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Partial Purification and Characterization of Glutaminase from *Lactobacillus reuteri* KCTC3594

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Abstract In this study, we attempted to purify and characterize glutaminase (EC. 3.5.1.2) from *Lactobacillus reuteri* KCTC3594. The glutaminase was purified approximately 21-fold from the cell-free extract of *L. reuteri* KCTC3594 by protamine sulfate treatment and chromatography methods including anion exchange and gel filtration. The sizes of two major bands of the enzyme were presumed to be 70 and 50 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The glutaminase activity of *L. reuteri* KCTC3594 was assayed in various ranges of pH, temperature, and salt concentrations. The enzyme activity was optimal at 40°C and pH of 7.5. It was shown that the glutaminase was salt-tolerant because the enzyme activity was maintained 50% at 15% (*w*/*v*) salt concentrations. On the other hand, the enzyme was strongly inhibited up to 80% by 6-diazo-5-oxo-L-norleucine (10 mM) and iodoacetate (50 mM) indicating that the purified enzyme represents typical characteristics of glutaminase.

Keywords *Lactobacillus* spp. · Glutaminase · Purification · Salt tolerance · Natural flavor

Introduction

Monosodium glutamate (MSG) has an "umami" taste known as the fifth basic taste following sweet, acid, salty, and bitter [1, 2]. The food industry has long used MSG as a flavor enhancer. However, MSG shows negative side effects such as wheezing, heart-rate changes, and breathing difficulty in some people, arousing controversy on its safety [2].

Much interest for public health is increasingly focused on the development of natural flavor enhancers as substitutes for MSG. Among the natural flavor enhancers currently

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available, glutaminase (EC. 3.5.1.2) is known as a key enzyme that enhances the flavor of fermented food like soy sauce. Glutaminase was studied in microorganisms as well as in mammalian cells [3]. In addition, glutaminase catalyzes the hydrolytic degradation of L-glutamine to L-glutamate, which is a high-flavor amino acid in foodstuffs [2, 4]. Also, it has been reported that it may be used as a possible therapeutic agent for the treatment of anti-leukemia when combined with asparaginase [3, 4]. In 1967, L-asparaginase was first introduced for clinical use [5], and it has been widely used for rapid remission of acute lymphocytic leukemia patients. Since, the substrate of glutaminase is similar to that of glutaminase–asparaginase, the amino acid sequences for them are not distinguishable [3]. We know that glutaminase has been clinically used based on the report that phosphate-activated glutaminase exhibits antineoplastic properties [6].

Among the microorganisms studied as sources of glutaminase, *Lactobacillus* spp. are probiotic bacteria and used in the food fermentation industry for manufacturing cheeses, buttermilk, sauerkraut, and yogurt [7, 8]. As promising probiotic organisms, they help maintain health [8]. As many lactobacilli do, *Lactobacillus reuteri* also produces an antimicrobial substance (reuterin) that inhibits pathogens in the gastrointestinal tract [9]. In addition, many *L. reuteri* strains were isolated from meat or dairy products and are widely used in the food industry. Therefore, *L. reuteri* is suitable for use in natural food materials. However, no publication has reported on the purification of glutaminase from lactobacilli until now. Here, we attempted to purify and investigate the enzymatic properties of glutaminase from *L. reuteri* KCTC3594.

Materials and Methods

Organism and Culture Conditions

L. reuteri KCTC3594 was cultured in MRS liquid medium or on solid plates with 1.5% agar at 37 °C under anaerobic conditions. MRS contained 20 g of glucose, 10 g of peptone NO. 3, 10 g of beef extract, 5 g of yeast extract, 5 g of sodium acetate, 2 g of potassium phosphate dibasic (K_2HPO_4), 1 g of Tween 80, and 2 ml of trace solution (5 g of $CaCl_2$, 145 mg of boric acid, 125 mg of iron sulfate ($FeSo_4$), 75 mg of calcium sulfate ($CaSo_4$), 4.3 mg of manganese sulfate ($MnSo_4$), 108 mg of zinc sulfate ($ZnSo_4$), 125 mg of Na-molybdate ($Na_2MoO_4 \cdot 2H_2O$), 7 g of nitrilacetate) per liter. The cultured cells were harvested after the stationary growth phase.

Preparation of Cell-Free Extract

To harvest huge amounts of *L. reuteri* KCTC3594 cells, the cells were serially cultured. One colony on a MRS agar plate was transferred to 5 mL of fresh MRS broth and incubated at 37 °C for 24 h. Then, 5 mL cultures of 10% (*v*/*v*) were inoculated into 70 mL MRS media and incubated up to the stationary phase. To prepare a large volume of culture broth, 70 mL cultures of 10% (*v*/*v*) were diluted into 3 L of fresh MRS media. After incubation using the same conditions at 37 °C for 24 h, the cells were harvested by centrifugation for 10 min at 8,000×*g*. The cell pellets were then washed twice with 60 mL of TE buffer (20 mM Tris-HCl; pH 7.5) and resuspended with 60 mL of TE buffer. The cells were homogenized three times using a high-pressure homogenizer (Emulsi Flex C-3, Avestin) at a pressure of 15,000 psi. The extracted cell suspensions were stored at −70 °C until use.

Enzyme Purification

Protamine Sulfate Treatment

A 2.5% (*w*/*v*) protamine sulfate solution was added to the crude cell extract up to a final concentration of 0.1 mg of protamine sulfate per 1 mg of protein and continuously stirred at 4 °C for 20 min. The mixture was then centrifuged at 8,000×*g* for 10 min at 4 °C, and the supernatant was frozen at −70 °C. After thawing at 37 °C for 30 min, the sample was centrifuged at 8,000×*g* for 20 min. Subsequently, the supernatant was filtered using a 0.22-μm-sized microfilter [10] and used for further study.

Weak Anion Exchange Chromatography

To purify the glutaminase enzyme, we used a FPLC system (Pharmacia). The filtrated supernatant was loaded on the DEAE (0.7×2.5 cm, Agen Bio HiTrap-DEAE-FF, GE Healthcare) column equilibrated by a TE buffer (50 mM Tris-HCl, and 5 mM EDTA; pH 7.5) at a flow rate of 1 ml/min for 30 min. The column was then eluted with a linear gradient of NaCl (from 0 to 0.25 M) at a flow rate of 1 mL/min for 40 min. The eluted sample was collected a total of 23 fractions, with each fraction contained in a 1-mL microcentrifuge tube and stored at 4 °C. The fractions at peaks were assayed for glutaminase activity. The fractions showing greater than 1 U/mL activity were pooled and concentrated to 5 ml by centrifugation in an Ultrafree 30 K NMWL membrane filter (Millipore, Bedford, USA).

Gel Filtration Chromatography

The glutaminase was further purified on a Superose 12HR (10×30 mm, Pharmacia Biotech) column which was equilibrated in TE buffer (20 mM Tris-HCl, 0.1 M NaCl, 10% ethylene glycol, and 5 mM EDTA; pH 7.5) at a flow rate of 0.3 mL/min for 1 h. Subsequently, the filtrated fractions (5 mL) were then loaded on the column. After loading, the column was eluted with TE buffer at a flow rate of 0.2 mL/min for 3 h. The collected fractions (1.5 mL) were then assayed for glutaminase activity.

Determination of Protein Concentration

The concentration of the total protein was measured by the Bradford method [11] using crystalline bovine serum albumin as the standard. In chromatography, the protein absorbance was monitored at 280 nm during FPLC.

Glutaminase Assay

Cultivated cells were centrifuged for 10 min at 8,000×*g* and harvested. Cell pellets were washed twice with 1 mL of TE buffer (50 mM Tris-HCl and 5 mM EDTA; pH 7.5) to remove remnant ingredients of media. The cells were then suspended with 1 mL of TE buffer and were subsequently homogenized through sonication for 1 min. The initiation solution (0.1 mL of fresh 2% L-glutamine solution and 0.2 mL of 100 mM Tris-HCl buffer; pH 7.5) was equilibrated for 5 min at 37 °C; 0.1 mL of cell extract was added to the initiation solution after the reaction started. The sample was then incubated for 10 min at 37 °C. In order to stop the reaction, the sample was boiled for 3 min and centrifuged for 5 min

at 8,000×*g*. An aliquot of 50 μL of the supernatant was added into the first reaction mixture with 1 mL of hydroxylamine buffer (0.25 M hydroxylamine and 20 mM EDTA, pH 8.0), 0.5 mL of 10 mM NAD^+ solution, 1 mL of distilled water, and 20 U of glutamate dehydrogenase. After incubation for 30 min at 37 °C, the glutaminase activity was found by measuring optical density at 340 nm with a spectrophotometer. One unit of glutaminase activity was defined as the amount of enzyme required to produce 1 μmol of NADH per min at 37 °C. To find the optimal temperature, pH, and salt concentration, the parameters in the initial equilibration steps were varied within the following ranges: 20 to 60 °C, pH 3 to 11, and 0% to 20% NaCl. The effect of potential inhibitors on the glutaminase activity of crude and purified samples were measured by similar glutaminase assays except for addition of 6-diazo-5-oxo-L-norleucine (DON; 10 mM) or iodoacetate (50 mM) in the initial equilibration step.

Electrophoresis

For the determination of the enzymatic molecular weight of glutaminase, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 12% polyacrylamide gel and stained with Coomassie blue R-250 [12].

Results and Discussion

Purification of Glutaminase

To find out where the glutaminase is located in the cell, glutaminase activity was assayed independently from cell pellets and the supernatant after two fractions were isolated from the crude cell extract. The glutaminase activity assayed in the cell pellet (14.185 U/mL) exhibited the most activity, while a relatively low activity of 2.48 U/mL was detected in the supernatant. From this data, it can be deduced that most of the glutaminase of *L. reuteri* KCTC3594 is bound to the cell membrane rather than in a soluble form in the cytoplasm.

The procedure and results of purifying glutaminase from the crude cell extract of *L. reuteri* KCTC3594 are summarized in Table 1. The enzyme purified from the wet cell mass of 35 g of crude cell extract exhibited specific activity of 0.79 U/mg. By protamine sulfate precipitation, the glutaminase was purified approximately 12 times more, and the yield rate was enhanced up to 96%. In other studies, the cell extract was centrifuged to remove cell debris before being precipitated by addition of protamine sulfate or ammonium sulfate [6, 10]. However, in this study, protamine sulfate was added directly to crude cell extract prior to centrifugation. To purify the enzyme further, weak anion exchange chromatography was carried out. A total of 10 fractions were eluted around two sharp peaks which were monitored with NaCl gradient ranges from 0.2 to 0.4 M. Among the fractions, the highest activity of 2.89 U/ml was detected at 0.35 M NaCl (Fig. 1a). On the other hand, no glutaminase activity was detected in the first peak in a range of 0.1 to 0.2 M NaCl, and the low activity of 0.164 U/mL was shown in the 0.2 M NaCl fraction. Using weak anion for isolation, the glutaminase activity was 15.37 U/mg and approximately 1.6-fold higher than when isolated using protamine sulfate. Finally, fractions with glutaminase activity over 1 U/mL were pooled and further purified using gel filtration chromatography. The purified glutaminase was eluted at two peaks by gel filtration. However, the peaks were not completely separated in contrast to the elution pattern of weak anion exchange chromatography. On the other hand, glutaminase activity was detected as 1.64 U/mL at

Table 1 Purification of glutaminase from *L. reuteri* KCTC3594.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
cell-free extract	941.9	747.41	0.79	100	1
Protamine sulfate treatment	72.7	716.48	9.86	96	12.4
Weak anion exchange	0.3	4.61	15.37	0.6	19.4
Gel filtration	0.1	1.64	16.4	0.2	20.7

the second peak (Fig. 1b). The purified glutaminase activity after gel filtration was 21-fold higher than that of crude cell extract. The yield and the final specific activity were 0.2% and 16.40 U/mg, respectively (Table 1). As reported by Dura et al., the glutaminase showed high instability especially during the gel-filtration step. This is the same result in our

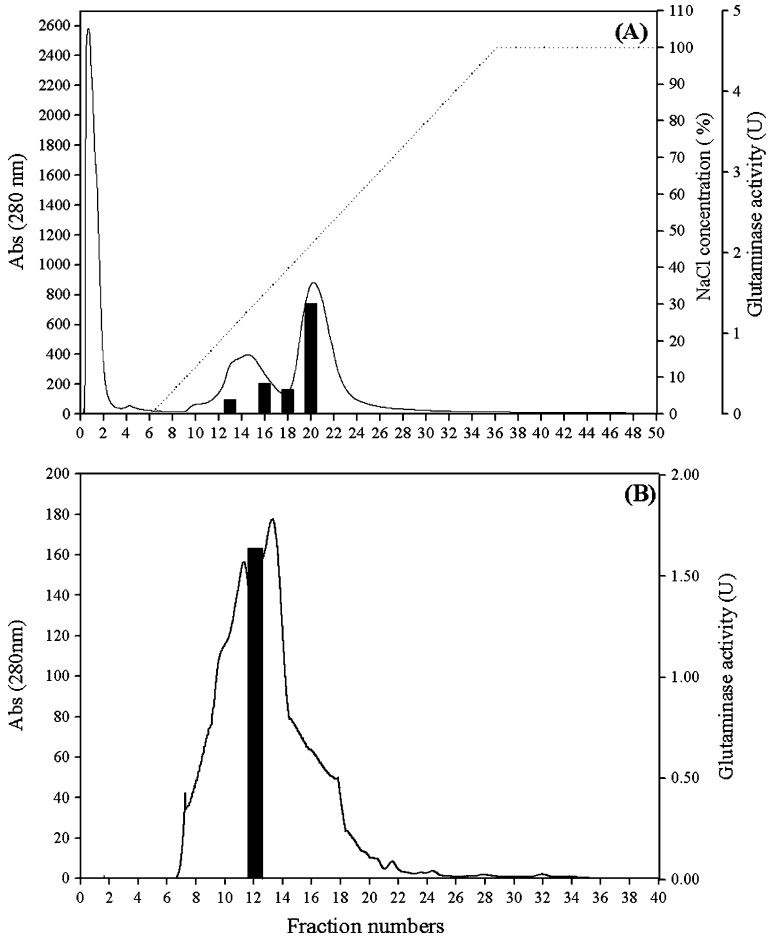

Fig. 1 Chromatographic purification of glutaminase from *L. reuteri* KCTC3594. **A** Weak anion exchange at pH 7.5 (0 to 0.25 M NaCl gradient) and **B** gel filtration. NaCl liner gradient (*dotted line*), absorbance 280 nm (*solid line*), glutaminae activity (*closed square*)

purification process for glutaminase from *L. reuteri* KCTC3594. It has been known that the instability of glutaminase is typical in mammalian or microbial organisms [10, 13]. Based on that feature, we also added to 10% ethylene glycol (FW 62.07) as a stabilizer. Nevertheless, the enzyme was not separated completely through several gel filtrations. On the other hand, the glutaminase activity was detected between the first two peaks (Fig. 1b).

The result of SDS-PAGE after the final step of purification revealed two major bands corresponding to molecular mass of approximately 70 and 50 kDa (Fig. 2). The enzyme preparation, however, still showed some minor impurities on the electrophoretogram. From these results, impurity seems to exist through SDS-PAGE because a glutaminase-associated enzyme appears together during the separation process due to strong binding. While the 70- and 50-kDa bands were shown as major bands during the separation process after precipitation by protamine sulfate, a minor band (40 kDa) decreased to 36 kDa due to possibly the changes of net electric charge of the protein which may have been affected during weak anion exchange chromatographic conditions. The result of decreased protein band from 40 to 36 kDa observed on the SDS-PAGE also showed similar tendency on gel filtration in which some impurity peaks appeared after glutaminase activity (Fig. 1b). This may also be due to an impurity which was strongly bound with glutaminase. Among them, 70 kDa showed an enzyme complex form in which glutaminase–asparaginase was combined or in the form of an isoenzyme of glutaminase. The glutaminase from *Pseudomonas* sp. appeared to synthesize two isoenzymes of 70 and 50 kDa [14], which closely correlate with the deduced molecular masses of those of *L. reuteri* KCTC3594. The glutaminase purified from *Micrococcus* was also determined to be 49.9 kDa [4]. The heterogenous structures of glutaminase or glutaminase–asparaginase have been described as tetramers, dimers, or monomers [6].

Properties of Glutaminase Activity of *L. reuteri* KCTC3594

We examined the effect of pH, temperature, and salt on the glutaminase activity of *L. reuteri* KCTC3594. The glutaminase was optimum at pH 7.5 (Fig. 3a). The optimum temperature was 40°C with 78% activity remaining after incubation for 10 min at 60°C

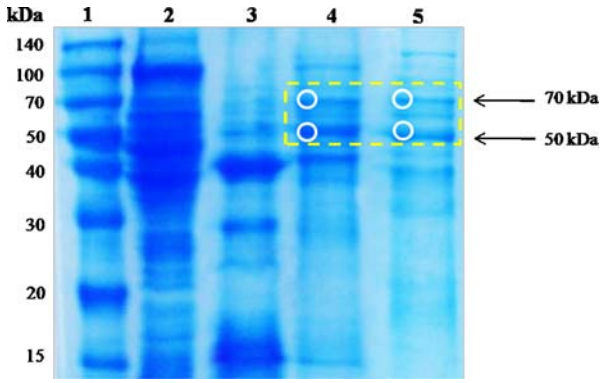

Fig. 2 Twelve percent SDS-PAGE of the purification process stained with Coomassie blue. The samples were loaded in duplicates. *Lane 1* molecular mass standard; *lane 2* cell-free extract; *lane 3* thawed extract of protamine sulfate treatment; *lane 4* sample after weak anion exchange; *lane 5* sample after gel filtration. The size of the molecular mass markers is indicated on the *right*. The major band was shown as subunits were exhibited at 70 and 50 kDa

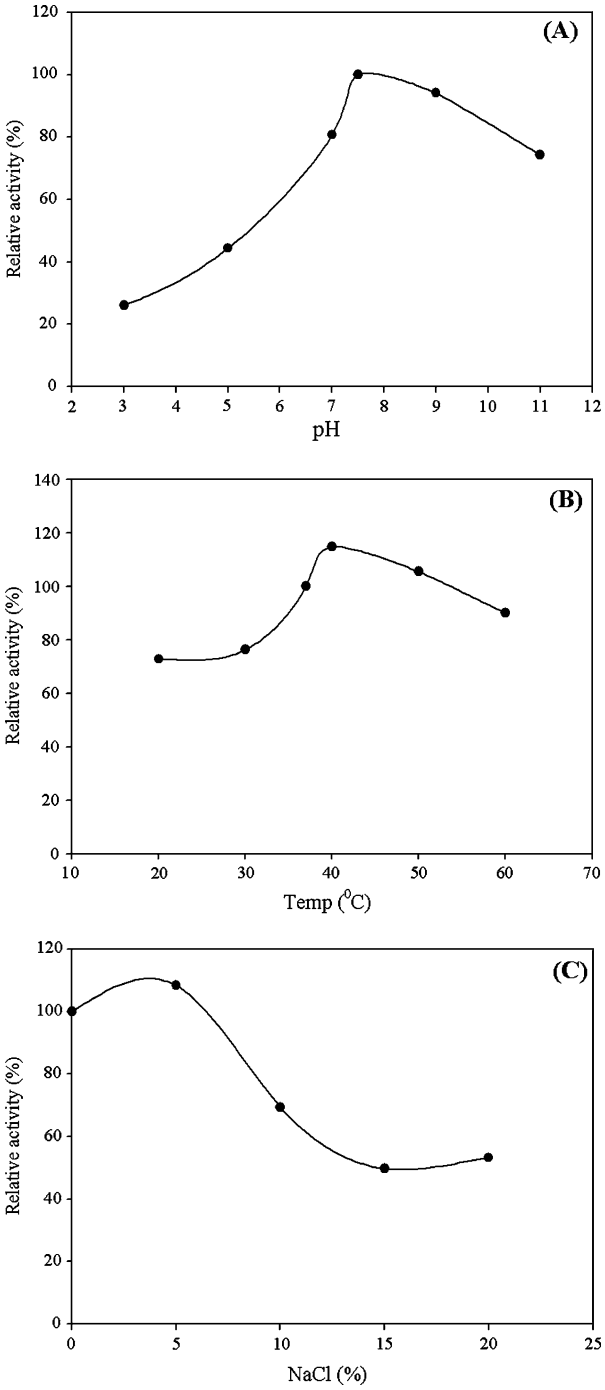

Fig. 3 Effect of pH, temperature, and salt concentrations on glutaminase activity of *L. reuteri* KCTC3594. Each experimental data point is the average of two determinations, and the activities are expressed as relative values observed in various ranges as follows: **A** pH 3–11; **B** 20–60 °C (temperature); **C** 0–20% NaCl (*w*/*v*)

(Fig. 3b). The activity was assayed in a reaction mixture of 0–20% (*w*/*v*) NaCl to determine the salt dependence of glutaminase activity. *L. reuteri* KCTC3594 was shown to be salt-tolerant with 110% relative activity under 5% NaCl and over 50% with presence of 20% NaCl (Fig. 3c).

Previous studies on glutaminases taken from *Micrococcus luteus* and *Stenotrophomonas maltophilia* reported a decrease in the activity of glutaminase with increasing salt concentration [15, 16]. However, regarding the glutaminase of *Lactobacillus rhamnosus*, the relative activity was 1.8 times higher under presence of 2.5% NaCl, exhibiting higher activity than the control without salt [8]. The *L. reuteri* KCTC3594 used in this study also showed similar osmotolerance as in *L. rhamnosus*. The above results suggest that glutaminase from lactobacilli can be more salt tolerant than other species that have been reported so far. In addition, the enzymes from *L. rhamnosus* [8] and *L. reuteri* KCTC3594 used in this study were also stable under wide ranges of pH and temperature (Fig. 3). The glutaminase activity was detected at a pH range between 5.0 and 11.0, which is broader than the range from 7.5 to 9 shown by *Debaryomyces* spp. [10] and the range from 6.0 to 9.0 by *L. rhamnosus* [8]. Therefore, glutaminase can increase the amount of glutamic acid through hydrolysis of glutamine under physical stress-reinforced conditions such as elevated salt concentration or temperature.

Inhibition of Enzyme

To test the effect of inhibitors on the glutaminase activity, two inhibitors were added to the enzyme assay reaction mixture at a final concentration of 10 and 50 mM for DON and iodoacetate, respectively. DON and iodoacetate showed approximately 80% enzyme inhibition at each concentration (Table 2). As a result of our experiment on the inhibition assay of glutaminase using DON and iodoacetate, both inhibitors inhibited the enzyme more strongly with a progress of separation steps than that of crude extract. DON and iodoacetate inhibited 75% activity of glutaminase from (microorganism) at even low concentrations of 0.1 and 5 mM, respectively [10] indicating the purified enzyme showed typical enzymatic properties of glutaminase. In *L. reuteri* KCTC3594; however, the inhibition concentrations were relatively higher than in *Debaryomyces* spp. showing 80% enzyme inhibition at the concentrations of 10 and 50 mM for DON and iodoacetate, respectively (Table 2).

In summary, many species of glutaminase have been isolated and characterized from microbial and mammalian origins; however, this is the first report for *Lactobacillus* spp. This paper shows the possibility of partial purification of glutaminase from lactobacilli.

Table 2 Effects of inhibitors on glutaminase activity at each purification step.

Purification step	Substance (mM) (DON)	Relative activity (%)	Substance (mM; iodoacetate)	Relative activity (%)
Control[a]	None	100	None	100
Cell-free extract		45		58
Protamine sulfate treatment	DON (10 mM)	34.1	Iodoacetate (50 mM)	44.3
Weak anion exchange		24		36
Gel filtration		15.5		22

[a] Control means the sample not treated with any inhibitor at each purification step

Therefore, we will continue to conduct experiments for complete purification of glutaminase because purification of glutaminase may affect not only the natural flavor market but also the pharmaceutical industry. In addition, it is also necessary to establish a detailed scale-up fermentation and processing procedure for the purpose of mass production of glutaminase.

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References

1. Farombi, E. O., & Onyema, O. O. (2006). *Human and Experimental Toxicology*, *25*, 251–259.
2. Tiziana, P., Sabrina, M., Simone, T., & Lanfranco, S. C. (2007). *Food Chemistry*, *104*, 1712–1717.
3. Nandakumar, R., Kazuaki, Y., Mamoru, W., & Mitsuaki, M. (2003). *Journal of Molecular Catalysis B*, *23*, 87–100.
4. Masuo, N., Yoshimune, K., Ito, K., Matsushima, K., Koyama, Y., & Moriguchi, M. (2005). *Journal of Bioscience and Bioengineering*, *100*, 576–578.
5. Davidson, L., Brear, D. R., Wingard, P., Hawkins, J., & Kitto, G. B. (1977). *Journal of Bacteriology*, *3*, 1379–1386.
6. Klein, M., Kaltwasser, H., & Jahns, T. (2002). *FEMS Microbiology Letters*, *206*, 63–67.
7. Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W. M., et al. (1998). *International Journal of Food Microbiology*, *44*, 93–106.
8. Weingand-Ziadé, A., Christiane, G. D., & Michael, A. (2003). *Enzyme and Microbial Technology*, *32*, 862–867.
9. Shornikova, A. V., Casas, I. A., Isolauri, E., Mykkanen, H., & Vesikari, T. (1997). *Journal of Pediatric Gastroenterology and Nutrition*, *24*, 399–404.
10. Durà, M. A., Flores, M., & Toldra, F. (2002). *International Journal of Food Microbiology*, *76*, 117–126.
11. Bradford, M. M. (1976). *Analytical Biochemistry*, *72*, 248–254.
12. Laemmli, U. K. (1970). *Nature*, *227*, 680–685.
13. Heini, H. G., Gebhardt, R., Brecht, A., & Mecke, D. (1987). *European Journal of Biochemistry*, *162*, 541–546.
14. Sabu, A., Nampoothiri, K. M., & Pandey, A. (2005). *Humana Press Inc., Totowa, NJ*, *17*, 75–90.
15. Yoshimune, K., Yamashita, R., Masuo, N., Wakayama, M., & Moriguchi, M. (2004). *Extremophiles*, *8*, 441–446.
16. Wakayama, M., Yamaqata, T., Kameumura, A., Bootim, N., Yano, S., Tachiki, T., et al. (2005). *Journal of Industrial Microbiology & Biotechnology*, *32*, 383–390.