sciendo

10.2478/aucft-2019-0002

EFFECTS OF CARBON AND NITROGEN SOURCES ON ACTIVITY OF CELL ENVELOPE PROTEINASE PRODUCED BY *LACTOBACILLUS PLANTARUM* LP69

– Research paper –

Fangfang CHENG*, He CHEN*[1], Ni LEI*, Meng ZHANG**, Hongchang WAN**

*School of Food and Biological Engineering, Shaanxi University of Science & Technology, Xi'an, 710021, China; **Shaanxi Yatai Dairy Co., Ltd., Xianyang, 713701, China

Abstract: In present study, the effects of carbon sources (glucose, lactose, sucrose, galactose, maltose and soluble starch) and nitrogen sources (casein peptone, whey protein, soy peptone, yeast, tryptone, beef extract and peptone) on activity of cell envelope proteinases (CEP), specific activity, protein content, OD_{600} value and pH in MRS broth fermented by *Lactobacillus plantarum* LP69 were investigated by individual factor experiment. The results indicated that carbon and nitrogen sources have significant influence on the activity of CEP and specific activitys of *L. plantarum* LP69, glucose, maltose, casein peptone and peptone are superior to other selected carbon and nitrogen sources. The optimum concentrations of glucose, maltose, casein peptone and peptone for *L. plantarum* LP69 are 2%, 2%, 1% and 1%; the activity of CEP are 19.52U/mL, 21.13U/mL, 13.49U/mL and 20.61U/mL, respectively.

Keywords: *Lactobacillus plantarum* LP69; cell-envelope proteinases; carbon sources; nitrogen sources

INTRODUCTION

Lactic acid bacteria (LAB) find a wide application as starters in fermented dairy products on account of their milk acidification and flavor development properties. (Fernandez-Espla et al., 2000). Some lactic acid bacteria are called probiotics, such as bifidobacteria and lactobacilli, traditionally defined as viable microorganisms that contribute to prevent or treat intestinal disorders when ingested by human (Rolfe, 2000; Tatsuya et al., 2015; Shu et al., 2016). Among the fermenting microorganisms of most fermented foods, lactic acid bacteria predominate and are used as probiotics to promote human body health. (Hammes et al., 2006). *Lactobacillus plantarum* is a type of common and beneficial Lactobacilli in nature, which plays an important role in the development of food flavor in the fermentation industry. Moreover, *Lactobacillus plantarum* is also an essential probiotic in the human gastrointestinal tract, which has an enormous influence on maintaining human health and preventing disease (Wu et al., 2019).

The cell envelope proteinase (CEP), produced by lactic acid bacteria, is a key class of proteolytic enzymes, because it acts on the first step of casein degradation which plays a part in developing sensory and texture properties of fermented foods (Chen et al., 2018; Sadat-Mekmene et al., 2011). Moreover, CEPs may release bioactive peptides with more health benefits than basic nutrition (Espeche et al., 2009). CEP is the core enzyme in the process of hydrolyzing goat milk protein to produce polypeptides. Prior studies by our group have shown that products of goat milk hydrolyzed by CEPs of *L. plantarum* LP69 have high antioxidant activity and angiotensin-I-converting enzyme (ACE) activity (Chen et al., 2012; Chen et al., 2015). Therefore, the more biochemical knowledge and commercial application of CEPs from *L. plantarum* is worth in-depth study, but these prospects are closely related to the medium optimization of CEPs production by *L.plantarum* which the CEP with higher activity can be obtained.

A main advantage of fermentation is that medium compositions and culture conditions affect cell growth and product synthesis — can be examined and controlled to improve the

[1] Corresponding author. E-Mail address: chenhe419@gmail.com

yield and quality of products, and the various types and sources of nutrients available in the medium can exert a profound influence on the fermentation yields (Danquah et al., 2007; Agyei et al., 2012). The use of a chemically defined medium is important to enhance the proteinase activity and support the growth of lactobacilli (Hebert et al., 2004). Chen et al. (Chen et al., 2012; Chen et al., 2013) screened various nitrogen sources, carbon sources/prebiotics to obtain the optimal substances for growth of et al., 2012) observed the influence of various sugars on CEPs production by *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313). In addition, the optimal medium for CEP production of *Lactobacillus casei* DI-1 and *Lactobacillus acidophilus* were studied and the proteinase activity was significantly increased (Wu et al., 2013; Ren et al., 2014). However, as far as we know, there is a lack of information on the medium optimization of CEPs production by *L. plantarum* LP69.

In our previous work, we screened and obtained *L. plantarum* LP69 which could fermented goat milk with ACE inhibitory activity. In the present study, the effects of carbon sources (glucose, lactose, sucrose, galactose, maltose and soluble starch) and nitrogen sources (casein peptone, whey protein, soy peptone, yeast, tryptone, beef extract and peptone) on activity of cell envelope proteinases (CEPs), specific activity, protein content, OD600 value and pH in MRS broth fermented by *Lactobacillus plantarum* LP69 were investigate by single factor experiment, which was to explore the carbon and nitrogen sources that can improve significantly the CEP activity of *L. plantarum* LP69 in culture medium. In the present work, we screened out the main factors that effecting the production of CEP of *L. plantarum* LP69 from various carbon sources and nitrogen sources, and further adopted single factor, which will provide a reference for the optimization of CEP-producing media.

MATERIAL AND METHODS

Microorganisms and Culture Media

L. plantarum LP69 was provided by the School of Food and Biological Engineering, Shaanxi University of Science & Technology and inoculated three generations in a row with MRS broth medium at 37℃ for 22 h. The Man Rogosa Sharpe (MRS) broth was purchased from hopebiol Technology Co., Ltd. (Tianjin, China). All culture medium were autoclaved at 121℃ for 15 min.

Harvesting of Crude CEPs

At 4℃, the culture was centrifuged at 4500 r/min for 20 min. The supernatant was decanted and the pellet was rinsed with 50 mM Tris-HCL buffer solution (pH 7.8) including 30 mM $CaCl_2$ three times. The suspension of washed whole cells was then suspended in 50 mM Tris-HCl supplemented with 50 mM EDTA-Na_2, pH 7.0. The reaction mixture was incubated at 37℃ for 1 h. The supernatant harvested by centrifugation (4500 r/min, 15 min, 4℃) designated as crude CEPs.

Culture pH and *Lactobacillus plantarum* LP69 Growth Assay

The pH values of the cultures were recorded with a pH meter with version PHS-3C. The growth of *Lactobacillus plantarum* LP69 was measured by optical density (OD) at 600 nm with SP-756PC ultraviolet spectrophotometer.

Measurement of Proteinase Activity

The enzyme activity was determined according to the prior study (Chen et al., 2018).

Protein Contents Assay

Using the bovine serum albumin as standard, protein concentration was measured by Bradford method (Bradford, 1976).

Determination of Specific Activity

Specific activity is the enzyme activity of per mg of enzyme protein. The formula for determining specific activity is as follows (Ngo et al., 2008):

$$\text{Specific activity (U/mg)} = \frac{\text{Total enzyme activity (U)}}{\text{Total protein (mg)}} \quad (1)$$

Statistical Analysis

Parallel experiments were conducted three times for each group, and the average values were taken as the final result. The Origin 9 software package and Microsoft Excel 2010 were used to process data.

RESULTS AND DISCUSSION

Establishment of bovine serum albumin and tyrosine standard curve

The linear regression equation of bovine serum standard curve is y=0.00581x+0.05795, R2=0.9585, and the protein has a good linear relationship between 0-100μg. The linear regression equation of the tyrosine standard curve is y=0.01063x-0.0955, R2=0.9988, and the protein has a good linear relationship between 10-50μg.

Effect of carbon sources on CEP activity of *L. plantarum* LP69

This test screened six carbon sources. The MRS broth was used as the base medium. The other components of the medium were kept unchanged, and the carbon source was changed to 2% glucose, lactose, sucrose, galactose, maltose and soluble starch, respectively. After inoculating with *L. plantarum* LP69, the cells were cultured at 37℃ for 22 h, and the OD_{600} value, pH value, CEP activity, protein content and specific activity were measured. The results are shown in Figure 1. CEP activity and specific activity of *L. plantarum* LP69 were the higher in culture medium with glucose and maltose as carbon sources. Therefore, glucose and maltose were selected as the preliminary screening of carbohydrates, and the appropriate concentration range was further determined by single factor experiment.

Figure 2 shows that with the increase of glucose concentration, the CEP activity, protein content and specific activity increased initially then started to drop. When glucose concentration was 2%, the enzyme activity was highest at 19.52U/mL and the protein content was the highest at 20.16mg/mL, as well as the specific activity reaching a maximum of 0.98U/mg.

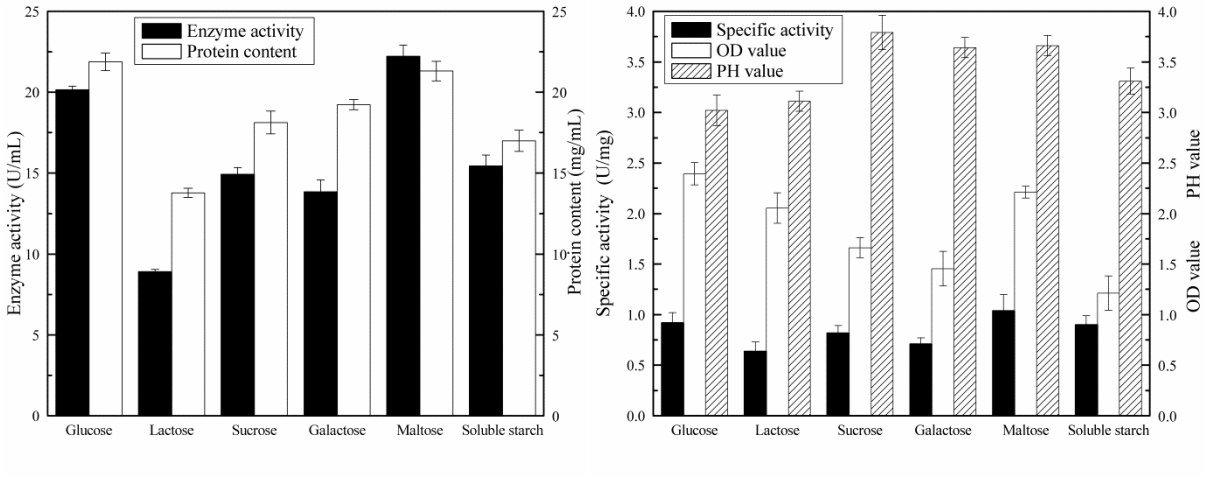

Figure 1. The effect of six carbon sources on CEP activity of *L. plantarum* LP69

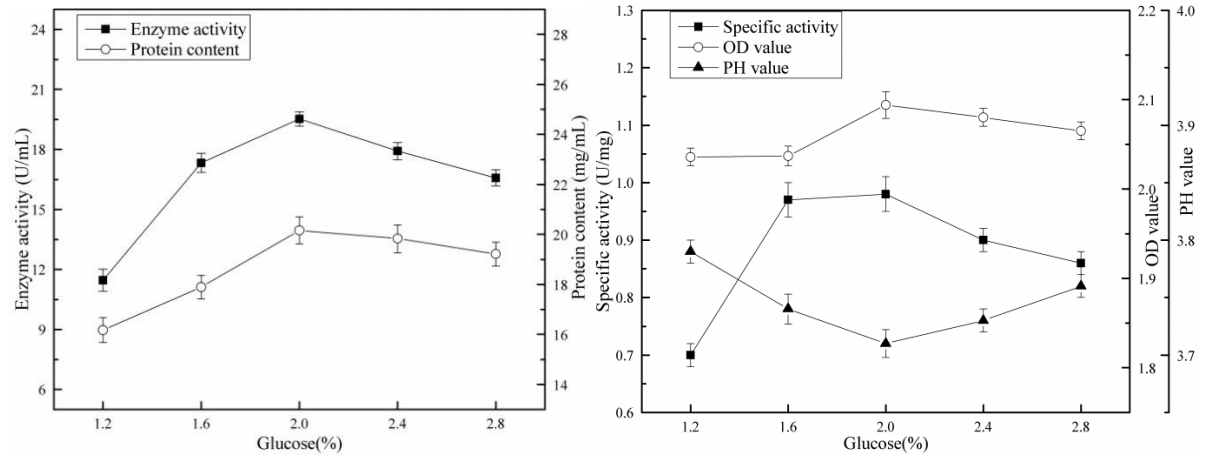

Figure 2. The effect of glucose on CEP activity of *L. plantarum* LP69

The OD value can reflect the growth of LP69. The larger the OD value, the more the number of viable cells. With the rising of glucose concentration, the OD value increased initially, followed by a decrease, peaked at the glucose concentration of 2%. The pH can reflect the metabolism of lactobacillus. The lower the pH value, the better the metabolic acid production. The Figure shows that the pH first decreased and then increased, and at the glucose concentration of 2%, the metabolism was optimal. These data indicated that a low concentration of glucose could promote the growth of *L. plantarum* LP69, but a high concentration of glucose has a passive effect. This maybe on account of excessive glucose produced a large amount of metabolite lactic acid, thus inhibited growth of *L. plantarum* LP69.

As figure 3 shows, the concentration of maltose increased, the CEP activity, protein content and specific activity all increased first and then decreased, with a maximum at the maltose concentration of 2%, which was 21.13U/mL, 14.57mg/mL and 1.45U/mg, respectively. The high proteinase activity observed for maltose could be accounted for by the action of maltose in maintaining the morphological integrity of the bacterial cell envelope **(**Agyei et al., 2012**).** From the aspect of strain LP69 growth, the bacterial density increased at first and then decreased, and the growth was best at the maltose concentration of 2%. The pH was the lowest point at 2% maltose, which indicated that the metabolism of strain LP69 was the best. Therefore, 2% were chosen as the optimum maltose concentration.

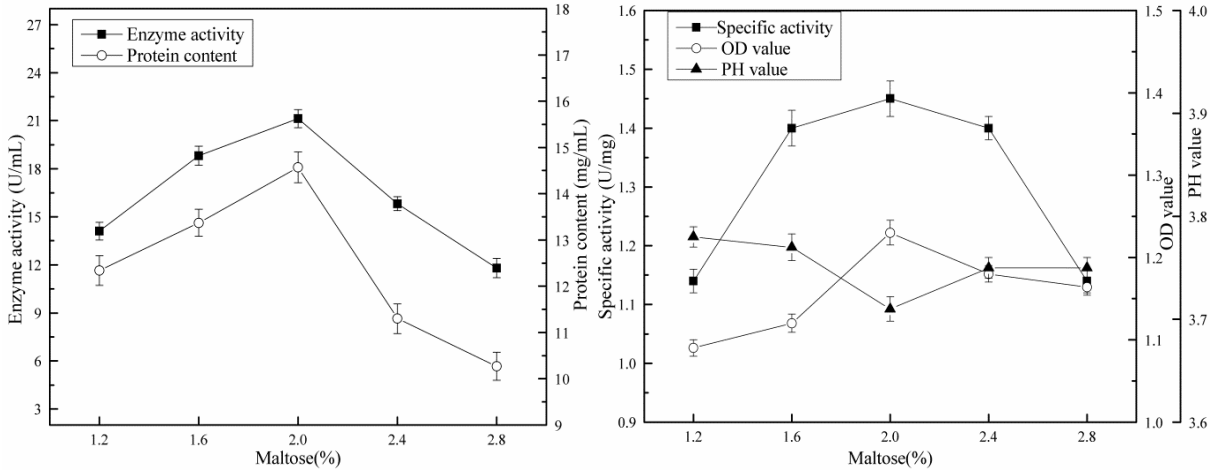

Figure 3. The effect of maltose on CEP activity of *L. plantarum* LP69

Carbohydrates are essential nutrients for the growth of microorganisms. All kinds of carbon sources can be absorbed and utilized by bacteria, and were used as raw materials for the synthesis of bacteria. Furthermore, carbon sources also served as the main energy source for bacterial metabolism. Chen et al. (2013) found that lactose promoted significantly the growth of *Streptococcus thermophilus*. A previous study identified that proteinase activity of strain LDL 313 was dependent on the carbon source, with 2% maltose recording the highest proteinase activity **(**Agyei et al., 2012**).** Moreover, 2% glucose and 1% beef peptone were considered to be the optimal combination for CEP production of *Lactobacillus acidophilus* (Ren et al., 2014). Gao et al. (2008) explored the effects of different carbon and nitrogen sources on the growth of *Lactobacillus casei* Zhang, and selected glucose as the best carbon source. Xu et al. (2011) studied the different carbon sources can improve the amount of antibacterial substance producing by strain LB-9, and glucose was chosen as the best carbon source. These demonstrate that higher activity of proteinase can be obtained from lactic acid bacteria by optimising the carbon compositions of medium.

Effect of nitrogen source on *L. plantarum* LP69 producing CEP

In this experiment, casein peptone, whey protein, soy peptone, yeast, tryptone, beef extract and peptone were screened, and other components of the basic medium were kept unchanged. Nitrogen source was changed to 1% of the above substances, respectively.

Figure 4 shows that among the seven nitrogen sources, the CEP activity and specific activity in media fermented by *L. plantarum* LP69 using casein peptone and peptone as nitrogen sources were higher. Thus the two nitrogen sources were selected as relatively good nitrogen sources for the next step to obtain a better concentration ratio by single factor experiment.

It can be seen from Figure 5 that as the proportion of casein peptone was gradually increased from 0.2% to 1.8%, the trends of enzyme activity, protein content and specific activity were all increased first and then decreased. When the concentration of casein peptone was 1%, the enzyme activity and specific activity reached a maximum value of 13.49U/mL and 0.75U/mg, respectively. The protein content obtained the maximum value of 21.49mg/mL at the casein peptone concentration of 1.4%. Since enzyme activity and specific activity were the main factors, when the concentration of casein peptone was 1%, the OD value was the highest, and the lower pH also reflected the better growth of LP69, therefore, 1% were chosen as the best casein peptone concentration.

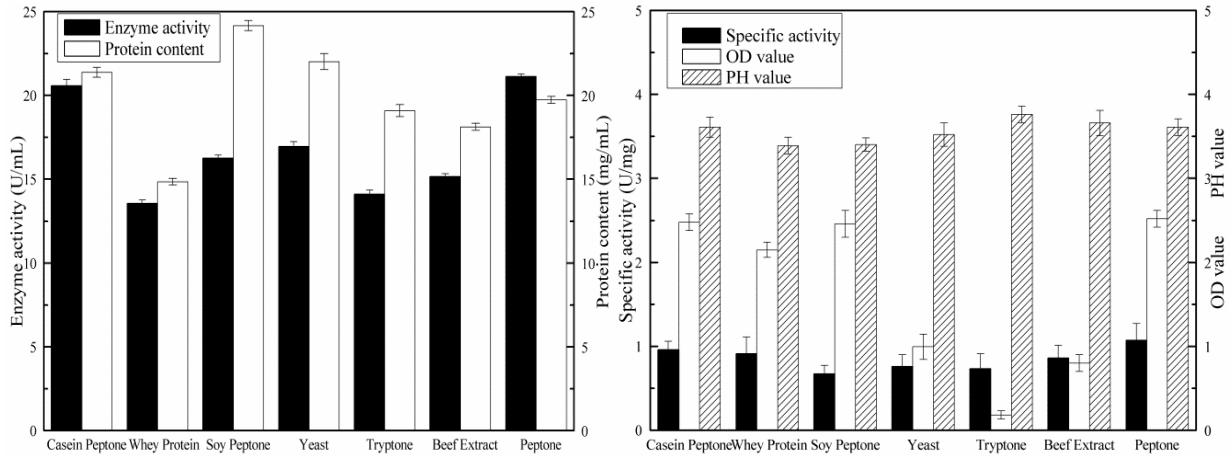

Figure 4. The effect of seven nitrogen source on CEP activity of *L. plantarum* LP69

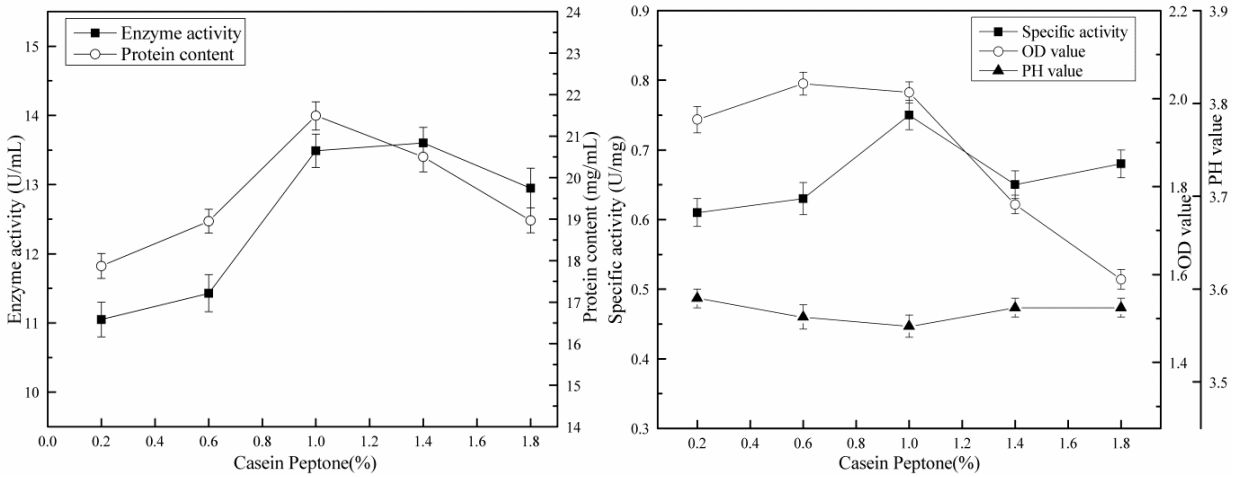

Figure 5. The effect of casein peptone on CEP activity of *L. plantarum* LP69

From the results in Figure 6, the enzyme activity, protein content and specific activity were basically the same, increased first and then decreased, with a maximum at the peptone concentration of 1%, which was 20.61U/mL, 20.18mg/mL and 0.97U/mg, respectively. The OD value and pH value also showed that when the peptone concentration was 1%, the *Lactobacillus plantarum* LP69 grew better, so 1% was selected as the optimal concentration of peptone.

Nitrogen-derived substances are significant factors in promoting microbial growth and are important substances that constitute the bacterial cell wall. LAB has a limited capacity to synthesize amino acids and is therefore dependent on the use of exogenous nitrogen sources for optimal growth (Hebert et al., 2000;

Espeche et al., 2009). Chen et al. (2016) reported that glutamate, soybean peptone and casein hydrolysate can improve the viable cells number of *Streptococcus thermophilus*. There is a significant effect on Soya peptone to the growth of *S. thermophilus* and the optimum soya peptone content was 30g/L (Chen et al., 2012). Similarly, it has been reported that the strain WH29-3-1 had the largest amount of thermoduric proteinases production when the cells had been grown in the medium containing glucose, peptone, beef extract, yeast powder (Fang et al., 2008). Therefore, in the process of lactobacillus producing proteases, different kinds and concentrations of nitrogen sources in the medium have a great influence on CEP activity and specific activity.

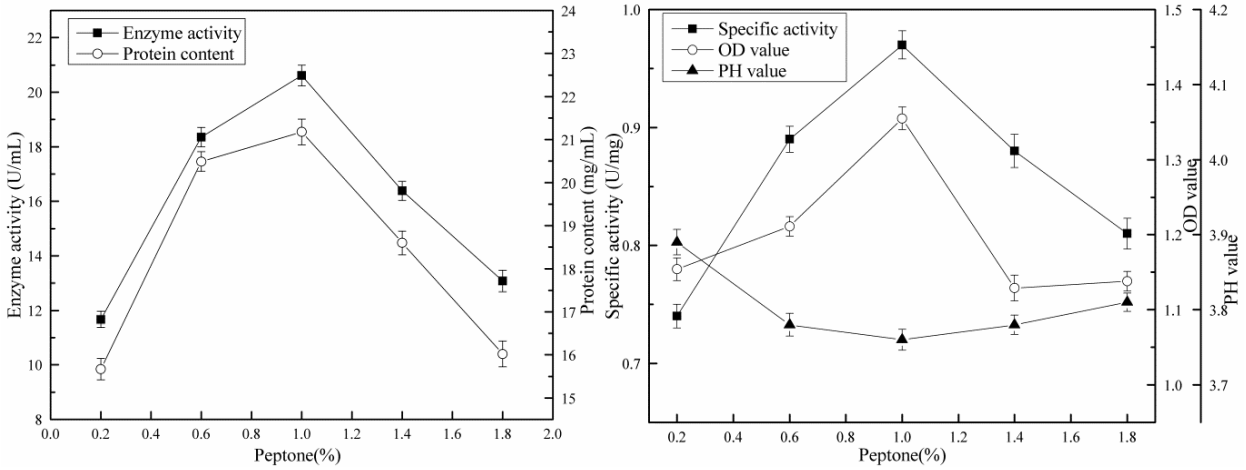

Figure 6. The effect of peptone on CEP activity of *L. plantarum* LP69

CONCLUSIONS

The selected carbon and nitrogen sources have significant influence on the activity of CEP from *Lactobacillus plantarum* LP69 in MRS broth medium. Glucose, maltose, casein peptone and peptone are superior to other selected carbon and nitrogen sources. The optimum concentration of Glucose, maltose, casein peptone and peptone for L. plantarum LP69 are 2%, 2%, 1% and 1%, the activity of CEP is 19.52U/mL, 21.13U/mL, 13.49U/mL and 20.61U/mL, respectively, which provided a reference for further optimizing of CEP-producing media of *L. plantarum* LP69.

ACKNOWLEDGEMENTS

The work was partially supported by the key industry innovation chain project of Science and Technology Department of Shaanxi Province (No.2018ZDCXL-NY-01-01), the science and technology project of Xi'an city [2017050NC/NY007(1)] and the Key Research and Development Program of Shaanxi (Program No. 2019ZDLNY06-02).

REFERENCES

1. Agyei, D. & Danquah, M.K. (2012). Carbohydrate Utilization Affects *Lactobacillus delbrueckii* subsp. *lactis* 313 Cell-enveloped-associated Proteinase Production. *Biotechnology and Bioprocess Engineering*, 17(4), 787-794. DOI: 10.1007/s12257-012-0106-2.
2. Agyei, D., Potumarthi, R. & Danquah, M.K. (2012). Optimisation of Batch Culture Conditions for Cell-Envelope-Associated Proteinase Production from *Lactobacillus delbrueckii* subsp. *Lactis* ATCC® 7830™. *Appl Biochem Biotechnol*, 168, 1035-1050. DOI: 10.1007/s12010-012-9839-9.
3. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, 72, 248-254. DOI: 10.1006/abio.1976.9999.
4. Chen, H., Chen, S.W., Chen, H.L., Wu, Y.Y. & Shu, G.W. (2014). Effects of carbon sources and

prebiotics added to growth media on proliferation and survival of *Lactobacillus bulgaricus* LB6 during freeze-drying. *Journal of Chemical and Pharmaceutical Research*, 6(6), 894-899.
5. Chen, H., Bao, C.J., Li, C.N., Wan, H.C. & Shu, G.W. (2016). Optimization of Culture Medium for Lactobacillus bulgaricus using Box-Behnken Design. *Carpathian Journal of Food Science and Technology*, 8(2), 38-46. DOI: 10.1515/aucft-2017-0001.
6. Chen, H., Chen, S.W., Li, C.N. & Shu, G.W. (2013). Screening of carbon sources/prebiotics and amino acids in the medium for *Streptococcus thermophilus* using Plackett-Burman design. *Journal of Chemical and Pharmaceutical Research*, 5(12), 975-980.
7. Chen, H., Huang, J., Cao, B.Y., Chen, L., Song, N. & Lei, N. (2018). Study of Extraction and Enzymatic Properties of Cell-Envelope Proteinases from a Novel Wild *Lactobacillus plantarum* LP69. *Catalysts*, 8(8). 325. DOI: 10.3390/catal8080325.
8. Chen, H., Ji, Z., Shu, G.W. & Xing, H. (2012). Effect of probiotic Lactobacillus strains on Angiotensin-I Converting enzyme inhibitory activity from fermented goat milk. *Adv. Mater. Res*, 531, 442-445. DOI: 10.4028/www.scientific.net/AMR.531.442.
9. Chen, H., Hui, Y.X., Chen, L., Wan, H.C., Shu, G.W. & Li, H. (2015). Effect of probiotic Lactobacillus strains on Antioxidant activity from fermented goat milk. *Carpathian Journal of Food Science and Technology*, 7(2), 109-114.
10. Chen, H., Li, C.N., Shu, G.W. & Wang, C.F. (2012). Screening of nitrogen sources in the medium for *Streptococcus thermophilus* using Plackett–Burman design. *Advanced Materials Research*, 531, 532-535. DOI: 10.4028/www.scientific.net/AMR.531.532.
11. Danquah, M.K. & Forde, G.M. (2007). Growth medium selection and its economic impact on plasmid DNA production. *Journal of Bioscience and Bioengineering*, 104(6), 490–497. DOI: 10.1263/jbb.104.490.
12. Espeche, T.M.B., Savoy de Giori, G. & Hebert, E.M. (2009). Release of the cell-envelope-associated proteinase of *Lactobacillus delbrueckii* subspecies *lactis* CRL 581 is dependent upon pH and temperature. *Journal of Agricultural and Food Chemistry*, 57(18), 8607-8611. DOI: 10.1021/jf901531q.
13. Fernandez-Espla, M.D., Garault, P., Monnet, V. & Rul, F. (2000). *Streptococcus thermophilus* cell wall-anchored proteinase: Release, purification, and biochemical and genetic characterization. *Applied and Environmental Microbiology*, 66(11), 4772–4778. DOI: 10.1128/AEM.66.11.4772-4778.2000.
14. Fang, F., Ji, L.L., Zhang, Y.B., Zhang, H.P. & Menghebilige. (2008). Screening of Thermoduric Proteinase-Producing Lactic Acid Bacteria，Conditions of Enzyme Production and Properties of Produced Thermoduric Proteinase. *Food Science*, 29(10), 375-379. DOI: 10.3321/j.issn:1002-6630.2008.10.087.
15. Gao, P.F., Li, Y., Zhao, W.J., Chen, X. & Cui, J.L. (2008). Study on the Optimization of Enrichment Medium of Lactobacillus casei Zhang. *Microbiology*, 35(4), 623-628. DOI: 10.13344/j.microbiol.china.2008.04.011.
16. Hebert, E.M., Raya, R.R. & De Giori, G.S. (2000). Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. *Appl. Environ. Microbiol*, 66(12), 5316–5321. DOI: 10.1128/AEM.66.12.5316-5321.2000.
17. Hammes, W.P. & Hertel, C. (2006). The Genera *Lactobacillus* and *Carnobacterium*. In Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H. & Stackebrandt, E.(Eds), *The Prokaryotes*(pp. 320–403). New York: Springer.
18. Hebert, E.M., Raya, R.R. & de Giori, G.S. (2004). Nutritional requirements of *Lactobacillus delbrueckii* subsp. *lactis* in a chemically defined medium. *Curr. Microbiol*, 49(5), 341–345. DOI: 10.1007/s00284-004-4357-9.
19. Ngo, L.T.A., Pham, T.L. & Le, V.V.M. (2008). Purification of Endopolygalacturonase from submerged culture of *Aspergillus awamori* L1 using a two-step procedure: Enzyme precipitation and gel filtration. *J. Food Res. Int*, 15, 135-140.
20. Ren, X.F., Pan, D.D., Zeng, X.Q., Zhao, Z.W. & Zhu, D.D. (2014). Optimization of Culture Conditions and Fermentation Conditions for Cell Wall Proteinase (CEP) Production by *Lactobacillus acidophilus*. J*ournal of Chinese Institute of Food Science and Technology*, 14(2), 146-153. DOI: 10.16429/j.1009-7848.2014.02.040.

21. Rolfe, R.D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *The Journal of nutrition*, 130(2S Suppl.), 396S–402S. DOI: 10.1093/jn/130.2.396S.
22. Sadat-Mekmene, L., Genay, M., Atlan, D., Lortal, S. & Gagnaire, V. (2011). Original features of cell-envelope proteinases of *Lactobacillus helveticus*. A review. *International Journal of Food Microbiology*, 146(1), 1-13. DOI: 10.1016/j.ijfoodmicro.2011.01.039.
23. Shu, G.W., Lei, N., Chen, H., Hu, M. & Yang, H. (2016). Application of central composite design to optimize the amount of carbon source and prebiotics for *Bifidobacterium bifidum* BB01. *Acta Universitatis Cibiniensis. Series E: Food Technology*, 20(1), 41-52. DOI: 10.1515/aucft-2016-0003.
24. Tatsuya, U., Jung-Hye, C., Hor-Gil, H. (2015). Changes in human gut microbiota influenced by probiotic fermented milk ingestion. *Journal Dairy Science*, 98(6), 3568-3576. DOI: 10.3168/jds.2014-8943.
25. Wu, Z. & Pan, D.D. (2013). Optimization of Culture Medium for the Production of Cell Envelope Proteinase by *Lactobacillus Casei* DI-1. *Journal of Chinese Institute of Food Science and Technology*, 13(2), 108-115. DOI: 10.16429/j.1009-7848.2013.02.004.
26. Wu, W.Q., Wang, L.L., Zhao, J.X., Zhang, H. & Chen, W. (2019). Research progress on physiological characteristics and health benefits of *Lactobacillus plantarum*. *Food and fermentation industries*, 45(1), 1-13. DOI: 10.13995/j.cnki.11-1802/ts.019602.
27. Xu, H.W., Ju, H.M., Sun, Q., Guo, W.Y. & Li, R. (2011). Optimization of carbon sources and nitrogen sources in ferment culture medium of bacterium producing small molecular antibacterial peptides. Chinese Journal of Health Laboratory Technology, 21(8), 1931-1935.