Stability of β-glucosidase Activity Produced by *Bifidobacterium* and *Lactobacillus* spp. in Fermented Soymilk During Processing and Storage

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ABSTRACT: Microorganisms possess endogenous enzymes, however the stability of these enzymes during storage in soymilk has not been studied. β-glucosidase is an important enzyme that could be used in the bioconversion of the predominant soy isoflavone glucosides to their bioactive aglycone forms. Fifteen probiotic microorganisms including *bifidobacterium*, *Lactobacillus acidophilus*, and *Lactobacillus casei* were screened for β-glucosidase activity using ρ-nitrophenyl-β-D-glucopyranoside as a substrate. Six strains were selected on the basis of β-glucosidase activity produced during fermentation of soymilk. The stability of the enzyme activity was assessed during incubation for up to 48 h and storage for 8 wk at frozen (–80 °C), refrigerated (4 °C), room (24.8 ° C), and incubation (37 °C) temperatures. *L. casei* strains showed the highest β-glucosidase activity after 24 h of incubation followed by *L. acidophilus* strains, whereas *bifidobacterium* strains showed least activity. However, β-glucosidase from *Bifidobacterium animalis* BB12 showed the best stability during the 48 h fermentation. Lower storage temperatures (–80 °C and 4 °C) showed significantly higher ($P < 0.05$) β-glucosidase activity and better stability than that at higher temperatures (24.8 °C and 37 °C). The stability of β-glucosidase from these microorganisms should be considered for enzymic biotransformation during storage of isoflavone β-glucosides to bioactive isoflavone aglycone forms with potential health benefits.

Keywords: *bifidobacterium*, *Lactobacillus*, soymilk, β-glucosidase, soy oligosaccharides

Introduction

The term "probiotic" was derived from the Greek meaning "for life." The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) have stated that there is an adequate scientific evidence to indicate that there is potential for probiotic foods to provide health benefits and that specific strains are safe for human use (FAO and WHO 2001). The expert panel commissioned by FAO and WHO defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Probiotics are normally marketed as capsules and powders or added to yogurts or yogurt-like products. The diverse range of "functional" or "pharma" food products currently available reflects the convenience of using food as a delivery system for probiotic microorganisms (Driessen and de Boer 1989). However, certain criteria need to be fulfilled to ensure the production of high quality foods that maximize their biotherapeutic potential. These include the incorporation of sufficient numbers of microorganisms into the product, the maintenance of viable populations during shelf life of the food and subsequent survival of these microbes through the gastrointestinal tract to attain intact delivery to the colon, which is the usual site of action (Berrada and others 1991; Sanders and others 1996).

Lactobacillus acidophilus, bifidobacterium, and *Lactobacillus casei* are among the predominant members of the intestinal microflora. There is an increasing evidence to suggest that some members of the lactic acid bacteria, such as *bifidobacterium* and lactobacilli, when consumed in sufficiently large numbers exhibit prophylactic and therapeutic properties in both humans and animals (Mitsuoka 1990). Morphologically, the genus *bifidobacterium* presents a Gram-positive, bacillar form and is anaerobic with an optimum growth temperature and pH of 37 °C and 6.8, respectively. *L. acidophilus* and *L. casei* strains are a large group of Gram-positive anaerobic rods. *Lactobacillus reuteri* is an obligate heterofermentative enterolactobacillus. *L. reuteri* strains isolated from different hosts have distinctive colony morphologies while retaining similar physiological and genetic characteristics. *Bifidobacterium* preferentially metabolizes hexose sugars for growth (Ballongue 1993). Due to their potential health benefits, selected strains of *bifidobacterium* are widely used in dairy preparations in conjunction with probiotic bacterium *L. acidophillus* (Shah 2000). The ability of lactobacilli and *bifidobacterium* to modify the gut microbiota and reduce the risk of cancer is in part due to their ability to decrease β-glucuronidase and carcinogen levels (Hosoda and others 1996).

L. acidophilus, bifidobacterium, and *L. casei* grow slowly in soymilk during product manufacture. Therefore, the established practice is to incorporate yogurt cultures (i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. It is reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested (Shah 2000). The enzymic activity within the cells is dependent on the viability of the microorganisms. Probiotic microorganisms possess β-glucosidase, β-galactosidase, and α-galactosidase, which play an important role in hydrolyzing isoflavone glucosides to bioavailable aglycones forms in fermented soymilk (Tochikura and others 1986).

Soymilk is a heterogenous source of carbohydrates, including predominantly raffinose, stachyose, and glucose (Liu 1997). The 2 principal oligosaccharides are the trisaccharide raffinose and the tetrasaccharide stachyose. They are not usually eliminated during preparations of soy meal. Hydrolytic digestion of soy oligosaccharides

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is minimal in the small intestine because mammals lack α-galactosidase necessary to hydrolyze the α-1-6 linkages (Slominski 1994).

To improve the biological activity of soymilk, the concentration of isoflavone aglycones have to be increased. Commercial β-glucosidases have been used in biotransformation of isoflavone glucosides (Park and others 2002, 2003) and β-glucosidase from *bifidobacterium* have been used in hydrolyzing the β-1,6 glucoside bonds to increase the concentration of bioactive isoflavone aglycones in soymilk (Tsangalis and others 2002, 2003). Therefore the objectives of the current study were to screen strains of *L. acidophilus, bifidobacterium*, and *L. casei* for optimal β-glucosidase activity in the soymilk and examine the stability of the enzyme derived from selected strains of the 3 groups of microorganisms during storage at different temperatures (–80 °C, 4 °C, 24.8 °C, and 37 °C) for 8 wk.

Materials and Methods

Bacteria

Pure cultures of *L. casei* ASCC 292, 1520, 2607, ASCC 290, 279, ASCC 1521, *L. acidophilus* ATCC 4356, ATCC 15820, 4962, 33200, 4461, *L. reuteri,* and *Bifidobacterium longum* 536 and 20099 were obtained from the Victoria Univ. Culture Collection (Werribee, Victoria, Australia). *B. animalis* BB12 was obtained from Chr Hansen Pty. Ltd. (Bayswater, Victoria, Australia). The purity of cultures was checked through Gram staining, and the organisms were stored at –80 °C in 40% glycerol.

Soymilk manufacture

SPI (SUPRO 590) supplied by Solae Co. (Chatswood, N.S.W., Australia) was used in the production of soymilk at 40 g/L of sterilized water. A 10 L batch of soymilk was manufactured and dispersed into 6 bottles of 1.6 L, then sterilized at 121 °C for 15 min. Each batch was then inoculated with each microorganism.

Fermentation and storage

The remaining 400 mL was aseptically dispersed into 6 equal volumes, inoculated by 6 selected strains, and incubated at 37 °C for up to 48 h and β-glucosidase activity was determined after 12, 24, 36, and 48 h of incubation. The other batch of 6 bottles containing volumes of 1.6 L each was inoculated (at 5% v/v; 10^7 to 10^8 colony-forming units/g) with each microorganism, incubated for 24 h at 37 °C, then aseptically dispersed into 4 sterile clean bottles (400 mL) each and stored at different temperatures (–80 °C, 4 °C, 24.8 °C, and 37 °C) for weekly β-glucosidase analysis for up to 8 wk.

Assay for β-glucosidase activity in soy milk

Fifteen strains of bacteria were each inoculated in 250 mL soy milk, then incubated at 37 °C for 48 h and screening for β-glucosidase activity was conducted at 0, 12, 24, 36, and 48 h of incubation. Based on β-glucosidase activity, 6 strains were selected for further enzymatic assay. The strains were activated in MRS broth (De Mann and others 1960) by inoculating 1% level at 37 °C for 20 h. The 4th inoculation was done in sterile soymilk from which 5% w/v of each active culture was inoculated in 200 mL of 6 bottles of soymilk. Fifty milliliters of aliquots were withdrawn aseptically from each sample at 12, 24, 36, and 48 h of incubation and β-glucosidase activity was determined using a modified method of Tsangalis and others (2002) by measuring the rate of hydrolysis of ρ-nitrophenyl β-D-glucopyranoside (ρNPG). For the storage study, 50 mL of aliquots were withdrawn aseptically from each sample at weekly intervals and β-glucosidase activity was determined.

One thousand microliters of 5 mM ρNPG prepared in 100 mM sodium phosphate buffer (pH 7.0) was added to 10 mL of each aliquot and incubated at 37 °C for 30 min (Scalabrini and others 1998). Five hundred microliters of 1 M cold sodium carbonate were added to stop the reaction. The aliquots were then placed in 1.8 mL Eppendorf centrifuge tubes followed by centrifugation (14000 × *g* for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Victoria, Australia). The amount of ρ-nitrophenol released was measured using a spectrophotometer (Pharmacia LKB®, Novospec II®, Uppsala, Sweden) at 420 nm. One unit of the enzyme activity was defined as the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from the substrate ρNPG per mL per min under assay conditions. The specific activity was expressed as units of enzyme per nanogram of the protein. The protein concentration was determined by a modified version of the Lowry method (Lowry and others 1951) and Rosenberge (1996). The supernatant was filtered through a 0.45 μm filter membrane to filter out ρ-nitrophenol. The ρNPG substrate and ρ-nitrophenol were purchased from Sigma Chemical Co. (Castle Hill, N.S.W., Australia).

Table 1—Peak enzyme activity in fermented soymilk at 24 h of incubation at 37 °-C

Microorganisms	Absorbance at 24 h (420 nm)	Units of enzyme[a]
L. acidophilus 4461	0.956	2.204
L. casei 2607	0.954	2.199
L. casei ASCC290	0.948	2.184
L. acidophilus ATCC4962	0.932	2.148
L. acidophilus ATCC4356	0.928	2.139
L. casei 1520	0.928	2.139
L. casei ASCC1521	0.923	2.128
L. casei ASCC279	0.921	2.122
L. casei ATCC15286	0.916	2.112
B. animalis BB12	0.909	2.095
L. casei ASCC292	0.906	2.088
L. acidophilus 33200	0.892	2.056
L. reuteri	0.885	2.039
B. longum 20099	0.867	1.998
B. longum 536	0.855	1.972

[a]Mean of units of enzyme (n = 6). One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.

Experimental design

The β-glucosidase assay during fermentation of soymilk of up to 48 h was determined in duplicate on 3 trials while 8 wk of storage at different temperatures was performed in triplicate on 2 trials. The data presented are means of 6 measurements, and the results are presented as mean ± standard error of 6 analyses.

Statistical analysis

To find the difference in β-glucosidase activity in soymilk, means were analyzed using one-way analysis of variance (ANOVA) and 95% confidence levels, using Microsoft Excel Statpro as described by Albright and others (1999) for fermentation period. However, β-glucosidase activity during storage at different temperatures was analyzed using two-way ANOVA and 95% confidence levels. All data with a $P < 0.05$ were classified as statistically significant.

Results and Discussion

Probiotic microorganisms show varying levels of β-glucosidase, β-galactosidase, and β-galactosidase activities (Tochikura and others 1986). According to Tsangalis and others (2003), some strains of *bifidobacterium* showed higher levels of β-galactosidase activity while others such as *B. animalis* BB12 exhibited lower levels of β-galactosidase but higher levels of β-glucosidase activity. Due to the fact that these 3 enzymes exist intercellularly in crude forms, assaying for a specific enzyme required the use of a specific substrate to determine the enzyme potential of a microorganism. Due to our interest in β-glucosidase activity, ρ-nitrophenyl β-D-glucopyranoside (πNPG)

Table 2—β-Glucosidase activity[1] of the selected probiotic microorganisms in soymilk incubated for 12, 24, 36, and 48 h

Incubation time (h)	*L. acidophilus* 33200	*B. animalis* BB12	*L. casei* 2607	*L. acidophilus* 4962	*L. acidophilus* 4461	*L. casei* ASCC290
12	$0.458^{a} \pm 0.019$	$0.257^{a} \pm 0.027$	$0.315^{a} \pm 0.048$	$0.317^{a} \pm 0.047$	$0.322^{a} \pm 0.062$	$0.338^{a} \pm 0.062$
24	$0.635^{acd} \pm 0.032$	$0.293^{a} \pm 0.027$	$0.609^{b} \pm 0.061$	$0.454^{ab} \pm 0.067$	$0.409^{a} \pm 0.041$	$0.537^{b} \pm 0.028$
36	$0.312^{ab} \pm 0.017$	$0.353^{a} \pm 0.034$	$0.295^{a} \pm 0.016$	$0.277^{a} \pm 0.012$	$0.333^{a} \pm 0.051$	$0.433^{ab} \pm 0.032$
48	$0.240^{b} \pm 0.078$	$0.275^{a} \pm 0.010$	$0.229^{a} \pm 0.021$	$0.216^{a} \pm 0.051$	$0.224^{a} \pm 0.008$	$0.323^{ab} \pm 0.047$

[1]One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as means ± standard error of units of enzyme ($n = 6$). [ab]Means in the same column with different letters are significantly different ($P < 0.05$). Statistical analysis by means of one-way ANOVA.

Table 3—β-Glucosidase activity[1] of *Lactobacillus acidophilus* 33200 in soymilk during storage at different temperatures

Storage temp.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
–80 °C	$1.371^{a,A} \pm 0.041$	$1.243^{a,A} \pm 0.041$	$1.255^{a,A} \pm 0.045$	$1.187^{a,A} \pm 0.037$	$1.190^{a,A} \pm 0.035$	$0.922^{ab,A} \pm 0.030$	$0.444^{bc,A} \pm 0.034$	$0.571^{bc,A} \pm 0.030$
4 °C	$1.225^{a,A} \pm 0.045$	$1.188^{a,A} \pm 0.027$	$1.187^{a,A} \pm 0.028$	$1.082^{a,A} \pm 0.017$	$1.090^{a,A} \pm 0.016$	$0.802^{b,A} \pm 0.010$	$0.546^{bc,A} \pm 0.015$	$0.526^{bc,A} \pm 0.023$
24.8 °C	$0.880^{a,B} \pm 0.029$	$0.871^{a,B} \pm 0.025$	$0.850^{a,B} \pm 0.025$	$0.432^{bc,B} \pm 0.015$	$0.345^{bd,B} \pm 0.012$	$0.345^{bd,B} \pm 0.011$	$0.139^{bcd,C} \pm 0.012$	$0.145^{bcd,B} \pm 0.007$
37 °C	$0.730^{a,C} \pm 0.026$	$0.682^{a,C} \pm 0.020$	$0.670^{a,B} \pm 0.025$	$0.461^{b,B} \pm 0.018$	$0.355^{bc,B} \pm 0.015$	$0.295^{bc,B} \pm 0.004$	$0.265^{bc,B} \pm 0.006$	$0.075^{bc,C} \pm 0.023$

[1]One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as mean ± standard error of units of enzyme ($n = 6$). Statistical analysis by means of two-way ANOVA. [abcd]Means in the same row with different lowercase letters are significantly different ($P < 0.05$). [ABCD]Means in the same column with different uppercase letters are significantly different ($P < 0.05$).

was used as the substrate. In Table 1, 15 strains are listed that show β-glucosidase activity in fermented soymilk after 24 h during a 48-h incubation period at 37 °C. Based on β-glucosidase activity in soymilk, it appeared that *L. acidophilus* and *L. casei* strains showed better β-glucosidase activity compared with that of *bifidobacterium*. Strains for further study were selected based on highest and lowest enzyme activities resulting in a representation of strains from *L. acidophilus, L. casei*, and *bifidobacterium*.

The β-glucosidase activity of the 6 selected strains over a 48-h incubation period at 37 °C is shown in Table 2. All microorganisms showed very low β-glucosidase activity under the assay conditions. As a result, the specific activity was expressed as the unit of enzyme activity per nanogram of the protein. There was a significant difference ($P < 0.05$) in the β-glucosidase activity over 12, 24, 36, and 48 h of incubation of *L. acidophilus* 33200, *L. casei 2607*, *L. acidophilus* 4962, and *L. casei* ASCC 290. The enzyme activities of *B. animalis* BB12 and *L. acidophilus* 4461 did not differ significantly ($P < 0.05$). These differences can be attributed to strain specificity.

In general, the 6 selected strains showed an increase in the β-glucosidase activity over incubation period of up to 24 h followed by a decline as fermentation progressed. The increase in enzyme activity between 12 h to 24 h was significant ($P < 0.05$) for strains of *L. acidophilus* 33200, *L. casei 2607*, *L. acidophilus* 4962, and *L. casei* ASCC 290. *L. acidophilus* 4461 had the highest activity at 24 h, but this was not significantly different from that at 12 h. *B. animalis* BB12 showed the highest activity after 36 h, but not significantly different ($P < 0.05$) from that at 12 h and at 24 h.

The increase in β-glucosidase activity and the subsequent decline apparently corresponded to the growth of these probiotic microorganisms in the soy media (growth results not shown). As reported by Tsangalis and others (2002), there was a direct correlation between β-glucosidase activity and the growth of *bifidobacterium*. Thus, it appears that, except *B. animalis* BB12, the strains of *L. acidophilus* and *L. casei* had exponential growth phase between 12 h and 24 h of incubation. *B. animalis* BB12, which had highest β-glucosidase activity at 36 h, appeared to have much slower growth than the other 5 microorganisms. These results are in agreement with those of Scalabrini and others (1998) and Tsangalis and others (2002). The growth of probiotic cultures in general is remarkably slow compared with that of yogurt starter cultures that take only 4 h to complete fermentation (Shah 2000).

Comparatively, *L. acidophilus* 33200 showed the highest β-glucosidase activity (0.635 units of enzyme) among the 5 strains, with peak enzyme activity at 24 h, whereas *B. animalis* BB12 with peak enzyme activity at 36 h showed least β-glucosidase activity (0.353 units of enzyme). During the period between 12 h and 24 h, the relative increase in β-glucosidase activities among the 6 microorganisms were highest at 0.294 units for *L. casei* 2607, followed by 0.199 units for *L. casei* ASCC 290, 0.177 units for *L. acidophilus* 33200, 0.137 units for *L. acidophilus* 4962, 0.087 units for *L. acidophilus* 4461, and 0.036 units for *B. animalis* BB12. *L. casei* strains, in general, showed greater β-glucosidase activity during the 12 h to 24 h of fermentation period compared with *L. acidophilus* strains, while *bifidobacterium* showed least β-glucosidase activity during the same period.

β-glucosidase stability during storage at different temperatures

Tables 3 to 8 show the β-glucosidase activity in fermented soymilk during 8 wk storage at different temperatures for *L. acidophilus* 33200, *B. animalis* BB12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461, and *L. casei* ASCC 290, respectively.

In general, lower storage temperatures (–80 °C and 4 °C) yielded higher β-glucosidase activity and better enzyme stability as compared with that at higher temperatures (24.8 °C and 37 °C). There was no significant difference ($P < 0.05$) in β-glucosidase activity and stability between storage at –80 °C and at 4 °C. This suggests that low temperatures (refrigerated or frozen) could be used for storage and distribution in the supply chain with minimal losses of β-glucosidase activity.

The storage temperature appeared to have an important influence on the β-glucosidase activity. According to Bruno and Shah (2003), viability of *bifidobacterium* cells is determined by the cellular activity and metabolism. Low temperatures restrict cellular activity and metabolism, therefore allowing very small energy losses, thus better stability of the cells and the enzyme within the cells. The lack of stability at higher storage temperatures could also be a result of faster change in the pH of the media compared with that at lower storage temperatures (pH data not shown). The faster change in pH at higher storage temperature is possibly due to the production of lactic and acetic acids.

As shown in the tables, there was, in general, an accelerated decrease in the β-glucosidase activity from the 6 strains after wk 5 of storage at all the temperatures, including the lower storage temperatures. It seems probable that viable cells will exhibit enzyme activity, and nonviable cells

Table 4—β-Glucosidase activity[1] of *Bifidobacterium animalis BB12* in soymilk during storage at different temperatures

Storage temp.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
–80 °C	$1.076^{a,A} \pm 0.021$	$1.048^{a,A} \pm 0.020$	$1.014^{ac,A} \pm 0.018$	$0.970^{ad,A} \pm 0.022$	$0.927^{bcd,A} \pm 0.037$	$0.692^{b,A} \pm 0.006$	$0.580^{b,A} \pm 0.002$	$0.614^{bc,A} \pm 0.005$
4 °C	$1.251^{a,A} \pm 0.015$	$1.203^{a,A} \pm 0.020$	$1.224^{a,A} \pm 0.022$	$1.229^{a,A} \pm 0.010$	$1.168^{a,A} \pm 0.019$	$0.831^{bc,A} \pm 0.006$	$0.894^{bd,A} \pm 0.006$	$0.652^{b,A} \pm 0.023$
24.8 °C	$1.447^{a,B} \pm 0.022$	$1.170^{bcd,B} \pm 0.024$	$0.992^{bc,B} \pm 0.026$	$0.973^{bc,B} \pm 0.027$	$0.797^{ac,B} \pm 0.050$	$0.645^{bdef,B} \pm 0.01$	$0.312^{bd,C} \pm 0.006$	$0.498^{bdef,B} \pm 0.012$
37 °C	$0.366^{a,B} \pm 0.058$	$0.167^{bcd,B} \pm 0.026$	$0.102^{bc,C} \pm 0.017$	$0.095^{bc,C} \pm 0.016$	$0.087^{bc,C} \pm 0.015$	$0.044^{bc,C} \pm 0.007$	$0.086^{bc,D} \pm 0.013$	$0.013^{bd,C} \pm 0.005$

[1]One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as mean ± standard error of units of enzyme ($n = 6$). Statistical analysis by means of two-way ANOVA. [abcdef]Means in the same row with different lowercase letters are significantly different ($P < 0.05$). [ABCD]Means in the same column with different uppercase letters are significantly different ($P < 0.05$).

Table 5—β-Glucosidase activity[1] of *Lactobacillus casei* 2607 in soymilk during storage at different temperatures

Storage temp	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
–80 °C	$1.232^{a,A} \pm 0.022$	$1.189^{a,A} \pm 0.017$	$1.158^{a,A} \pm 0.009$	$1.016^{b,A} \pm 0.011$	$1.065^{b,A} \pm 0.014$	$0.826^{bc,A} \pm 0.011$	$0.392^{bcd,A} \pm 0.010$	$0.335^{bcd,A} \pm 0.013$
4 °C	$1.174^{a,A} \pm 0.020$	$1.104^{ac,A} \pm 0.014$	$1.084^{ad,A} \pm 0.023$	$1.012^{bcd,A} \pm 0.011$	$1.021^{bcd,A} \pm 0.022$	$0.793^{b,A} \pm 0.011$	$0.306^{be,A} \pm 0.013$	$0.346^{bef,A} \pm 0.005$
24.8 °C	$1.117^{a,A} \pm 0.023$	1.084a,A ± 0.026	$0.961^{a,AB} \pm 0.013$	$0.927^{a,AB} \pm 0.017$	$0.882^{b,B} \pm 0.027$	$0.827^{b,A} \pm 0.022$	$0.111^{bcd,BC} \pm 0.012$	$0.068^{bcd,B} \pm 0.008$
37 °C	$0.640^{a,B} \pm 0.016$	$0.632^{a,B} \pm 0.010$	$0.426^{b,C} \pm 0.014$	$0.511^{b,C} \pm 0.019$	$0.411^{bde,C} \pm 0.026$	$0.347^{be,B} \pm 0.002$	$0.153^{bcf,B} \pm 0.009$	$0.042^{bc,B} \pm 0.006$

[1]One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as mean ± standard error of units of enzyme ($n = 6$). Statistical analysis by means of two-way ANOVA. [abcdef]Means in the same row with different lowercase letters are significantly different ($P < 0.05$). [ABCD]Means in the same column with different uppercase letters are significantly different ($P < 0.05$).

Table 6—β-Glucosidase activity[1] of *Lactobacillus acidophilus* 4962 in soymilk during storage at different temperatures

Storage temp.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
–80 °C	$1.236^{a,A} \pm 0.004$	$1.192^{a,A} \pm 0.005$	$1.153^{ab,A} \pm 0.006$	$1.112^{ab,A} \pm 0.006$	$1.079^{b,A} \pm 0.004$	$0.869^{bc,A} \pm 0.008$	$0.444^{bcd,A} \pm 0.006$	$0.341^{bcde,A} \pm 0.018$
4 °C	$1.258^{a,A} \pm 0.029$	$1.127^{b,A} \pm 0.012$	$1.058^{b,A} \pm 0.018$	$1.106^{b,A} \pm 0.025$	$1.019^{b,A} \pm 0.023$	$0.887^{bc,A} \pm 0.013$	$0.455^{bcd,A} \pm 0.014$	0.308^{bcde},A ± 0.010
24.8 °C	$0.862^{a,B} \pm 0.007$	$0.789^{b,B} \pm 0.006$	$0.701^{bc,B} \pm 0.001$	$0.633^{cd,B} \pm 0.015$	$0.615^{cd,B} \pm 0.012$	$0.611^{bc,B} \pm 0.005$	$0.121^{bef,B} \pm 0.007$	$0.276^{befg,B} \pm 0.001$
37 °C	$0.607^{a,C} \pm 0.010$	$0.567^{a,C} \pm 0.016$	$0.382^{b,C} \pm 0.010$	$0.270^{bc,C} \pm 0.010$	$0.253^{bc,C} \pm 0.022$	$0.261^{bc,C} \pm 0.004$	$0.075^{bcd,C} \pm 0.005$	$0.040^{bc,C} \pm 0.004$

[1]Means ± standard error of units of enzyme per nanogram of proteins. One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as mean ± standard error of units of enzyme ($n = 6$). Statistical analysis by means of two-way ANOVA. [abcde]Means in the same row with different lowercase letters are significantly different ($P < 0.05$). [ABCD]Means in the same column with different uppercase letters are significantly different ($P < 0.05$).

would not show any enzyme activity, which could as well be an index of cell viability. From the product development perspective, the shelf life of a functional food product manufactured by use of these probiotic microorganisms in soymilk would be approximately 5 to 6 wk.

Table 3 shows the β-glucosidase activity of *L. acidophilus* 33200 in soymilk during 8 wk storage at different temperatures. The enzyme activities were significantly higher ($P < 0.05$) at –80 °C and at 4 °C of storage as compared with those at 24.8 °C and 37 °C after 1 wk of storage. In general, the enzyme activity reduced during storage. Although there was a general decrease in the β-glucosidase activity, the values for lower storage temperatures were not significantly ($P < 0.05$) different. The same trend of higher and better stability of β-glucosidase at lower storage temperatures (–80 °C and 4 °C) compared with higher storage temperatures (24.8 °C and 37 °C) was observed for *B. animalis* BB12 (Table 4), *L. casei* 2607 (Table 5), *L. acidophilus* 4962 (Table 6), *L. acidophilus* 4461 (Table 7), and *L. casei* ASCC 290 (Table 8). It has been reported that proteins generally show better stability at ≤ 4 °C. Storage at room temperature often leads to protein degradation and/or inactivity (Pierce Technology Inc. 2003). For short-term storage of 1 d to a few weeks, many proteins (including enzymes) may be stored at 4 °C. These findings suggest that low temperature storage may be suitable for better stability of β-glucosidase enzyme.

The values of β-glucosidase activity (units of enzymes) during low temperature storage remained generally higher compared with those at higher temperatures throughout the entire storage period. At the end of wk 8, the enzyme activity was marginally higher at –80 °C compared with that at 4 °C for *L. acidophilus* 33200, *L. acidophilus* 4962, and *L. acidophilus* 4461, while marginally higher at 4 °C as compared with at –80 °C, for *B. animalis* BB12, *L. casei* 2607, and *L. casei* ASCC 290. Although the values were different between storage at 4 °C and at –80 °C, the differences were not statistically significant ($P < 0.05$).

Stability of β-glucosidase activity at different temperatures for 5 wk for 6 probiotic microorganisms

Stability of β-glucosidase activity during the first 5 wk of storage would be critical as this is the period most likely to be the shelf life of the functional soy-based product. Figure 1 to 6 show the stability of β-glucosidase activity produced by *L. acidophilus* 33200, *B. animalis* BB12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461, and *L. casei* ASCC 290, respectively. Stability of β-glucosidase activity could be significant in the stability of soy isoflavone aglycones, which are transformed from their inactive glucoside forms by these enzymes. The enzyme stability was determined by drawing a regression trendline of β-glucosidase activity during 5 wk of storage at –80 °C, 4 °C, 24.8 °C, and 37 °C.

The trendlines showed a negative slope indicating a general decrease in the β-glucosidase activity during the 5 wk of storage. The slope magnitude varied with strain and storage temperature. The slope was indicative of the stability of the enzyme; smaller gradient indicated better stability while a bigger one signified less stability. Lower storage temperatures generally showed smaller gradient/slope in contrast to higher storage temperatures that gave a comparatively larger gradient. Figure 1 shows the regression trendlines of β-glucosidase activity of *L. acidophilus* 33200 in soymilk at different temperatures. Storage at –80 °C and 4 °C had a gradient of 0.0418 and 0.0376 units of enzyme per week, respectively. On the other hand, storage at higher temper-

atures of 24.8 °C and 37 °C had higher slope magnitudes of 0.1509 and 0.0973 units of enzyme per week, respectively, indicating less stability of the enzyme at higher temperatures. The same trend was observed with other strains, as shown in Figure 2 to 6. Amongst the 6 strains, *B. animalis* BB12 exhibited the best stability of β-glucosidase activity during storage at 4 °C, showing a slope of 0.014 units of enzyme per week. The enzyme activity was, however, most unstable during storage at 24.8 °C where the slope magnitude was 0.1546. Thus, stability of β-glucosidase could be attributed to strain specificity, and is also influenced by storage temperature and the duration of storage.

Table 7—β-Glucosidase activity[1] of *Lactobacillus acidophilus* 4461 in soymilk during storage at different temperatures

Storage temp.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
–80 °C	$1.269^{a,A} \pm 0.012$	$1.159^{b,A} \pm 0.008$	$1.080^{bc,A} \pm 0.013$	$1.147^{b,A} \pm 0.005$	$1.121^{b,A} \pm 0.005$	$1.008^{bcd,A} \pm 0.008$	$0.722^{bcde,A} \pm 0.008$	$0.602^{bcde,A} \pm 0.006$
4 °C	$1.420^{a,A} \pm 0.040$	$1.335^{abc,A} \pm 0.020$	$1.291^{bc,A} \pm 0.018$	$1.301^{bc,A} \pm 0.037$	$1.294^{bc,A} \pm 0.044$	$1.021^{b,A} \pm 0.023$	$0.502^{bd,A} \pm 0.019$	$0.392^{bd,A} \pm 0.002$
24.8 °C	$0.538^{a,B} \pm 0.009$	$0.451^{ab,B} \pm 0.017$	$0.441^{ab,B} \pm 0.002$	$0.404^{bc,B} \pm 0.009$	$0.370^{cd,B} \pm 0.007$	$0.254^{cde,B} \pm 0.018$	$0.179^{cdf,B} \pm 0.014$	$0.061^{def,B} \pm 0.028$
37 °C	$0.253^{a,C} \pm 0.067$	$0.184^{ab,C} \pm 0.087$	$0.015^{ad,C} \pm 0.009$	$0.011^{bcd,C} \pm 0.007$	$0.008^{bcd,C} \pm 0.001$	$0.008^{bcd,C} \pm 0.012$	$0.006^{bce,C} \pm 0.011$	$0.006^{bce,C} \pm 0.010$

[1]Means ± standard error of units of enzyme per nanogram of proteins. One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as mean ± standard error of units of enzyme ($n = 6$). Statistical analysis by means of two-way ANOVA. ^{abcde}Means in the same row with different lowercase letters are significantly different ($P < 0.05$). ^{ABCD}Means in the same column with different uppercase letters are significantly different ($P < 0.05$).

Table 8—β-Glucosidase activity[1] of *Lactobacillus casei* ASCC 290 in soymilk during storage at different temperatures

Storage temp.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
–80 °C	$1.345^{a,A} \pm 0.024$	$1.342^{a,A} \pm 0.009$	$1.279^{ab,A} \pm 0.005$	$1.271^{ab,A} \pm 0.014$	$1.243^{bc,A} \pm 0.009$	$1.125^{bc,A} \pm 0.008$	$0.642^{cde,AB} \pm 0.003$	$0.540^{de,AB} \pm 0.014$
4 °C	$1.175^{a,A} \pm 0.022$	$1.152^{ab,A} \pm 0.016$	$1.127^{ab,A} \pm 0.015$	$1.101^{acd,A} \pm 0.004$	$1.076^{bcd,A} \pm 0.017$	$0.852^{b,B} \pm 0.003$	$0.823^{b,A} \pm 0.003$	$0.739^{be,A} \pm 0.008$
24.8 °C	$0.629^{a,B} \pm 0.034$	$0.623^{a,B} \pm 0.017$	$0.618^{a,B} \pm 0.012$	$0.507^{ab,B} \pm 0.033$	$0.469^{bc,B} \pm 0.026$	$0.386^{cd,C} \pm 0.013$	$0.311^{cde,C} \pm 0.020$	$0.232^{cdf,C} \pm 0.009$
37 °C	$0.546^{a,B} \pm 0.017$	$0.477^{ab,BC} \pm 0.008$	$0.343^{b,C} \pm 0.012$	$0.255^{bc,C} \pm 0.031$	$0.202^{bcd,C} \pm 0.031$	$0.133^{bce,D} \pm 0.005$	$0.118^{cde,D} \pm 0.025$	$0.067^{def,D} \pm 0.016$

[1]One unit of enzyme is the amount of β-glucosidase that released 1 nanomole of ρ-nitrophenol from ρNPG per mL/min at 37 °C.
Results expressed as mean ± standard error of units of enzyme ($n = 6$). Statistical analysis by means of two-way ANOVA. ^{abcde}Means in the same row with different lowercase letters are significantly different ($P < 0.05$). ^{ABCD}Means in the same column with different uppercase superscripts are significantly different ($P < 0.05$).

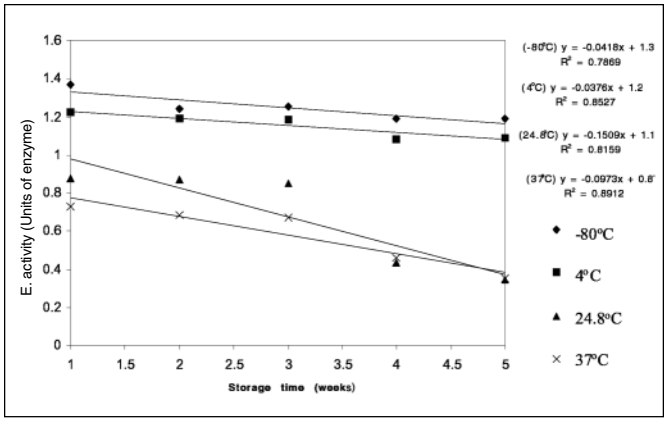

Figure 1—β-Glucosidase activity in soymilk by *L. acidophilus* 33200 during storage at different temperatures

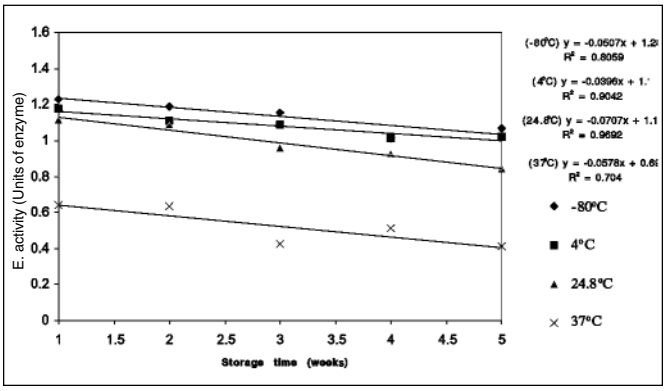

Figure 3—β-Glucosidase activity of *L. casei* 2607 in soymilk at different storage temperatures

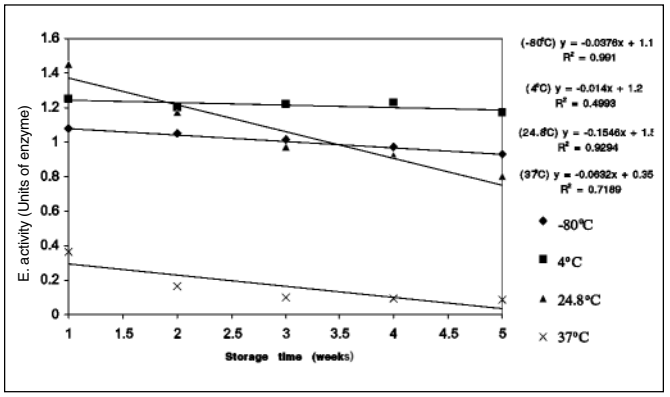

Figure 2—β-Glucosidase activity of *B. animalis* BB12 in soymilk during storage at different temperatures

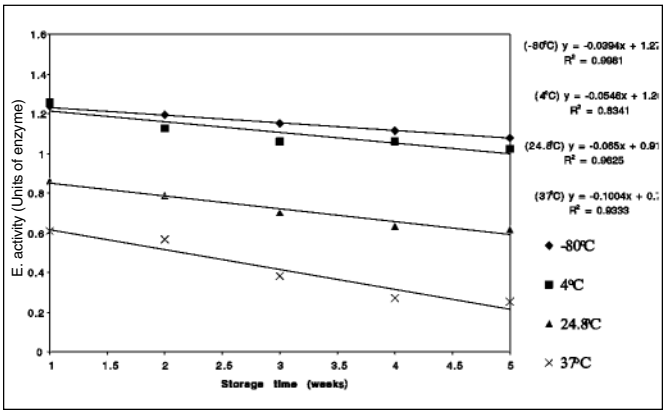

Figure 4—β-Glucosidase activity of *L. acidophilus* 4962 in soymilk at different storage temperatures

The equations in Figures 1 to 6 also show the y intercept, which is the initial β-glucosidase activity in soymilk. The initial enzyme activity is higher for storage at lower storage temperatures than that at higher storage temperatures. It appears reasonable to assume that 5 wk of storage as shelf-life for soy-based probiotic functional food would be dependent on β-glucosidase activity and the stability of the enzyme. There was no significant difference ($P < 0.05$) in initial β-glucosidase activity for all 6 microorganisms during storage at lower temperatures of 4 °C and –80 °C.

The order among the 6 strains for best stability at 4 °C was given by *B. animalis* BB12 with a slope magnitude of 0.014 units enzyme per week, followed by *L. casei* ASCC 290, *L. acidophilus* 4461, *L. acidophilus* 33200, *L. casei* 2607, and *L. acidophilus* 4962 whose regression trendline magnitudes are 0.0249, 0.0284, 0.0376, 0.0396, and 0.0546 units of enzyme per wk, respectively. Thus, the choice of microorganism for use in the soy-based functional food will depend on the β-glucosidase activity and its stability at the chosen storage temperature.

Conclusions

L. acidophilus 33200, *B. animalis* BB12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461, and *L. casei* ASCC 290 produced varying levels of β-glucosidase activity and stability at different storage temperatures. The β-glucosidase activity declined over time. For storage of a product for up to 5 wk for maintaining appreciable levels of β-glucosidase activity and better stability of the enzyme, storage at 4 °C would be ideal as was observed from the slope calculated during storage. Even at the end of 8 wk storage, it was observed that 3 strains had marginally higher β-glucosidase activity at 4 °C compared with that at –80 °C, but the values were not significantly different ($P < 0.05$). Thus for very long-term storage, a temperature of –80 °C may be preferred. β-Glucosidase enzyme is important in the biotransformation of the predominant inactive isoflavone glucosides in soymilk to their bioactive isoflavone aglycone constituents. Thus, the enzyme activity and its stability during storage may influence conversion of inactive isoflavone glucosides to bioactive isoflavone aglycones. The health benefits of bioactive isoflavone aglycones in postmenopausal women have been well documented. A correct storage temperature is therefore important for maintaining high level of β-glucosidase activity from probiotic microorganisms as well as stability of the enzyme in soymilk.

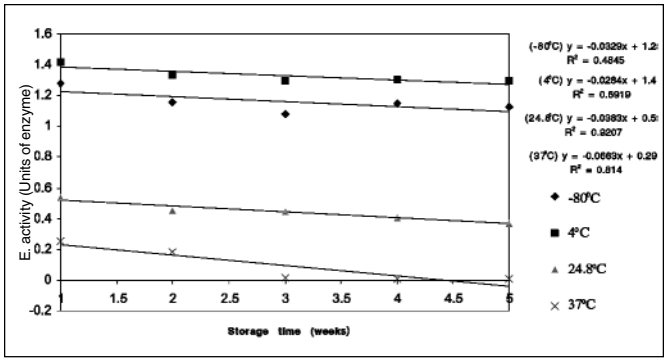

Figure 5—β-Glucosidase of *L. acidophilus* 4461 in soymilk during storage at different temperatures

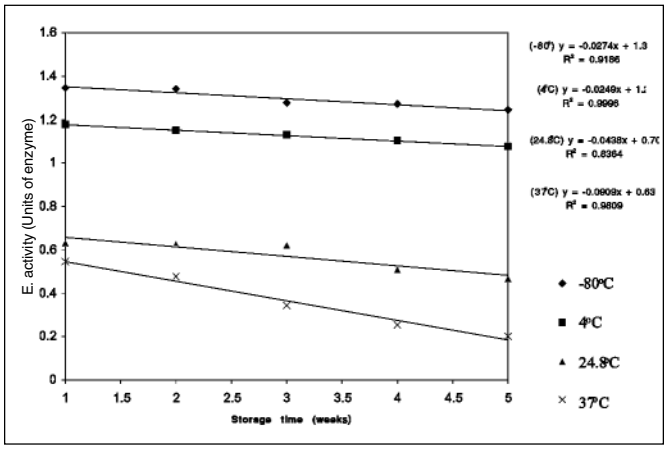

Figure 6—β-Glucosidase activity of *L. casei* ASCC 290 in soymilk at different storage temperatures

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References

Albright SC, Winston WL, Zappe C. 1999. Data analysis and decision making with Microsoft Excel. Pacific Grove, Calif.: Brooks/Cole Publishing Co. 996 p.

Ballongue J. 1993. Bifidobacteria and probiotic action. In: Salminen S, Von Wright A, editors. Lactic acid bacteria. New York: Marcel Dekker. p 519–87.

Berrada N, Lemeland JF, Laroche G, Thouvenot P, Piaia M. 1991. *Bifidobacterium* from fermented milks: Survival during gastric transit. J Dairy Sci 74:409–13.

Bruno FA, Shah NP. 2003. Viability of two freeze-dried strains of *Bifidobacterium* and commercial preparations at various temperatures during prolonged storage. J Food Sci 68:7:2336–9.

De Mann JC, Rogosa M, Sharpe ME. 1960. A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–5.

Driessen FM, de Boer R. 1989. Fermented milks with selected intestinal bacteria: a healthy trend in new products. Neth Milk Dairy J 43:367–82.

[FAO and WHO] Food and Agriculture Organization of the United Nations and World Health Organization. 2001. Regulatory and clinical aspects of dairy probiotics. FAO and WHO Expert Consultation Report. FAO and WHO working group report. www.fao.org. Accessed Nov 15, 2004.

Hosoda MH, Hashimoto FH, Morita H, Hosono A. 1996. Effect of administration of milk fermented with *Lactobacillus acidophilus* LA-2 on fecal mutagenicity and microflora in the human intestine. J Dairy Sci 79:745–9.

Liu KS. 1997. Soybeans: chemistry, technology and utilization. New York: Chapman and Hall. 532 p.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with folin phenol reagent. J Agric Food Chem 47:2697–704.

Mitsuoka T. 1990. Role of intestinal flora in health with special reference to dietary control of intestinal flora. In: Nga BH, Lee YK, editors. Microbiology applications in food biotechnology. Essex: Elsevier. 135 p.

Park YK, Aguiar CL, Alencar SM, Mascrenhas HAA, Scamparini ARP. 2002. Conversion of malonyl β-glucoside isoflavones into glycoside isoflavones found in some cultivars of Brazilian soybeans. Cienc Technol Aliment 22:130–5.

Park YK, Lui MCY, Aguiar CL. 2003. Production of enriched isoflavone aglycones during processing of soy protein isolates and soy protein concentrates [Abstract]. In:2003 IFT Annual Meeting Book of Abstracts; 12–16 July 2003; Chicago, Ill.: Inst. of Food Technologists. p 215.

Pierce Technology Inc. 2003. Technical resource, protein stability and storage. Rockford, Ill.: Pierce, a Perbio Science Co. Available from: www.piercenet.com. Accessed Nov 19, 2004.

Rosenberge IM. 1996. Protein analysis and purification: Benchtop Techniques. Boston: Birkhauster Publishing. p 105–7.

Sanders ME, Walker DC, Walker KM, Aoyama K, Klaenhammer TR. 1996. Performance of commercial cultures in fluid milk applications. J Dairy Sci 79:943–55.

Scalabrini P, Rossi M, Spettoli P, Matteuzi D. 1998. Characterisation of *Bifidobacterium* stains for use in soymilk fermentation. Intern J Food Microbiol 39:213–9.

Shah NP. 2000. Probiotic bacteria: Selective enumeration and survival in dairy foods. J Dairy Sci 83:894–907.

Slominski BA. 1994. Hydrolysis of galactooligosaccharides by commercial preparations of α-galactosidase and β-fructofuranose: Potential for use as dietary additives. J Sci Food Agric 65:323–30.

Tochikura T, Sakai K, Fujiyoshi T, Tachiki T, Kumagai H. 1986. π-Nitrophenoyl glycoside-hydrolysing activities in bifidobacteria and characterization of β-D-galactosidase of *Bifidobacterium longum* 401. Agric Biol Chem 50(9):2279–86.

Tsangalis D, Ashton JF, McGill AEJ, Shah NP. 2002. Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase producing bifidobacteria. J Food Sci 67:8:3104–13.

Tsangalis D, Ashton JF, McGill AEJ, Shah NP. 2003. Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented withD-glucose and L-cysteine. J Food Sci 68:623–31.