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Research Article

Effect of Organic Nitrogen Source on Angiotensin I Converting Enzyme (ACE) Inhibitory Peptides Fermented by *Lactobacillus bulgaricus* LB6 from Goat Milk

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Abstract: Angiotensin I Converting Enzyme (ACE) plays an important physiological role in the regulation of hypertension. ACE inhibitors can lower hypertension. Lactic acid bacteria are known to produce ACE inhibitors during fermentation. Effect of fermentation time and organic nitrogen source including whey powder, casein hydrolyses, casein peptone, soybean peptone and casein on ACE inhibitory peptides fermented from goat milk by *Lactobacillus bulgaricus* LB6 was investigated using single factor test. The adding of whey powder and casein hydrolyses were both 0.50, 0.60, 0.70, 0.80 and 0.90%, casein peptone and soybean peptone were both 0.10, 0.30, 0.50, 0.70 and 0.90%, casein was 0.10, 0.20, 0.30, 0.40 and 0.50%, respectively. The results were as follows: The optimal fermentation time for ACE inhibitory peptide was 12 h, whey powder, casein peptone, soybean peptone and casein could promote ACE inhibition significantly increase ($p<0.05$) and optimal concentration was 0.70, 0.90, 0.30 and 0.20%, respectively. Casein hydrolyses could promote growth of *L. bulgaricus* LB6, but inhibit the production of ACE inhibitory peptide.

Keywords: ACE inhibitory peptide, casein hydrolyses organic nitrogen source, casein peptone, goat milk, *Lactobacillus bulgaricus*, soybean peptone, whey powder

INTRODUCTION

Hypertension defined as high systolic and diastolic blood pressures is a major chronic disease, which leads to stroke, coronary heart disease, kidney dysfunction, disability and death (López-Fandiño *et al*., 2006). The angiotensin converting enzyme (ACE, EC. 3.4.15.1) plays an important physiological role in regulating blood pressure and raise blood pressure by converting angiotensin-I to the potent vasoconstrictor angiotensin-II (Leclerc *et al*., 2002; Ondetti *et al*., 1977; Skeggs *et al*., 1956). Therefore, inhibition of ACE activity is considered to be a useful therapeutic approach in the treatment of hypertension. Although synthetic ACE inhibitors are effective as antihypertensive drugs, they have certain side effects. In this respect, functional foods with blood pressure-lowering properties have recently received considerable attention (Mohamed *et al*., 2010; Roy *et al*., 2010).

Recently, certain functional foods containing ACE inhibitory peptides have been shown to act as an additional or alternative treatment in hypertension. Fermented milks containing many ACE-inhibitory and antihypertensive peptides have been produced using different lactic acid bacteria, such as *Lactobacillus helveticus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. lactis* ssp. lactis, *L. lactis* ssp. Cremoris and *Enterococcus faecalis* (Algaron *et al*., 2004; Ashar and Chand, 2003; Fuglsang *et al*., 2003b; Gobbetti *et al*., 2000; Herna´ndez-Ledesma *et al*., 2004; Leclerc *et al*., 2002; Muguerza *et al*., 2006; Nakamura *et al*., 1995; Quiro´ s *et al*., 2007; Robert *et al*., 2004; Rodríguez-Figueroa *et al*., 2010; Rokka *et al*., 1997; Shuangquan and Taku, 2008; Vermeirssen *et al*., 2003; Yamamoto *et al*., 1994a, b, 1999). In our previous study, 28 probiotic Lactobacillus strains were used to ferment goat milk to screen for production ACE-inhibitory peptides, the results showed that 20 strains had ACE inhibitory activity and among them of 4 strains including *Lactobacillus reuteri*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus* and *Lactobacillus helveticus* were especially significant as producers of ACE-inhibitory peptides (He *et al*., 2012). In this study, Effect of five kinds of organic nitrogen source including whey powder, casein hydrolyses, casein peptone, soybean peptone and casein on ACE inhibitory peptides fermented from goat milk by *Lactobacillus bulgaricus* LB6 was investigated to obtain the optimum adding of nitrogen source and provided reference for further optimization.

MATERIALS AND METHODS

Materials and reagents: Whole goat milk powder was purchased from a milk shop (Shaanxi Redstar Dairy Co.,

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Ltd., Weinan, China). Hippuryl-histidyl-leucine (Hip-His-Leu) and ACE (extracted from rabbit lung acetone powder) were bought from Sigma Chemical Co. (St Louis, MO, USA), whey powder, casein hydrolyses, casein peptone, soybean peptone and casein were purchased from Xi'an Luosenbo Technology Co., Ltd. (Xi'an, China), All chemicals used were of analytical grade unless otherwise specified.

Microorganisms and their activation: Pure cultures of *Lactobacillus bulgaricus* LB6 was obtained from the College of Life Science and Engineering, Shaanxi University of Science and Technology. Stock cultures were stored at -20°C in freeze-dried powder. The microorganism were activated successively three times in rehydrated de Mann Rogosa Sharpe (MRS) broth (Haibo media, Qindao, China) at 37°C for 24 h prior to use.

Preparation of fermented goat milk: Reconstituted skim goat milk was pasteurized, inoculated with the starter culture containing *Lactobacillus bulgaricus* LB6 and fermented at 37°C until coagulated. The viable counts of *L. bulgaricus* LB6 in the fermented milk was counted using de Man, Rogosa, Sharpe (MRS) agar (Haibo media, Qindao, China).

Measurement of ACE inhibitory activity: The whey fraction from the fermented milk was used for testing the ACE inhibitory effect. Aliquots of the fermented milk were collected, vigorously stirred and centrifuged at 1000×g for 20 min to obtain the corresponding whey fractions. The supernatants collected were filtered through a Xinhua filter and used to determine their ACE inhibitory activity. ACE inhibitory activity was measured by a spectrophotometric assay according to the method of Cushman and Cheung (1971) with some modifications. Added 80 μL of each sample to 200 μL sodium borate buffer (0.1 mol/L, pH 8.3) containing NaCl (0.30 mol/L) and HHL (5 mmol/L). Then, ACE (20 μL, 0.1 U/mL) was added and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 250 μL 1 mol/L HCl. Adding 1.7 mL ethyl acetate to extract the hippuric acid formed and evaporated at 120°C for 30 min, redissolved in 2 mL deionized water after cooled at room temperature, then the absorbance was measured at an optical density of 228 nm. The activity of each sample was tested in triplicate and done averaging. The ACE inhibitory rate was calculated using the following equation: ACE inhibition (%) = (A - B) / (A - C) ×100%, where A is the optical density without the whey fraction, B is the optical density without ACE and C is the optical density in the presence of both ACE and the whey fraction.

Measurement of viable cell counts, pH and titration acidity: Serial dilutions of the fermented goat milk samples made in saline water (0.9%, w/v, NaCl) containing 0.1 g/L peptone were spread onto MRS agar plates and incubated for 48 h at 37°C. All dilutions were plated in triplicate. Enumeration was performed by manual counting, whenever possible the mean numbers from two different dilutions were used and results were expressed as colony forming units per milliliter (CFU/mL) of fermented milk (He *et al*., 2013). The pH in fermented goat milk was directly evaluated through a pH-meter (pHS-3C) at the room temperature and titration acidity was determined according to the sodium hydroxide titration method and Jill Nieer degrees (°T) described, respectively.

RESULTS AND DISCUSSION

Effect of incubation time on ACE inhibitory peptides fermentated by *L. bulgaricus* LB6 from goat milk: The activated *Lactobacillus bulgaricus* LB6 at inoculum size 5% was transferred into pasteurized reconstituted goat milk in anaerobic tube and cultured at 37°C for 24 h. The samples were taken out for determining pH

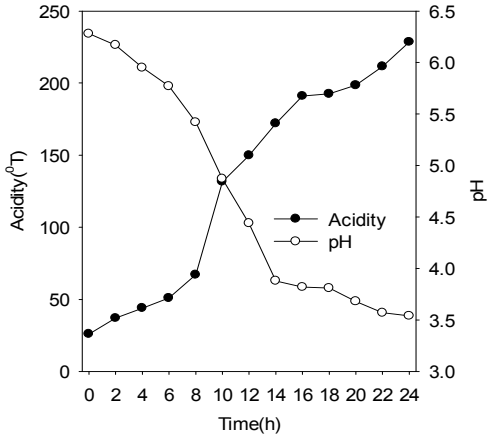

Fig. 1: Effect of incubation time on acidity and pH in fermented goat milk

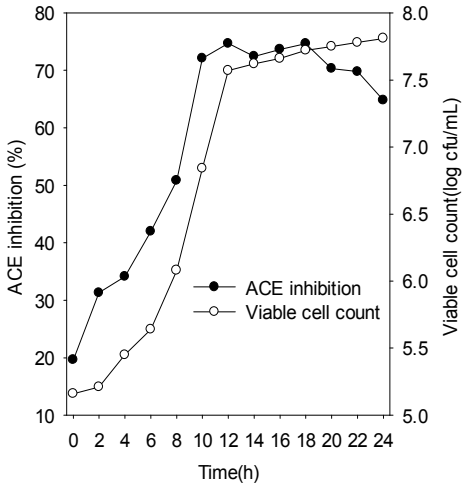

Fig. 2: Effect of incubation time on ACE inhibition and viable counts in fermented goat milk

value, titration acidity, ACE inhibition and viable count every 2 h. The results were shown in Fig. 1 and 2.

The growth curve of *L. bulgaricus* LB6 showed as "S" type, 0-4 h was a period of adjustment, the viable counts of *L. bulgaricus* LB6 slowly increased, pH value decreased and the titration acidity increased slowly; 4-12 h was logarithmic phase for cell growth of *L. bulgaricus* LB6, the numbers of viable counts increased rapidly, changes of pH and acidity is large, when the pH value decreased to 4.7-5.0, the fermented milk began to appear curd; then *L. bulgaricus* LB6 grew into the stable phase from 12 h. The viable count remained almost unchanged because the growth rate and death rate of *L. bulgaricus* LB6 is basically the same, the value of pH continues to decline and titration acidity continued to increase, but the trend tended to be smooth.

During the 24 h fermentation, the ACE inhibition in fermented goat milk increased rapidly in 0-12 h, tended to be smooth in 12-18 h, then decreased gradually from the beginning of 18 h, which was consistent with the growth regularity of *L. bulgaricus* LB6. The ACE inhibition increased with the increase in the number of live bacteria in the first 12 h when *L. bulgaricus* LB6 was in logarithmic phase, the possible reasons was that the ACE inhibitory peptide content reached the maximum for the hydrolysis of goat milk protein by proteases or peptidase produced by *L. bulgaricus* LB6, but the proteases or peptidase was inhibit or the ACE inhibitory peptide was break down when the titration acidity increased after 12 h, which lead to ACE inhibition decreased.

The ACE inhibition in fermented goat milk reached to maximum (74.70%) at 12 h and the viable count, pH and titration acidity was 3.72×10^7 CFU/mL, 4.44 and 149.8°T, Therefore, the 12 h is chosen as the fermentation time for further research on ACE inhibitory peptide produced by *L. bulgaricus* LB6.

Effect of whey powder on ACE inhibitory peptides fermentated by *L. bulgaricus* LB6 from goat milk: The whey powder was added to pasteurized reconstituted goat milk and the concentration were 0.50, 0.60, 0.70, 0.80 and 0.90%, respectively. The inoculum size was 5% and cultured at 37°C for 12 h. The samples were taken out for determining pH value, titration acidity, ACE inhibition and viable count. The results were shown in Fig. 3 and 4.

As shown in Fig. 3, the viable counts and ACE inhibition in fermented goat milk first increased and then decreased with the concentration of whey powder increasing, the viable counts reached maximum value (5.01×10^8 CFU/mL) at whey powder 0.80%, but ACE inhibition reached maximum value (84.42%) at whey powder 0.70%. The ACE inhibition gradually decreased in the cow milk fermented by *Lactobacillus casei* with the concentration of whey powder increasing (Jiang *et al*., 2011), which may be because the structure and content of whey protein in bovine and goat milk is

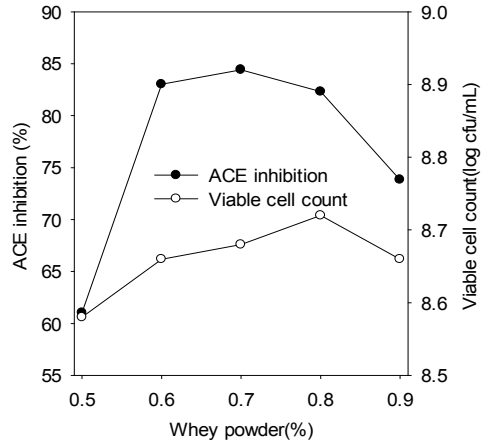

Fig. 3: Effect of whey powder on ACE inhibitory rate and viable cell count in fermented goat milk

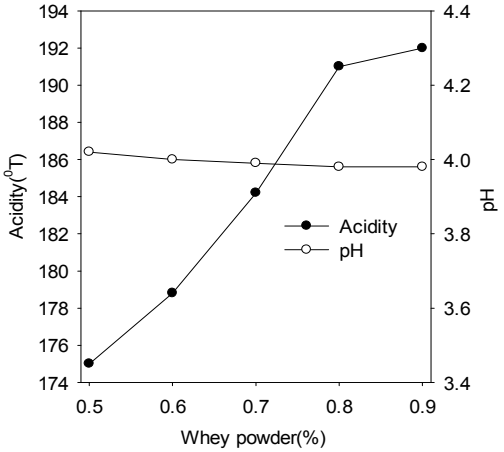

Fig. 4: Effect of whey powder on acidity and pH in fermented goat milk

different. As shown in Fig. 4, the pH decreased and titration acidity increased with the increase of the concentration of whey powder. The titration acidity gradually increased from 175°T at whey powder 0.50% to 192°T at whey powder 0.90%, but the pH variation had no significant difference ($p>0.05$), which showed that goat milk has a good buffer capacity.

Effect of casein hydrolyses on ACE inhibitory peptides fermentated by *L. bulgaricus* LB6 from goat milk: The casein hydrolyses were added to pasteurized reconstituted goat milk and the concentration were 0.50, 0.60, 0.70, 0.80 and 0.90%, respectively. The results were shown in Fig. 5 and 6.

The viable counts of *L. bulgaricus* LB6 and ACE inhibition showed opposite changes with the increase of casein hydrolyses concentration from Fig. 5, The viable counts of *L. bulgaricus* LB6 increased from 3.63×10^8 CFU/mL at casein hydrolyses 0.5% to 8.13×10^8 CFU/mL at casein hydrolyses 0.8%, while ACE

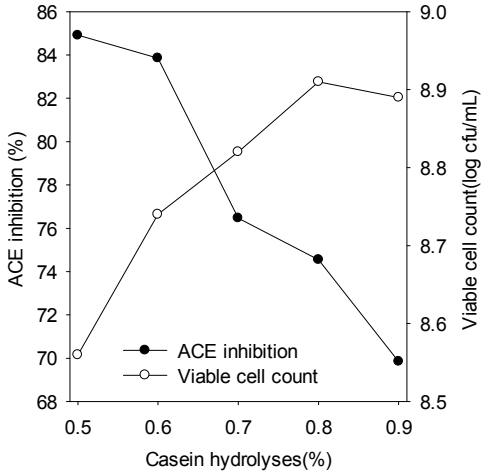

Fig. 5: Effect of casein hydrolyses on ACE inhibitory rate and viable cell count in fermented goat milk

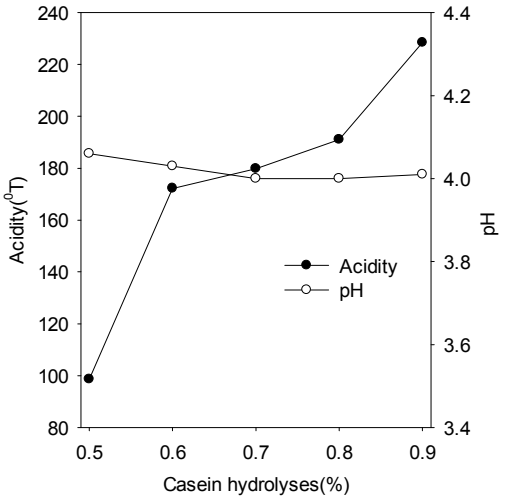

Fig. 6: Effect of casein hydrolyses on acidity and pH in fermented goat milk

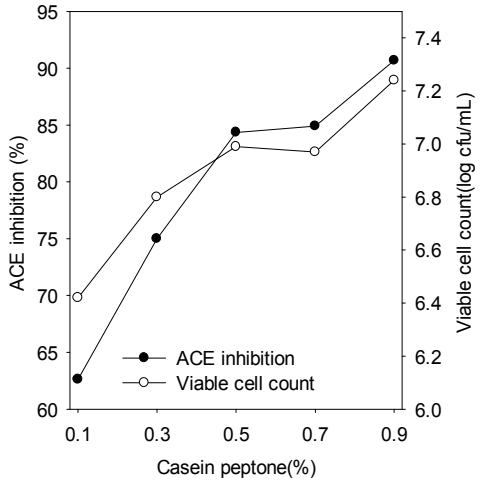

Fig. 7: Effect of casein peptone on ACE inhibitory rate and viable count

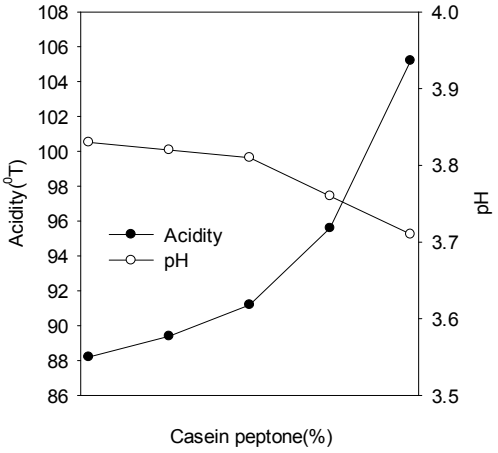

Fig. 8: Effect of casein peptone on acidity and pH in fermented goat milk

inhibition decreased from 84.91% at casein hydrolyses 0.5% to 69.85 at casein hydrolyses 0.9%, which indicated that addition of casein hydrolyses could promote growth of *L. bulgaricus* LB6, but inhibit the production of ACE inhibitory peptide. The decline reason of ACE inhibition may be due to some components in casein hydrolyses was the product of hydrolysis of protease produced by *L. bulgaricus* LB6, which increased product concentration and generated feedback inhibition for enzyme activity, thereby led to weaken the hydrolysis. With casein hydrolyses increasing, the titration acidity in fermented goat milk increased significantly ($p<0.05$) while the pH value had no significant change ($p>0.05$), which showed that goat milk has a good buffer capacity.

Effect of casein peptone on ACE inhibitory peptides fermentated by *L. bulgaricus* LB6 from goat milk: The casein peptone was added to pasteurize reconstituted goat milk and the concentrations were 0.10, 0.30, 0.50, 0.70 and 0.90%, respectively. The results were shown in Fig. 7 and 8.

The viable counts of *L. bulgaricus* LB6 and ACE inhibition both increased with the concentration of casein peptone increasing from Fig. 7, the viable counts increased from 2.63×10^6 CFU/mL at casein hydrolyses 0.10% to 1.74×10^7 CFU/mL at casein hydrolyses 0.5% and ACE inhibition increased from 62.62% at casein hydrolyses 0.10% to 90.68 at casein hydrolyses 0.5%, the trend between growth trend of *Lactobacillus bulgaricus* LB6 and ACE inhibition are similar, which indicated that addition of casein peptone could promote growth of *L. bulgaricus* LB6 and the production of ACE inhibitory peptide. With casein peptone increasing, the titration acidity in fermented goat milk increased significantly ($p<0.05$) while the pH value had no significant change ($p>0.05$) from Fig. 8, the titration acidity increased from 88.20 to 105.20 and pH from 3.83 to 3.71.

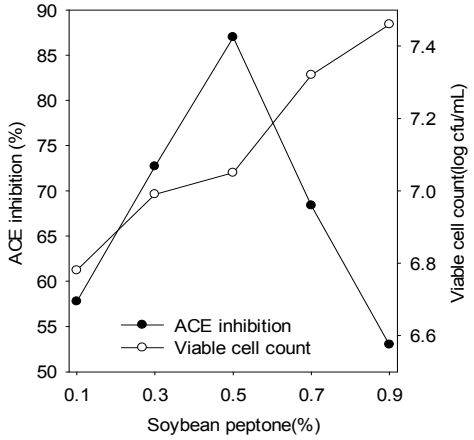

Fig. 9: Effect of soy peptone on ACE inhibitory rate and viable count

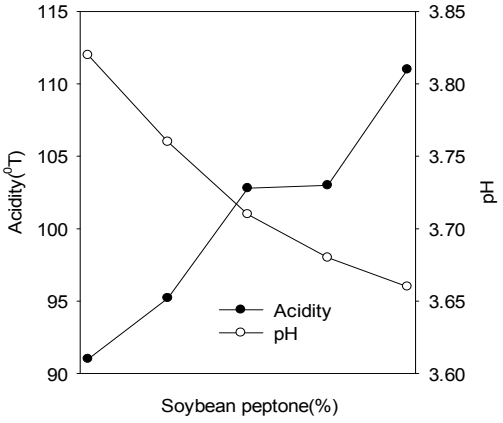

Fig. 10: Effect of soy peptone on acidity and pH in fermented goat milk

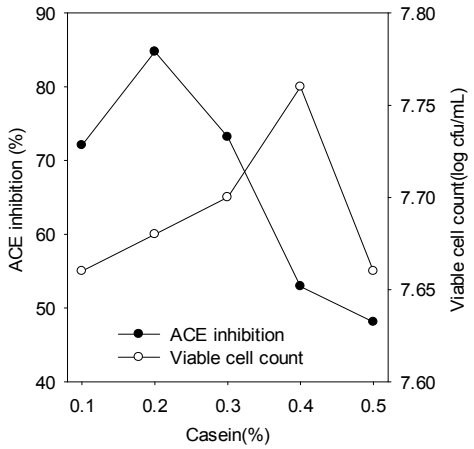

Fig. 11: Effect of casein on ACE inhibitory rate and viable count

Effect of soybean peptone on ACE inhibitory peptides fermentated by *L. bulgaricus* LB6 from goat milk: The soybean peptone was added to pasteurized reconstituted goat milk and the concentrations were 0.10, 0.30, 0.50, 0.70 and 0.90%, respectively. The results were shown in Fig. 9 and 10.

The addition of soybean peptone could promote growth of *L. bulgaricus* LB6 from Fig. 9. The viable counts of *L. bulgaricus* LB6 increased from 6.03×10^6 CFU/mL at soybean peptone 0.10% to 2.88×10^7 CFU/mL at soybean peptone 0.90%, while ACE inhibition first increased from 57.77% at soybean peptone 0.10 to 86.98% at soybean peptone 0.50% and then decreased to 53.00% at soybean peptone 0.90% with the concentration of soybean peptone increasing. The reasons for this trend may be that adding of soy peptone at 0.10-0.50% increased the content of total protein in goat milk and the protein content which can be hydrolyzed into ACE inhibitory peptides, therefore the ACE inhibition increased in a certain range. With soy peptone concentration continued to increase from 0.50 to 0.90%, the ACE inhibition decreased, which may be due to by incomplete enzymatic hydrolysis for total protein in goat milk was too much. With soybean peptone increasing, the titration acidity in fermented goat milk increased significantly ($p<0.05$) while the pH value decreased from Fig. 10, the titration acidity increased from 91.00 to 111.00 and pH from 3.82 to 3.66, which indicated adding of soybean peptone promote acid-producing by *L. bulgaricus* LB6.

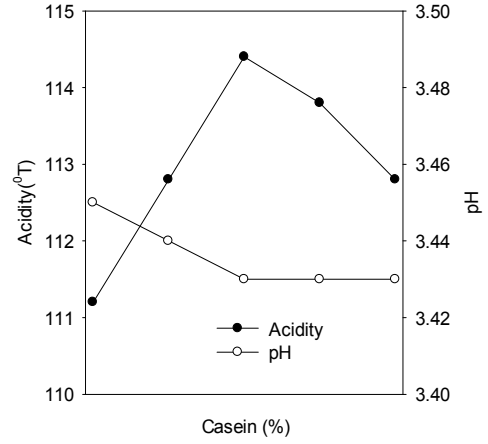

Fig. 12: Effect of casein on acidity and pH in fermented goat milk

Effect of casein on ACE inhibitory peptides fermentated by *L. bulgaricus* LB6 from goat milk: The results were shown in Fig. 9 and 10. The casein was added to pasteurized reconstituted goat milk and the concentrations were 0.10, 0.20, 0.30, 0.40 and 0.50%, respectively. The results were shown in Fig. 11 and 12.

The viable counts of *L. bulgaricus* LB6, ACE inhibition and titration acidity all first increased and then decreased with the concentration of soybean peptone increasing. The viable counts increased from

4.57×10^7 CFU/mL at casein 0.10% to 5.75×10^7 CFU/mL at casein 0.40% and decreased to 4.55×10^7 CFU/mL at casein 0.50%. The ACE inhibition increased from 72.06% at casein 0.10% to 84.75 at casein 0.20% and decreased to 48.10 at casein 0.50%. The titration acidity increased from 11.20 at casein 0.10% to 114.40 at casein 0.30% and decreased to 112.80 at casein 0.50%. The pH value in fermented goat milk changed not obviously from Fig. 12.

CONCLUSION

The optimal fermentation time for ACE inhibitory peptide was 12 h, four kinds of organic nitrogen source including whey powder, casein peptone, soybean peptone and casein could promote ACE inhibition significantly increase ($p<0.05$) and optimal concentration was 0.70, 0.90, 0.30 and 0.20%, respectively. Casein hydrolyses could promote growth of *L. bulgaricus* LB6, but inhibit the production of ACE inhibitory peptide.

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