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Effect of indigenous lactic acid bacteria fermentation on enrichment of isoflavone and antioxidant properties of *kerandang* (*Canavalia virosa*) extract

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Abstract

Five strains indigenous lactic acid bacteria i.e. *Lactobacillus plantarum-pentosus* T14, *Lactobacillus plantarum-pentosus* T20, *L. plantarum* T32, *L. plantarum* T33 and *L. plantarum-pentosus* T35 were tested for their capabilities to transform isoflavone glucosides to aglycones in the kerandang crude extract. Changes in growth, pH, titratable acidity (TA), β-glucosidase activity was investigated during fermentation at 37ºC for 24 h. Isoflavone transformation was analyzed using UPLC (Ultra Performance Liquid Chromatography). The antioxidant properties were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferrous ion-chelating ability method. The result showed that initial cell population of 10^6 - 10^7 CFU/ml rapidly increased and reached 10^9 CFU/ml in MRS-kerandang extract after 9 to 12 h of fermentation and constant to 24 h fermentation. All five strains produced lactic acid followed by the decreasing of pH. *Lactobacillus plantarum-pentosus* T14 showed the highest β-glucosidase activity is 558 ± 9.8 mU/ml culture at 12 h fermentation. All five strains were able to transform isoflavone glucoside to aglycone. *L. plantarum-pentosus* T14 have a better ability to transform, followed by *L. plantarum-pentosus* T20, *L. plantarum* T32, *L. plantarum* T33 and *L. plantarum-pentosus* T35.

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Introduction

Kerandang (*Canavalia virosa*) seeds are known contain bioactive compounds, such as isoflavone whose beneficial effects need to be explored. *Kerandang* beans are a rich source of protein, yet predominantly kerandang foods are not widely accepted mainly which their were beany flavor and also because of the belief that they cause flatulence. The effects of numerous process, such as soaking, germination, hydrothermal processing and fermentation, *kerandang* bean have been developed to lessen of undesirable flavors during processing (Sridhar and Seena, 2005; Djaafar *et al.*, 2010).

Some investigations showed that isoflavone consumption has been associated with a reduced risk of most hormone-associated health. The intake of isoflavone genistein and daidzein has been shown to provide protection against oxidative modification of low density lipoprotein (LDL) in human volunteers (Kerry and Abey, 1998). Mitchell *et al.* (1998) reported that the isoflavones were relatively poor hydrogen donors compared with the others polyphenols compounds such as kaempferol. Some investigations related to soyfood fermentation for isoflavone and the action of isoflavone in antioxidant properties have been reported (Pyo *et al*., 2005; Boue *et al*., 2008).

Fermentation of beans with lactic acid bacteria is known to enhance the antioxidant content, especially isoflavones aglycones (Tsangalis *et al.*, 2002; Pyo *et al*., 2005; Chun *et al.*, 2007). This is associated with β-glucosidase production by lactic acid bacteria. Pyo *et al*. (2005), that was reported that the *Lactobacilli* and *Bifidobacterium* strains possess β-glucosidase activity in soybean fermentation. The reduction of daidzin and genistin content into their aglycones may be based on the hydrolytic reaction catalyzed by β-glucosidase produced lactic acid bacterial. Fermentation of soybean by lactic acid bacterial producing β-glucosidase for 48 h at 37°C, namely *Lactobacillus plantarum* KFRI 00144, *Lactobacillus delbrueckii* subsp. *latis* KFRI 01181, *Bifidobacteria thermophilum* KFRI 00748 and *Bifidobacteria breve* K-101 were resulted in a significant increase ($P < 0.05$) in the antioxidant capacity expressed as both Trolox equivalent antioxidant capacity (TEAC) (mM) and percent scavenging activity (Pyo *et al*., 2005).

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Lactic acid bacterias are known producing β-glucosidase which play an important role in transformation of isoflavone glucoside to aglycone (Tsangalis *et al*., 2002; Pyo *et al*., 2005). Scientific study related to kerandang fermentation to increase the level of isoflavone aglycones and antioxidant properties has been reported. The objectives of research were examined whether the levels isoflavone aglycones and antioxidant activity which could increase in fermented kerandang by indigenous lactic acid bacteria-producing β-glucosidase.

Material and Methods

Kerandang beans

Kerandang beans were obtained from the beach land in the Bugel Village, Panjatan District, Kulon Progo Regency, Yogyakarta. Harvesting was conducted by picking old brown pods from the trees. Pods were then peeled and dried to reach 10% of water content. Their epidermal seeds were further removed mechanically by using an abrasive peeler to produce clean yellowish peeled beans (Figure 1).

Cultures

Pure culture of five strains of lactic acid bacteria were obtained from FNCC (Food Nutrition Culture Collection) Gadjah Mada University, Yogyakarta, as shown in Table 1. The stock cultures were grown and maintained in MRS (De Mann Rogosa Sharpe) agar medium. MRS broth medium has been used to propagate the organisms, before it was used to ferment the *kerandang* extract.

Preparation of kerandang extract

Kerandang extract preparation was refers to the methods by John and Shahidi (2010). Ten grams of *kerandang* bean which have been treated by extracted using 80% methanol (100 ml) in an ultrasonic shaker for 30 min. The slurries producing by the extraction and centrifugation at 4,000 rpm; 4ºC for 10 min that supernatants were collected. The residue was re-extracted under the same conditions. The supernatants were evaporated using a rotary vacuum evaporator (IKA Brand basic HB10) at 40ºC for 30 min. Stock of extract stored at 4ºC until used for fermentation.

Fermentation of kerandang extract

The inoculum was prepared by transferring the cultures from MRS Broth medium into MRS-kerandang extract steril medium, so subcultured in the same medium twice, incubated at 37ºC for 20-24 h. The MRS-kerandang crude extract medium was prepared consist of (per liter) proteose peptone No. 3 (10 g), beef extract (10 g), yeast extract (5 g), tween 80 (1 g), ammonium citrate (2 g), sodium acetate (5 g), magnesium sulfate (0.1 g), manganese sulfate (0.05 g), dipotassium phosphate (2 g), *kerandang* crude extract (10 g).

Table 1. Cultures of lactic acid bacteria

Source of cultures	Name of cultures
Tempe	*Lactobacillus plantarum-pentosus* T14
Asinan rebung	*Lactobacillus plantarum-pentosus* T20
Gatot	*Lactobacillus plantarum* T32
Asinan terong	*Lactobacillus plantarum* T33
Asinan terong	*Lactobacillus plantarum-pentosus* T35

Figure 1. Peeled *kerandang* beans

Fifty milliliters of MRS-*kerandang* extract steril medium inoculated with single culture (0.2%, v/v) and then incubated at 37ºC for 24 hours. A sample would be taken from each bottle aseptically at interval 6 h during fermentation. Sample directly analyzed to measure pH using pH meter, acid production (total lactic acid), growth using plate count method on MRS agar (Fardiaz, 1992), antioxidant activity using DPPH method (Pyo *et al.*, 2005; Xu and Chang, 2007; Ye *et al.*, 2009), ferrous ion-chelating ability method (Wang *et al.*, 2009) and β-glucosidase activity (Tsangalis *et al.*, 2002). Isoflavones were analyzed using UPLC with UV-Vis detector (Tsangalis *et al.*, 2002).

Determination of pH

The pH of the withdrawn aliquots every 6 h during the fermentation has been monitored using a microprocessor pH meter (Thermo Scientific, Orion 3 Start) at 27ºC after calibrated with fresh pH 4.0 and 7.0 standard buffers.

Determination of acid production and cell growth

Titratable acidity was determined by the method of Fardiaz (1992) by titration with 0.1N NaOH solution and expressed as percent lactic acid. Cell number was measured in triplicate using pour plate method (Fardiaz, 1992) with lactobacilli MRS media (Oxoid). Fermented sample (1 ml) was serially diluted with 0.85% NaCl solution and then 100 µl of diluted samples were taken into sterile plates. MRS medium containing 1.5% agar and 0.8% $CaCO_3$ was poured into the plate and mixed carefully. After incubation at 37ºC for 24 h, single colonies were counted.

Assay of β-glucosidase activity

Preparation of crude enzyme was conducted following that lactic acid bacteria cells have been harvested by centrifuging 15 ml fermented medium (4,500 rpm for 10 min; 4ºC). The supernatant was used for the analysis of β-glucosidase activity. The β-glucosidase activity of lactic acid bacteria strains was assayed by determining the rate of hidrolysis of the ρ-nitrophenyl-β-D-glucopyranoside substrate (ρNPG). Five hundred microliters of crude enzyme was added to 1000 µl of 5 mM ρNGP, prepared in 100 mM sodium phosphate buffer (pH 7) and incubated at 37ºC for 30 min. The reaction was terminated by adding 1000 µl of 1M cold (4ºC) sodium carbonate. The amount of ρ-nitrophenol released was measured with a spectrophotometer UV-Vis (Shimadzu, UV-1656 PC) at 401 nm. One unit of enzyme was defined as the amount of enzyme that released 1 µmol of ρ-nitrophenol from the substrate ρ-NPG per ml per min under assay condition. ρ-Nitrophenol was used as standard in the enzyme assay.

Determination of antioxidant activity

DPPH free radical-scavenging assay, which the ability of the extracts to scavenge the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was detected using spectrophotometer (Pyo *et al*., 2005; Xu and Chang, 2007; Ye *et al*., 2009). A 200 µL aliquot of each extract was mixed in a test tube with 3.8 mL of 1 mM DPPH, incubated for 1 h and then measured the absorbance at 517 nm with a spectrophotometer UV-Vis (Shimadzu, UV-1656 PC). All measurements were performed in triplicate. Free radical-scavenging activity was calculated by the following equation:

$$\text{Scavenging } \% = [1 - (A_{sample} - A_{blank})/A_{control}] \times 100\%$$

where A = absorbance, methanol (3.8 ml) plus sample solution (0.2 ml) was used as a blank.

Ferrous ion-chelating ability assay, that the ferrous ion-chelating ability of sample was determined according to method of Wang *et al.* (2009). One thousand micro liters of extract were mixed with 200 µl of 5 mM ferozzine, 50 µl of 2 mM $FeCl_2$ and 2.75 ml of distilled water in a tube. The solutions were well mixed and allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm with a spectrophotometer UV-Vis (Shimadzu, UV-1656 PC). All measurements were performed in triplicate. The ferrous ion-chelating ability was calculated as follows:

$$\text{Ferrous ion- chelating ability } (\%) = [A_0 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

UPLC analysis of isoflavone

Extraction quantification of isoflavone glucosides and aglycones from fermented *kerandang* extract were performed according to the methods of Tsangalis *et al.* (2002) and Pyo *et al.* (2005). A supernatant from lactic acid fermentation of the *kerandang* extract was filtered through a bond elute C-18 (VARIAN) and then eluted with 2 ml of 80% methanol. The insoluble residue was separated by centrifugation (Centrifuge 5804 R) at 4000 rpm, 4ºC for 10 min and filtered through Millex-HV PVDF 0.45 µm prior to transferring to UPLC vials.

Transformation of isoflavone was carried out using liquid chromatography with a quaternary pump, a diode array ultraviolet visible (UV-Vis) detector and vaccum degasser. The UPLC ACQUITY™ PDA-ELS system was equipped with PDA eλ Detector, Binary Solvent Manager, Sampel Manager and an Acquity UPLC® BEH C18 1.7 mm (2.1 x 100 mm) reverse-phased column which was set thermostatically at 25ºC. It was used to separate the isoflavone isomers. UPLC was used for isoflavones determination. UPLC linier gradiation was used to isolate the isoflavones for detection composed of 10% (v/v) acetonitrile and 0.1% (v/v) formic acid in water (solvent A) and 100% acetonitrile containing 0.1% formic acid (solvent B). The pump was set at a flow rate of 0.4 mL/min. After the 25 µL injection of sample or isoflavone standard onto the column, solvent A was set at 100% for 1 min, reduced to 60% over 5 min and finally 100% for 1 min prior to the next injection. The diode array UV-Vis detector was set at wavelength 260 nm to detect the isoflavone glucosides and aglycones. Mixed standards containing all isoflavone glucosides and aglycones were used for quantification of isoflavone. Single standard was also prepared for peak identification.

Results and Discussion

Growth of lactic acid bacteria in kerandang extract

Changes in viable cell number of the 5 indigenous lactic acid bacteria in kerandang extract during fermentation at 37ºC are shown in Figure 2. All five strains were showed relatively having a good growth in kerandang extract. The initial cell growth rate varied slightly depending on the species. Initial cell population of 10^6 - 10^7 CFU/ml rapidly increased and reached 10^9 CFU/ml in MRS-kerandang extract after 9 to 12 h of fermentation and constant to 24 h fermentation. Mital and Steinkraus (1974) suggested that strain of *L. acidophilus* ATCC No. 4356, *L.*

Table 2. β-glucosidase activity of indigenous lactic acid bacteria during fermentation in kerandang extract at 37ºC

Indigenous lactic acid bacteria	Bacteria source	Enzyme activity (mU/ml culture)/ fermentation time (hours)		
		6	12	24
Lactobacillus plantarum-pentosus T14	*Tempe*	ND	**558± 9.8**	**2±0.8**
Lactobacillus plantarum-pentosus T20	*Asinan rebung*	ND	**398 ± 9.0**	ND
Lactobacillus plantarum T32	*Gatot*	ND	**430 ± 9.8**	189 ± 7.3
Lactobacillus plantarum T33	*Asinan rebung*	ND	**20 ± 1.6**	9 ± 1.6
Lactobacillus plantarum-pentosus T35	*Asinan rebung*	ND	**18 ± 2.4**	ND

Inoculum number at initial fermentation = 0,2% (10^6 CFU/ml);
ND = No activity detected.

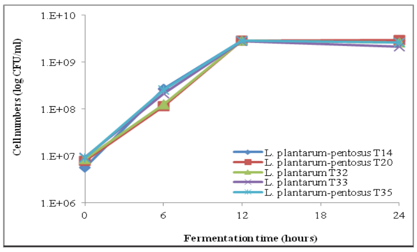


Figure 2. Growth of lactic acid bacteria in MRS-*kerandang* extract at 37ºC

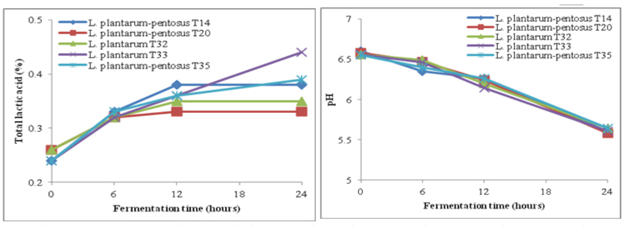
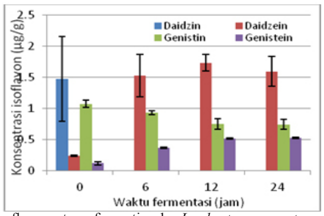


Figure 3. Lactic acid production and pH changes in MRS-*kerandang* extract during fermentation at 37ºC

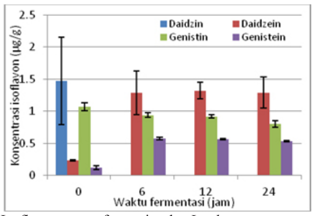
cellobiosis NRRL-B-1840 and *L. plantarum* B-246 (10^9 CFU/ml) attained higher maximum populations in soymilk than *L. bulgaricus* (Marshall) (10^6 CFU/ml). In addition, Chun *et al.* (2007) also reported that after 9 to 12 h of fermentation, cell population was the highest in soymilk inoculated with *L. paraplantarum* KM or *E. durans* KH than those with *S. salivarius* HM or *W. confusa* JY.

Lactic acid production by lactic acid bacteria

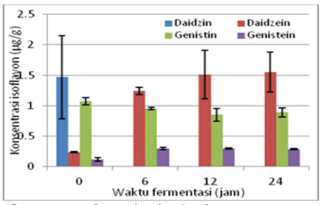
Lactic acid production and change of pH during fermentation of *kerandang* extract at 37ºC shown in Figure 3. All five strains produced lactic acid followed by the decreasing of pH. *L. plantarum* T33 produced the highest amount of lactic acid than the other strains. Although *L. plantarum-pentosus* T20 was well grown in MRS-*kerandang* extract medium, however lactic acid production was lower. Lactic acid bacteria which was inoculated in *kerandang* extract will grow and use the available carbohydrates as energy source to produce lactic acid. *Kerandang* beans contained of sucrose is about 1,684 ppm (Djaafar *et al*., 2012). Since sucrose is the major fermentable sugar, organisms utilized it as energy source to produce acid during fermentation (Mital and Steinkraus, 1974).



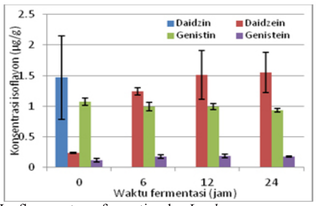
Isoflavone transformation by *L. plantarum-pentosus* T14 in extract of *Kerandang* at 37ºC



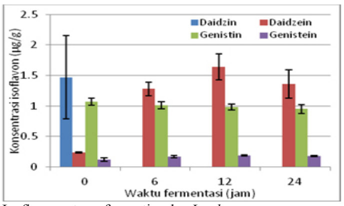
Isoflavone transformation by *L. plantarum-pentosus* T20 in extract of *Kerandang* at 37ºC



Isoflavone transformation by *L. plantarum-pentosus* T32 in extract of *Kerandang* at 37ºC



Isoflavone transformation by *L. plantarum-pentosus* T33 in extract of *Kerandang* at 37ºC



Isoflavone transformation by *L. plantarum-pentosus* T35 in extract of *Kerandang* at 37ºC

Figure 4. Isoflavone transformation by indigenous lactic acid bacterian in *kerandang* extract at 37ºC

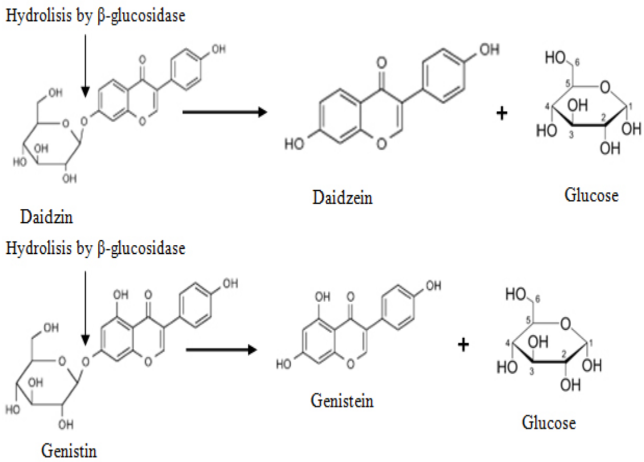


Figure 5. Structure of isoflavone and transformation by β-glucosidase producing lactic acid bacteria (Sumber : Tsangalis *et al*., 2002; Pyo *et al*., 2005; Pham and Shah, 2007)

Glucosidase activity

The glucosidase activity of five strains of indigenous lactic acid bacteria was showed in Table 2. All five strains were shown detectable level of β-glucosidase activity at 12 h fermentation in MRS-kerandang extract under the assayed condition. *Lactobacillus plantarum-pentosus* T14 showing the highest enzyme activity was 558 mU/ml culture at 12 h fermentation whreas *Lactobacillus plantarum-*

Table 3. Radical Scavenging Activity of *Kerandang* extract fermented at 37ºC

Lactic acid bacteria	Source of lactic acid bacteria	Radical Scavenging Activity (%)/fermentation time (hours)			
		0	6	12	24
Lactobacillus plantarum-pentosus T14	Tempeh	36.56 ± 0.03ᵃ	51.31 ± 0.04ᶜ	53.66 ± 0.01ᶜ	54.22 ± 0.01ᵈ
Lactobacillus plantarum-pentosus T20	Bambooshoot pickle	36.56 ± 0.03ᵃ	49.71 ± 0.01ᵃ	54.16 ± 0.02ᵈ	50.76 ± 0.02ᵃ
Lactobacillus plantarum T32	*Gatot*	36.56 ± 0.03ᵃ	52.17 ± 0.02ᵈ	52.52 ± 0.02ᵇ	50.72 ± 0.01ᵃ
Lactobacillus plantarum T33	Bambooshoot pickle	36.56 ± 0.03ᵃ	50.67 ± 0.02ᵇ	54.26 ± 0.03ᵉ	51.15 ± 0.04ᵇ
Lactobacillus plantarum-pentosus T35	Bambooshoot pickle	36.56 ± 0.03ᵃ	51.33 ± 0.05ᶜ	50.69 ± 0.03ᵃ	53.96 ± 0.02ᶜ

Different letter in the same coloum indicated significant difference (P < 0,05)

Table 4. Ferrous ion-chelating Ability of *Kerandang* extract fermented at 37ºC

Lactic acid bacteria	Source of lactic acid bacteria	Ferrous ion-chelating ability (%)/fermentation time (hours)			
		0	6	12	24
Lactobacillus plantarum-pentosus T14	Tempeh	47.50 ± 0.15ᵃ	91.48 ± 0.22ᵉ	97.53 ± 0.17ᵉ	95.18 ± 0.02ᵉ
Lactobacillus plantarum-pentosus T20	Bambooshoot pickle	47.50 ± 0.15ᵃ	87.25 ± 0.04ᵈ	82.80 ± 0.11ᵇ	81.52 ± 0.32ᵇ
Lactobacillus plantarum T32	Gatot	47.50 ± 0.15ᵃ	86.72 ± 0.07ᶜ	91.51 ± 0.03ᵈ	84.04 ± 0.01ᶜ
Lactobacillus plantarum T33	Bambooshoot pickle	47.50 ± 0.15ᵃ	81.20 ± 0.14ᵃ	77.60 ± 0.17ᵃ	86.45 ± 0.20ᵈ
Lactobacillus plantarum-pentosus T35	Bambooshoot pickle	47.50 ± 0.15ᵃ	81.82 ± 0.06ᵇ	84.54 ± 0.20ᶜ	78.64 ± 0.28ᵃ

Different letter in the same coloum indicated significant difference (P < 0,05)

pentosus T35 has the lowest enzyme activity of 18 mU/ml culture (Table 2). This suggests that *L. plantarum-pentosus* T14 was able to grow in the extract of *kerandang* and produce β-glucosidase.

The enzyme activity increased up to 12 h fermentation, but then decreased after 24 h fermentation. It was showed in line with the growth of bacteria in the exponential phase, up to 12 h fermentation, afterward the growth of bacteria turned into stationary phase (Figure 2). Pyo *et al.* (2005) reported that the activity of β-glucosidase *L. plantarum* KFRI 00,144 was strongly correlated with the high exponential growth phase. Tsangalis *et al.* (2002) also found that the β-glucosidase enzyme activity in line with the growth of bifidobacteria. Thus highest enzyme activity was performed during exponential phase (growth phase), so decreased while the stationary phase.

Isoflavone concentration

Isoflavone glucosides and isoflavone aglycones concentration during fermentation of the *kerandang* extract were shown in Figure 4. All five strains were able to transform isoflavone glucosides to aglycones. *L. plantarum-pentosus* T14 have a better ability than *L. plantarum-pentosus* T20 *L. plantarum* T32, *L. plantarum* T33 and *L. plantarum-pentosus* T35. It was in line with β-glucosidase activity of *L. plantarum-pentosus* T14 that increased at 12 h fermentation of the *kerandang* extract (Table 2).

Isoflavone are present in legume as glucosides, with the glucose conjugated at the 7 position of isoflavone. β-glucosidase producing lactic acid bacteria during fermentation catalysed the hydrolysis of isoflavone glucoside and increased isoflavone aglycone concentration (Figure 5) (Tsangalis *et al.*, 2002; Pyo *et al.*, 2005; Pham and Shah, 2007). Pyo *et al.* (2005) reported that β-glucosidase of *L. plantarum* KFRI 00144 was able to hydrolyze isoflavone glucosides become isoflavone aglycones in soybean fermentation. According to Chun *et al.* (2007), the aglycone concentration in soymilk fermented with *L. plantarum* KM were 6-fold and 7-fold higher than the initial levels of daidzein and genistein, respectively, after 6 h fermentation.

Antioxidant activity in fermented kerandang extract

The free radical scavenging activity and ferrous ion-chelating ability for each sample were shown in Table 3 and Table 4. Fermentation of kerandang extract with five strains of lactic acid bacteria showed that it could enchance radical scavenging activity. All five strains were having high antioxidant activity by DPPH assay, were *L. Plantarum-pentosus* T14 was having the highest antioxidant activity than the others strain. The increasing of radical scavenger activity by means of fermentation with *L. plantarum-pentosus* T14 and *L. plantarum-pentosus* T35 were 1.48 times for 24 h; *L. plantarum-pentosus* T20 and *L. plantarum* T32 were 1.39 times; *L. plantarum* T33 was 1.40 times compared to radical scavenging activity at initial time (0 h) of fermentation. Similarly, the increasing of ferrous ion-chelating ability during fermentation was also relatively high, namely *L. plantarum-pentosus* T14 was 2.00 times; *L. plantarum-pentosus* T20 was 1.72 times; *L. plantarum* T32 was 1.77 times; *L. plantarum* T33 was 1.82 times, and *L. plantarum-pentosus* T35 was 1.66 times compared to the ferrous ion-chelating ability at initial time of fermentation (Table 3 and Table 4). Pyo *et al.* (2005) explained that using the DPPH and ABTS radical scavenging assay, the antioxidant activity of each extract was following the order of *B. thermophilum* KFRI 00144 > *L. Delbrueckii* subsp. *lactis* KFRI 01181 > *L. plantarum*

KFRI 00748 > *B. breve* K-101.

These results suggested that transformation isoflavone glucosides into isoflavone aglycones contributing such a high antioxidant activity in fermentation of *kerandang* extract by lactic acid bacteria, due to the addition of a hydroxyl group on the atom C-7 of isoflavone (Otieno *et al*., 2005; Tsangalis *et al.*, 2002) are responsible for increased antioxidant activity. Thereby forming stable free radical that do not initiate or propagate further oxidation of lipids.

Conclusion

All five strains of indigenous lactic acid bacteria showed relatively to have a good growth in the *kerandang* extract and acid production. Hydrolysis of the glycoside moiety depended on the strain of lactic acid bacteria. Overall, *L. plantarum-pentosus* T14 showed the best growth rate, acid production, β-glucosidase activity and isoflavone hydrolysis. *L. plantarum-pentosus* T14 seems to be a promising strain as a starter for production of bioactive fermentation of *kerandang* based on its growth rate, acid production and isoflavone transformation capabilities in a short time. Effects of the usage of mixed culture with different organisms and establishment of the optimum fermentation condition in terms of production of desirable isoflavone compounds, are some valuables that worth for further investigation in the future.

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