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ORIGINAL PAPER

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EFFECT OF PREBIOTICS, INORGANIC SALTS AND AMINO ACIDS FOR CELL ENVELOPE PROTEINASE PRODUCTION FROM *LACTOBACILLUS PLANTARUM* LP69

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ABSTRACT

Background. Cell-envelope proteinases (CEPs) can improve the texture and organoleptic characteristics of dairy products, and may also cause the release of bioactive peptides, which contribute to improving the host's health. Thus, the CEPs with high activity produced by *L. plantarum* has great potential in the development of functional dairy products.

Methods. A single factor experiment was used to investigate the effects of prebiotics (inulin, stachyose, isomaltooligosaccharide, xylooligosaccharides, galactooligosaccharides and fructooligosaccharides), inorganic salts (Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4, CH_3COONa and $C_6H_{14}N_2O_7$) and amino acids (arginine, leucine, serine, lysine, alanine and glutamic acid) on the activity of cell envelope proteinases (CEPs), specific activity, protein content, OD_{600} value and pH in MRS broth fermented by *Lactobacillus plantarum* LP69.

Results. The optimum concentration of inulin (0.7%), isomaltooligosaccharide (0.5%), Na_2HPO_4 (0.4%), CH_3COONa (0.4%), leucine (20 mg/L), serine (20 mg/L) for *L. plantarum* LP69 was estimated with the activity of CEP in the range 17.36–21.47 U/mL, protein content in the range 19.18–22.53 mg/mL, specific activity in the range 0.77–1.12 U/mg.

Conclusion. Inulin, isomaltooligosaccharide, Na_2HPO_4, CH_3COONa, leucine and serine are superior to other selected substances, and have a significant influence on the CEP activity and specific activity of *L. plantarum* LP69. This would provide a reference for further optimization of CEP-producing media of *L. plantarum* LP69.

Keywords: *Lactobacillus plantarum* LP69, cell-envelope proteinases, prebiotics, inorganic salts, amino acids, single-factor test

INTRODUCTION

Using lactic acid bacteria (LAB) in the fermentation industry has a long history that applies especially to fermented dairy products such as cheese, yogurt and milk powder. Lactobacillus is the largest genera in LAB, with the widest technological and industrial application, and is applied as probiotic culture to

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promote the host's health (Hammes and Hertel, 2006). The CEP, anchored on the LAB surface, is a class of extracellular proteolytic enzymes obtained easily from lactic acid bacteria. In the food industry, it is well known that cell-envelope proteinases (CEPs) can improve the texture and sensory characteristics of dairy products, as well as causing the release of bioactive health-beneficial peptides from milk protein (Tsakalidou et al., 1999). Cell-envelope proteinases – CEPs have enormous biotechnological potential and thus the production of profuse CEPs with high activity is necessary to satisfy the needs of the food industry.

Lactobacilli are extremely fastidious organisms adapted to complex growth media (Hebert et al., 2004). Not only do they need carbohydrates as an energy and carbon source, they also require nucleotides, amino acids, vitamins, salts and other nutriments for their growth and products synthesis in medium (Hebert et al., 2000). The production of CEPs from *Lactobacilli* is affected not only by the medium compositions but also by temperature and pH, and these fermentation parameters may determine the yield and activity of the enzymes produced (Espeche Turbay et al., 2009). Most studies have delineated the isolation, purification and properties of CEP from some lactobacilli species (Chen et al., 2018; Laloi et al., 1991; Scolari et al., 2006). Agyei et al. (2012) optimized the batch culture conditions for CEP production from *Lactobacillus delbrueckii* subsp. *lactis* 313 and obtained maximum proteinase activity. Ren et al. (2014) explored the optimal medium components and fermentation conditions in MRS broth for CEP production by *Lactobacillus acidophilus*. In addition, Samartsev et al. (2000) found that pH and substances of the medium had a significant impact on the production of cell-wall-bound enzymes by *Bifidobacterium adolescentis* 94-BIM. However, there is very little literature on the optimization of media for CEP production by *Lactobacillus plantarum*.

Lactobacillus plantarum is distributed widely in nature, and has widespread application in foods, animal feeds, industrial production, health care and other fields for its great physiological characteristics and probiotic functions (Wu et al., 2019). In previous studies, our lab found that products of goat milk hydrolyzed by CEPs of *L. plantarum* LP69 have strong ACE-inhibitory activity and antioxidant activity (Chen et al., 2012; 2015). Therefore, in order to carry out further study of the biotechnological characteristics of CEP from the strain LP69, it is necessary to obtain CEPs with high activity by manipulating culture medium conditions. This study aimed to investigate the effect of selected prebiotics, inorganic salts and amino acids added to the MRS medium on CEP activity, specific activity and protein content of *L. plantarum* LP69, which provided insights for the further optimization of CEP-producing mediums.

MATERIALS AND METHODS

Bacterial strain and media

L. plantarum LP69 was obtained from the School of Food and Biological Engineering, Shaanxi University of Science and Technology. The deMan, Rogosa and Sharpe (MRS) broth was obtained from hopebiol Technology Co., Ltd. (Tianjin, China) and autoclaved at 121°C for 15 min. The strain LP69 was inoculated 5% (v/v) into MRS broth and activated for three generations at 37°C for 22 h.

Preparation of crude CEPs

Different concentrations of selected substances were added to the activated strain LP69, and these were fermented in MRS medium at 37°C for 22 h under anaerobic conditions with an inoculum size of 5%. Following this, the culture was centrifuged (4500×g, 20 min, 4°C) and the harvested cells were washed three times with 50 mM Tris-HCL buffer solution (pH 7.8) containing 30 mM $CaCl_2$, after which the cell pellets were obtained by centrifugation (4500×g, 20 min, 4°C). A mixture of buffer (50 mM Tris-HCL buffer, 50 mM EDTA-Na_2, pH 7.0) and sediments was incubated at 37°C for 1 h. The crude CEPs was obtained after centrifugation (4500 r/min for 15 min at 4°C).

Culture conditions

A modified culture media was used in this study, which was based on the ingredients of a standard MRS-broth. This modification mainly concerned the change of inorganic salts, the addition of prebiotics and amino acids, while other components of the medium remained unchanged. The prebiotics, inorganic salts, amino acids were added to MRS broth at 0.5%, 0.4%, 20 mg/L, respectively. After being cultured with a strain LP69 at 37°C for 22 h, the samples collected

were taken out for measuring enzyme activity, protein content, specific activity, OD_{600} value and pH value, and the optimal nutrients were selected for further concentration-screening experiments under the same culture conditions.

Culture pH and *L. plantarum* LP69 growth analysis

The pH of the culture was measured with a pH meter (PHS-3C) and the absorbance at 600 nm was determined by UV spectrophotometer with version SP-756PC. The bacterial growth was measured after cultivating for 22 h under anaerobic conditions.

Measurement of proteinase activity

Enzyme activity was measured according to a previous study, which based on the Folin method (Chen et al., 2018).

Protein quantification

The amount of protein was estimated by means of the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Specific activity assay

Specific activity was expressed as the number of units of enzyme activity per mg protein (Ngo et al., 2008):

$$\text{Specific activity, U/mg} = \frac{\text{total enzyme activity, U}}{\text{total protein, mg}}$$

Statistical analysis

Data from three replicated trials for each treatment are presented as means with standard deviation (mean ±SD). Statistical analysis was performed using the Origin 9 software package (Origin Lab Inc., Alexandria, VA, USA) and Microsoft Excel 2010 (Redmond, WA, USA). *P*-values < 0.05 represented significant differences.

RESULTS AND DISCUSSION

Effect of prebiotics on *L. plantarum* LP69 producing CEP

The level of prebiotic added was a critical factor in improving the viability of lactobacillus during fermentation (Paloma et al., 2019). It can be seen from Figure 1 that when inulin or isomaltooligosaccharide was added to the medium, the CEP produced by *L. plantarum* LP69 had higher enzyme activity, specific activity and optical density ($p < 0.05$). Different prebiotics were added to the medium, and there was no significant change in pH values ($p > 0.05$). Therefore, inulin and isomaltooligosaccharide were selected for further study.

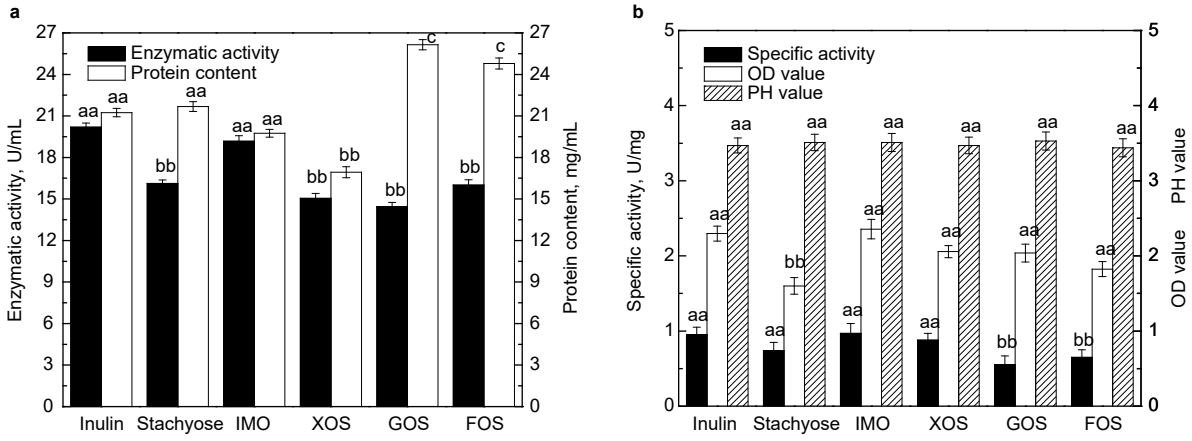

Fig. 1. The effect of different prebiotics on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH; IMO – isomaltooligosaccharide, XOS – xylooligosaccharides, GOS – galactooligosaccharides, FOS – fructooligosaccharides. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

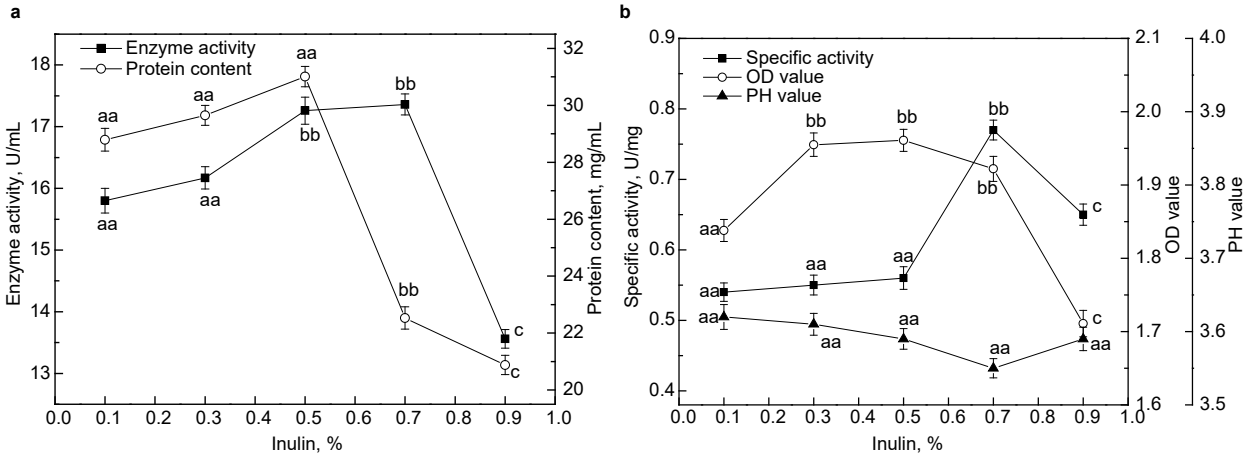

Fig. 2. The effect of inulin on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

The inulin was added to the MRS medium at 0.1%, 0.3%, 0.5%, 0.7% and 0.9%. As shown in Figure 2, as the inulin concentration increased, enzyme activity, protein content and specific activity first increased and then decreased ($p < 0.05$). The enzyme activity was highest at 17.36 U/mL at the inulin concentration of 0.7%, and the specific activity was 0.77 U/mg. When the inulin concentration was 0.5%, the protein content reached its maximum value of 31.01 mg/mL and the OD value also reached the maximum. The pH value reached the lowest point at 0.7% inulin concentration, indicating that bacteria had good metabolic acid production capacity. Since the enzyme activity and specific activity were the primary indicators, 0.7% was selected as the optimum concentration of inulin.

Adebola et al. (2014) found that inulin can markedly promote the growth of lactobacilli probiotics, which was consistent with the results of this study. However, inulin in high concentrations inhibited cell growth, and the possible reason was that fructose produced by

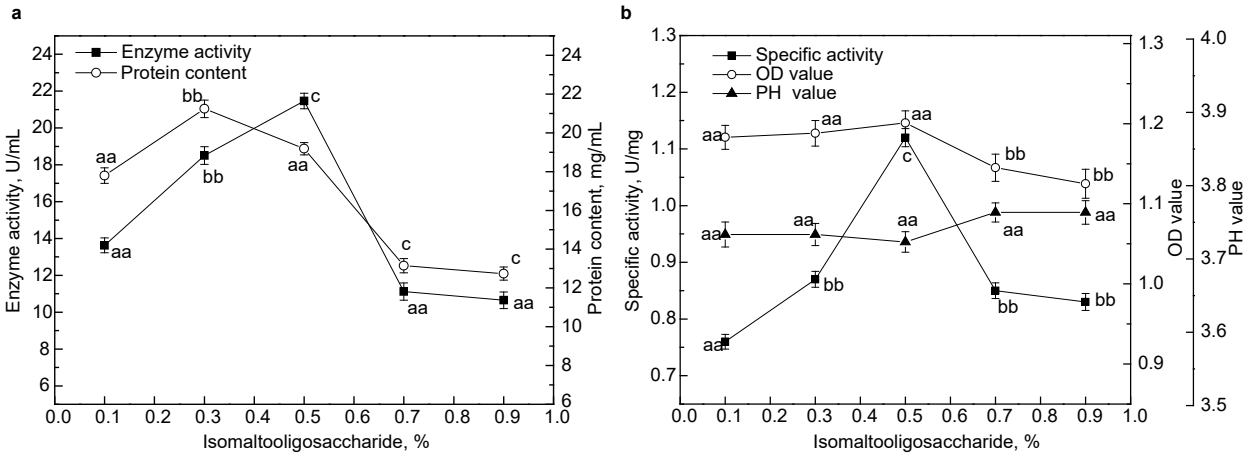

Fig. 3. The effect of isomaltooligosaccharide on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

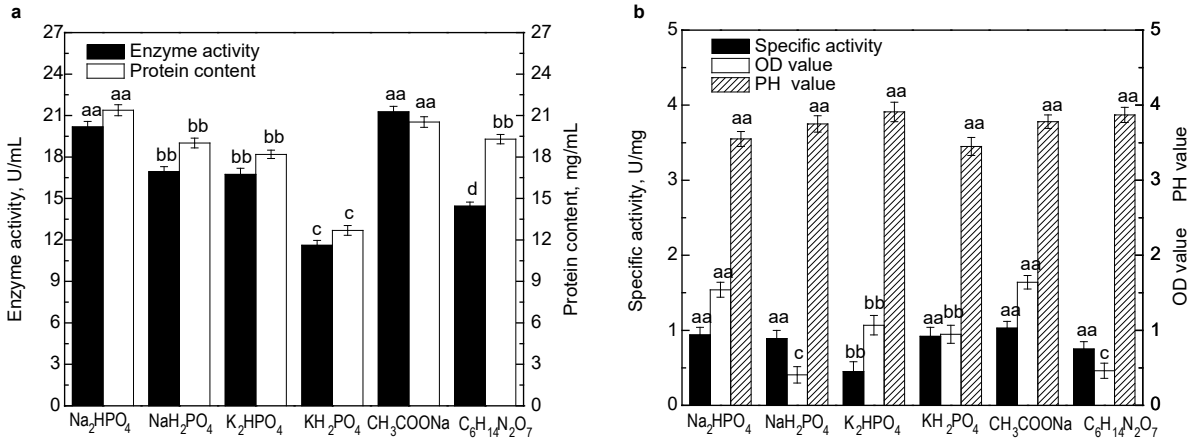

Fig. 4. The effect of different inorganic salts on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

partial inulin hydrolysis acted as an additional carbon source, increasing the osmotic pressure of the cells (Oliveira et al., 2012). Enzyme activity changed with an increasing inulin concentration, indicating that the expression of CEP by *L. plantarum* LP69 may be linked with the medium composition. These results demonstrated that suitable inulin content in the MRS medium improved both *L. plantarum* LP69 growth and levels of products.

The isomaltooligosaccharide (IMO) was added to the MRS medium at 0.1%, 0.3%, 0.5%, 0.7% and 0.9%. As shown in Figure 3, enzyme activity and specific activity increased with an increasing concentration of the IMO, and then dropped, with a maximum at the IMO concentration of 0.5%, which was 21.47 U/mL, 1.12 U/mg, respectively ($p < 0.05$). The protein content increased first and then decreased, and reached the maximum value of 21.24 mg/mL at the IMO concentration 0.3%. In addition, the OD value and pH value were respectively taken to the maximum and minimum values at 0.5% IMO concentration. Therefore, 0.5% was selected as the optimum concentration of IMO.

Consumption of IMO can stimulate the growth of lactobacillus (Chen et al., 2011). The addition of IMO could effectively improve the CEP activity and specific activity of *L. plantarum* LP69, but a high concentration of IMO inhibited the bacterial growth and CEP production. This may be because IMO produced a large amount of glucose during metabolism (Hu et al., 2013), which inhibited bacteria proliferation and changed the composition of the medium. These results suggested that appropriate IMO content in MRS broth stimulated both *L. plantarum* LP69 growth and CEP activity.

Effect of inorganic salts on *L. plantarum* LP69 producing CEP

The addition of appropriate buffer salts in medium can adjust the pH of the growth environment and the osmotic pressure of the cells (Yao et al., 2017), which promotes the growth of bacteria and improves the level of the product (Tong et al., 2012). It can be seen from Figure 4 that when Na_2HPO_4 or CH_3COONa was added to the medium, *L. plantarum* LP69 had higher enzyme activity, protein content, specific activity and OD value ($p < 0.05$). There was no significant change in pH values when different inorganic salts were added into the MRS medium ($p > 0.05$). Therefore, Na_2HPO_4 and CH_3COONa were selected for further study.

The Na_2HPO_4 was added to MRS medium at 0%, 0.2%, 0.4%, 0.6% and 0.8%. As shown in Figure 5, by increasing the Na_2HPO_4 percentage in the medium, the enzyme activity, protein content and specific activity first increased and then decreased ($P < 0.05$), which were consistent with the trend of optical density. When the concentration of Na_2HPO_4 was 0.4%, the enzyme activity, protein content and specific activity

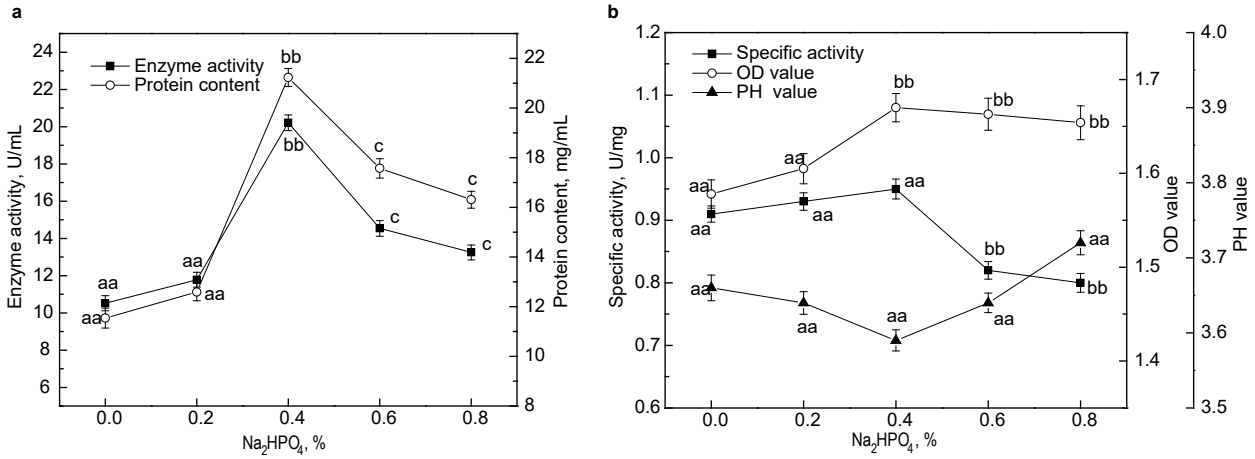

Fig. 5. The effect of Na_2HPO_4 on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

reached a maximum of 20.21 U/mL, 21.23 U/mg, and 0.95 U/mg, respectively, and pH reached the minimum value. Therefore, 0.4% was selected as the best concentration of Na_2HPO_4.

Fang et al. (2008) reported that K_2HPO_4 and Na_2HPO_4 can greatly enhance the activity of thermoduric protease produced by LAB, and the effect of K_2HPO_4 concentration on enzyme production is also especially remarkable. This research proved that a suitable content of Na_2HPO_4 could improve the CEP activity of *L. plantarum* LP69, but an excessive concentration of Na_2HPO_4 has a negative effect on CEP production. The trend of bacterial density change was in accordance with the trend of CEP activity. These results suggest that the expression of CEP by *L. plantarum* LP69 and bacterial growth is probably related to the medium composition.

The CH_3COONa was added to the MRS medium at 0%, 0.2%, 0.4%, 0.6% and 0.8%. The results (Fig. 6) show that the enzyme activity, protein content and

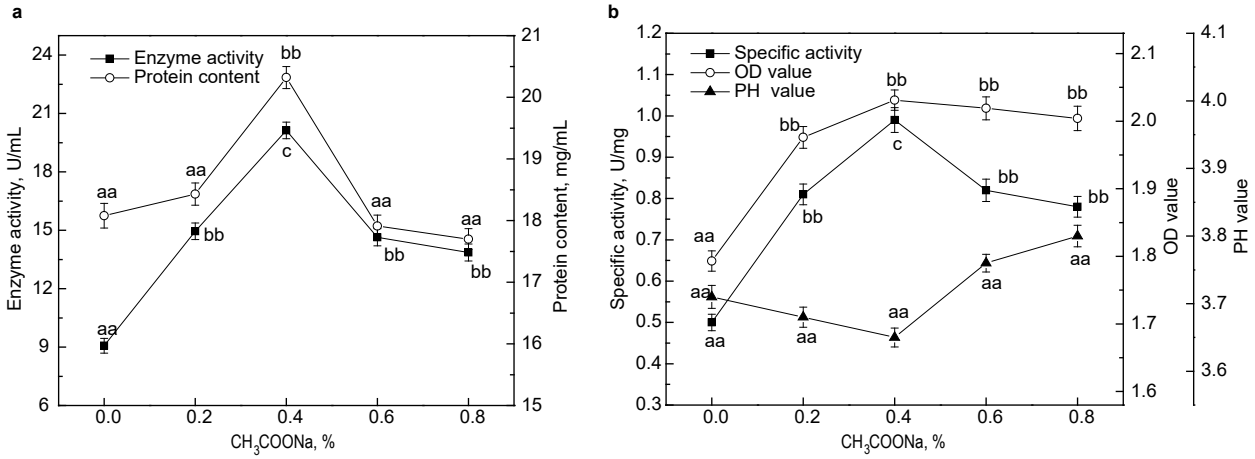

Fig. 6. The effect of CH_3COONa on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

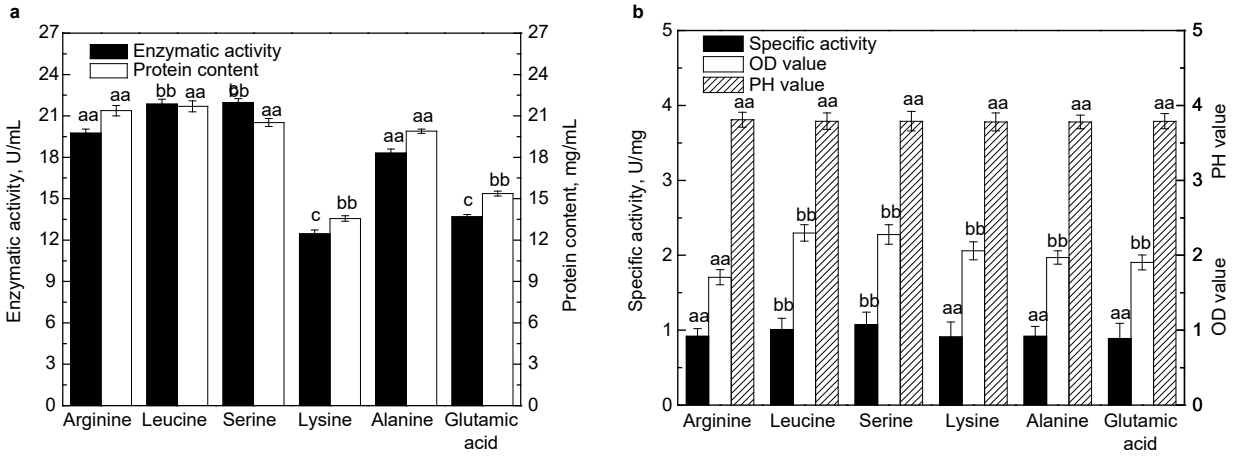

Fig. 7. The effect of different amino acids on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

specific activity first increased and then decreased ($P < 0.05$) with the increase in CH_3COONa concentration, and all reached the highest value when the concentration of CH_3COONa was 0.4%, with a maximum value of 20.13 U/mL, 20.32 mg/mL, 0.99 U/mg, respectively. The OD value and pH value also obtained good values respectively when the CH_3COONa concentration was 0.4%. Therefore, the CH_3COONa concentration of 0.4% was selected.

Wu and Pan (2013) delineated that CH_3COONa significantly improved the CEP activity of *Lactobacillus casei* DI-1 in the medium, which is in accordance with the results observed for strain LP69, but CH_3COONa in a high concentration had a passive effect on proteinase activity. Meanwhile, it was found that a suitable content of CH_3COONa could greatly improve the density of *L. plantarum* LP69. This study showed that medium composition and concentration could influence microbial growth and metabolite production (Li et al., 2006).

Effect of amino acids on *L. plantarum* LP69 producing CEP

Amino acids could induce proteinase production of bacteria. It can be seen from Figure 7 that when leucine or serine was added to the medium, *L. plantarum* LP69 had higher enzyme activity, specific activity and OD value ($p < 0.05$). There was no obvious change in the pH of medium when different amino acids were added into the MRS broth ($p > 0.05$). Therefore, leucine and serine were selected for further study.

The leucine was added to the MRS medium at 10, 15, 20, 25 and 30 mg/L. As shown in Figure 8, as the leucine concentration increased, enzyme activity, protein content, specific activity and OD value first increased and then decreased ($P < 0.05$). When the concentration of leucine was 20 mg/L, the enzyme activity, protein content and specific activity reached a maximum of 19.98 U/mL, 19.58 mg/mL and 1.02 U/mg, respectively. The OD value and pH value were taken to the maximum and minimum value when the leucine concentration was 20 mg/L. Therefore, 20 mg/L was selected as the best concentration of leucine.

The serine was added to the MRS medium at 10, 15, 20, 25 and 30 mg/L. Figure 9 shows that when the serine concentration increased from 10 mg/L to 30 mg/L, the enzyme activity, protein content and specific activity all first increased and then decreased ($P < 0.05$), and reached the maximum at a serine concentration of 20 mg/L. The maximum values were 19.39 U/mL, 20.86 mg/mL, 0.92 U/mg, respectively. The OD value and pH value also indicated that *L. plantarum* LP69 grew better at 20 mg/L serine concentration. Therefore, 20 mg/L was selected as the optimum content of serine.

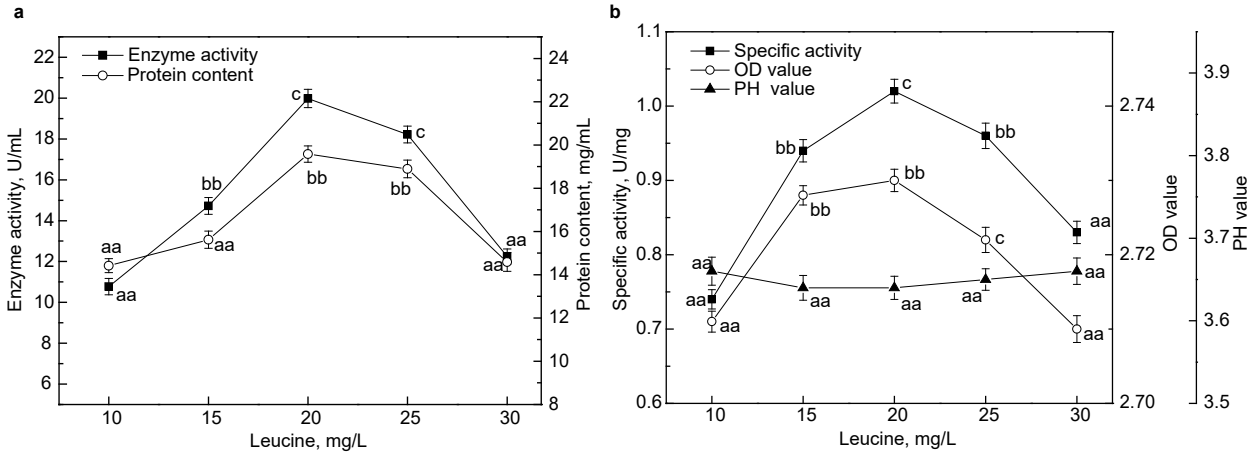

Fig. 8. The effect of leucine on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

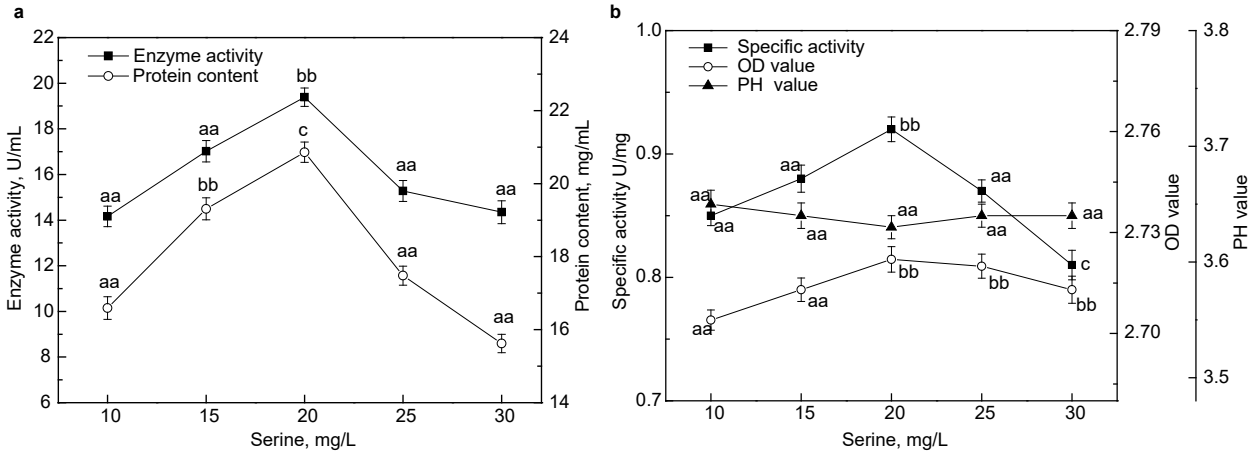

Fig. 9. The effect of serine on CEP production by *L. plantarum* LP69: **a** – enzyme activity, protein content, **b** – specific activity, OD_{600} value, pH. Mean values relating to the same series, expressed in different letters are significantly different ($p < 0.05$)

A study indicated that serine and leucine had remarkable promotion effects on the growth of *Lactobacillus bulgaricus* (An et al., 2009). The results of the present study show that serine and leucine also stimulated the growth of *L. plantarum* LP69. Fairbairn and Law (1986) reported that leucine is an effective factor to induce proteinase production. Moreover, it was found that serine had an important role in increasing the CEP activity of *Lactobacillus casei* DI-1 (Wu and Pan, 2013). These findings are identical with those observed for strain LP69. The excess accumulation of amino acids in turn reduced the protease synthesis, which failed to improve CEP production (Rahman et al., 2003).

CONCLUSIONS

The effect of prebiotics, inorganic salts and amino acids on the CEP production by *Lactobacillus plantarum* LP69 was investigated. The results showed that inulin, isomaltooligosaccharide, Na_2HPO_4, CH_3COONa, leucine and serine were superior to other selected

substances. The optimum concentration of inulin, isomaltooligosaccharide, Na_2HPO_4, CH_3COONa, leucine and serine for CEP production were 0.7%, 0.5%, 0.4%, 0.4%, 20 mg/L, 20 mg/L, respectively, and the maximum levels of CEP activity were obtained. These results provide a reference for further optimization of CEP-producing media of *L. plantarum* LP69.

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