoflavone (daidzein, genistein, factor 2) concentration by 69.69% to highest level. The amount of daidzein, genistein, and factor 2 increased to 739,165 µg (75.74%), 805,855 µg (69.99%) and 91,542 µg (31.22%) per gram of defatted soybean powder respectively after 48 h. *M. luteus* inoculation at 0 h, 6 h, 12 h, 24 h, and 36 h of fermentation process increased the soybean free radical scavenging activity to 64.28%, 65.03%, 77.54%, 57.81% and 82.55%, respectively. This study shows that inoculation time of *M. luteus* plays an important role in the production of daidzein, genistein and factor 2 at the end of soybean fermentation and affects free radical scavenging activity of soybean.

Keywords: daidzein, genistein, free radical scavenging, *Glycine max*, glucosides, Indonesia.

Introduction

Soybean (*Glycine max*) contains high protein levels and some bioactive compounds including isoflavones [1]. Isoflavone is the main phenolic compound in soybean which possesses many beneficial functions in health such as preventing chronic diseases including cardiovascular, osteoporosis, breast and prostate cancer [2]. Isoflavone also acts as an antioxidant compound [3] because it can prevent cells from oxidative damage, therefore, lowering the risk of many degenerative diseases. Isoflavones in soy beans are available in 12 different isoforms which are classified as glucosides (daidzin, genistin, and glycitin), acetylglucosides (acetyldaidzin, acetylgenistin, and acetylglycitin), malonylglucosides (malonyldaidzin, malonylgenistin, and malonylglyicitin) and aglycones (daidzein, genistein, and glycitein) [4]. Among other forms of

isoflavones, the aglycones are the most important compound because of their higher biological activity when compared to the glucoside forms [5].

Rhizopus oligpsporus, a fungi-producing tempe, produces extracelullar enzyme β-glucosidase during soybean tempe fermentation process. Enzyme β-glucosidase is also contained in soybean and has been active since the soaking process [6, 7]. The enzyme hydrolizes β-glucosidic bond in the glucoside forms, liberating the aglycone forms and sugar groups. This structural change of isoflavones increases their bioavailability to human and biological activity as well as their antioxidative activity [6, 8].

A bacteria species isolated from tempe, *Micrococcus luteus*, is known to be able to transform glycitein to a new isoflavone identified as factor 2 (6,7,4'-trihydroxy isoflavone) by O-demethylation reaction [9]. The factor 2 has not been found in unfermented soybean and other natural sources. Factor 2 was proved to exhibit antioxidant activity stronger than other forms of isoflavones [10, 11]. The 6,7-ortho-dihydroxy group in this compound seems to be essential to its strong antioxidative activity. This activity contributes to its ability in preventing cancer, cardiovascular diseases and tumors.

In a previous study [12], the use of *R. oligosporus* and *M. luteus* in mixed cultured soybean fermentation with ratio 1:1 showed higher levels in the production of total isoflavones, especially factor 2. On the other hand, with the addition of *M. luteus*, poor growth of mycelium was observed. This study was conducted to determine the inoculation time of *M. luteus* to maximize the yield of daidzein, genistein and factor 2 in soybeans and to enhance soybean antioxidant activity.

Materials and Methods

Inoculum preparation

R. oligosporus derived from Microbiology Laboratory of SITH ITB was inoculated on potato dextrose agar (PDA) slant and incubated at room temperature for 7 days. The spore suspension (10^6 spores/mL) was prepared by washing the PDA slant with the mixture of NaCl 0.85% solution and Tween 80 (0.1%). *M. luteus* from Microbiology Laboratory of SITH ITB was inoculated on nutrient agar (NA) slant and incubated at room temperature for 48 h. After incubation, *M. luteus* was transferred to nutrient broth (NB). The *M. luteus* culture was shaken at 140 rpm and incubated at room temperature for 24 h. The bacterial suspension was adjusted to 10^6 cfu/mL (optical density 0.1).

Soybean fermentation

Soybeans were fermented according to the method described by Kasmidjo [13] with modification. About 1.5 kg of yellow-seeded soybean were sorted, washed and then soaked in boiled water for 24 h at ambient temperature. The soaking water was discarded and soybeans were dehulled manually. The dehulled soybeans were steamed for about 1 h and drained. After cooling, the cooked bean was inoculated with 10% (v/v) of *R. oligosporus* and *M. luteus* at a ratio of 1:1. *R. oligosporus* was inoculated at the beginning of fermentation stage, followed by *M. luteus* inoculation together with *R. oligosporus*, after 6 h, 12 h, 24 h and 36 h fungal fermentation and then incubated in petri dishes for 48 h at 30ºC.

Extraction of isoflavone aglycone

After incubation for 48 h, fermented soybeans were dried in an oven at 50ºC for about 12 h prior to grinding. About 45 g powdered soybean was extracted using n-hexane for 8-12 h until the whole fat had been removed. Defatted tempe powder (30 g) was then extracted using 300 mL of 80% aqueous methanol (1:10 w/v). The mixture was kept at 4ºC for 12-24 h. After 12-24 h, the mixture

was filtered using filter paper and the solvent was evaporated under low pressure at 45ºC using rotary evaporator. The resulting residue was then dissolved in 120 mL 80% aqueous methanol and was filtered using Whatman filter paper (no.42). The solvent was reevaporated using rotary evaporator at the same condition until it had been reduced to about half of its original volume and then chilled at -20ºC for 20 min. The mixture was filtered using Whatman filter paper (No.42) and reevaporated at the same condition and the paste formed was redissolved in 10 mL of absolute methanol [14].

Isoflavone aglycone content analysis using HPLC

Soybean extracts from the previous step were diluted in methanol PA 200 times and filtered by using syringe and organic filter paper. Soybean extracts (20 µL) were injected into Lichrosorb RP-18 column, combined with a solvent gradient system. The flow rate was maintained at 1.0 mL/min. The two solvent used as mobile phase were solvent A (water : acetic acid, 97:3) and solvent B (methanol : acetic acid, 97:3). For the first 5 min, the mobile phase used was 100% of solvent A, and then the proportion of solvent B was increased linearly to 100% in 55 min and decreased gradually to 0% over 5 min period. The detector used in this analysis was UV detector at 260 nm [14].

Antioxidant activity measurement

The antioxidant activity of the fermented soybean extracts were evaluated based on the ability to capture stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The evaluation method was adopted from Su and Chien [15]. The concentration of DPPH solution in methanol absolute was made to be 0,03 g/L and 5 mL of DPPH solution were added to 0.1 mL of soybean extracts. The mixture was homogenated and incubated at room temperature for 30 min in the dark. The blank solution was made by mixing 5 mL of DPPH solution with 0.1 mL of absolute methanol. After 30 min incubation, the absorbance of each reaction was measured with a UV spectrophotometer at ʎ 517 nm. The perentages of DPPH scavenging activity were defined by $(A_{blank}-A_{sample}) : A_{blank}$ x 100%.

Results and Discussion

According to the data in Table 2, *M. luteus* addition after 24 h fungal fermentation produced the highest level of daidzein (739,17 µg/g), genistein (805,86 µg/g) and factor 2 (91,54 µg/g). Fungal fermentation within 24 h before the inoculation of *M. luteus* was able to maximize the production of daidzein and genistein at concentration 602,630 µg/g and 585,309 µg/g, respectively (Table 1). High production of daidzein and genistein after 24 h incubation with singel culture of *R. oligosporus* might be due to *R. oligosporus* β-glucosidase higher degree of activity. Nakajima *et al* [16] reported that isoflavone aglycone content increased with fermentation time and its concentration was twofold after 24 h fermentation. Glycitein amount in soybean after 24 h fungal fermentation also probably increased to maximum amount. Since *M. luteus* was able to transform glycitein into factor 2 by O-demethylation reaction, factor 2 production from glycitein also increased to highest level at the end of incubation period.

Daidzein, genistein, and factor 2 in soybean extract were detected by comparing their retention time with the isoflavone standards run under the same condition. HPLC chromatogram of fermented soybean with *M. luteus* added at 24 h is shown in Figure 1. Daidzein, genistein and factor 2 had retention time at 39.459 min, 41.786 min, 37.503 min, respectively. From the chromatogram, it can be seen that factor 2 was produced in the least amount.

M. luteus inoculation at 12 h enhanced total isoflavones (daidzein, genistein, factor 2) concentration higher than those produced with *M. luteus* inoculated at the beginning, 6 h, and 36 h. The major

compounds were daidzein (556,50 μg/g) and genistein (623,24 μg/g). The yield of factor 2 was lower than the initial concentration by 37.58% (Table 2). The amount of daidzein and genistein increased gradually after 6 h fungal fermentation and reached their maximum concentration at 24 h (Table 1). In tempe making process, Fereirra *et al* [17] suggested that the amount of isoflavone glucosides did not change significantly within 6 h fermentation process but thereafter decreased gradually by 25% during 12 h fermentation then followed by an increased amount of isoflavone aglycones. Lower concentration of factor 2 at the end of fermentation (43,55 μg/g) was probably due to either the higher factor 2 transformation rate into other isoflavone compounds than factor 2 formation rate or low amount of glycitein in soybean as factor 2 precursor. This suggests that *M. luteus* inoculation after 12 h fungal fermentation did not increase factor 2 concentration in soybeans.

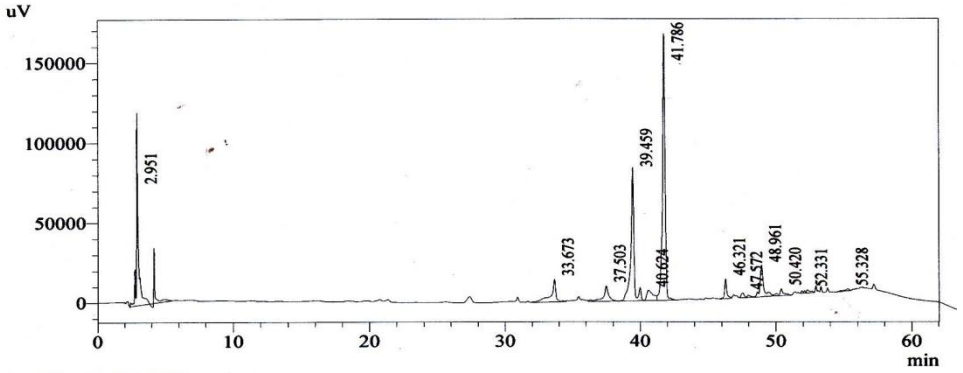

Figure 1. HPLC chromatogram of soy isoflavone in fermented soybean with *M. luteus* inoculated at 24 h of fermentation process.
(Factor 2 = 37,503; Daidzein=39,459; Genistein=41,786)

Soybeans fermented using mixed culture with *M. luteus* inoculated together with *R. oligosporus* at the beginning stage of fermentation showed higher increase in total isoflavone concentration (15.22%) than those fermented with *M. luteus* inoculated at 6 h (8.78%) and 36 h (6.45%) (Table 2, Figure 2a). When mixed culture was applied from the beginning, transformation of the existing glycitein into factor 2 occurred for the rest of fermentation process (48 h). Factor 2 concentration (80,22μg/g) was higher than those fermented with *M. luteus* inoculated at 6 h (23,31 μg/g) and 12 h (43,55 μg/g) (Table 2). Daidzein and genistein concentration were not obtained at maximum level and this was probably due to longer mixed fermentation duration than duration of fermentation solely with *R. oligosporus*, which led to higher pH at the beginning step of fermentation, thereby inhibiting fungal growth and its β-glucosidase activity.

Although it provided longer duration for fungal fermentation, the addition of *M. luteus* after 36 h fungal fermentation produced the lowest yield of total isoflavone (Table 2). The activity of β-glucosidase probably decreased just after 24 h and the rate of other isoflavones formation from isoflavone aglycone might be higher than the formation of isoflavone aglycone itself.

Table 1. Isoflavone aglycone and factor 2 content in soybean fermented with single culture of *R. oligosporus* for 6 h, 12 h, 24 h, and 36 h / prior to *M. luteus* inoculation. Soybeans were fermented with initial pH 5.35 and incubation temperature was 30°C.

Fermentation time	Factor 2		Daidzein		Genistein		Final pH
	Concentration (µg/g)	Yield (%)	Concentration (µg/g)	Yield (%)	Concentration (µg/g)	Yield (%)	
Control	**69,76**		**420,61**		**474,07**		**5,35**
6 h	**58,75**	**-15,78**	**273,03**	**-35,09**	**303,41**	**-36**	**6,77**
12 h	**49,19**	**-29,48**	**400,22**	**-4,85**	**490,60**	**3,49**	**5,92**
24 h	**39,52**	**-43,34**	**602,63**	**43,28**	**585,31**	**23,47**	**6,55**
36 h	**43,79**	**-37,23**	**297,00**	**-29,39**	**393,41**	**-17,01**	**6,51**

Table 2. Isoflavone aglycone and factor 2 content in mixed-fermented soybean after 48 h incubation with *M. luteus* added at various times. The isoflavone concentration analysis was performed at 48 h. Soybeans were fermented with initial pH 5.35 and incubation temperature was 30°C.

M. luteus inoculation time	Factor 2		Daidzein		Genistein		Final pH
	Concentration (µg/g)	Yield (%)	Concentration (µg/g)	Yield (%)	Concentration (µg/g)	Yield (%)	
Control	69,76		420,61		474,07		5,35
0 h	80,22	14,99	506,31	20,37	524,66	10,67	8,51
6 h	23,31	-66,59	438,80	4,32	586,98	23,82	6,26
12 h	43,55	-37,58	556,50	32,31	623,24	31,47	8,59
24 h	91,54	31,23	739,17	75,74	805,86	69,99	9,3
36 h	0,00	-100,00	420,24	-0,09	606,41	27,92	8,78

M. luteus introduction into soybean fermentation process caused change in soybean pH to be more alkaline, from 5.35 up to 9.3 due to fast degradation of protein further into ammonia (Table 2). In this study, the production of ammonia was supported by the emerging of ammoniacal odour at the end of fermentation process. *M. luteus* inoculation before the optimum *R. oligosporus* biomass production and isoflavone aglycones conversion could occur, would have probably affected the fungal growth and its β-glucosidase activity significantly due to alkaline pH condition. Graham *et al* [18] and Sparringa and Owens [19] suggested that *R. oligosporus* grew rapidly at pH less than 6. Sparringa and Owens [19] also suggested that the optimum growth of *R. oligosporus* NRRL 2710 occurred at pH 5.5. *R. oligosporus* β-glucosidase activity during soybean tempe fermentation reached its optimum activity at pH 5 (60°C) but it was stable at pH ranging from 2-9 at 20°C [20].

In this study, the antioxidant activity of fermented soybean with single culture of *R. oligosporus* increased with duration of fermentation as previously reported by Chaiyasut *et al* [21] (Table 1). Antioxidant activity of fermented soybean was reported as DPPH scavenging activity percentage. Isoflavones are considered as polyphenol compounds with high antioxidant activity due to their chemical structure [22]. Soybean extract with the highest concentration of daidzein, genistein and factor 2 did not exhibit the most improved ability to quench free radical DPPH.

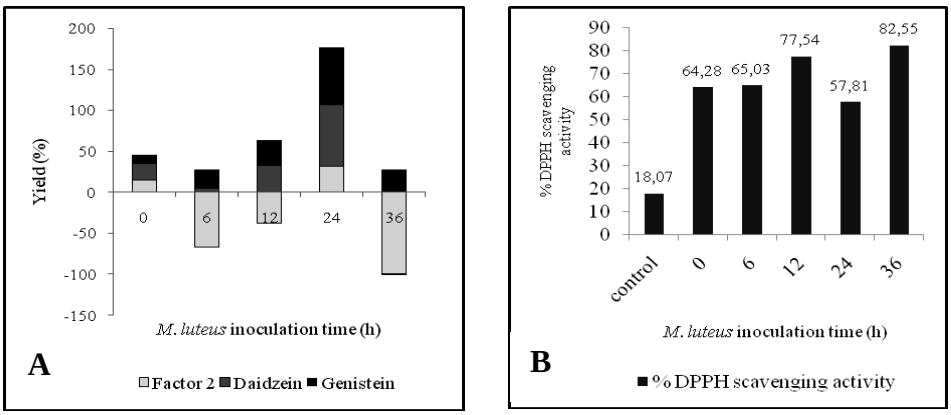

Figure 2. Factor 2, daidzein and genistein yields and DPPH free radical scavenging activity of soybean after 48 h fermentation began with single culture fermentation with *R. oligosporus* followed by mixed culture fermentation with the addition of *M. luteus* at various time of fermentation (0 h, 6 h, 12 h, 24 h, 36 h) at 30^{o}C. Initial pH of soybean was 5.35 and final pH was ranging from 6.62-9.3.

(a) Maximum yield of factor 2, daidzein, and genistein was obtained when *M. luteus* was added at 24 h (mixed fermentation duration 24 h), (b) Maximum percentage of DPPH scavenging ability was obtained when *M. luteus* was inoculated at 36 h (mixed fermentation duration was 12 h)

The highest amount of total isoflavone was obtained in soybean fermented with *M. luteus* inoculated at 24 h, but the highest DPPH scavenging activity was exhibited in soybean when *M. luteus* was inoculated at 36 h. This was probably due to the antagonist effect of isoflavone compounds contained in soybean extract with the highest level of daidzein, genistein and factor 2. There might also be other molecules produced during fermentation that contributed more significantly to the soybean antioxidant activity, such as peptides and free amino acids. The increases in peptides and free amino acids during fermentation through fungal and bacterial proteolytic activity might result in higher soybean antioxidant activity [23]. Thus, various inoculation times of *M. luteus* may affect the isoflavone composition and free amino acid amounts in soybean which leads to different free radical scavenging activity.

Conclusion

Solid state fermentation of soybeans with mixed culture of *Rhizopus oligosporus* and *Micrococcus luteus* increases soybean isoflavone content. Maximum increase of daidzein, genistein and factor 2 was obtained if *M. luteus* was added at 24 h of fermentation period. High concentration of daidzein, genistein and factor 2 in fermented soybean extract did not show higher radical scavenging activity. The highest DPPH free radical scavenging activity was demonstrated in fermented soybean extract with the addition of *M. luteus* after 36 h fungal fermentation.

References

1. Božanić, R. and Proizvodnja (2006). Svojstva i fermentacija sojinog mlijeka. ***Mljekarstvo***, 56, 233-254.

2. Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E. and Etherton, T.D. (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. ***The American Journal of Medicine***, 113, 71-88.

3. Arora, A., Nair, M.G. and Strasburg, G.M. (1998). Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. ***Archives of Biochemistry and Biophysics***, 356, 133–141.

4. Kudou, S., Fleury, Y. and Welt, D. (1991). Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). ***Agricultural and Biological Chemistry***, 55, 2227–2233.

5. Izumi, T., Piskula, M.K., Osawa, S., Obata, A., Tobe, K. and Saito, M. (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. ***Journal of Nutrition***, 130, 1695-1699.

6. Matsuura, M., Obata, A. and Fukushima, D. (1989). Objectionable flavor of soymilk developed during the soaking of soybeans and its control. ***Journal Food Science***, 54(3), 602-605.

7. Ha, E.Y.W., Morr, C.V. and Seo, A. (1992) Isoflavone aglycones and volatile organic compounds in soybean: effects of soaking treatments. ***Journal of Food Science***, 57, 414-417.

8. Randhir, R., Vattem, D. and Shetty, K. (2004) Solid-state bioconversion of fava bean by *Rhizopus oligosorus* for enrichment of phenolic antioxidants and L-DOPA. ***Innovative Food Science and Emerging Technologies***, 5,235-244.

9. Klus, K., Borger-Papendorf, G., and Barz, W. (1993) Formation of 6,7,4''-trihydroxyisoflavone (factor 2) from soybean seed isoflavones by bacteria isolated from tempe, ***Phytochemistry***, 34, 979-981.

10. Gyorgy, P., Murata, K. and Ikehata, H. (1964) Antioxidants isolated from fermented soybeans (tempeh), ***Nature***, 203, 870-874.

11. Murata, K. (1985) *Asian Symposium on Non-Salted Soybean Fermentation.*

12. Aditiawati, A. and Devanthi, P.V.P. (2012) Optimization of the length of soybean soaking time and the inoculum ratio of *Rhizopus oligosporus* and *Micrococcus luteus* for isoflavone factor 2 production by solid state fermentation. *International Conference on Women's Health in Science and Engineering.*

13. Kasmidjo (1990) Tempe, Pusat Antar Universitas Pangan dan Gizi. Yogyakarta: Universitas Gadjah Mada.

14. Iskandar, Y.M. and Priatni, S. (2005) Biokonversi senyawa isoflavonoida oleh *Rhizopus oryzae* L16 pada hasil fermentasi kedelai, ***Teknologi Indonesia***, 28, 11-19.

15. Su, M.S. and Chien, P.J. (1991) Antioxidant activity, anthocyanins, and phenolics of rabbiteye blueberry (*Vaccinium ashei*) fluid products as affected by fermentation, ***Food Chemistry***, 104, 182–187.

16. Nakajima, N., Nozki, N. and Ishihara, K. (2005) Analysis of isoflavone content in tempeh, a fermented soybean, and preparation of a new isoflavone-enriched tempeh. ***Journal of Bioscience and Bioengineering***, 100, 685-687.

17. Ferreira, M.P., Oliveira, M.C.N., Mandarino, J.M.G., Silva, J.B., Ida, E.I. and Carrão-Panizzi, M.C. (2011) Changes in the isoflavone profile and in the chemical composition of tempeh during processing and refrigeration, ***Pesquisa Agropecuária Brasileira***, 46(11).

18. Graham, D.C.W., Steinkraus, K.H. and Hackler, L.R. (1976) Factors affecting production of mold mycelium and protein in synthetic media. ***Applied and Environmental Microbiology***, 32, 381–387.

19. Sparringa, R.A. and Owens, J.D. (1999) Protein utilization during soybean tempeh fermentation. ***Journal of Agricultural and Food Chemistry***, 47, 4375-4378.

20. Ebata, J., Fukuda, Y., Hirai, K. and Murata, K. (1972) B-glucosidase involved in the antioxidant formation in tempeh, fermented soy-beans. ***Journal of the Agricultural Chemical Society of Japan***, 46, 323-329.

21. Chaiyasut, C., Kumar, T., Tipduangta, P. And Rungseevijit prapa, W. (2010) Isoflavone content and antioxidant activity of thai fermented soybean and its capsule formulation. ***African Journal of Biotechnology***, 9(26), 4120-4126.

22. Heim, K.E., Tagliaferro, A. R. and Bobilya, D.J. (2002) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships, ***The Journal of Nutritional Biochemistry***, 13, 572–584.

23. Pyo, Y.H., Lee, T.C. and Lee, Y.C. (2005) Effect of lactic acid fermentation on enrichment of antioxidant properties and bioactive isoflavones in soybean. ***Journal of Food Science***, 70, S215-S220.