Role of the Bifidobacteria in Soymilk Fermentation

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Soy-based foods are rich in high quality proteins, essential amino acids, minerals, vitamins etc. thus they provide a range of health benefits. In this study, growth and $\alpha$-galactosidase activity of bifidobacteria ($Bifidobacterium\ lactis\ Bb-12$, $B.\ longum\ Bb-46$, $B.\ bifidum\ B3.2$, $B.\ bifidum\ B7.1$ and $B.\ breve\ B9.14$) were investigated. All tested bifidobacteria strains showed $\alpha$-galactosidase activity that catalyses hydrolysis of soybean galacto-oligosaccharides. The highest enzyme activity (23.94 U/L) was assayed in the case of $B.\ lactis\ Bb-12$ strain. Soymilk without any additional nutrients was good medium for growth of $Bifidobacterium$ strains. Moreover, $10^4$-$10^7$ cfu/ml initial cell concentrations resulted in $10^8$ cfu/ml after 8-12 h of incubation in soymilk, and were kept viable up to the end of fermentation (48 h). Due to intensive metabolism, the titratable acidities of the fermented soymilk were in the range of 14 and 29 SH° after 24h of fermentation. Production the lactic and acetic acid were in the range of 23–60 mmol/L and 2.4-5.6 mmol/L, respectively. Molar ratios of acetate to lactate in all tested strains varied from 0.08-0.1 that are very promising for further technological development of probiotic fermented soy-based food products.

1. Introduction

Soybean ($Glycine\ max.\ L.\ Merr$) is one of the most important oleaginous seeds in the world due to availability and rich in high quality proteins, essential amino acids, calcium, phosphorus, iron, vitamins (especially A and B) and vegetable oil [Genta et al. 2002]. Soy-based foods may provide a range of health benefits through hypolipidemic, anticholesterolemic and antiatherogenic properties as well as reduction in allergenicity [Favaro Trindade et al. 2001] and risk of most hormone-associated health disorders [Kurzer 2000]. Moreover, soybean contains all the amino acids essential to human nutrition, thus it should be good food base for substitution of milk for those who are vegetarians or lactose-intolerance [Scalabrini et al. 1998]. However, consumption of soymilk is hindered due to the presence of unpleasant off-flavours carried over from soybean as well as various oligosaccharides including raffinose and stachyose that may cause a gastrointestinal discomfort known as flatulence to consumers [Scalabrini et al. 1998]. Raffinose and stachyose are non-digestible $\alpha$-galactosidic oligosaccharides due to lack of $\alpha$-galactosidase in the human gastrointestinal track. These matters can be addressed and eliminated by treatment using external $\alpha$-galactosidase enzyme or/and fermentation with microorganism possessed high $\alpha$-galactosidase activity. The later concept should be more attractive in all scientific, technical and nutritional points of view, because in one processing step it gives possibility to remove off-flavour effects causing by $n$-hexanal and form probiotic products with high nutritional values. In the last few decades, intensive research dealing with fermentation of soymilk using $Lactobacillus$ strains was carried out worldwide [Wang et al. 2003; Wang et al. 2009]. Hydrolysis of isoflavones as well as production of flavourful lactic acid during fermentation of soymilk was reported by Bordignon and co-workers in 2004. Reduction
of galacto-oligosaccharides (raffinose, stachyose, soygalacto-oligosaccharides etc.) various lactic acid bacteria (L. cellobiosis, L. plantarium, L. curvatus, L. fermentum, L. pentosaceus etc.) have also been reported by several authors [Yoon & Hwang 2008].

Nowadays, several *Bifidobacterium* strains are well known as probiotics with many health-promoting effects, and they play an important role in the microbial ecology of the human and animal gut. Moreover, some strains were also reported about capability of this bacterium to metabolise α-galactosyl type galacto-oligosaccharides [Kamaly 1997; Kullen et al. 1998], thus soymilk that contains sucrose, raffinose, stachyose, proteins, vitamins etc. is should be good medium for the growing bifidobacteria [Kamaly 1997]. Interestingly, Bordignon and co-workers (2004) reported that *B. bifidum* JCM 1255, *B. breve* JCM 1922 and *B. infantis* JCM 1222 strains rather prefer galacto-oligosaccharides than sucrose during fermentation of soymilk. This finding led to conclude high α-galactosidase activity of these strains. Although, due to this activity *Bifidobacteria* are able to cleave α-galactosidic bounds, very few data are available in the literature related to properties of α-galactosidase enzyme from this micro-organism. Generally, *Bifidobacteria* utilise glucose through so called “bifidus pathway” resulted high level of acetic acid that causes odour effects in final product, thus it should be main drawback of application of this bacteria in fermentation system. No doubt that the molar ratio of lactic and acetic acid varies species to species, even strains to strains or fermentation conditions. In the case of lactic acid bacteria, some authors [Kwon et al., 2000; Nancib et al., 2001] reported that increase in supplement of nitrogen source resulted higher concentration of lactic acid, thus flavour of product should be better. In our previous study [Kun et al. 2008], this effect was also observed when doing fermentation of carrot juice with several *Bifidobacterium* strains. In this study, role of bifidobacteria in soymilk fermentation was aimed to investigate.

2. Materials and methods

2.1. Media

*Laboratory media*: Trypticase–Phytone–Yeast medium (TPY) contained (per litre) trypticase (BBL) 10 g, phytone (BBL) 5 g, glucose 5 g, yeast extract (Difco) 2.5 g, Tween 80 1 ml, L-cysteine HCl 0.5 g, K₂HPO₄ 2 g, MgCl₂*6H₂O 0.5 g, ZnSO₄*7H₂O 0.25 g, CaCl₂ 0.15 g, FeCl₃ 0.03 g. Its pH was about 6.0.

*Soymilk*: Soybeans were washed and soaked in water for one day at room temperature. Soaking water was drained and it was boiled in fresh water (its quantity was the quadruple of soybean) for 30 minutes, then the whole amount was crushed with mixer for 5 minutes and filtered through double-layer cloth to yield soymilk. The cake was extracted several times to gain approximately 4.5-5 litre soymilk per 500 g soybean. It was autoclaved at 121°C for 15 minutes.

2.2. Microorganisms and their cultivation

*Bifidobacterium lactis* Bb-12 and *B. longum* Bb-46 were purchased from Chr. Hansen A/S (Hørsholm, Denmark). *Bifidobacterium bifidum* B3.2, *B. bifidum* B7.1 and *B. breve* B9.14 were isolated from human faeces and classified (Mayer et al., 2003). All *Bifidobacterium* strains were pre-cultured anaerobically (in Bugbox anaerobic chamber, Ruskinn Technology) in TPY medium at 37°C for approximately 24 h.
2.3. Fermentation

Fermentation was initiated with $10^6$-$10^7$ CFU (colony forming unit)/ml concentration of the relevant *Bifidobacterium* strains. All trials were carried out under anaerobe conditions in Anaerobe Jar+GasPak System (OXOID) or in Bugbox anaerobic chamber at 37°C. Fermentation was followed by counting the colony forming units and measuring pH and titratable acidity.

2.4. Analytical procedures

**Titratable acidity (SH°) and pH:** The titratable acidity was determined with Soxhlet-Henkel method by titration. During the cultivation, the main metabolic products are organic acids, particularly lactic and acetic acids. The pH changes in batches of soymilk or TPY media were monitored during fermentation using a SevenMulti pH-meter (Metler Toledo).

**Living cell number:** The plate counts of bifidobacteria were determined applying Beeren’s agar. Samples from the fermented broth were diluted by 10-fold serial dilution and aliquots were transferred into Petri dishes, mixed with the medium. After solidification, the plates were incubated under anaerobic conditions at 37°C. The colonies were counted after 48-h or 72-h of incubation.

**Determination of organic acids:** The concentration of organic acids were determined with Waters HPLC System consisting of W610 pump Waters HPLC Controller, 717 plus autosampler W410 refraktor index (RI), and photodiode array (PDA) detectors. A thermostatically controlled column compartment set at 45°C containing Aminex HPX-87H ion exclusion column was used at a flow rate of 0.6 ml/min using 5 mM H$_2$SO$_4$ as the mobile phase. The data acquisition and integration were performed using the MillenniumTM 4.0 software package. Each sample was injected three times. Standards (external and internal) of organic acids (lactic, acetic, malic, citric, succinic, H$_2$SO$_4$ and oxalic) were used to identify and quantify the components in the samples.

**Assay of α-galactosidase enzyme activity:** The α-galactosidase enzyme activity was assayed in the reaction mixture containing 0.3 mL of McIlvaine buffer (100mM, at pH 6.6) and 0.5 mL of 15 mM *p*-nitrophenyl α-D-galactopyranoside (pNPαGal) substrate. Intracellular enzyme fraction was liberated from cell culture using chemical method (Cetyl trimethylammonium bromide, CTAB). Buffered substrate was pre-incubated at the relevant temperature for 5 min and the enzyme reaction was started by adding 0.2 mL of adequately diluted enzyme solution. After 5 min the enzyme reaction was stopped by adding 5 mL of 0.1M Na$_2$CO$_3$ solution. The released *p*-nitrophenol was determined spectrophotometrically at 405 nm using calibration linear prepared with *p*-nitrophenol under the same conditions.

One unit (U) of enzyme activity was defined as the amount of enzyme that releases one µmol *p*-nitrophenol per min under the relevant conditions.
3. Results and discussion

3.1. α-Galactosidase activity of different Bifidobacteria

The α-galactosidase activity of different *Bifidobacterium* strains was investigated in laboratory media supplemented with 1% raffinose. The results are shown in Figure 1. The best α-galactosidase producer was *B. lactis* Bb-12, it was 23.94 U/L. Similar α-galactosidase activity was observed the *B. bifidum* B7.1 strain (22.57 U/L). Other two tested *Bifidobacterium* strains (*B. longum* Bb-46 and *B. breve* B9.14) were produced 5-fold lower galactosidase enzyme as *B. lactis* Bb-12. Desjardins and co-workers (1990) observed that bifidobacteria possessed high activity of α–galactosidase and β-galactosidase. Tochikura and co-workers (1986) however, found that bifidobacteria exhibit higher hydrolyzing activity toward various p-nitrophenyl glycosides than other intestinal bacteria. In case of *B. bifidum* B3.2 low α-galactosidase activity was detected.

Figure 1. The α-galactosidase activity of *Bifidobacterium* in TPY medium with 1% raffinose

3.2. Growth behaviour

The changes of cell numbers of bifidobacteria during fermentation of soymilk are presented in Figure 2. Generally, maximum counts of cell number occurred at 12th hour of fermentation except *B. bifidum* B3.2 strain, even inoculation the soymilk with about $10^7$ cfu/ml or $5.10^4$ cfu/ml initial cell concentration. These counts of most strains varied from $5.10^7$ to $10^8$ cfu/ml, in the case of *B. bifidum* B7.1 about $5.10^8$ cfu/ml were counted as in the case of *B. bifidum* B3.2 at 24 h of fermentation of soymilk. These results are in difference from those reported by Hou and co-workers (2000) or Wang and co-workers (2003) when they studied growth of *B. infantis* and *B. longum* in soymilk. They found that both *B. infantis* CCRC 14633 and *B. longum* B6 strains have to take at least 24 hours for reach maximum cell numbers in native soymilk.
3.3. Changes in titratable acidity and pH

During fermentation of soymilk, the titratable acidity (TA) increased from about 3 SH° to the range 14.27 - 29.32 SH° depending on the used strain, meanwhile pH value decreased from 6 to 4.5 (Figure 3). The highest acidity (29 SH°) was observed in case of B. bifidum B3.2 strain. Intensive growth of bacteria was also confirmed by drop of pH in the first 8 hours of fermentation (Figure 3). In general, pH values dropped from 6.0 to 5.0 or below. In 2003, Wang and co-workers reported that in similar situation, B. infantis CCRC 14633 had to take about 40 hours and 48 hour of fermentation to reach pH 5.04 and pH 4.61, respectively. Bifidobacterium breve JCM 1192 strain was reported to achieve better fermentation profile (drop in pH from 6.2 to 5.1 after 16 h), while B. adolescentis JCM 1275 and B. bifidum JCM 1255 produced less acid during fermentation of soymilk [Bordignon et al., 2004]. Our results are in comparable with some profiles produced by lactic acid bacteria such as Lactobacillus delbrueckii subsp. bulgaricus IFO 13953 [Bordignon et al., 2004], L. casei subsp. rhamnosus FNCC 098, L. plantarum SMN 25, L. plantarum pentosus SMN 01 (Sumarna, 2008).
Figure 3. The profile of the acidity and pH of the soymilk fermentation with bifidobacteria

3.4. Changes in lactic, acetic and propionic acid concentration

It is well known that lactic acid is one of the most important compounds in formation of flavor of fermented products such as soymilk. One unique aspect of bifidobacteria is that all the lactic acid produced is in the L(+) form which is more easily metabolized by infants than the D(-) form [Ishibashi & Shimamura 1993]. Theoretically, utilisation of carbohydrates through “bifidus” pathway, bifidobacteria produces more acetic acid than lactic acid (punctually 3:2 in molar ratio) [Scardovi, 1981]. Changes of some organic acids in soymilk during the fermentation of soymilk with four applied *Bifidobacterium* strains are summarized in Table 1. Due to intensive metabolic activity, both *B. lactis* Bb-12 and *B. bifidum* B3.2 strains produced high amount of lactic and acetic acids in ferment broth even after 6 hours (in the case of Bb-12 52.52 mmol/L lactic and 4.44 mmol/L acetic acid, in the case of B3.2, 43.61 mmol/L and 3.43 mmol/L, respectively). Other two strains (Bb-46 and B 7.1) also showed good results in production of short chain fatty acids after 12 h of fermentation of soymilk. These results were about ten times higher than those reported by Donkor and co-workers (2007). In their study, *B. lactis* B94 and *B. longum* B1536 produced 0.02 and 0.03 mg/ml lactic acid and 0.05 mg/ml acetic acid concentration at 12th hours of soymilk fermentation. Higher acetic acid concentration were published by Hou and co-workers (2000), where they found that 11.32 mmol/L and 11.42 mmol/L acetic acid concentration were determined at 12 h of fermentation using *B infantis* CCRC 14633 and *B. longum* B6 strains, respectively. Based on molar ratio of acetic to lactic acid calculated, in the first stage of fermentation these values were about 1.8-1.9, thus *B infantis* CCRC 14633 and *B. longum* B6 did not follow only bifidus pathway. Interestingly, all of investigated strains in our study produced much more lactate than acetate in molarity (0.08-1.34 molar ratio). Our present results confirm data reported in our previous study [Kun et al. 2008], when fermentation of carrot juice. Some studies dealing with nutrients necessary for lactic acid fermentation have established [Kwon et al. 2000; Nancib et al. 2001]. They found that supplement of more nitrogenous components resulted higher concentration of lactic acid. Soymilk is rich in protein and amino acid content, thus it may result in change of molar ratio of acetate to lactate during fermentation. Furthermore, these results demonstrated that the molar ratio of acetic and lactic acids produced by bifidobacteria varies depending on numerous parameters such as applied strains, culture medium, the fermentation time, even fermentation conditions etc.
Table 1. Changes of lactic, acetic and propionic acid in soymilk fermented with different bifidobacteria strains

<table>
<thead>
<tr>
<th>Fermentation time (h)</th>
<th>Content (mmol/L)</th>
<th>Molar ratio (acetic/lactic)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Lactic acid</td>
<td>Acetic acid</td>
</tr>
<tr>
<td><strong>B. lactis Bb-12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.22</td>
<td>0.72</td>
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<tr>
<td>6</td>
<td>52.52</td>
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<tr>
<td>12</td>
<td>43.85</td>
<td>3.55</td>
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<tr>
<td><strong>B. longum Bb-46</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.22</td>
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<tr>
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<td>4.22</td>
<td>0.72</td>
</tr>
<tr>
<td>6</td>
<td>43.61</td>
<td>3.43</td>
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<tr>
<td>12</td>
<td>58.14</td>
<td>5.57</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>4.22</td>
<td>0.72</td>
</tr>
<tr>
<td>6</td>
<td>1.98</td>
<td>2.65</td>
</tr>
<tr>
<td>12</td>
<td>59.55</td>
<td>4.59</td>
</tr>
</tbody>
</table>

4. Conclusion

All investigated *Bifidobacterium* strains (*B. lactis* Bb-12, *B. longum* Bb-46, *B. bifidum* B3.2, *B. bifidum* B7.1 and *B. breve* B9.14) were capable to grow and ferment native soymilk without any nutrient supplementation. Due to α-galactosidase activities, they are able to hydrolyse α-glycosidic galacto-oligosaccharides in soymilk eliminating flatulent effects. These strains produce high level of lactic acid in fermented soymilk resulted titratable acidity in range of 14 and 29 SH°. Moreover, the molar ratios of acetate to lactate concentration at 12h of fermentation varied from 0.08 to 0.1 that are very good results from technological points of views, due to high concentration of acetic acid causes odour effect of final product. Overall, our results are very promising and may serve as base for development of technology for production of probiotic fermented soymilk.

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References


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