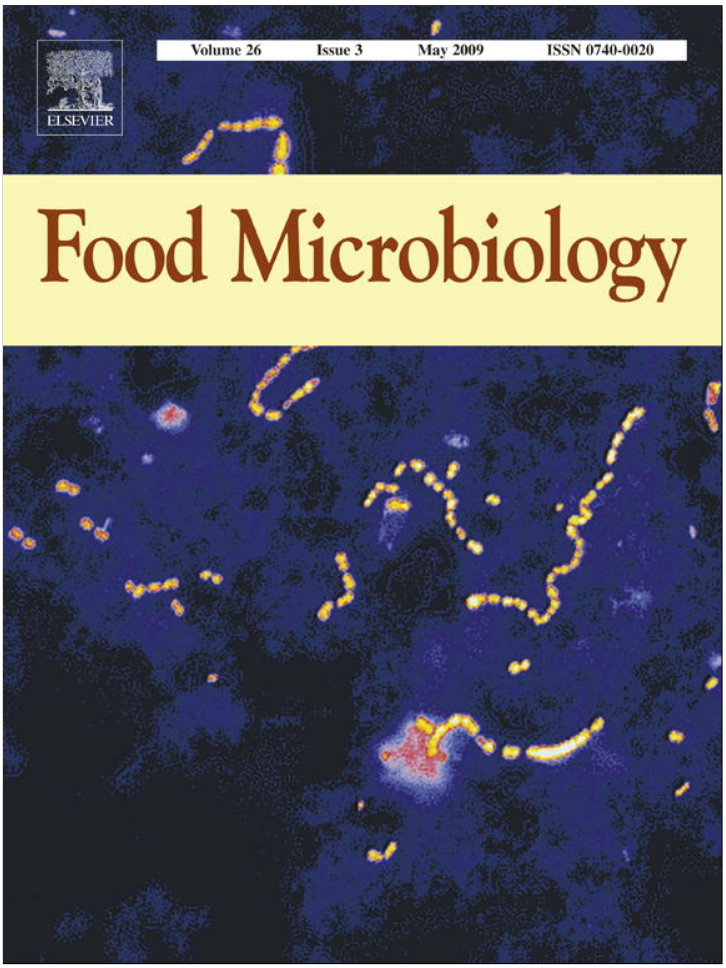

Food Microbiology 26 (2009) 333–339

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation

Jose A. Marazza [a], Marisa S. Garro [a,*], Graciela Savoy de Giori [a,b]

[a] Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, (T4000ILC) San Miguel de Tucumán, Tucumán, Argentina
[b] Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, UNT, Tucumán, Argentina

ARTICLE INFO

Article history:
Received 7 August 2008
Received in revised form
19 November 2008
Accepted 27 November 2008
Available online 16 December 2008

Keywords:
Soymilk fermentation
Lactic acid bacteria
β-Glucosidase enzyme
Sugars and isoflavones

ABSTRACT

Lactobacillus rhamnosus CRL981 showed the highest levels of β-glucosidase and was selected to characterize this enzyme system, among 63 strains of different *Lactobacillus* species. The maximum activity was obtained at pH 6.4 and 42 °C. The enzyme showed weak resistance to thermal inactivation maintaining only 20% of the initial activity when it was exposed at 50 °C for 5 min. It also, showed stability when stored at 4 °C for 60 days. Afterwards, *L. rhamnosus* was evaluated for hydrolysis of isoflavones to aglycones, cell population, residual sugars and organic acid produced during fermentation on soymilk (37 °C for 24 h). Higher viable counts were obtained after 12 h of fermentation (8.85 log CFU ml^{-1}) followed by a drop of pH and an increase of acidity during fermentation due the production of organic acids. *L. rhamnosus* CRL981 was able to proliferate in soymilk and produce a high β-glucosidase activity achieving a complete hydrolysis of glucoside isoflavones after 12 h of fermentation. The present study indicates that *L. rhamnosus* CRL981 could be used in the development of different aglycone-rich functional soy beverages.

1. Introduction

Interest in soybeans and soy-based products has grown significantly in the last decade due to their reported nutritional and health-promoting benefits. Researchers have credited phytochemicals in soybeans, especially isoflavones, for some of these beneficial health effects. Soy isoflavones are reported to play a role in the prevention of osteoporosis, cardiovascular disease, and several hormone dependent cancers (Setchell and Cassidy, 1999; Adlercreutz, 2002; Omoni and Aluko, 2005). Soybean and soy foods contain great amounts of isoflavones which are found as a complex mixture of glucoside conjugates the most abundant being the glucosides of genistein and daidzein, known as genistin and daidzin (Setchell et al., 2002). The relative proportions of glucoside conjugates can vary considerably between different soy foods depending on species, geographical and environmental conditions, and the extent of industrial processing (Wang and Murphy, 1994a,b). In the case of soymilk, the isoflavones are present as either aglycones or glucosides depending on the soy source used in manufacture (raw soybean or soy protein isolate) (Tsangalis et al., 2004; Wei et al., 2007). The metabolic fate of soy isoflavones after consumption, as well as their biological activities depends on their chemical structure (Cassidy et al., 2006). After ingestion, the isoflavone glucosides are hydrolyzed by both intestinal mucosal and bacterial β-glycosidase releasing the aglycones (Setchell, 1998). Several studies have demonstrated that lactic acid bacteria with β-glucosidase activity are able to increase the aglycone content during soymilk fermentation (Chien et al., 2006; Otieno et al., 2006; Donkor and Shah, 2008).

Previously, in an attempt to develop a probiotic adjunct culture that overcomes the undesirable beany flavor, and to reduce the level of raffinose and stachyose, contributors of flatulence, a number of series of studies has been performed on the fermentation of soymilk with lactic acid bacteria and bifidobacteria (Garro et al., 1998, 1999, 2004a; LeBlanc et al., 2004a). It was observed that the fermented soymilk containing probiotic bacteria possessed a reduced content of stachyose and raffinose due to its high α-galactosidase activity (LeBlanc et al., 2004b,c), and was able to stimulate an immune response. These results suggest that a probiotic adjunct culture for soymilk can be developed through the fermentation with lactic acid bacteria and bifidobacteria.

Recently, several strains of *Lactobacillus* (*L.*) *acidophilus, L. casei, L. plantarum, L. fermentum, Bifidobacterium* (*B.*) *animalis subsp lactis* and *B. longum* have been reported as being able to increase the

* Corresponding author. Tel.: +54 0381 431 0465; fax: +54 0381 400 5600.
E-mail address: mgarro@cerela.org.ar (M.S. Garro).

0740-0020/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2008.11.004

aglycone isoflavone content in fermented soymilk (Otieno et al., 2006; Wei et al., 2007; Chun et al., 2007; Pham and Shah, 2008a,b). *L. rhamnosus* could be used to increase isoflavone concentrations in fermented soy foods since it possesses β-glucosidase activity, however, few authors have reported using this strain (Matsuda et al., 1992, 1994). Moreover, due the great biodiversity of lactic acid bacteria, the β-glucosidase production as well as the performance on bioconversion of isoflavones during soy food fermentation is variable and characteristic of each strain.

In this context, the aim of this work was to evaluate the biotechnologically relevant characteristics of β-glucosidase-producing lactic acid bacteria strains in order to determine their potential in the development of new starter cultures for functional soymilk-based foods enriched with bioactive isoflavone aglycones. To achieve this goal, the screening of bacterial strains with high β-glucosidase activity as well as a partial biochemical characterization of this enzyme of one strain was conducted. The effect of selected strain on the hydrolysis of isoflavone to aglycones, residual sugars and organic acids produced in soymilk fermented at 37 °C for 24 h was also determined.

2. Materials and methods

2.1. Microorganisms and growth conditions

A total of 63 strains of lactic acid bacteria belonging to the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina) were used in this study. The species involved were *Lactobacillus (L.) fermentum* (10 strains), *L. helveticus* (6 strains), *L. paracasei* subsp. *paracasei* (11 strains), *L. delbrueckii* subsp. *lactis* (8 strains), *L. reuteri* (4 strains), *L. plantarum* (13 strains), and *L. rhamnosus* (11 strains). *Bifidobacterium* (5 strains) with high β-glucosidase activity were used as positive control. Before experimental use, cultures were propagated (2%, v/v) twice in MRS medium (De Man et al., 1960) for *Lactobacillus* and modified MRS medium for *Bifidobacterium* and incubated at 37 °C for 18 h without agitation in microaerophilic conditions. Modified MRS medium consisted of MRS broth with 1% sucrose, supplemented with 0.05% L-cysteine hydrochloride, 0.0005% hemin and 0.00005% vitamin K. All solutions were sterilized separately (0.22 μm filtration), and then added to the MRS base.

2.2. Preparation of cell-free extracts

Cells at the end of the exponential phase of growth (1 ml initial volume) were collected by centrifugation (10,000*g*, 10 min, 4 °C), washed twice with 100 mM McIlvaine buffer (Na_2HPO_4–citric acid, pH 5.8; McIlvaine, 1921), and resuspended in 150 μl of the same buffer. The cells were mixed with 200 mg of glass beads (0.10–0.11 mm, Sigma, USA) and disrupted by shaking on a vortex mixer at maximum speed ten times during 20 min with 1-min intervals on ice. Cellular debris was removed by centrifugation (10,000*g*, 10 min, 4 °C). The supernatant fluid was used as a crude enzyme extract.

2.3. β-glucosidase activity assay

Enzyme activity was measured as described previously (Garro et al., 2006) by following the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlu) (Sigma). One unit of enzyme (UE) was defined as the amount of enzyme required to liberate 1.0 μmol of *p*NP per ml per min under the assay conditions. Specific activity was expressed as UE per mg of protein. The enzymatic activity was determined in the supernatant of the cultures and in the cells free extract.

Protein concentration was determined by using the Bio-Rad Protein Assay based on the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Determination of optimal pH, temperature and thermal stability of β-glucosidase

The effect of pH on β-glucosidase activity was determined in 100 mM McIlvaine buffer with pH ranging from 3.0 to 8.0.

The influence of temperature on enzymatic activity was determined by incubating the assay mixture for 15 min at temperatures from 10 to 50 °C.

Thermal stability was determined by incubating the enzyme extract at temperatures from 50 to 100 °C for 5 and 10 min. The substrate was then added and the solution incubated for an additional 15 min at 37 °C in order to measure residual enzymatic activity.

2.5. Storage stability of the enzyme

Enzyme suspensions from each microorganism were stored at 4 °C and −20 °C during 2 months. The residual enzyme activity was determined throughout this period.

2.6. Preparation of soymilk

Whole soybeans were washed and soaked overnight in distilled water. The swollen soybeans were manually dehulled and then ground with water. The ratio of dry soybeans (200 g) to water (1 l) used for grinding was 1:5 (w/v). The slurry was cooked at 80 °C for 15 min. The cooked slurry was filtered through a double layered cheese cloth in order to separate insoluble residues. The liquid was transferred into glass bottles and sterilized by autoclaving at 121 °C for 15 min. The resulting soymilk was cooled; sucrose (previously sterilized by filtration) was then added to a final concentration of 1% (v/v) and stored in a refrigerator (4 °C) before use.

2.7. Soymilk fermentation

Five hundred milliliters of soymilk were inoculated (4% v/v) with *L. rhamnosus* CRL981 previously activated in soymilk. The inoculated soymilk was fermented at 37 °C for 24 h. Samples were taken aseptically at 0, 3, 6, 9, 12 and 24 h and immediately cooled on ice to determine cell viability, pH, titratable acidity, organic acids, residual sugars, isoflavones and β-glucosidase activity. Non-inoculated soymilk incubated in the same experimental conditions was used as a control.

2.7.1. Analytical assays

Cell viability was determined by the plate dilution method using MRS agar (De Man et al., 1960). Serial dilutions of each fermented soymilk sample were plated in duplicate and the plates were incubated at 37 °C for 48 h. The results were expressed as colony forming units per milliliter (CFU ml^{-1}).

The pH of the samples was measured with a pH meter (Sartorius PT-10, Germany).

Titratable acidity expressed as percent of lactic acid (%TA) was measured by titration with 0.11 N NaOH.

For evaluation of organic acids and residual sugars samples were centrifuged at 10,000*g* for 10 min at 4 °C and supernatants were stored at −20 °C until analysis.

Lactic and acetic acids were determined by HPLC (LKB, model 2142, Bromma, Sweden) using an REZEX ROA-Organic Acid column (200 × 7.8 mm, Phenomenex, Torrance, CA, USA) at room

Table 1
β-Glucosidase activity of *Lactobacillus* and *Bifidobacterium* strains used in the present study.

Species	Strains	β-Glucosidase activity
L.[a] *fermentum*	CRL220, CRL250, CRL251, CRL345, CRL646, CRL661, CRL722, CRL944, CRL955, CRL973	–
L. helveticus	CRL974, CRL1062, CRL1176, CRL1177, CRL1178, CRL1179	–
L. paracasei subsp. *paracasei*	CRL59, CRL66, CRL72, CRL75, CRL76, CRL206, CRL207, CRL232, CRL678, CRL997, CRL1004	+
L. delbrueckii subsp *lactis*	CRL564, CRL581, CRL654, CRL655, CRL960, CRL1207, CRL1226, CRL1236	–
L. reuteri	CRL1099, CRL1101, CRL1097, CRL1100	–
L. plantarum	CRL41, CRL120, CRL219, CRL428, CRL680, CRL700, CRL759, CRL785, CRL794, CRL972, CRL1073, CRL1076, CRL1093	–
L. rhamnosus	CRL74, CRL179, CRL186, CRL 193, CRL201, CRL204, CRL228, CRL627, CRL627, CRL932, CRL981	+
B.[b] *longum*[c]	CRL849, CRL1401	+
B. bifidum[c]	CRL1399, CRL1396	+
B. adolescentis[c]	CRL1400	+

[a] *L.*: *Lactobacillus.*
[b] *B.*: Bifidobacterium.
[c] Control.

temperature, with a flow rate of 0.5 ml min^{-1} of 5 mM sulfuric acid following absorbance at 210 nm.

Stachyose, raffinose, sucrose and their hydrolysis products (galactose, glucose and fructose) were quantified by HPLC coupled to a differential refractometer (LKB, model 2142, Bromma, Sweden) using REZEX RSO oligosaccharides column (200 × 10 mm, Phenomenex, Torrance, CA, USA) at a column temperature of 70 °C, using HPLC grade water as the eluant at a flow rate of 0.3 ml min^{-1}. Samples were deproteinized before sugar determination as described previously (Garro et al., 2004b).

2.7.2. Isoflavone assays

For isoflavones analysis, the soymilk samples were freeze-dried and stored at −20 °C until used. The extraction of isoflavones, including β-glucosides, and aglycones from fermented and non-fermented soymilk were carried out according to Lee et al. (2004) with some modification. Briefly, a 250 mg of each freeze-dried sample was extracted with 100 mM HCl (1 ml), acetonitrile (3.5 ml), and bidistilled water (1.5 ml) for 2 h with shaking at room temperature. The insoluble residue was separated by centrifugation (10,000*g*, 5 min, 4 °C). The supernatant was then filtered with a syringe filter (0.45 μm PVDF membrane, Millipore, USA) for HPLC analysis.

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed with KNAUER Smartline System (Germany), using PORSUIT XRs C18 column (150 mm × 4.6 mm, VARIAN) and Smartline multiwavelength UV detector. The mobile phase was composed of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). The solvents flow rate was 0.8 ml min^{-1}, using a gradient of 80% A (20% B) at 0 min, steady for 2.5 min 80% A, decreasing to 70% A for 7.5 min, 65% A for 10 min, 60% A for 5 min, steady at 60% A for 12 min, decreasing to 15% A for 7 min, steady at 15% A for 3 min and then increasing to 80% A for 2 min and steady at 80% for A until completing the gradient program of 60 min. Samples were monitored from 254 to 262 nm and area responses were integrated. Glicitein (16 mg l^{-1}) was used as internal standard (the soymilk used in this study does not contain this glucoside). Isoflavones were identified by retention time and multiwavelength UV spectra were compared with those of standards. Standards of glucosides (daidzin, and genistin) and aglycones (daidzein, and genistein) were obtained from Fluka and Sigma, respectively.

2.7.3. β-Glucosidase assay in soymilk

Soymilk samples (1.5 ml) were centrifuged at 10,000 *g* for 10 min at 4 °C and the cell pellet was washed once and resuspended with 100 mM McIlvaine buffer (Na_2HPO_4–citric acid, pH 5.8). This suspension was permeabilized by adding 50 μl of toluene–acetone (1:9) per ml of cell suspension. The mixture was stirred for 5 min. The permeabilized samples were kept at 4–6 °C before the enzymatic analysis. The β-glucosidase activity was measured by the method described by Garro et al. (2006), and expressed as the amount of enzyme required to liberate 1.0 μmol of *p*NP per ml per min. Specific activity was expressed as UE per mg of protein.

2.8. Statistical results

All results presented in this paper are the average of three independent assays. The variation among results was less than 10%. Results were expressed as mean ± standard deviation, and their significance was analyzed using Student's *t* test.

3. Results and discussion

3.1. Screening of β-glucosidase producing microorganism

The ability to produce β-glucosidase was evaluated in 63 strains of lactobacilli (Table 1). The activity was detected in the cell-free extract and was not found in culture supernatant. Only strains of *Lactobacillus (L.) rhamnosus* and *L. paracasei* subsp. *paracasei,* among the seven analyzed species of lactobacilli, were able to express β-glucosidase activity upon its specific substrate, *p*NPGlu. *L. rhamnosus* strains generally tended to show higher β-glucosidase specific activity compared to that of *L. paracasei* subsp. *paracasei* strains (Fig. 1a and b). Only 18% of *L. rhamnosus* strains showed lower enzymatic activity (<1.4 UE mg^{-1}) whereas 55% of *L. paracasei* subsp. *paracasei* showed this characteristic. However, a wide variation in the enzyme activity was found among the microorganisms studied. The specific activity was in the range of 0.5–22.93 UE mg^{-1} for *L. rhamnosus* and between 0.28 and 7.99 UE mg^{-1} for *L. paracasei* subsp. *paracasei.* The highest values of β-glucosidase activity were obtained with *L. paracasei* subsp. *paracasei* CRL678 and *L. rhamnosus* CRL981 which had 2.86 times higher activity values than the former. None of *L. plantarum* and *L. delbrueckii* subsp. *lactis* strains were able to hydrolyze the *p*NPGlu substrate under the assay conditions used in this study, bifidobacteria were used as positive controls. In general, several studies showed that lactic acid bacteria and bifidobacteria are able to produce the enzyme β-glucosidase (Otieno et al., 2006; Chun et al., 2008; Donkor and Shah, 2008), but most of these were carried out in soymilk and not in MRS medium; therefore the behavior of the microorganisms as well as the production of the enzyme could depend, to some extent, on the culture medium employed. Tsangalis et al. (2002) reported that *B. longum*-b showed a highest β-glucosidase activity when it was grown in MRS–glu (4.625 U mg^{-1}).

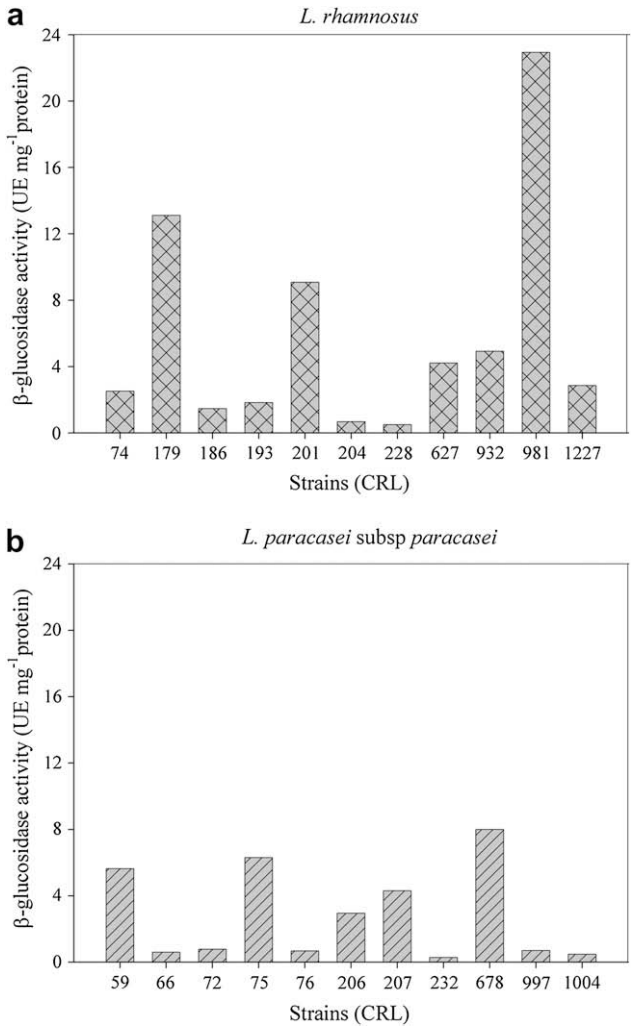

Fig. 1. β-Glucosidase activity produced by crude enzyme extracts of strains a) *Lactobacillus (L.) rhamnosus* and b) *L. paracasei* subsp. *paracasei* obtained in MRS medium at 37 °C during 18 h.

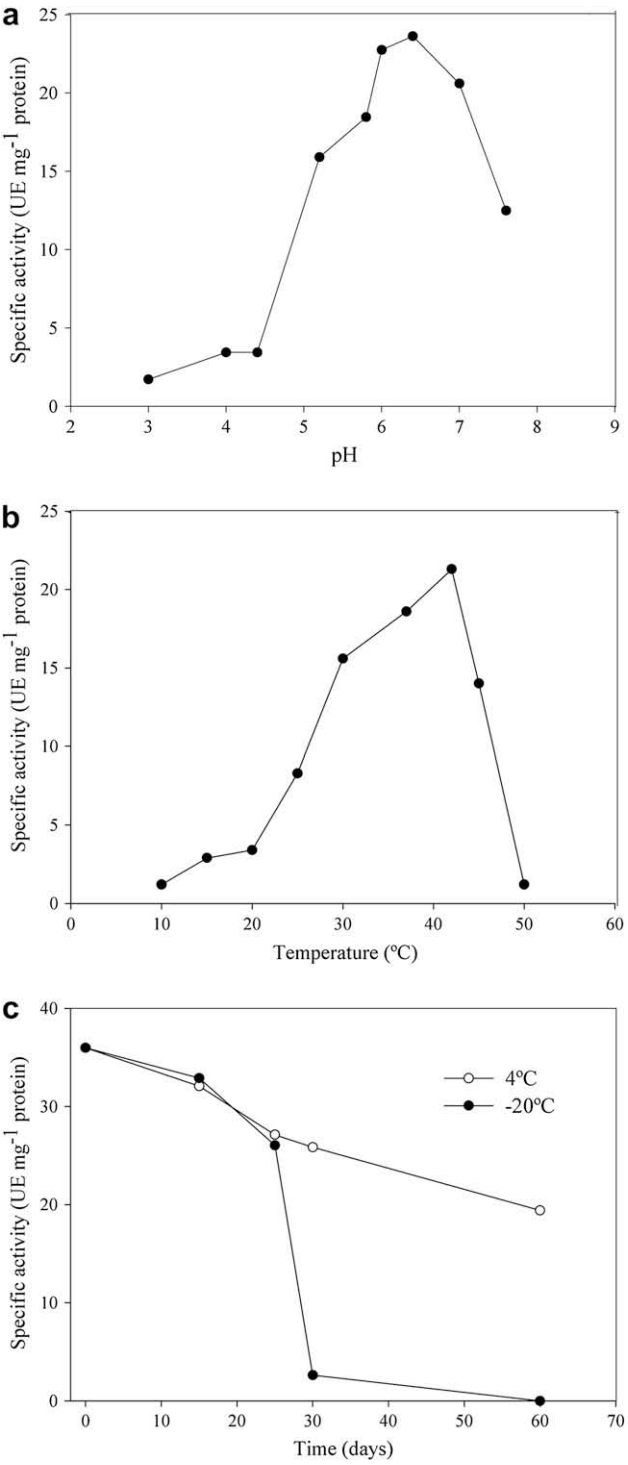

Fig 2. Enzymatic characterization of β-glucosidase produced by *L. rhamnosus* CRL981. a) Effect of pH; b) the effect of the temperature; c) effect of storage at 4 to −20 °C.

It has been reported that bifidobacteria shows higher β-glucosidase activities than other intestinal bacteria (Desjardins et al., 1990), however, we demonstrated that *L. rhamnosus* CRL981 was able to express 4.9 times higher activity than that produced by *B. longum*-b reported by Tsangalis et al. (2002). It is known that this enzymatic activity depends on the strain, growth medium and culture conditions; moreover, the enzyme plays a key role in the bioconversion of isoflavones. Although it has been reported that several strains of lactic acid bacteria express β-glucosidase activity (Wei et al., 2007; Chun et al., 2007), few are related to *L. rhamnosus*, moreover, our results demonstrated that *L. rhamnosus* CRL981 was able to express the highest values among lactobacilli strains examined so far and was selected for further studies.

3.2. Biochemical characterization of β-glucosidase

The effect of pH on enzyme activity is shown in Fig. 2a. The cell-free extract exhibited activity in a range of pH between 5.2 and 7.0; the optimum pH being 6.4 where a specific activity of 23.6 UE mg^{-1} protein was detected. These results are different from reported by Sestelo et al. (2004) who found pH 5.0 to be the optimal pH for β-glucosidase activity in *L. plantarum* strain isolated from wine although it was active in the similar pH range. All cell-extracts were inactive at pH levels below 4.5. At pH values above 7.0 the enzyme activity decreased by 47.1% in comparison with that observed at the optimal pH.

The effect of the temperature on the β-glucosidase activity of *L. rhamnosus* CRL981 is shown in Fig. 2b. Greater activity was observed when the cell-free extract was incubated at temperatures between 30 and 45 °C being 42 °C the optimal temperature. Similar behavior was also reported by Coulon et al. (1998) who observed

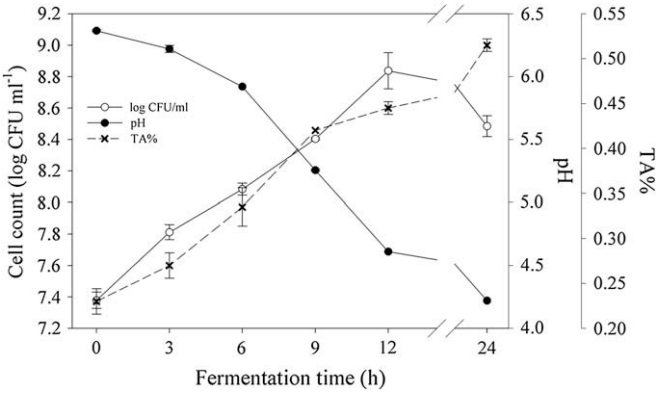

Fig. 3. The changes in cell viability, pH and titratable acidity (TA) during soymilk fermentation by *L. rhamnosus* CRL981.

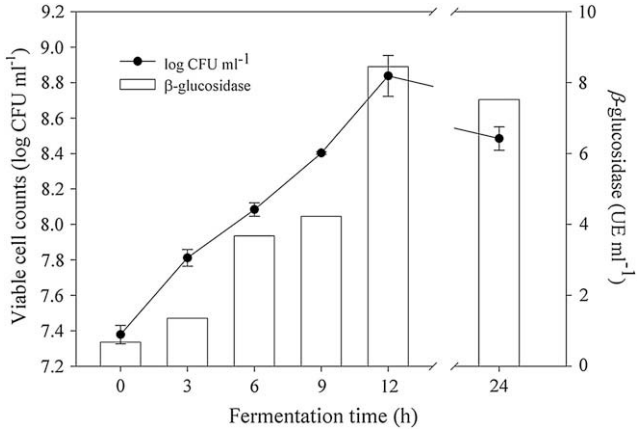

Fig. 5. Growth and production of β-glucosidase enzyme during soymilk fermentation by *L. rhamnosus* CRL981 at 37 °C for 24 h.

that the β-glucosidase activity of *L. casei* ATCC 393 was maximal at 35 °C. The effect of the temperature on the β-glucosidase activity of *L. rhamnosus* CRL981 is shown in Fig. 2b. Greater activity was observed when the cell-free extract was incubated at temperatures between 30 and 45 °C being 42 °C the optimal temperature. Similar behavior was also reported by Coulon et al. (1998) who observed that the β-glucosidase activity of *L. casei* ATCC 393 was maximal at 35 °C. When the enzyme was exposed at 50 °C for 5 min, only 20% of the activity was detected with respect to the control. Sestelo et al., 2004 reported that β-glucosidase activity in *L. plantarum* strain was reduced by 50% when it was heated at 50 °C during 5 min. At temperatures above to 50 °C (75 °C and 100 °C) no activity was detected. Similar results were reported by Grimaldi et al., 2005, who observed that β-glucosidase activity of *Lactobacillus* and *Pediococcus* strains was reduced when exposed at temperatures above 60 °C. These results suggest that *L. rhamnosus* CRL981 is a better source of enzyme for use of β-glucoside bonds at moderate temperatures.

Stability of β-glucosidase is also influenced by storage times and temperatures (Fig. 2c). The enzyme retained about a 72% of residual activity after storage at 4 and −20 °C for 25 days. However, the enzyme activity was most unstable at −20 °C showing a complete activity loss at 60 days. In contrast to these results Otieno et al. (2005) reported great storage stability for β-glucosidase from *L. casei* strains grown on soymilk, since its enzyme retained higher residual activity at −20 °C.

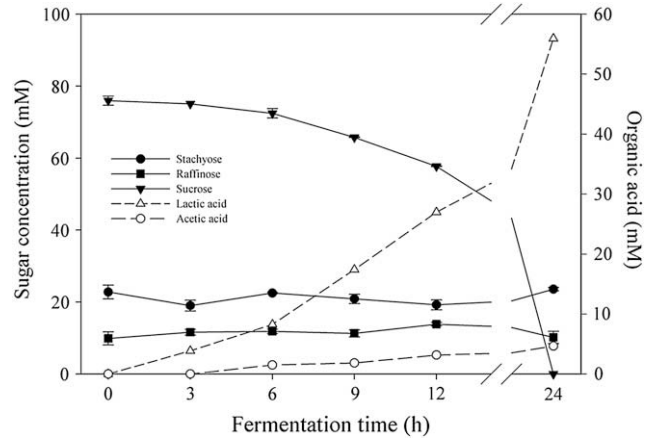

Fig. 4. Sugar consumption and organic acids production in soymilk fermented with *L. rhamnosus* CRL981.

3.3. Soymilk fermentation

3.3.1. Growth pattern

The growth kinetics of the microorganisms in soymilk at 37 °C is shown in Fig. 3. *L. rhamnosus* CRL981 started to multiply immediately after inoculation. Viable counts increased from 7.38 log CFU ml^{-1} to 8.85 log CFU ml^{-1} after 12 h of incubation, which is in agreement with results reported by Chun et al. (2007). These authors showed that different lactic acid bacteria strains exhibited higher cell population in soymilk (7–9 log CFU ml^{-1}). The drop in pH was most pronounced between 6 and 12 h of fermentation (from 5.9 to 4.6) while %TA increased during fermentation reaching twice the initial value (0.52% at 24 h) (Fig. 3). The acidification of soymilk observed was higher than those reported by Wang et al. (2002) for *Streptococcus (S.) thermophilus* CCRC14085 and *B. longum* B6. This pH reduction caused coagulation and the appearance of this soymilk changed after fermentation. The characteristic of the formed coagulum was similar to a fragile and weak curd. Coagulation of soy proteins occurs at a wide range a pH, beginning at 6.4. The 7S globulin precipitates at pH values between 4.0 and 5.6, whereas 11S fraction precipitates between 4.4 and 6.4 (Liu, 1997). In general, the characteristic of coagulum depends on the pH and thermal processing involved in the fermentation.

3.3.2. End-products formation and sugar consumption

The concentration of organic acids and sugar consumption in soymilk fermentation are shown in Fig. 4. Under the conditions used in this study, *L. rhamnosus* CRL981 produced principally lactic acid and lower amounts of acetic acid in soymilk. At the end of fermentation (24 h) 55.9 mM of lactic acid and 4.7 mM of acetic acid were produced. The molar ratio of lactate:acetate changed throughout the fermentation period, between 5.5:1.0 and 11.9:1.0 at 6 and 24 h, respectively. These results were similar to those reported by Garro et al. (1998) who used a strain of *L. casei*. Sucrose was preferentially hydrolyzed; a 24% of residual carbohydrate was found after 12 h and was not detected at 24 h. In contrast, stachyose and raffinose remained relatively unchanged throughout the incubation period because *L. rhamnosus* CRL981 does not possess α-galactosidase activity, which acts upon gal–gal bonds in these galacto-oligosaccharides (data not shown). Glucose and fructose (products of sucrose hydrolysis) were scarcely detected, which suggests that they were consumed by the microorganism during growth. These results were similar to those observed by Garro et al. (1998) who used strains of *S. thermophilus* and *L. casei* in soymilk.

Table 2
Changes in soymilk isoflavones during fermentation with *Lactobacilus (L.) rhamnosus* CRL981 (37 °C for 24 h).

Soymilk/Strain	Fermentation time (h)	Isoflavone (mg l^{-1})			
		Glucosides		Aglycones	
		Daidzin	Genistin	Daidzein	Genistein
Control[a]	0	4.56 ± 0.12	22.15 ± 0.10	3.62 ± 0.28	7.95 ± 0.16
	3	4.02 ± 0.42	23.45 ± 0.08	3.59 ± 0.20	8.02 ± 0.07
	6	3.98 ± 1.05	22.96 ± 0.15	3.26 ± 0.62	8.40 ± 0.06
	9	3.89 ± 0.98	22.91 ± 0.13	3.81 ± 0.05	8.10 ± 0.04
	12	4.36 ± 0.20	23.05 ± 0.10	3.30 ± 0.57	7.90 ± 0.13
	24	4.51 ± 0.09	20.80 ± 0.20	3.45 ± 0.32	8.20 ± 0.10
L. rhamnosus CRL981	0	4.80 ± 0.12	20.13 ± 0.15	3.34 ± 0.20	8.41 ± 0.22
	3	4.33 ± 0.21	18.01 ± 0.35	3.48 ± 0.18	9.43 ± 0.12
	6	3.26 ± 0.29	23.06 ± 0.17	4.39 ± 0.31	13.17 ± 0.29
	9	2.09 ± 0.18	3.19 ± 0.23	12.52 ± 0.12	29.99 ± 0.18
	12	0	0	14.53 ± 0.17	32.96 ± 0.30
	24	0	0	13.53 ± 0.20	31.94 ± 0.16

[a] Non-fermented soymilk.

3.4. β-Glucosidase activity and bioconversion of isoflavones

Changes of β-glucosidase activity and isoflavones content during fermentation of soymilk with *L. rhamnosus* CRL981 at 37 °C for 24 h were studied. As shown in Fig. 5, the enzyme activity increased during exponential growth phase (between 3 and 12 h), and reached a maximum level of 8.45 UE ml^{-1}. During the next 12 h of fermentation, the activity decreased by 11.1% with respect to the maximum value. These results were higher (almost 40 times) than reported by Chien et al. (2006), who showed that one strain of *S. thermophilus* only was able to produce 0.197 UE ml^{-1} after 24 h of fermentation. The change of enzyme levels during fermentation was followed by an increase of population in soymilk. *L. rhamnosus* CRL981 showed a higher correlation (0.95) between β-glucosidase activity and growth at 12 h of fermentation on soymilk. This rate was better than reported by Donkor and Shah (2008). These authors reported correlations of 0.70 for *L. acidophilus* L10 (at 36 h) and 0.79 for *B. lactis* B94 (at 12 h) with lower values in the enzyme production (maximum 0.12 UE/ml). The results obtained with *L. rhamnosus* CRL981 demonstrated that the values are higher than that reported in the literature independently of the media used for growth (MRS or soymilk).

In addition, changes of glucoside and aglycone isoflavone contents, as a result of enzyme activity, are shown in Table 2. The non-fermented soymilk (control), with an initial concentration of 68% of β-glucoside forms (daidzin and genistin) did not present changes in the isoflavones rate during all the incubation period. In contrast, the glucoside isoflavones concentrations decreased during the fermentation of soymilk with *L. rhamnosus* CRL981 due to β-glucosidase activity, followed by an increase of aglycone isoflavones content. A similar trend in the change of β-glucosidase activity, glucoside isoflavone, and aglycone isoflavone content was also observed in soymilk fermented by *S. thermophilus* (Chien et al., 2006). The observed phenomenon was in agreement with the reports of Esaki et al. (1994) and Sheih et al. (2000). Our results show that glucoside isoflavones decreased rapidly between 6 and 9 h disappearing at 12 h while an increase in aglycones through the fermentation was observed. Genistein was the main aglycone isomer found in fermented soymilk because in non-fermented soymilk there are large amounts of its corresponding glucoside (genistin). Similar results were reported by Chun et al. (2007) who analyzing the behavior of different lactic acid bacteria species during fermentation of soymilk, observed the highest rate of hydrolysis of β-glucosides occurred during 6 h of fermentation time reaching conversion values of 90 and 100% for daidzin and genistin, respectively.

In the present study, the β-glucosidase system of *L. rhamnosus* CRL981 was partially characterized, showing high β-glucosidase activity in MRS as well as in soymilk. The data presented in this study add new knowledge to current literature, and will be helpful in improving the use of this microorganism in order to obtain fermented products from soybean. On the other hand, this strain was able to increase bioactive isoflavones during soymilk fermentation, to achieve 100% of bioconversion at 12 h. The ability to hydrolyze the β-glucoside isoflavones present in soymilk may have nutritional benefits due to the increase of the bioability of isoflavones, responsible in part for the health benefits associated to soy food consumption. *L. rhamnosus* CRL981 could be used as a starter culture to produce different aglycone-rich functional soy beverage.

Acknowledgements

This study was partly supported by grants from Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPyCT-FONCYT), and Consejo de Ciencia y Técnica de la Universidad Nacional de Tucumán (CIUNT), Argentina. The authors are grateful to Dr. Jorge Palacios and Dra. Mónica Locascio for their technical assistance in the HPLC analyses, and to Dr. Jean Guy LeBlanc for constant discussion and suggestions.

References

Adlercreutz, H., 2002. Phytoestrogens and breast cancer. J. Steroid Biochem. Mol. Biol. 83 (1–5), 113–118.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Cassidy, A., Brown, J.E., Hawdon, A., Faughnan, M.S., King, L.J., Millward, J., Zimmer-Nechemias, L., Wolfe, B., Setchell, K.D.R., 2006. Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. J. Nutr 136, 45–51.
Chien, H.L., Huang, H.Y., Chou, C.C., 2006. Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. Food Microbiol. 23, 772–778.
Chun, J., Kim, G., Lee, K., Choi, D., Kwon, G.H., Park, J.Y., Jeong, S.J., Kim, J.S., Kim, J.H., 2007. Conversion of isoflavone glucosides to aglycones in soymilk by fermentation with lactic acid bacteria. J. Food Sci. 72 (2), M39–M44.
Chun, J., Kim, J.S., Kim, J.H., 2008. Enrichment of isoflavone aglycones in soymilk by fermentation with single and mixed cultures of Streptococcus infantarius 12 and Weissella sp. 4. Food Chem. 109, 278–284.

Coulon, S., Chemarin, P., Gueguen, Y., Arnaud, A., Galzy, P., 1998. Purification and characterization of an intracellular beta-glucosidase from *Lactobacillus casei* ATCC 393. Appl. Biochem. Biotechnol. 74 (2), 105–114.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23, 130–135.

Desjardins, M., Roy, D., Goulet, G., 1990. Growth of bifidobacteria and their enzyme profiles. J. Dairy Sci. 73, 299–307.

Donkor, O.N., Shah, N.P., 2008. Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus, Bifidobacterium lactis*,and *Lactobacillus casei* in soymilk. J. Food Sci. 73 (1), M15–M20.

Esaki, H., Onozaki, H., Osawa, T., 1994. Antioxidative activity of fermented soybean products. In: Huang, M.T. (Ed.), Food Phytochemicals for Cancer Prevention I, Fruits and Vegetables. American Chemical Society, Washington, DC, pp. 353–360.

Garro, M.S., de Valdez, G.F., Oliver, G., de Giori, G.S., 1998. Growth characteristics and fermentation products of *Streptococcus salivarius* subsp. *thermophilus, Lactobacillus casei* and *L. fermentum* in soymilk. Z. Lebensm. Unters Forsch. A 206, 72–75.

Garro, M.S., de Valdez, G.F., Oliver, G., de Giori, G.S., 1999. Hydrolysis of soya milk oligosaccharides by *Bifidobacterium longum* CRL849. Z. Lebensm. Unters. Forsch. A 208, 57–59.

Garro, M.S., de Valdez, G.F., de Giori, G.S., 2004a. Temperature effect on the biological activity of *Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251 in pure and mixed cultures grown in soymilk. Food Microbiol. 21, 511–518.

Garro, M.S., de Valdez, G.F., de Giori, G.S., 2004b. Determination of oligosaccharides in fermented soymilk products by high-performance liquid chromatography. In: Spencer, J.F.T., Ragout de Spencer, A.L. (Eds.), Environmental Microbiology: Methods and Protocols. Humana, New Jersey, pp. 135–138.

Garro, M.S., Aguirre, L., de Giori, G.S., 2006. Biological activity of *Bifidobacterium longum* in response to environmental pH. Appl. Microbiol. Biotechnol 70, 612–617.

Grimaldi, A., Bartowsky, E., Jiranek, V., 2005. Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. J. Appl. Microbiol. 99, 1061–1069.

LeBlanc, J.G., Garro, M.S., Savoy de Giori, G., 2004a. Effect of pH on *Lactobacillus fermentum* growth, rafinose removal, α-galactosidase activity and fermentation products. Appl. Microbiol. Biotechnol. 65, 119–123.

LeBlanc, J.G., Garro, M.S., Silvestroni, A., Connes, C., Piard, J.-C., Sesma, F., de Giori, G.S., 2004b. Reduction of α-galctooligosaccharides in soyamilk by *Lactobacillus fermentum* CRL 722: in vitro and in vivo evaluation of fermented soyamilk. J. Appl. Microbiol. 97, 876–881.

LeBlanc, J.G., Garro, M.S., Savoy de Giori, G., Font de Valdez, G., 2004c. A novel functional soy-based food fermented by lactic acid bacteria: effect of heat treatment. J. Food Sci. 69, M246–M250.

Lee, J., Renita, M., Fioritto, R.J., Martin, S.K., Schwartz, S.J., Vodovotz, Y., 2004. Isoflavone characterization and antioxidant activity of Ohio soybeans. J. Agric. Food Chem. 52, 2647–2651.

Liu, K., 1997. Soybeans: Chemistry, Technology and Utilization. Chapman and Hall, New York, pp. 415–421.

McIlvaine, T.C., 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. 49, 183–186.

Matsuda, S., Miyazahi, T., Matsumoto, Y., Ohba, R., Teramoto, Y., Ohta, N., Ueda, S., 1992. Hydrolysis of Isoflavones in soybean cooked Syrup by *Lactobacillus casei* subsp. *rhamnosus* IFO 3425. J. Ferment. Bioeng. 74 (5), 301–304.

Matsuda, S., Norimoto, F., Matsumoto, Y., Ohba, R., Teramoto, Y., Ohta, N., Ueda, S., 1994. Solubilization of a novel isoflavone glycoside-hydrolyzing β-glucosidase from *Lactobacillus casei* subsp. *rhamnosus*. J. Ferment. Bioeng. 77 (4), 439–441.

Omoni, A.O., Aluko, R.E., 2005. Soybean foods and their benefits: potential mechanisms of action. Nutr. Rev. 63 (8), 272–283.

Otieno, D.O., Ashton, J.F., Shah, N.P., 2005. Stability of β-glucosidase activity produced by *Bifidobacterium* and *Lactobacillus* spp. in fermented soymilk during processing and storage. J. Food Sci. 70 (4), 236–241.

Otieno, D.O., Ashton, J.F., Shah, N.P., 2006. Evaluation of enzymic potential for biotransformation of isoflavones phytoestrogen in soymilk by *Bifidobacterium animalis, Lactobacillus acidophilus* and *Lactobacillus casei*. Food Res. Int. 39, 394–407.

Pham, T.T., Shah, N.P., 2008a. Effect of lactulose on biotransformation of isoflavone glycosides to aglycones in soymilk by *Lactobacilli*. J. Food Sci. 73 (3), M158–M165.

Pham, T.T., Shah, N.P., 2008b. Effects of lactulose supplementation on the growth of bifidobacteria and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk. J. Agric. Food Chem. 56 (12), 4703–4709.

Sestelo, A.B.F., Poza, M., Villa, T.G., 2004. β-Glucosidase activity in a *Lactobacillus plantarum* wine strain. World J. Microbiol. Biotechnol. 20, 633–637.

Setchell, K.D.R., 1998. Phytoestrogens: the biochemistry, physiology, and implication for human health of soy isoflavones. Am. J. Clin. Nutr. 68 (suppl), 1333S–1346S.

Setchell, K.D.R., Cassidy, A., 1999. Dietary isoflavones: biological effects and relevance to human health. J. Nutr. 129, 758S–767S.

Setchell, K.D.R., Brown, N.M., Zimmer-Nechemias, L., Brashear, W.T., Wolfe, B.E., Kirschner, A.S., Heubi, J.E., 2002. Evidence of lack of absorption of soy isoflavones in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am. J. Clin. Nutr. 76, 447–453.

Sheih, I.C., Wu, H.Y., Lai, Y.J., Lin, C.F., 2000. Preparation of high free radical cavenging temp by a newly isolated Rhizopus sp. R-69 from Indonesia. Food Sci. Agric. Chem. 2, 35–40.

Tsangalis, D., Ashton, J.F., McGill, A.E.J., Shah, N.P., 2002. Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase-producing bifidobacteria. J. Food Sci. 67, 3104–3113.

Tsangalis, D., Ashton, J.F., Stojanovska, L., Wilcox, G., Shah, N.P., 2004. Development of an isoflavone aglycone-enriched soymilk using soy germ, soy protein isolate and bifidobacteria. Food. Res. Int 37, 301–312.

Wang, H., Murphy, P.A., 1994a. Isoflavone content in commercial soybean foods. J. Agric. Food Chem. 42, 1666–1673.

Wang, H., Murphy, P.A., 1994b. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J. Agric. Food Chem. 42, 1674–1677.

Wang, Y.C., Yu, R.C., Chou, C.C., 2002. Growth and survival of bifidobacteria and lactic acid bacteria during the fermentation and storage of cultured soymilk drinks. Food Microbiol. 19, 501–508.

Wei, Q.K., Chen, T.R., Chen, J.T., 2007. Using of *Lactobacillus* and *Bifidobacterium* to product the isoflavone aglycones in fermented soymilk. Int. J. Food Microbiol. 117, 120–124.