Malaysian Journal of Microbiology, Vol 6(1) 2010, pp. 30-40
http://dx.doi.org/10.21161/mjm.17709

Hydrolysis of bioactive isoflavone in soymilk fermented with β-glucosidase producing lactic acid bacteria from local fermented foods of Indonesian

Sumarna*

Department of Food Science and Biotechnology,
Technical Implementation Unit for Development of Chemical Engineering Processes,
Indonesian Institute of Sciences, Yogyakarta, Indonesia.
Email: marna_smn@yahoo.com

Received 9 April 2009; received in revised form 23 July 2009; accepted 30 July 2009

ABSTRACT

This study investigated the possible application of β-glucosidase producing lactic acid bacteria isolated from local fermented foods as a functional starter culture to obtain the bioactive isoflavones, genistein and daidzein, in fermented soymilk. Seven strains of bacteria, *Lactobacillus plantarum pentosus* SMN 001*, Lactobacillus casei* subsp *rhamnosus* FNCC 098, *Lactobacillus casei* subsp *rhamnosus* FNCC 099*, Lactobacillus casei* subsp *rhamnosus* FNCC 113*, Lactobacillus delbrueckii subsp. delbrueckii* FNCC 045*, Lactobacillus plantarum* SMN 025 *and Lactobacillus plantarum pentosus* FNCC 235 exhibited variable β-glucosidase activity. *L. plantarum* SMN 025 and *L. casei* subsp *rhamnosus* FNCC 098 exhibited the highest β-glucosidase activity of 0.653 and 0.643 U/mL respectively. Acid development, viable populations, β-glucosidase activity and quantification of isoflavone using HPLC were performed at 0, 6, 12, 18, 24 and 30 h of incubation at 41 °C. Seven β-glucosidase-producing strains are able to increase aglycones in fermented soymilk, however, each of the strain produces significantly different bioconversion (p<0.05) of the glucoside isoflavones into their bioactive aglycones. During this fermentation period, with *L. plantarum* SMN 025, and *L. casei* subsp *rhamnosus* FNCC 098, the high level reduced from 150.62 µg/mL (at 24 h) to 142.58 µg/mL and from 150.62 µg/mL (at 24 h) to 144.71 µg/mL, respectively. The indicate that two β-glucosidase producing lactic acid bacteria have great potential for enrichment of bioactive isoflavones in soymilk fermentation.

Keywords: lactic acid bacteria, isoflavone contents, β-glucosidase, soymilk

INTRODUCTION

Isoflavones are phytochemicals present in leguminous plants, especially in soybeans. Soy isoflavones have been implicated in health benefits, including the potential to reduce the risk of age-related and hormone-related diseases including cancer, menopausal symptoms, cardiovascular disease, and osteoporosis (Jacobsen *et al*., 1998; FDA, 1999; Gerhauser *et al*., 2003; Hermansen *et al*., 2003; Omoni *et al*., 2005). Researches indicate that differences in the chemical structure of isoflavones may result in variable bioavailabilities in biological systems (Brown, 1998; Xu *et al*., 1994). In general, isoflavones in soybeans exist mainly as glucoside forms and rarely as aglycone forms unless they have been fermented (Wang and Murphy, 2006). It has been reported that certain intestinal bacteria play major roles in the hydrolysis of isoflavone glucosides and promote their absorption in the intestine (Setchell *et al*., 2002). Biotransformation and the production of metabolites of isoflavones in the intestinal tract are highly dependent on the nature of intestinal microflora .

In soybeans, most isoflavones exist as glycoside, acetylglycoside, and malonylglycoside forms and, to a lesser extent, in the form of aglycones. However, it is recognized that the readily bioavilable isoflavones are aglycones rather than glycosides. The glucoside conjugates of isoflavones are converted into aglycones during soybean processing by the effect of β-glucosidase. β-glucosidase is considered to be key enzyme for the conversion of isoflavone form in fermented soybean foods. We have shown β-glucosidase to be effective in converting isoflavone glycoside to aglycones (Pandjaitan *et al*., 2000a; 2000b). β-glucosidase has superior activity for hydrolyzing acetylglycoside and malonylglycoside isoflavones. If β-glucosidase can effectively convert acetylglycoside and malonylglycoside to their aglycones, it can lead to an enrichment of isoflavone aglycones in soy food such as soy milk fermented. Depending on processing technique, soybean products present different forms and concentrations of isoflavones. Fermented products contain higher levels of aglycones forms (Coward *et al*., 1998 and Wang and Murphy, 2006). Lactic acid fermentation (Wang and Murphy, 2006) and pre-soaking treatments during processing activate the enzymatic process of β-glucosidase (Matsuda *et al.*, 1994 Matsuura *et al.*, 1993; 1998). Wang *et al.*, 1996; 2006) observed that the optimum pH for production of aglycones, daidzein and genistein, in soymilk was around 6.0 at 50 °C temperature. Two isoforms of β-glucosidase

***Corresponding author**

brought nearly all the hydrolyzing action into daidzin and genistin (Matsuura *et al*., 1993). The enzyme showed optimum activity at pH 4.5 and 45 °C, and its pH range of action was 3.2-7.0. The isoflavones daidzein and genistein occur naturally in most soyfoods, conjugated almost exclusively to sugars.

Probiotic microorganisms possess β-glucosidase, β-galactosidase and α-galactosidase (Tochikura *et al.*, 1986), which play an important role in the hydrolysis isoflavone glycosides to the bioavailable aglycones forms. Commercial β-glucosidases have been used in biotransformation of isoflavone glycosides (Park *et al.,* 2002; 2003) and β-glucosidase from *Bifidobacterium* have been used in hydrolysing the β-1,6-glycoside bonds in order to increase the concentration of bioactive isoflavone aglycones in soymilk (Tsangalis *et al.*, 2002; 2003). Microorganisms possess endogenous β-glucosidases which can be utilised to hydrolyse predominant isoflavone glucosides in soymilk to improve biological activity. It has been reported that probiotic organisms including *Bifidobacterium*, *Lactobacillus acidophilus* and *L. casei* increased the concentration of bioactive isoflavone aglycones in soymilk during fermentation. It has been reported that many bifidobacteria and some other lactic acids (Bordignon *et al*., 2004; Pyo *et al*., 2005; Chien *et al*., 2006) hydrolyse isoflavone glycosides into corresponding aglycones. *Bifidobacterium breve*, *Bifidobacterium bifidum* and *L. casei* subsp. *rhamnosus* strains produced the yogurt-like fermented soybean milk containing high concentrations of isoflavone aglycones. Therefore, our objectives were to examine the effectiveness of β-glucosidase producing lactic acid bacteria isolated from local fermented foods as a functional starter cultures to increases the bioactive isoflavones in fermented soymilk.

MATERIALS AND METHODS

Materials and chemicals used

Soybeans (Glycine max. L. Merr) of cultivar wilis, high-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Hanover Park, IL). The aglycone standards of genistein, daidzein and glycitein as well as flavone were purchased from Sigma (Merck, Oxoid), while the β -glucoside standards of genistin, daidzin and glycitin as well as daidzein were purchased from Alfa Chemical. Genistein, genistin, flavone, daidzein were prepared in HPLC grade methanol, and daidzin, glycitein and glycitin were prepared in ethanol due to their varied solubility characteristics. All others chemicals and reagents were of analytical grade.

Strains and culture conditions

Sevens strains of lactic acid bacteria were isolated from local fermented foods such as fermented cassava (gatot, and growol), tempeh, bamboo shoot, muromi ketchup, Yoghurt *L. plantarum pentosus* SMN 001, *L. casei* subsp. *rhamnosus* FNCC 098*, L. casei* subsp. *rhamnosus* FNCC 099*, L. casei* subsp. *rhamnosus* FNCC 113, *L. delbrueckii* subsp. *delbrueckii* FNCC 045, *L. plantarum* SMN 025 and *L. plantarum pentosus* FNCC 235 (Sumarna, 2008) were obtained from food and nutrition culture collection Centre for Food and Nutrition studies, Gadjah Mada University. Culture stock were kept in 10% glycerol and 10% skim milk with the ratio 1:1 kept in sterile 1.5 mL polyethylene srew cap tubes at –40 °C. The strains were rejuvenated in MRS broth (Merck, Oxoid) at 37 °C for 24 h.

Soymilk preparation

Soybean grains (500 g) were soaked in cold water for 12 h at room temperature (27 °C). After soaking, water was discarded and the grains were then ground in a blender, for 3 min, in 4000 mL of hot water at 60 °C. Soymilk was extracted by filtration through a cotton cloth. The slurry was cooked for 10 min, filtered and boiled at 70 °C for 30 min.

Fermentation of soymilk with lactic acid bacteria isolated from local fermented foods

Two sets of 6 glass bottles each containing 300 mL sterile soymilk were aseptically inoculated with active culture of *L. plantarum pentosus* SMN 001, *L. casei* subsp. *rhamnosus* FNCC 098, *L. casei* subsp. *rhamnosus* FNCC 099, *L. casei* subsp. *rhamnosus* FNCC 113, *L. delbrueckii* subsp. *delbrueckii* FNCC 045, *L. plantarum* SMN 025, and *L. plantarum pentosus* FNCC 235 strains at 5% (v/v) and incubated at 41 °C for 24 h. The bottles were labeled 0, 6, 12, 18, 24, and 30 h in order to facilitate withdrawing of aliquots at 0, 6, 12, 18, 24, and 30 h of fermentation. Aliquots of 50 mL from each of the 6 bottles were taken at 0, 6, 12, 18, 24, and 30 h of incubation. Each aliquot was divided into 20 and 30 mL portions in sterile 50 mL screw top falcon tubes. The 20 mL portions were used for determination of β-glucosidase activity, and pH, and enumeration of cell counts, whereas the 30 mL portions were freeze dried for the analysis of isoflavones using high-performance liquid chromatography (HPLC).

pH measurements

Changes in pH were monitored during fermentation of soymilk at 0, 6, 12, 24, and 30 h using a pH meter (HANNA Instruments).

Enumeration of bacterial population

Cell populations of *L. plantarum pentosus* SMN 001*, L. casei* subsp. *rhamnosus* FNCC 098, *L. casei* subsp. *rhamnosus* FNCC 099, *L. casei* subsp. *rhamnosus* FNCC, 113, *L. delbrueckii* subsp. *delbrueckii* FNCC 045, *L. plantarum* SMN 025 and *L. plantarum pentosus* FNCC 235 was determined as described previously. Briefly, 1 g of each fermented soymilk sample was added to 9 mL of sterile 0.15% (w/v) bacteriological peptone (Oxoid) and

water diluent and vortexed for 30 s. The resulting suspension was serially diluted in sterile 0.15% (w/v) peptone water (Oxoid) and 1 mL of the appropriate dilution was used for selective enumeration by the pour plate technique. The cell growth of each organism was assessed by enumerating bacterial population after 6, 12, 24, 36 and 48 h of fermentation of soymilk on MRS agar (Amyl media). Anaerobic jars and gas generating kits were used for creating anaerobic conditions. Plates containing 25 - 250 colonies were counted and recorded as colony forming units (CFU) per gram of the fermented soymilk.

Analysis of β-glucosidase activity soymilk

Seven strains of lactic acid bacteria were each inoculated in 50 mL soymilk, and incubated at 37 °C for 30 h and screening for β-glucosidase activity was conducted at 0, 12, 18, 24, and 30 h of incubation. Based on β-glucosidase activity, seven strains were selected for further enzymatic assay. The strains were activated in MRS broth by inoculating 1% level at 41 °C for 24 h. The 5 inoculations were done in sterile soymilk from which 5% w/v of each active culture was inoculated in 300 mL of 6 bottles of soymilk. Fifty milliliters of aliquot was withdrawn aseptically from each sample at 0, 6, 12, 18, 24, and 30 h of incubation and β-glucosidase activity was determined using modified method of Tsangalis *et al*. (2002) by measuring the rate of hydrolysis of *p*-nitrophenyl-*β*-D-glucopiranoside 1 mM (*ρ*NPG). One thousand microliters of 5 mM (*ρ*NPG) prepared in 100 mM sodium phosphate buffer (pH 7) was added to 10 mL of each aliquot and incubated at 41 °C for 30 min (Scalabrini *et al*., 1998). Five hundred microliters of 1 M cold sodium carbonate was added to stop the reaction. The aliquots were then placed in 1.8 mL eppendorf centrifuge tubes followed by centrifugation (14000 x *g*) for 30 min using an eppendorf centrifuge (model 5415). The amount of *ρ*-nitrophenol released was measured using a spectrophotometer at 420 nm. One unit of the enzyme activity was defined as the amount of β-glucosidase that released 1 micromole of ρ-nitrophenol from substrate *ρ*NPG per mL per min under assay conditions. The specific activity was expressed as a unit of enzyme per microgram of the protein. The protein concentration was determined by a modified version of the Lowry method. The supernatant was filtered through a 0.45 µm filter membrane to filter out *ρ*-nitrophenol. The *ρ*NPG substrate and *ρ*-nitrophenol were purchased from Sigma Chemical Co. Jakarta, Indonesia.

Extraction of isoflavones for HPLC analysis

The extraction of isoflavones, including malonyl-, acetyl-, β-glycosides, and aglycones from fermented and non-fermented soymilk was performed in triplicate, using a modified version of the method described by Tsangalis *et al.* (2002). A 1 g freeze-dried sample was added to 50 mL of methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman No. 1 filter paper into a 100 mL volumetric flask. The remaining dried soy matter was washed with the filtered portion and then refiltered into the same flask. A 5 mL aliquot was mixed with 60 µL of internal standard flavone solution (10 mg/50 mL) and dried under a stream of nitrogen using a Techne Sample Concentrator. The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and acetonitrile (50:50) solution and centrifuged (14,000 × *g*) for 30 min using an Eppendorf centrifuge (model 5415C) to precipitate undissolved matter prior to transferring to HPLC vials.

HPLC analysis of isoflavones

HPLC gradient elution for isolating the isoflavones for detection was acetonitrile (Solvent A) and 10 mM ammonium acetate buffer containing 0.1% trifluoro-acetic acid (Solvent B) set at a flow rate of 1 mL/min (Setchel, 1998). After 20 µL injection of sample or isoflavone standard into the column, solvent B was set at 100% for 2 min, reduced to 60% over 22 min and finally 100% for 5 min prior to the next injection. A diode array UV-visible detector was set at dual wavelengths of 260 nm to detect the malonyl-, acetyl-, and β-glycosides, aglycones, and the flavone. Single standards were prepared for identification of peak. Malonyl- and acetyl-glycoside conjugates were quantified with respect to their β-glycoside equivalent response factors and corrected according to molecular weight. Isoflavone concentrations were calculated back to wet basis (µg isoflavones/mL soymilk).

Statistical analysis

The enzyme activity in soymilk and isoflavone concentrations during incubation were obtained in triplicate on two occasions and presented as means ± standard error of 3 replicates. The analysis was conducted using one-way analysis of variance (ANOVA) and 95% confidence levels, using Microsoft Excel. ANOVA data with a $p<0.05$ was classified as statistically significant.

RESULTS AND DISCUSSION

Proximate composition and total isoflavone contents of local developed and cultivated soybean variety wilis, soymilk.

Results of analysis Proximate composition and total isoflavone contents are given in Table 1.

Table 1: Proximate composition (g /100g dry weight) and total isoflavone contents

Composition	Wilis soybeans	Soymilk
Moisture	11.0 ± 0.40	88.90 ± 1.80
Protein	34.0 ± 1.00	3.6 ± 0.12
Fat	21.6 ± 0.20	1.3 ± 0.03
Carbohydrates	28.1 ± 1.20	2.08 ± 0.01
Raffinose (mg)	987 ± 2.80	850 ± 1.30
Stachyose (mg)	475 ± 1.50	350 ± 1.10
Ash	2.97 ± 0.10	0.46 ± 0.01
Glucoside		
Daidzin	57.02 ± 3.12	27.06 ±1.12
Genistin	62.64 ± 3.14	32.65 ± 1.14
Glycitin	21.03 ± 0.47	11.03 ± 0.47
Malonylglucoside		
Daidzin	27.06 ± 2.21	7.06 ± 0.12
Genistin	11.65 ± 1.14	5.65 ± 0.14
Glycitin	13.03 ± 1.18	3.03 ± 0.12
Acetylglucoside		
Daidzin	24.26 ± 2.42	14.26 ± 0.42
Genistin	22.56 ± 1.74	12.56 ± 0.74
Glycitin	14.33 ± 1.31	9.33 ± 0.31
Aglycone		
Daidzein	7.36 ± 0.32	15.16 ± 0.32
Genistein	3.46 ± 0.44	8.46 ± 0.44
Glycitin	2.37 ± 0.13	4.37 ± 0.13
Total	266.77 ± 5.43	150.62 ± 5.43

The pH change and growth of with strains lactic acid bacteria were isolated from local fermented foods

All the seven strains showed lowering pH of culture media and production of lactic acid are essential for manufacturing soygurt. In the soymilk, most strains tested could lower pH and produce lactic acid. However, *L. plantarums* SMN 025 *and L. casei* subsp. *rhamnosus* FNCC 098 showed lowering pH of the culture medium or production of lactic acid, and the highest viable counts during fermentation occurred at 24 h at 41 °C in the soymilk. Changes in the pH and population cell of with strains lactic acid bacteria were isolated from local fermented foods in soymilk during fermentation at 0, 6, 12, 18, 24, and 30 h at 41 °C are shown in Figure 1 and 2. Subsequently, fermentation at 24 h the populations declined slowly for all strains growth. The initial pH of all soymilk samples after inoculation was 6.4. At 41 °C, the fastest pH decline was observed in soymilk with culture *L. plantarums* SMN 025 *and L. casei* subsp. *rhamnosus* FNCC 098. After 24 h of fermentation, the pH values of soymilk reached 3.7.and 3.8, respectively. Seven strains of lactic acid bacteria tested in this study could grow in the soybean milk medium with a maximum viable cell count greater than 10^9 CFU/ mL.

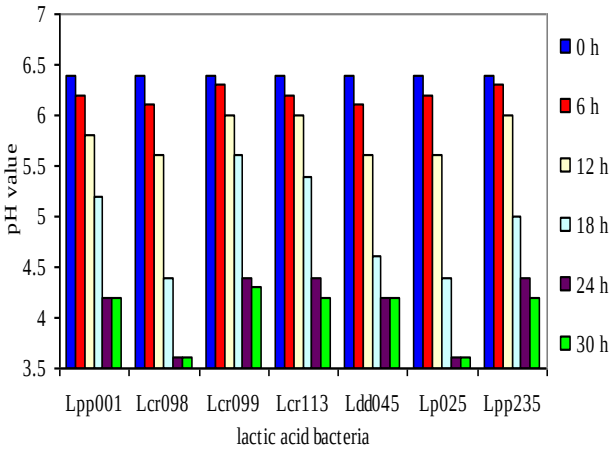

Figure 1: pH changes in soymilk during fermentation with each of the seven strains lactic acid bacteria at 41°C (Lpp001=*L. plantarum pentosus* SMN 001; Lcr098= *L. casei* subsp. *rhamnosus* FNCC 098; Lcr099=*L. casei* subsp. *rhamnosus* FNCC 099; Lcr113=*L. casei* subsp. *rhamnosus* FNCC 113; Ldd045=*L. delbrueckii* subsp. *delbrueckii* FNCC 045; Lp025=*L. plantarum* SMN 025, and Lpp235=*L. plantarum pentosus* FNCC 235)

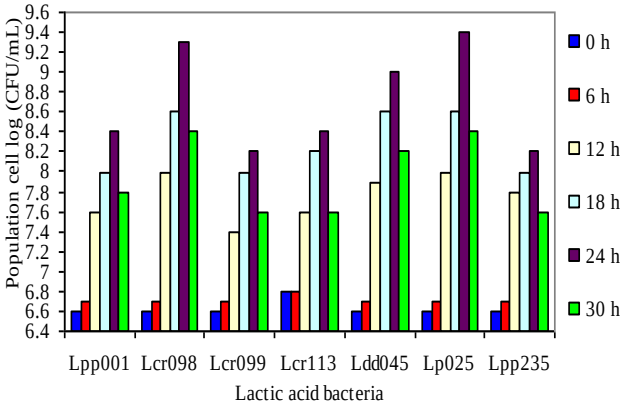

Figure 2: Population cell in soymilk during fermentation with each of the seven strains lactic acid bacteria at 41°C (Lpp001=*L. plantarum pentosus* SMN 001; Lcr098= *L. casei* subsp. *rhamnosus* FNCC 098; Lcr099=*L. casei* subsp. *rhamnosus* FNCC 099; Lcr113=*L. casei* subsp. *rhamnosus* FNCC 113; Ldd045=*L. delbrueckii* subsp. *delbrueckii* FNCC 045; Lp025=*L. plantarum* SMN 025, and Lpp235=*L. plantarum pentosus* FNCC 235)

β-Glucosidase activity in fermented soymilk

Due to the fact that these glucosidase enzymes exist intercellularly in crude forms, assaying for a specific enzyme required the use of a specific substrate to determine the glucosidase enzyme potential of seven strain. Due to our interest in β-glucosidase activity, *ρ*-nitrophenyl-*β*-D-glucopyranoside (*p*NPG) was used as the substrate.

Seven strains showing glucosidase activity in fermented soymilk at 12 and 24 h incubation period at 41 °C are listed. β-glucosidase activity of the seven strains of lactic acid bacteria were isolated from local fermented foods in fermented soymilk during incubation period of 12 and 24 h is shown in Table 2. All the seven strains showed detectable levels of the enzyme activity when grown in soymilk. There was a significant difference ($p< 0.005$) in the β-glucosidase activity over 12, and 24 h of incubation for all strains. All the seven strains showed an increase in β-glucosidase activity up to 24 h followed by a decline as fermentation progressed (data not showed). The increase in β-glucosidase activity between 12 and 24 h was significant ($p<0.05$) for with *L. plantarum pentosus* SMN 001, *L. casei* subsp. *rhamnosus* FNCC 098*, L. casei subsp rhamnosus* FNCC 099*, L. casei* subsp. *rhamnosus* FNCC 113, *L. delbrueckii* subsp. *delbrueckii* FNCC 045, *L. plantarum* SMN 025 and *L. plantarum pentosus* FNCC 235*.*

However, *L. plantarum* SMN 025 had the highest β-glucosidase activity at 24 h, though this was significantly different ($p<0.05$) to the activity during the entire incubation process.

The β-glucosidase enzyme is responsible for the breakdown of β-1-6-glycosidic linkage, which conjugates the pran ring of isoflavone and the sugar moieties. In hydrolysing the glycosidic bond, the isoflavone glucosides are broken down to their bioactive aglycone forms. β-glucosidase was reported to catalyse the hydrolysis of glucoside isoflavones with the formation of aglycones (Esaki *et al*., 2004). In general, the bioconversion of isoflavone glucosides into the bioactive aglycone forms followed the same pattern of β-glucosidase activity during the incubation of all the seven strains. Based on β-glucosidase activity in soymilk, it appeared that *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098 strains showed better β-glucosidase activity. Strains for further study were selected based on the highest enzyme activities resulting in representation of strains from that *L. plantarum* SMN 025*, L. casei* subsp. *rhamnosus* FNCC 098. The soymilk fermented with *L. plantarum* SMN 025, *L. casei* subsp. *rhamnosus* FNCC 098 exhibited the highest β-glucosidase activity of 0.653; 0.643 U/ mL respectively.

Table 2: β-glucosidase activity of seven lactic acid bacteria in fermented soymilk at 12 and 24 h of incubation at 41 °C*

Microorganisms	Source of fermented food	Incubation time	Unit of enzyme
L. plantarum pentosus SMN 001	Cassava (patilo)	12	0.321 ± 0.048^{b}
		24	0.463 ± 0.027^{d}
L. plantarum SMN 025	Bamboo shoot	12	0.352 ± 0.015^{c}
		24	0.653 ± 0.031^{f}
L. plantarum pentosus FNCC 235	Moromi soybean	12	0.329 ± 0.016^{b}
		24	0.587 ± 0.028^{e}
L. casei subsp. *rhamnosus* FNCC 098	Cassava (growol)	12	0.354 ± 0.012^{c}
		24	0.648 ± 0.053^{f}
L. casei subsp. *rhamnosus* FNCC 099	Cassava (gatot)	12	0.287 ± 0.013^{a}
		24	0.472 ± 0.025^{d}
L.casei subsp. *rhamnosus* FNCC 113	Tempeh	12	0.327 ± 0.011^{b}
		24	0.486 ± 0.028^{d}
L.delbrueckii subsp.*delbrueckii* FNCC 045	Yoghurt	12	0.329 ± 0.016^{b}
		24	0.617 ± 0.028^{e}

* From means standard deviation of three duplicates for each sample
abcdef Values in a column with different letters are significantly different ($p \leq 0.05$)
One unit of enzyme is the amount of β-glucosidase that released 1 micromole of *ρ*-nitrophenyl-*β*-D-glucopyranoside (*ρ*NPG) from *ρ*NPG per mL/ min at 41 °C

Changes of isoflavone content during fermentation with strains lactic acid bacteria were isolated from local fermented foods

During fermentation of soymilk with strains lactic acid bacteria were isolated from local fermented foods exhibiting β-glucosidase activity, a concomitant enzymatic hydrolysis of isoflavone glucosides occurs, leading to changes in the concentration of all the isoflavone forms in soymilk. Table 3 show the changes in isoflavone concentration occurring in soymilk during 24 h fermentation with *L. plantarum pentosus* SMN 001, *L. casei* subsp. *rhamnosus* FNCC 098*, L. casei subsp rhamnosus* FNCC 099*, L. casei* subsp. *rhamnosus* FNCC 113, *L. delbrueckii* subsp. *delbrueckii* FNCC 045, *L. plantarum* SMN 025 and *L. plantarum pentosus* FNCC 235 respectively. All the seven microorganisms caused a significant increase (p<0.05) in the concentration of isoflavone aglycones via the β-glucosidase catalysed hydrolysis of isoflavone glucoside conjugates. Contents of glucosides, malonylglucosides and acetylglucoside isoflavones decreased slightly, while contents of aglycone isoflavones increased high during 24 h of fermentation in the soymilk fermented with strains lactic acid bacteria were isolated from local fermented foods. However, a optimun increase in the content of the bioactive aglycone isoflavones was noted in soymilk after 24 h of fermentation. Simultaneously, the contents of glucoside isoflavones underwent the most significant reduction. During this fermentation period, with *L. plantarum* SMN 025, the high level reduced from 150.62 µg/mL (at 24 h) to 142.58 µg/mL and with *L. plantarum pentosus* FNCC 235, the lower level reduced from 150.62 µg/mL (at 24 h) to 149.74 µg/mL respectively. A similar trend in the changes of these isoflavones was also found when soymilk was fermented with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098*.*

Isoflavones content in soymilk fermented with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098

Result HPLC chromatograms of isoflavones in soymilk fermentation of soymilk with *L. plantarum* SMN 025. Contents of glucosides, malonylglucosides and acetylglucoside isoflavones decreased slightly, while contents of aglycone isoflavones increased slightly during the first 12 h of fermentation in the soymilk fermented with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098. During this fermentation period, the level reduced from 150.62 µg/mL (at 18 h) to 145.74 µg/mL (86%) and from 150.62 µg/mL (at 18 h) to 145.94 µg/mL, (86%) respectively. A similar trend in the changes of these isoflavones was also found when soymilk was fermented with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098*.* The changes in isoflavone concentration in relation to increasing bacteria population in soymilk during 24 h fermentation at 41 °C with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098*,* the high level reduced from 150.62 µg/mL (at 24 h) to 142.58 µg/mL (105%) and from 150.62 µg/mL (at 24 h) to 144.71 µg/mL (95%), respectively, is shown in Figure 3 and 4. Simultaneously, the contents of glucoside isoflavones underwent the most significant reduction.The maximum concentration of aglycones produced corresponded to the highest β-glucosidase activity and the maximum cell population of each microorganism. In general, the concentration of isoflavone aglycones increased and at the same time, the concentration of isoflavone glucosides were reduced during fermented soymilk. However, a seen increase in the content of the bioactive aglycone isoflavones was noted in soymilk after 24 h of fermentation was no significant change (p>0.05) (data not shown).

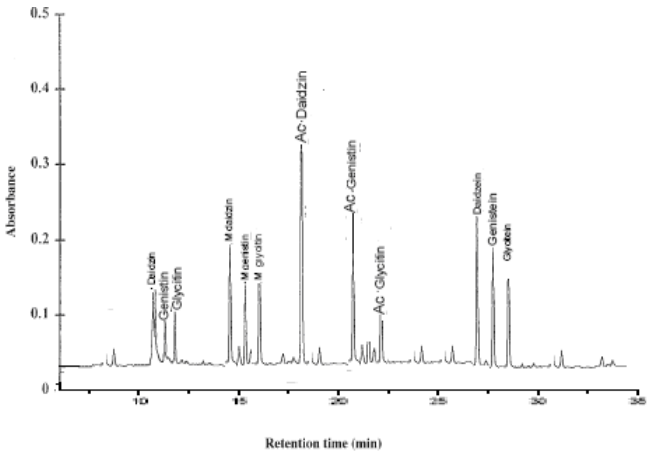

Figure 3: HPLC chromatograms of isoflavones in soymilk fermentation at 24 h of incubation at 41 °C with *L. plantarum* SMN 025

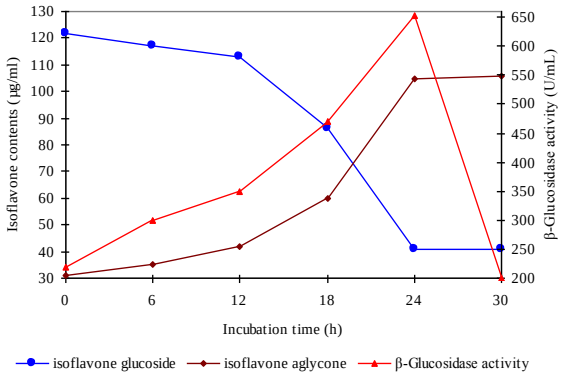

Figure 4: Changes in aglycone and glucoside isoflavone contents and β-glucosidase activity during the fermentation of soymilk with *L. plantarum* SMN 025

Table 3: Isoflavone contents of various fermented soymilk*

Isoflavone	Content of isoflavones (µg/mL) in soymilk fermented with culture							
	Control	***L. plantarums* SMN 025**	***L. plantarum pentosus* SMN 001**	***L. plantarum pentosus* FNCC 235**	***L. casei* subsp. *rhamnosus* FNCC 098**	***L. casei* subsp. *rhamnosus* FNCC 099**	***L. casei* subsp. *rhamnosus* FNCC 113**	***L. delbrueckii subsp. delbrueckii* FNCC 045**
Glucoside								
Daidzin	27.06 ± 1.14[a]	2.86 ± 1.02[b]	15.97 ± 1.12[c]	15.77 ± 1.13[c]	3.56 ± 1.01[d]	15.87 ± 1.18[c]	15.86 ± 1.16[c]	7.60 ± 1.08[e]
Genistin	32.65 ± 1.13[a]	3.65 ± 1.11[b]	11.42 ± 1.10[c]	12.52 ± 1.11[c]	3.75 ± 1.08[b]	12.52 ± 1.14[c]	11.56 ± 1.11[c]	11.36 ± 1.04[d]
Glycitin	11.03 ± 0.47[a]	3.23 ± 0.42[b]	5.71 ± 0.41[c]	5.93 ± 0.37[c]	3.52 ± 0.27[b]	5.73 ± 0.24[c]	6.03 ± 0.67[c]	8.13 ± 0.44[d]
Malonylglucoside								
Daidzin	7.06 ± 0.12[a]	4.03 ± 0.12[b]	7.47 ± 0.15[c]	7.46 ± 0.12[c]	4.26 ± 0.12[b]	7.46 ± 0.18[c]	6.96 ± 0.12[c]	6.62 ± 0.08[d]
Genistin	5.65 ± 0.14[a]	3.45 ± 0.11[b]	5.74 ± 0.06[c]	5.75 ± 0.31[c]	3.55 ± 0.24[b]	5.75 ± 0.14[c]	5.45 ± 0.13[c]	4.35 ± 0.10[d]
Glycitin	3.03 ± 0.12[a]	2.03 ± 0.08[b]	3.16 ± 0.17[a]	3.12 ± 0.19[a]	2.23 ± 0.10[b]	3.14 ± 0.22[a]	3.13 ± 0.14[a]	2.19 ± 0.12[b]
Acetylglucoside								
Daidzin	14.26 ± 0.42[a]	10.26 ± 1.12[b]	13.29 ± 1.23[a]	13.19 ± 1.26[a]	11.62 ± 1.02[b]	13.29 ± 1.11[a]	11.22 ± 1.01[b]	10.36 ± 1.10[b]
Genistin	12.56 ± 0.74[a]	7.16 ± 0.78[b]	11.34 ± 0.97[c]	11.05 ± 0.57[c]	7.45 ± 0.84[b]	11.35 ± 0.67[c]	12.16 ± 0.74[c]	8.86 ± 0.76[d]
Glycitin	9.33 ± 0.31[a]	4.33 ± 1.02[b]	9.83 ± 1.10[a]	9.93 ± 1.08[a]	4.31 ± 1.12[b]	9.93 ± 1.14[a]	10.34 ± 1.06[a]	5.53 ± 1.11[d]
Aglycone								
Daidzein	15.16 ± 0.32[a]	42.70 ± 1.12[b]	23.16 ± 1.12[c]	23.26 ± 1.12[c]	43.96 ± 1.12[b]	23.16 ± 1.12[c]	24.27 ± 1.12[c]	33.26 ± 1.12[d]
Genistein	8.46 ± 0.44[a]	34.61 ± 0.74[b]	22.44 ± 0.74[c]	22.15 ± 0.74[c]	32.67 ± 0.74[b]	22.25 ± 0.74[c]	21.32 ± 0.74[c]	29.86 ± 0.74[d]
Glycitin	4.37 ± 0.13[a]	24.27 ± 1.12[b]	19.31 ± 1.12[c]	18.92 ± 1.12[c]	23.73 ± 1.12[b]	19.31 ± 1.12[c]	18.21 ± 1.12[c]	19.83 ± 1.12[c]
Total	150.62 ± 1.02	142.58 ± 0.60	148.81 ± 1.03	149.05 ± 1.01	144.71 ± 0.50	149.74 ± 1.08	148.80 ± 1.10	147.95 ± 0.30

* From means standard deviation of three duplicates for each sample
[abcde] Means in the same row with different small letter superscripts are significantly different ($p<0.05$)
Fermented soymilk with sevens strains of lactic acid bacteria were isolated from local fermented foods were incubated at 41 °C for 24 h

Table 4: Changes in isoflavone content during the fermentation of soymilk with *L. plantarum* SMN 025*

Isoflavone	Content of isoflavones (µg/mL) in fermented of soymilk was incubated at 41 °C				
	0 h	6 h	12 h	18 h	24 h
Glucoside					
Daidzin	27.06 ± 1.16^{a}	27.01 ± 1.02^{a}	26.05 ± 1.15^{b}	18.16 ± 1.02^{c}	2.86 ± 1.12^{d}
Genistin	32.65 ± 1.14^{a}	32.55 ± 1.24^{a}	32.45 ± 1.16^{a}	12.65 ± 1.14^{b}	3.65 ± 1.14^{c}
Glycitin	11.03 ± 0.47^{a}	10.05 ± 0.47^{a}	10.02 ± 0.43^{a}	9.03 ± 0.47^{b}	3.23 ± 0.47^{c}
Malonylglucoside					
Daidzin	7.06 ± 0.12^{a}	7.04 ± 0.11^{a}	6.09 ± 0.09^{b}	5.06 ± 0.12^{c}	4.03 ± 0.10^{d}
Genistin	5.65 ± 0.14^{a}	4.35 ± 0.24^{a}	4.15 ± 0.16^{a}	4.05 ± 0.14^{a}	3.45 ± 0.14^{b}
Glycitin	3.03 ± 0.12^{a}	3.83 ± 0.10^{a}	3.03 ± 0.11^{a}	2.83 ± 0.12^{b}	2.03 ± 0.12^{c}
Acetylglucoside					
Daidzin	14.26 ± 0.42^{a}	13.86 ± 1.12^{b}	12.26 ± 1.16^{c}	12.12 ± 1.12^{c}	10.26 ± 1.12^{b}
Genistin	12.56 ± 0.74^{a}	11.05 ± 0.74^{b}	10.32 ± 0.54^{b}	10.56 ± 0.74^{b}	7.16 ± 0.74^{c}
Glycitin	9.33 ± 0.31^{a}	8.03 ± 1.12^{b}	8.63 ± 1.11^{b}	8.43 ± 1.12^{b}	4.33 ± 1.12^{b}
Aglycone					
Daidzein	15.16 ± 0.32^{a}	16.26 ± 1.12^{b}	17.16 ± 1.02^{c}	30.76 ± 1.18^{d}	44.70 ± 1.12^{e}
Genistein	8.46 ± 0.44^{a}	10.56 ± 0.74^{b}	11.26 ± 0.64^{b}	20.16 ± 0.74^{c}	34.61 ± 0.74^{d}
Glycitin	4.37 ± 0.13^{a}	5.33 ± 1.12^{a}	6.53 ± 0.92^{b}	11.93 ± 1.12^{c}	24.27 ± 1.12^{d}
Total	150.62 ± 5.43	149,92 ± 4.52	147,95 ± 4.34	145,74 ± 3.21	142.58 ± 3.13

* From means standard deviation of three duplicates for each sample
^{abcde} Means in the same row with different small letter superscripts are significantly different (p<0.05)
Fermented soymilk containing *L. plantarum* SMN 025 was incubated at 41°C for 24 h

Table 5: Changes in isoflavone content during the fermentation of soymilk with *L. casei* subsp. *rhamnosus* FNCC 098*

Isoflavone	Content of isoflavones (µg/mL) in fermented of soymilk was incubation at 41 °C				
	0 h	6 h	12 h	18 h	24 h
Glucoside					
Daidzin	27.06 ± 1.12^{a}	27.01 ± 1.02^{a}	25.05 ± 1.15^{b}	18.16 ± 1.22^{c}	3.56 ± 0.12^{d}
Genistin	32.65 ± 1.14^{a}	32.55 ± 1.24^{a}	32.25 ± 1.16^{a}	12.65 ± 1.04^{b}	3.75 ± 0.14^{c}
Glycitin	11.03 ± 0.47^{a}	10.05 ± 0.47^{b}	10.02 ± 0.43^{b}	9.03 ± 0.37^{c}	4.52 ± 0.47^{d}
Malonylglucoside					
Daidzin	7.06 ± 0.12^{a}	7.04 ± 0.11^{a}	6.09 ± 0.09^{b}	5.06 ± 0.13^{c}	5.26 ± 0.17^{d}
Genistin	5.65 ± 0.14^{a}	4.35 ± 0.24^{b}	4.15 ± 0.16^{b}	4.05 ± 0.14^{b}	4.25 ± 0.14^{b}
Glycitin	3.03 ± 0.12^{a}	3.83 ± 0.10^{b}	3.03 ± 0.11^{a}	2.83 ± 0.11^{b}	2.53 ± 0.12^{b}
Acetylglucoside					
Daidzin	14.26 ± 0.42^{a}	13.76 ± 1.12^{b}	13.26 ± 1.16^{b}	12.12 ± 1.10^{c}	11.62 ± 1.12^{d}
Genistin	12.56 ± 0.74^{a}	11.05 ± 0.74^{b}	11.32 ± 0.54^{b}	10.56 ± 0.79^{c}	9.45 ± 0.74^{d}
Glycitin	9.33 ± 0.31^{a}	8.03 ± 1.12^{b}	8.63 ± 1.11^{b}	11.43 ± 1.13^{c}	4.31 ± 0.12^{d}
Aglycone					
Daidzein	15.16 ± 0.32^{a}	16.26 ± 1.12^{b}	17.16 ± 1.02^{c}	30.76 ± 1.18^{d}	41.96 ± 2.12^{e}
Genistein	8.46 ± 0.44^{a}	10.46 ± 0.74^{b}	11.26 ± 0.64^{b}	19.26 ± 0.94^{c}	32.77 ± 1.74^{d}
Glycitin	4.37 ± 0.13^{a}	5.33 ± 1.12^{a}	6.43 ± 0.92^{b}	9.93 ± 1.02^{c}	20.73 ± 1.52^{d}
Total	150.62 ± 5.32	149.72 ± 4.12	148.65 ± 4.31	145.94 ± 3.23	144.71 ± 3.15

* From means standard deviation of three duplicates for each sample
^{abcde} Means in the same row with different small letter superscripts are significantly different (p<0.005). Fermented soymilk containing *L. casei* subsp. *rhamnosus* FNCC 098 was incubated *at* 41 °C for 24 h

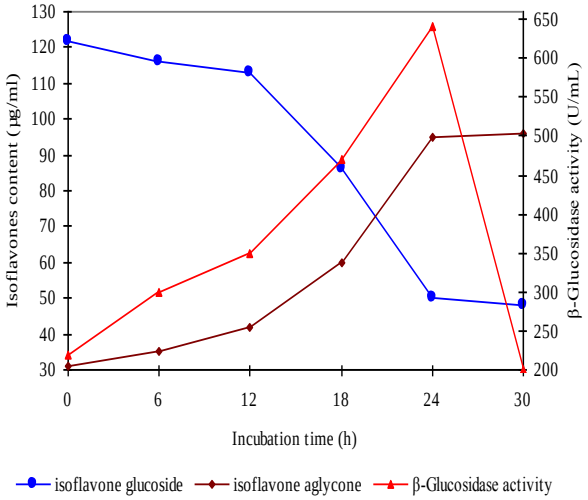

Figure 5: Changes in aglycone and glucoside isoflavone contents and β-glucosidase activity during the fermentation of soymilk with *L. casei* subsp. *rhamnosus* FNCC 098

Changes of β-glucosidase activity, aglycone and glucoside isoflavone contents during fermentation

Changes in the content of β-glucosidase activity, isoflavone glucosides and aglycones in soymilk fermented with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098 showed in Figure 4 and 5, respectively. Decrease in the concentration of isoflavone glucosides with a corresponding significant increase in the concentration of isoflavone aglycones during incubation with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098. Activity of β-glucosidase in soymilk fermented with *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098 exhibited the highest of 0.653; 0.643 U/mL respectively. In addition, changes of glucoside and aglycone isoflavone contents expressed as a percent of total isoflavone, obtained by dividing the content of individual isoflavone with total isoflavones. The highest β-glucosidase activity in the *L. plantarum* SMN 025 and *L. casei* subsp. *rhamnosus* FNCC 098 fermented in soymilk increase in the concentration of isoflavone aglycones. There appeared to be correlations between the level of cell population and β-glucosidase activity of each strain, and the hydrolysis of conjugated isoflavones in soymilk fermentation. The observed phenomenon was in agreement with the reports of Esaki *et al.* (1994) and Choi, *et al.* (2002). Wang *et al.* (2006) reported that fermentation with lactic acid bacteria and bifidobacteria, individually and in combination, increased the antioxidative activity of soymilk.

CONCLUSIONS

Seven strains of bacteria, *L. plantarum pentosus* SMN 001, *L. casei* subsp. *rhamnosus* FNCC 098, *L. casei* subsp. *rhamnosus* FNCC 099, *L. casei* subsp. *rhamnosus* FNCC 113, *L. delbrueckii* subsp. *delbrueckii* FNCC 045, *L. plantarum* SMN 025, *and L. plantarum pentosus* FNCC 235 exhibited variable β-glucosidase activity. *L. plantarum* SMN 025*, L. casei subsp rhamnosus* FNCC 098 exhibited the highest β-glucosidase activity of 0.653; 0.643 U/ mL respectively. Seven β-glucosidase-producing strains are able to increase aglycones in fermented soymilk, however, each of the strain produces significantly different bioconversion (p<0.05) of the glucoside isoflavones into their bioactive aglycones. During this fermentation period, with *L. plantarum* SMN 025, and *L. casei* subsp. *rhamnosus* FNCC 098*, the* high level reduced from 150.62 µg/mL (at 24 h) to 142.58 µg/mL and from 150.62 µg/ mL (at 24 h) to 144.71 µg/mL, respectively. The present study indicates that two β-glucosidase producing lactic acid bacteria has great potential for enrichment of bioactive isoflavones in soymilk fermentation.

ACKNOWLEDGEMENT

The author would like to thank the Indonesian Institute of Sciences and the Centre for Food and Nutrition Studies, Gadjah Mada University for an excellent collaboration and Prof. Ferry Sandra for helpful discussions.

REFERENCES

Bordignon, J. R., Nakahara, K., Yoshihashi, T. and Nikkuni, S. (2004). Hydrolysis of isoflavone and consumption oligosaccharides during lactic acid fermentation of soybean, Food and Technology Division, (JIRCAS), Tsukuba, Ibaraki 305-8686, Japan. http://www.Jircas.affrc.go.jp

Brouns, F., (2002). Soya isoflavones: A new and promising ingredient for the health food sector. *Food Research International* **35, 187-193.**

Brown, J.P., (1988). Hydrolysis of glycosides and esters. *In:* Role of the gut flora in toxicity and cancer. Rowland R. (ed.). Academic Press, San Diego, CA, **pp. 109–144.**

Chien, H. L., Huang, H. Y. and Chou, C. C. (2006). Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. *Journal of Food Microbiology* **23, 772-778**.

Choi, Y. B., Kim, K. S. and Rhee, J. S. (2002). Hydrolysis of soybean isoflavone glucosides by lactic acid bacteria. *Journal Biotechnology* **24, 2113-2116.**

Coward, L., Smith, M., Kirk, M., & Barnes, S. (1998). Chemical modification of isoflavones in soyfoods during cooking and processing. *American Journal of Clinical Nutrition* **68, 1486S-1491S.**

Esaki, H., Watanabe, R., Hishikawa, N., Osawa, T., and Kawakishi, S. (2004). Utility of isoflavone

preparations from soy sauce cake as antioxidant materials. *Journal of Japan* Society, *Food Science Technology* **51, 47-53.**

Fukutake, M., Takahshi, M. and Ishida, K. (1996). Quantification of genistein and genistin in soybeans and soybean products. *Journal Food Chemistry and Toxicology* **34, 457-461.**

Food and Drug Administration (1999). Food labeling, health claims, soy protein, and coronary heart disease. Food and Drug Admin., 21 Code of Federal Regulations, Part 101, Oct 26.

Gerhauser, C., Klimo, K., Heiss, E., Neumann, I., Gamal-Eldeen, A., Knauft, J., Liu, G. Y., Sitthimonchai, S. and Frank, N. (2003). Mechanism-based *in vitro* screening of potential cancer chemopreventive agents. *Journal of Mutation Research* **524, 163-172.**

Hargreves, D. F., Potten, C. S., Harding, C., Shaw, L. E., Morton, M. S., Roberts, B.A., Hawell, A. and Bundred, N. J. (1999). Two-week dietary soy supplementation has an estrogenic effect on normal premenopause breast. *Journal of Clinical Endocrinology Metabolism* **84, 4017-4024.**

Hermansen, K., Dinesen, B., Hoie, L. H., Morgenstern, E. and Gruenwald, J. (2003). Effects of soy and other natural products on LDL:HDL ratio and other lipid parameters: A literature review. *Journal of Advisior Theraphy* **20, 50-78.**

Jacobsen, B. K., Knutsen, S. F. and Fraser, G. E. (1998). Does high soy milk intake reduce prostate cancer incidence. The Adventist Health Study (United States). Journal of *Cancer Causes Control* **9, 553-557.**

King, **R. A. and Bignell, C. M. (2000).** Concentration of isoflavone phytoestrogens and their glucosides in Australian soya beans and soya foods. *Australian Journal of Nutrition and Dietetics,* **57, 70-78.**

Liggins, J., Bluck, L. J., Runswick, S., Atkinson, C., Coward, W. A. and Bingham, S. A. (2000). Daidzein and genistein contents of vegetables. *Journal of Nutrition*, **84, 717-725.**

Matsuda, S., Norimoto, F., Matsumoto, Y., Ohba, R., Teramoto, Y., Ohta, N. and Ueda, S. (1994). Solubilization of a novel isoflavone glucoside-hydrolyzing β-glucosidase from *Lactobacillus casei* subsp. *rhamnosus*. *Journal of Fermentation Bioengineering* **77, 439-441.**

Matsuura, M., and Obata, A. (1993). β-Glucosidases from soybeans hydrolyze daidzin and genistin. *Journal of Food Science* **58, 144-147.**

Mastuura, M., Obata, A., & Fukushima, D. (1998). Objectionable flavor of soymilk developed during soaking of soybeans and its control. *Journal of Food Science* **54, 602-607.**

Messina, M., Gugger, E. T. and Alekel, D. L. (2001). Soy protein, soybean isoflavones, and bone health: A review of the animal and human data. *In:* Handbook of nutraceuticals and functionl foods. Wildman, R. (ed.). CRC Press, Boca Raton, FL. **pp. 77-98.**

Murphy, P. A., Barua, K. and Hauck, C. C. (2002). Solvent extraction selection in the determination of isoflavones in soy foods. *Journal of Chromatography B* **777, 129-138.**

Omoni, A. O. and Aluko, R. E. (2005). Soybean foods and their benefits: Potential mechanisms of action. *Journal of Nutrition* **63, 272-283.**

Pandjaitan, N., Hettiarachchy, N. and Ju, Z., Y. (2000a). Enrichment of genistein in soy protein concentrate with beat β-glucosidase. *Journal Food Science* **65, 403-407.**

Pandjaitan, N., Hettiarachchy, N., Ju, Z. Y., Crandall, P., Seneller, C. and Ombek, D. (2000b). Enrichment of Genistein in soy protein concentrate with hydrocolloids and β-glucosidase. *Journal Food Science* **65, 591-595.**

Park, Y. K., Aguiar, C. L., Alencar, S. M., Mascrenhas, H. A. A. and Scamparini, A. R. P. (2002). Conversion of malonyl ß-glucoside isoflavones into glycoside isoflavones found in some cultivars of Brazilian soybeans. *Journal of Science Technology Alimentacion*, **22, 130-135.**

Park, Y. K., Lui, M. C. Y. and Aguiar, C. L. (2003). Production of enriched isoflavone aglycones during processing of soy protein isolates and soy protein concentrates. *In: 2003 IFT Annual Meeting Book of Abstracts*. 12 – 16 July 2003. Institute of Food Technologists, Chicago, IL. **pp. 215.**

Pyo, Y. H., Lee, T. C. and Lee, Y. C. (2005). Enrichment of bioactive isoflavones in soymilk fermented with β-glucosidase-producing lactic acid bacteria. *Journal of Food Research International* **38, 551-559.**

SAS (2001). SAS User's Guide: Statistics (8.01 Ed.). SAS Institute Inc., Cary, NC.

Scalabrini, P., Rossi, M., Spettoli, P., & Matteuzi, D. (1998). Characterisation of Bifidobacterium stains for use in soymilk fermentation. *Internal Journal of Food Microbiology* **39, 213-219.**

Setchell, K. D. R. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *American Journal of Clinical Nutrition* **68, 1333S-1346S.**

Setchell, K, D. R., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T., Wolfe, B. E., Kirschner, A. S. and Heubi, J. E. (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *American Journal of Clinical Nutrition* **76, 447-453.**

Sumarna. (2008). Changes of raffinose and stachyose in soy milk fermentation by lactic acid bacteria from local fermented foods of Indonesian. *Malaysian Journal of Microbiology* **4(2), 26-34.**

Tochikura, T., Sakai, K., Fujiyoshi, T., Tachiki, T., and Kumagai, H. (1986). *Para*-nitrophenyl glycoside-hydrolyzing activities in bifidobacteria and characterization of *beta*-galactosidase of *Bifidobacterium longum* 401. *Journal Agricultural and Biological Chemistry* **50(9), 2279-2286.**

Tsangalis, D., Ashton, J. F., McGill, J. and Shah, N. P. (2002). Enzymatic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase producing bifidobacteria. *Journal of Food Science* **67, 3104-3113.**

Tsangalis, D., Ashton, J. F., McGill, A, E. J. and Shah, N. P. (2003). Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented with D-glucose and L-cysteine. *Journal of Food Science* **68, 623-631.**

Wang HJ, Murphy PA. (1994) Isoflavone content in commercial soybean foods. *Journal of Agricultural and Food Chemistry* **42, 1666-1673.**

Wang, H. J. and Murphy, P. A. *(*1996). Mass balance study of isoflavones during soybean processing. *Journal of Agricultural and Food Chemistry* **44, 2377-2383.**

Wang, Y. C., Yu, R. C., Yang, H. Y. and Chou, C. C. (2003). Sugar, acid and B-vitamin contents in soymilk fermented with lactic acid bacteria alone or simultaneously with bifidobacteria. *Journal of Food Microbiology* **20, 333-338.**

Wang, Y. C., Yu, R. C. and Chou, C. C. (2006). Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. *Journal of Food Microbiology* **23, 128-135.**

Xu, X., Wang, H. J. and Murphy, P. A. (1994). Daidzein is a more bioavailable soymilk isoflavone than genistein in adult woman. *Journal of Nutrition* **124, 825-832.**