sciendo

10.2478/aucft-2019-0003

OPTIMIZATION OF FERMENTATION CONDITIONS FOR THE PRODUCTION OF ANGIOTENSIN-CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES FROM COW MILK BY *LACTOBACILLUS BULGARICUS* LB6

– Research paper –

He CHEN[*], Lin MA[*], Jinna QI[*], Jili CAO[1**], Yuan TAN[**]

**School of Food and Biological Engineering, Shaanxi University of Science & Technology, Xi'an, 710021, China; ** Xi'an Oriental Dairy Co., Ltd., Xi'an, Shaanxi 710027, China*

Abstract: The purpose of this research was to screen out the optimal -producing peptide conditions for cow milk fermented by *Lactobacillus bulgaricus* LB6. The effects of temperature, inoculation size, time and skim milk concentration on the ACE inhibition rate of fermented milk were investigated by single factor experiment, and the optimal fermentation conditions were determined by orthogonal experiment. The conditions of the single factor experiment were: Temperatures were 37° C, 39° C, 42° C, 44° C and 46° C. The inoculation amount was 1%, 3%, 5%, 7% and 9%, the time was 8h and 10h. At 12h, 14h and 16h, the concentration of skim milk was 8%, 10%, 12%, 14% and 16%, respectively. The results showed that the optimal fermentation conditions for ACE inhibitory peptide produced by *Lactobacillus bulgaricus* LB6 were 4% inoculation, 13h in time, 42°C in temperature and 13% in skim milk. Under this condition, the ACE inhibition rate reached 76.50% and the OD value was 0.330. The titration acidity was 116.4°T, the pH was 4.62, and the sensory evaluation was 75 scores.

Keywords: ACE inhibitory peptide, *Lactobacillus bulgaricus,* fermentation, Skimmed milk powder

INTRODUCTION

Hypertension is a clinical syndrome characterized by elevated arterial systolic or diastolic blood pressure, often accompanied by cardiovascular disease, which poses a serious threat to human health (López-Fandiño et al., 2006; Rey et al., 2014). At present, most drugs for treating hypertension are chemically synthesized, and long-term use may cause harm to other functions of the human body (Assimes et al., 2010; Liou et al., 2008). Studies find that angiotensin-converting enzyme (ACE) inhibitory peptide has a blood pressure lowering function, and taking a food containing the peptide does not affect normal blood pressure of a person or an animal, and has high safety and small side effects (Bao et al., 2016 ;Mohamed et al., 2010; Roy et al., 2010; Du et al., 2013).

In recent years, food-borne antihypertensive peptides have attracted much attention. People with hypertension benefit from eating foods containing ACE- inhibitory peptide. There are already many lactic acid bacteria capable of producing ACE inhibitory peptides in fermented milk, such as *Lactobacillus plantarum*, *Lactobacillus helveticus*, *L. lactis* ssp. *lactis*, *L. casei*, *L. lactis* ssp. *cremoris and L. acidophilus*. (Ahtesh et al., 2017;Algaron et al., 2004; Chen et al., 2018; Elvira et al., 2010 Georgalaki et al., 2017; Jiang et al., 2011; Nakamura et al., 1995; Nielsen et al., 2009; Pihlanto et al., 2010; Pritchard et al., 2018; Quiro´ s et al., 2007; Robert et al., 2004; Rodríguez-Figueroa et al., 2010; Shuangquan et al., 2008;Yamamoto et al., 1994a, b, 1999; Yanli et al., 2016).

Previous studies have found that 20 strains can produce ACE inhibitory peptides in fermented goat milk, mainly including *Lactobacillus reuteri* LT33, *Lactobacillus bulgaricus* L6, *Lactobacillus rhamnosus* LR22 *and Lactobacillus helveticus* LH69 (He et al., 2012,2013). In this study, the effects of culture temperature, culture time, inoculation size and reconstituted milk concentration on the ACE inhibition rate of fermented milk were investigated by single factor test. The effects of OD, acidity and pH on fermented milk were also investigated. The main factors affecting the screening were tested, and the optimal fermentation conditions were determined by verification, which laid a foundation for subsequent experiments.

[1] Corresponding author. E-Mail address: xiandfcjl@gmail.com

MATERIALS AND METHOD

Materials: Skim milk powder was purchased from New Zealand (Anchor, New Zealand). Hippuryl-histidyl-leucine (Hip-His-Leu) and ACE were bought from Sigma Chemical Co. (St Louis, MO, USA),.MRA liquid medium was purchased from Beijing Aoboxing Biotechnology Co., Ltd.(Beijing China). Unless otherwise stated, all chemicals agents used are analytical grade.

Microorganisms and their activation: A small amount of *L. bulgaricus* LB6 freeze-dried powder was inoculated into MRS liquid medium under aseptic processing conditions, mixed and cultured at 37°C for 18-24 h, according to 5% inoculation amount, continuous transfer activation 3 times, The activated bacteria were transferred to 14% sterilized reconstituted milk by 5% inoculation, cultured at 37 °C for 12 h, and continuously transferred twice to prepare a starter.

Preparation of fermented milk: After the skim milk powder was prepared to a concentration of 14%, the *L. bulgaricus* LB6 was inoculated at a rate of 5%, and the OD value, acidity, and pH were measured after incubation at 42 °C for 12 hours.

Measurement of ACE inhibitory activity: Aliquots of fermented milk were collected, vigorously stirred and centrifuged at 1000 × g for 20 minutes to obtain the corresponding whey, and the collected supernatant was passed through a filter to determine its ACE inhibitory activity, respectively. The method of determination is based on previous research (Cushman et al.,1971; Guowei et al., 2013)
The ACE inhibitory rate was calculated using the formula (1):

$$ACE \text{ inhibition } (\%) = \frac{(OD_A - OD_B)}{(OD_A - OD_C)} \times 100\% \quad (1)$$

In the formula (1), OD_A represents the optical density without the whey fraction, OD_B represents the optical density without ACE, and OD_C represents the optical density in the presence of ACE and whey fraction.

Determination of OD value: The 1 mL of fermented milk and 9 ml of 0.2% EDTA were added to the test tube, and the pH was made alkaline (pH 11-12) with sodium hydroxide, magnetic stirring evenly, and then allowed to stand for 5 minutes. The unfermented blank milk sample was used as a reference, the absorbance was measured at 640 nm, and the biomass of the lactic acid bacteria was evaluated based on the absorbance.

pH determination: Direct measurement at room temperature using a pHS-3C acidity meter.

Acidity determination: The sodium hydroxide titration method is expressed in terms of Jill Nieer degrees (°T).

Sensory evaluation: Tasting by at least five tasters, the fermented milk samples were evaluated in terms of color, smell, taste and tissue state. The distribution of specific sensory project weights is shown in Table 1 (China Standard, 2000).

Table.1 Fermented milk sensory evaluation standard

project	Color(10 scores)	Odor(30 scores)	Taste(30 scores)	Organizational status(30 scores)
Poor	Color anomaly (0-2.5 scores)	Almost no aroma(0-7.5 scores)	Mildew and astringency (0-7.5 scores)	Poor curd, serious whey precipitation (0-7.5 scores)
medium	Uneven color (2.5-5.0 scores)	Lighter aroma(7.5-15.0 scores)	Sour is too thick or too light, slightly astringent (7.5-15.0 scores)	Uneven milk, whey precipitation (7.5-15.0 scores)
good	Uniform color, white or yellowish (5.0-7.5 scores)	Have a pure yogurt flavor (15.0-22.5 scores)	Moderate sour, slightly astringent (15.0-22.5 scores)	Better solidification a small amount of whey precipitated (15.0-22.5 scores)
excellent	Uniform color, milky white (7.5-10.0 scores)	Pure and yoghurt flavor(22.5-30.0 scores)	Moderate sour, no astringency, no astringency (22.5-30.0 scores)	Fine texture, very good coagulation, no whey (22.5-30.0 scores)

RESULTS AND DISCUSSION

Effect of temperature on ACE inhibition rate of *L. bulgaricus* LB6 fermented milk

L. bulgaricus LB6 was placed in the basic fermentation medium according to the inoculation amount of 5%, and then cultured at 37°C, 39°C, 42°C, 44°C, 46°C for 12 h, and then taken out to determine the ACE inhibition rate and OD of the fermented milk. Value, titration acidity, pH value, the results are shown in Figures 1 and 2.

As shown in Figure 1, as the temperature increases, both the ACE inhibition rate and the OD value increase first and then decrease. The peak OD value at 42°C indicates that the optimal growth temperature of *L. bulgaricus* LB6 in milk is 42°C. At 42°C, the ACE inhibition rate reached a peak of 63.1%. The increase or decrease in temperature caused a significant decrease in the inhibition rate, indicating that 42°C is the optimum temperature for ACE inhibitory peptide production by *L. bulgaricus* LB6. The reason for this phenomenon may be that temperature has an effect on the activity of proteolytic enzymes and the growth of bacteria. When the temperature is low, the activity of the enzyme is not strong, the metabolic activity of the cells is weak, and the growth of the cells is slow; when the temperature is slowly increased, the activity of the enzyme is enhanced, and the growth rate of the bacteria is accelerated; the temperature continues to rise when the enzyme is reached. At the optimum temperature for solution, the enzyme activity is the strongest and the degree of hydrolysis is the most sufficient; when the optimum temperature for cell growth is reached, the number of viable cells is the largest; but when the temperature exceeds the optimum enzymatic hydrolysis temperature and optimum growth temperature The various enzymes in *L. bulgaricus* LB6 are inhibited or even inactivated, and the hydrolysis of protease and the growth of bacteria are reduced.

As can be seen from Figure 2, the temperature has a great effect on the titration acidity and pH of the fermented milk. The titration acidity is from 47°T to 96°T, and the variation range is large, and the pH range (4.32-4.78) is also large. Starting from 42°C, the titration acidity and pH changes tend to be gentle. The sensory evaluation of the fermented milk was the highest at 39°C to 42°C, and the sensory evaluation of the fermented milk at 42°C was the highest. The reason for this phenomenon may be the time during which the flavoring substance in the fermented milk is formed during the process from 39°C to 42°C.

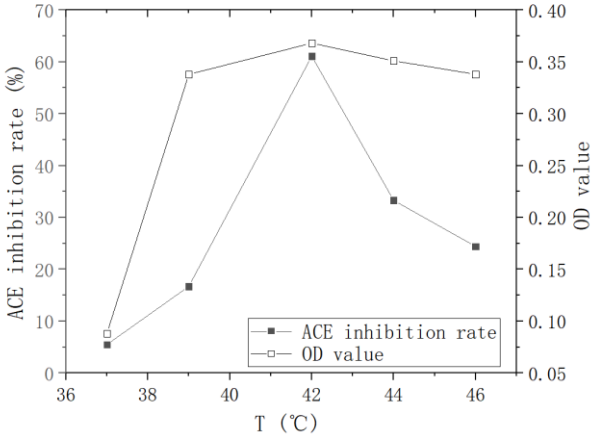

Figure1. Effect of temperature on ACE inhibition rate and OD value of fermented milk

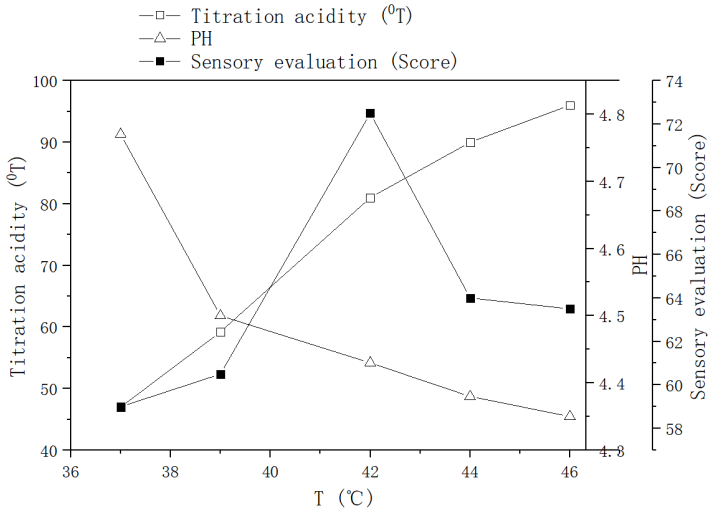

Figure 2. Effect of temperature on fermented acidity and pH and sensory evaluation

Effect of inoculation amount on ACE inhibition rate of *L. bulgaricus* LB6 fermented milk

L. bulgaricus LB6was inoculated into the basal medium at an inoculation amount of 1%, 3%, 5%, 7%, and 9%, and various indexes were measured. The results are shown in the Figures 3 and 4. As Figure 3 indicates, the ACE inhibition rate is the highest at 3% of the inoculation amount, and the OD value is the largest at 5%, indicating that the enzyme and the substrate reach the optimum ratio at the inoculation amount of 3%. With the increase of inoculation amount, the ACE inhibition rate and OD value of *L. bulgaricus* LB6 fermented milk gradually decrease. The reason for this

phenomenon may be that when the concentration of the substrate is constant, the substrate in the fermentation broth cannot meet the growth of *L. bulgaricus* LB6 as the inoculation amount increases, and some of the fermentation products are reused by *L. bulgaricus* LB6 to be decomposed into smaller ones. The peptides are even individual amino acids, resulting in a decrease in the content of small ACE inhibiting peptides in the fermentation broth.

As shown in the Figure 4, the titration acidity of the fermented milk increases with the increase of the inoculation amount, and the maximum titration acidity is obtained at the inoculation amount of 9%. pH is negatively correlated with the inoculation amount. At the 5% inoculation amount, the sensory evaluation score was the highest. The reason for this may be that under a certain substrate concentration, 5% inoculation amount is most suitable for *L. bulgaricus* LB6 metabolism, producing some flavor substances, so that the flavor of fermented milk is better.

Effect of time on ACE inhibition rate of *L. bulgaricus* LB6 fermented milk

They were taken out after fermentation at 8h, 10h, 12h, 14h and 16h, and the indexes were measured. The results are shown in Figures 5 and 6.

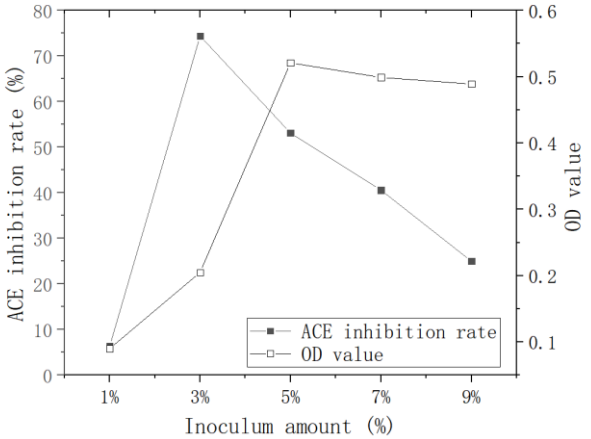

Figure3. Effect of inoculation amount on ACE inhibition rate and OD value of fermented milk

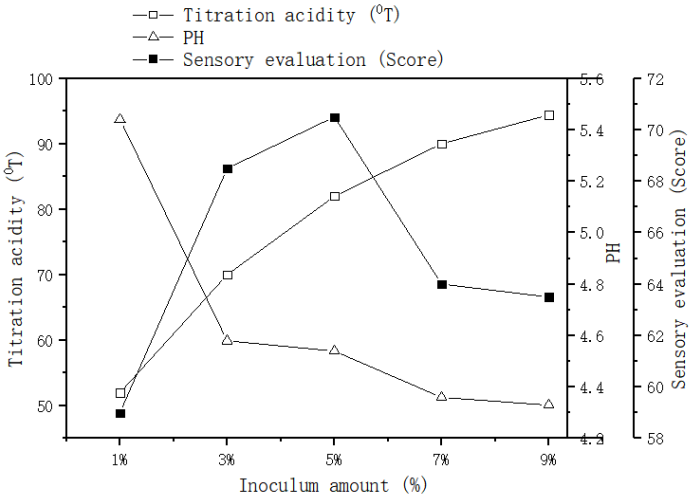

Figure 4. Effect of inoculation amount on fermentation acidity and pH value and sensory evaluation

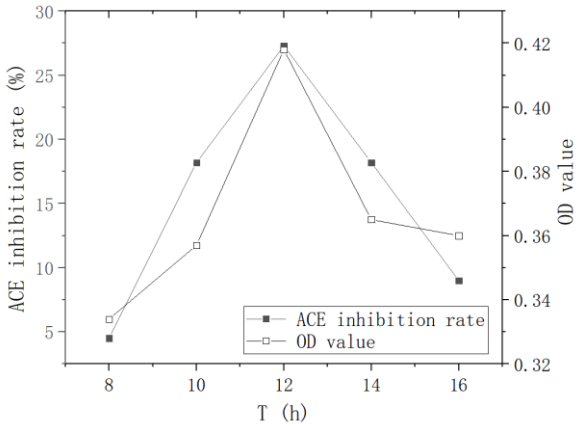

Figure 5. Effect of time on ACE inhibition rate and OD value of fermented milk

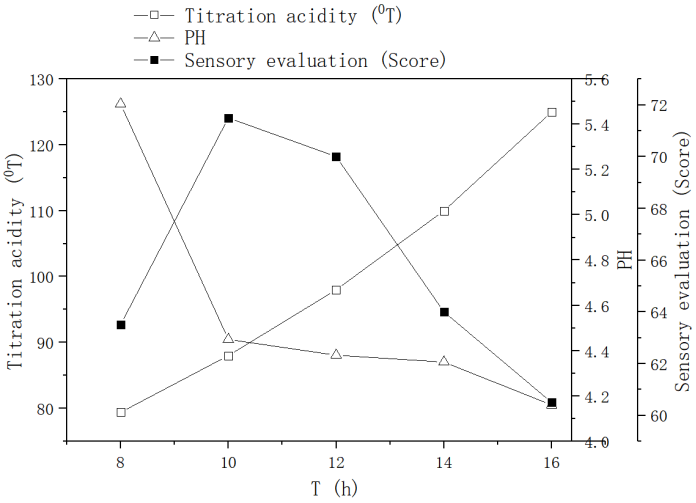

Figure 6. Effect of time on fermentation acidity and pH value and sensory evaluation

With the increase of fermentation time, the ACE inhibition rate and OD value both increased first and then decreased, reaching a maximum at 12h (Figure 5). The reason for this may be that as the fermentation time is prolonged, the substrate concentration is insufficient to supply all the nutrients needed for the growth of the strain. The strain grows slowly and the activity is reduced, so the ability to degrade the cow protein is reduced, so the

ACE is produced. The inhibitory peptide is reduced. The ACE inhibition rate of fermented milk reached the highest (26.97%) at 12h, at which time the OD value was 0.418, the fermented milk had a pH of 4.38, the acidity reached 84°T, and the acidity was strong.

It can be seen from the Figure 6 that when the fermentation time is 8-10h, the sensory evaluation of fermented milk has the highest change rate, so 8-10h may be the best time for the formation of flavor substances. With the long fermentation time, the time change rate is small, and the fermentation proceeds to 10h. At the time, the fermented milk has the highest sensory evaluation score and the best flavor.

Effect of skim milk concentration on ACE inhibition rate of *L. bulgaricus* LB6 fermented milk

Reconstituted milk was prepared at 8%, 10%, 12%, 14% and 16%, respectively. After fermentation, the indicators were determined and the results are shown in the Figures 7 and 8.

As shown in Figure 7, as the skim milk concentration increase, the ACE inhibition rate of the fermented milk first increased rapidly, and the inhibition rate reached the maximum at the concentration of 14%, and then decreased. The reason for this is that when the concentration of the recovered milk is within a certain range (10%-14%), as the concentration of the milk increases, the protein substrate for enzymatic hydrolysis increases, and the resulting ACE inhibitory peptide is also formed. Increased; and then due to excessive milk protein content, the inhibition of the substrate causes the ability of the bacterial enzyme to decompose the milk protein to produce peptides, the ACE inhibitory peptide produced decreases, and the ACE inhibition rate also decreases. As the concentration of reconstituted milk increased, the change in OD value was large, indicating that the increase in milk concentration promoted the growth of *L. bulgaricus* LB6.

Figure 8 indicates that with the increase of the concentration of recovered milk, the titration acidity and pH of the fermented milk first increase and then decrease, and the change range is obvious. There is no opposite change in titration acidity and pH, which may be related to the buffering effect of cow's milk. The sensory evaluation score of the fermented milk at a degreasing concentration of 14% is high. This may be because the substrate concentration is too low or too high, which is not conducive to the *L. bulgaricus* LB6 value-added metabolism, and the flavor substances produced are less, so that the flavor of the fermented milk is tasted not suitable.

Optimization of Peptide Conditions for *L. bulgaricus* LB6 Fermented Milk by Orthogonal Test

The inoculation amount (A), temperature(B), time(C) and skim milk concentration(D) were selected as the investigation factors. The test results were determined by reference to the single factor analysis test results. The four-factor and three-level orthogonal experiments were carried out on the above factors to further optimize the fermentation conditions. As shown in the Table 2, experimental results and analysis are presented, where Y_1 is the ACE inhibition rate ,Y_2 is the OD value, Y_3 is the titration acidity, Y_4 is the pH value ,Y_5 is the sensory evaluation.

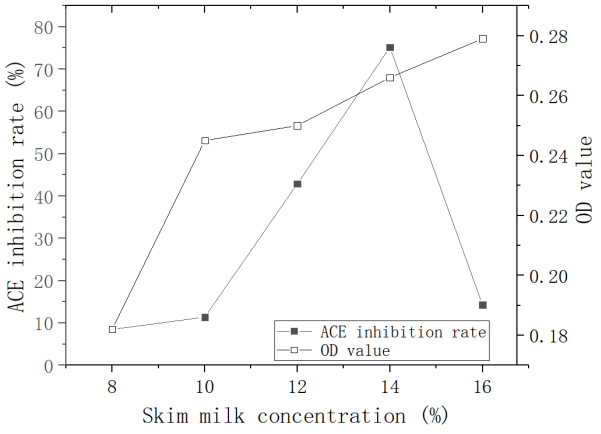

Figure 7. Effect of skim milk concentration on ACE inhibition rate and OD value of fermented milk

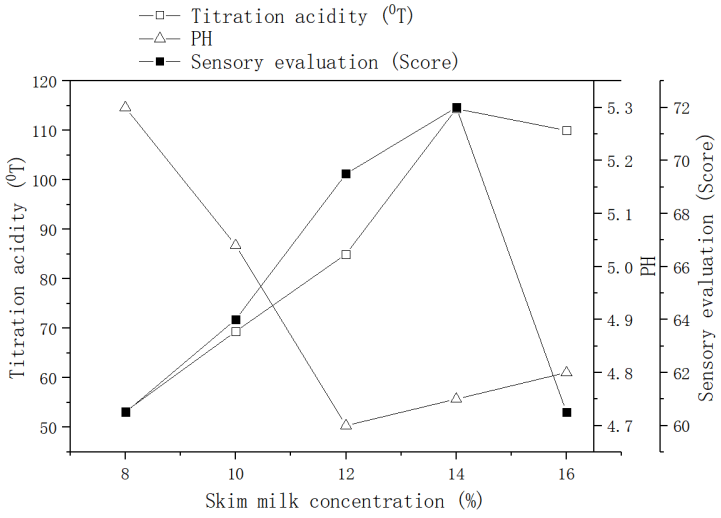

Figure 8. Effect of skim milk concentration on fermentation acidity and pH value and sensory evaluation

Table 2. Orthogonal test results of peptide conditions for LB6 fermented milk

Trial No.		Factor number				Y_1(%)	Y_2	Y_3(^{0}T)	Y_4	Y_5
		A/%	B/℃	C/h	D/%					
1		1(3)	1(40)	1(11)	1(12)	74	0.127	55	5.09	65
2		1	2(42)	2(12)	2(13)	75.8	0.453	77	4.68	70
3		1	3(44)	3(13)	3(14)	71.2	0.234	70.4	4.79	66
4		2(4)	1	2	3	70	0.163	58	5.01	68
5		2	2	3	1	67.7	0.494	86	4.23	76
6		2	3	1	2	68.5	0.265	68.4	4.89	69
7		3(5)	1	3	2	72.6	0.18	61	4.78	70
8		3	2	1	3	75.3	0.492	97	4.74	78
9		3	3	2	1	61.6	0.39	63.4	4.72	73
Y_1/%	k_1	75.333	72.200	72.600	67.767					
	k_2	68.733	74.600	70.800	73.967					
	k_3	69.833	67.100	70.500	72.167					
	R	6.600	7.500	2.100	6.200					
Y_2/%	k_1	0.271	0.157	0.295	0.287					
	k_2	0.307	0.480	0.285	0.299					
	k_3	0.304	0.246	0.303	0.296					
	R	0.036	0.323	0.018	0.012					

The mean is represented by k, and k_1, k_2, and k_3 are the sum of the indicators having factor level 1, level 2, and level 3, respectively. R represents the difference between the maximum value and the minimum value in k_1, k_2, and k_3. The magnitude of the extreme difference R reflects the magnitude of the action of each factor in the experiment. The larger the R, the greater the influence of the corresponding factor on the experimental index, which is an important condition. The highest level of k for each factor constitutes the most fermentable condition. It can be seen from the table that the significance of each factor on the ACE inhibition rate is B>A>D>C, that is, temperature>inoculation amount> skim milk concentration>time, and the best combination is B2A1D2C1. The influence degree of each factor on OD value is B>A>C>D, that is, temperature>inoculation amount>skimmed milk concentration>time, and the best combination is B2A2C3D2.The above two combinations are not in the 9 sets of experiments that have been done, so the two combinations are verified and the results of B2A2C3D2 are better than those of B2A1C2D1. Therefore, the optimal fermentation conditions are combined into B2A2C3D2, that is, the optimum fermentation conditions are temperature 42°C, inoculation amount 4%, time 13 h, and skim milk concentration 13%. Pan found that the optimal inoculum condition for the maximum yield of ACE-inhibiting peptides produced by *Lactobacillus helveticus* LB10 was 4%(Pan *et al*., 2010), which is consistent with our results. Shu showed that the best peptide-producing condition was obtained when the milk powder concentration was 14%(Guowei, S *et al*., 2013), and our results showed that the optimal peptide-producing condition was obtained when the milk powder concentration was 13%, and the ACE inhibition rate reached 76.50%.

CONCLUSION

The optimal fermentation conditions for ACE inhibitory peptide produced by *Lactobacillus bulgaricus* LB6 are 4% inoculation, 13h in time, 42°C in temperature and 13% in skim milk. Under this condition, the ACE inhibition rate reaches 76.50% and the OD value is 0.330. The titration acidity is 116.4°T, the pH was 4.62, and the sensory evaluation was 75.

ACKNOWLEDGEMENTS

The work was partially supported by the science and technology project of Xi'an city [No.XJR1506-(10)] and the project of Baqiao district [No.2016-(7)].

REFERENCES

1. Ahtesh, F. B. , Apostolopoulos, V. , Stojanovska, L. , Shah, N. P. , & Mishra, V. K. . (2017). Effects of fermented skim milk drink by\r, *kluyveromyces marxianus*\r, laf4 co-cultured with lactic acid bacteria to release angiotensin-converting enzyme inhibitory activities. *International Journal of Dairy Technology*.
2. Algaron, F., G. Miranda, D. Le Bars and V. Monnet. 2004. Milk fermentation by *Lactococcus lactis* with modified proteolytic systems to accumulate potentially bio-active peptides. *Lait*, 84: 115-123.
3. Assimes, T. L., Elstein, E., Langleben, A., & Suissa, S. (2010). Long-term use of antihypertensive drugs and risk of cancer. *Pharmacoepidemiology & Drug Safety, 17*(11), 1039-1049.
4. Bao, C., Chen, He., Chen, Li., Cao, J., & Meng, J. (2016). Comparison of ACE inhibitory activity in skimmed goat and cow milk hydrolyzed by alcalase, flavourzyme, neutral protease and proteinase K, *Acta Universitatis Cibiniensis. Series E: Food Technology*, *20*(1), 77-84.
5. Chen, L. , Zhang, Q. , Ji, Z. , Shu, G. , & Chen, H. . (2018). Production and fermentation characteristics of angiotensin-i-converting enzyme inhibitory peptides of goat milk fermented by a novel wild *lactobacillus plantarum* 69. *LWT, 91*.
6. China Standard Press. (2000). National Standard Application Guide for Sour Milk and Dairy Products.GB2746-1999. Beijing China.
7. Cushman, D.W. and H.S. Cheung, 1971. Spectrophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. *Biochem. Pharmacol.*, 20: 1637-1648.
8. Guowei, S., Hui, Y., He, C., Zhe, J., and Hongni, X. , 2013. Effect of ascorbic acid, incubation temperature and inoculum size on ACE inhibitory activity in fermented goat milk by *Lactobacillus bulgaricus* LB6. J. Chem. Pharm. Res. 5(12):988-995.
9. Du, L. , Fang, M. , Wu, H. , Xie, J. , Wu, Y. , & Li, P. , et al. (2013). A novel angiotensin i-converting enzyme inhibitory peptide from phascolosoma esculenta water-soluble protein hydrolysate. *Journal of Functional Foods, 5*(1), 475-483.
10. Elvira María Hebert, Saavedra, L. , & Ferranti, P. . (2010). *Bioactive Peptides Derived from Casein and Whey Proteins*. *Biotechnology of Lactic Acid Bacteria: Novel Applications*. Wiley‐Blackwell.
11. Georgalaki, M. , Zoumpopoulou, G. , Mavrogonatou, E. , Van Driessche, G. , Alexandraki, V. , & Anastasiou, R. , et al. (2017). Evaluation of the antihypertensive angiotensin-converting enzyme inhibitory (ace-i) activity and other probiotic properties of lactic acid bacteria isolated from traditional greek dairy products. *International Dairy Journal*, S0958694617301516.
12. He, C., J. Zhe, S. Guowei and X. Hongni, 2012. Effect of probiotic *Lactobacillus* strains on angiotensin I converting enzyme inhibitory activity from fermented goat milk. *Adv. Mater. Res*., 531: 442-445.
13. He, C., W. Juan, L. Qian and S. Guowei, 2013. Effect of NaHCO3, MgSO4, sodium Ascorbate, sodium glutamate, phosphate buffer on survival of *Lactobacillus bulgaricus* during freeze-drying. *Adv. J. Food Sci. Technol.*, 5: 771-774.
14. Jiang, Z.M., G. Wu, G.C. Huo and B. Tian, 2011. Study on external conditions of angiotensin converting enzyme inhibitory peptide derived from fermented milk. *Sci. Technol. Food Ind.*, 32: 106-108.
15. Liou, Y. S. , Ma, T. , Tien, L. , Chien, C. , Chou, P. , & Jong, G. P. . (2008). Long-term effects of antihypertensive drugs on the risk of new-onset diabetes in elderly taiwanese hypertensives. *International Heart Journal, 49*(2), 205-211.
16. López-Fandiño, R., J. Otte and J. van Camp, 2006. Physiological, chemical technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *Int. Dairy J.*, 16: 1277-1293.

17. Mohamed, H., M. Ons, S. Nabil, T. Yosra, K. Sadok and N. Moncef, 2010. Chemical composition, angiotensin I-converting enzyme (ACE) inhibitory, antioxidant and antimicrobial activities of the essential oil from Periploca laevigata root barks. *Food Chem.*, 121: 724-731.
18. Nakamura, Y., N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki and T. Takano, 1995. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.*, 78: 777-783.
19. Nielsen, M. S. , Martinussen, T. , Bénédicte Flambard, S?Rensen, K. I. , & Otte, J. . (2009). Peptide profiles and angiotensin-i-converting enzyme inhibitory activity of fermented milk products: effect of bacterial strain, fermentation ph, and storage time. *International Dairy Journal, 19*(3), 0-165.
20. Pan, D., & Guo, Y. (2010). Optimization of sour milk fermentation for the production of ace-inhibitory peptides and purification of a novel peptide from whey protein hydrolysate. *International Dairy Journal, 20*(7), 472-479.
21. Pihlanto, A. , Virtanen, T. , & Korhonen, H. . (2010). Angiotensin i converting enzyme (ace) inhibitory activity and antihypertensive effect of fermented milk. *International Dairy Journal, 20*(1), 0-10.
22. Pritchard, S. R. . (2018). Isolation and characterisation of bioactive peptides derived from milk and cheese. *Journal of Virology, 62*(10), 3832–3839.
23. Quiro´ s, A., M. Ramos, B. Muguerza, M.A. Delgado, M. Miguel, A. Aleixandre and I. Recio, 2007. Identification of novel antihypertensive peptides in milk fermented with Enterococus faecalis. *Int. Dairy J.*, 17: 33-41.
24. Rey, O. L. . (2014). Cognitive and linguistic impairment associated with hypertension. *Dissertations & Theses - Gradworks*.
25. Robert, M.C., A. Razaname, M. Mutter and M.A. Juillerat, 2004. Peptides derived from sodium caseinate hydrolysates produced by *Lactobacillus helveticus* NCC 2765. J. Agr. *Food Chem.*, 52: 6923-6931.
26. Rodríguez-Figueroa, J.C., R. Reyes-Díaz, A.F. González-Córdova, R. Troncoso-Rojas, I. VargasArispuro and B. Vallejo-Cordoba, 2010. Angiotensin-converting enzyme inhibitory activity of milk fermented by wild and industrial *Lactococcus lactis* strains. *J. Dairy Sci.*, 93: 5032-5038.
27. Roy, F., J.I. Boye and B.K. Simpson, 2010. Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. *Food Res. Int.*, 43: 432-442.
28. Shuangquan, H.T. and M. Taku, 2008. Angiotensin Iconverting enzyme inhibitory peptides in skim milk fermented with *lactobacillus helveticus* 130B4 from camel milk in Inner Mongolia, China. *J. Sci. Food Agric.*, 88: 2688-2692.
29. Yamamoto, N., A. Akino and T. Takano, 1994a. Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. Biosci. *Biotechnol. Biochem.*, 58: 776-778.
30. Yamamoto, N., A. Akino and T. Takano, 1994b. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci*., 77: 917-922.
31. Yamamoto, N., M. Maeno and T. Takano, 1999. Purification and characterization of an antihypertensive peptide from a yoghurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.*, 82: 1388-1393.
32. Yanli, Tuba, Akal, H. C. , Yeti?Emiyen, A., Hayaloglu, A. A. . (2016). Influence of adjunct cultures on angiotensin-converting enzyme (ace)-inhibitory activity, organic acid content and peptide profile of kefir. *International Journal of Dairy Technology*.