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EFFECT OF MEDIUM COMPOSITION ON CELL ENVELOPE PROTEINASE PRODUCTION BY *LACTOBACILLUS PLANTARUM* LP69

– Research paper –

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Abstract: Cell envelope proteases (CEPs) can break down milk protein into peptides with different functions, which are of great benefit to human health. Therefore, the high-yield CEPs of *Lactobacillus plantarum* have the potential to produce functional dairy products. In previous experiments, we found that Na_2HPO_4, inulin, casein peptone and leucine have significant effects on CEP production by *Lactobacillus plantarum* LP69. So we proceeded to optimize the composition of the CEP-producing culture medium of *L. plantarum* through Box-Behnken design and response surface methodology. The protease activity, protein content and specific activity of CEPs produced by *L. plantarum* by inulin (0.2, 0.3, 0.4 %), casein peptone (0.4, 0.6, 0.8 %), Na_2HPO_4 (0.50, 0.52, 0.54 %) and leucine (14, 16, 18 mg/L) were evaluated. The optimal ratio of medium is 0.4 % inulin, 0.66 % casein peptone, 0.5 % Na_2HPO_4 and 14.04 mg/L Leucine. The final enzyme activity reached (24.46±0.81) U/mL, and the specific activity reached (1.41±0.46) U/mg.

Keywords:*Lactobacillus plantarum* LP69; cell-envelope proteinases; medium optimization; Box-Behnken design

INTRODUCTION

Lactic acid bacteria (LAB), as a large number and rich variety of probiotics in nature, are a type of microorganisms that can use carbohydrates to ferment to produce organic acids. When we add lactic acid bacteria to dairy products,it can change the texture and flavor of dairy products(Cheng.,2019,Tao et al.,2021, Spano.,2021). Lactic acid bacteria can colonize the human body, effectively inhibit the growth of harmful bacteria. Therefore, they are used as a probiotic, which can be used to prevent and treat some gastrointestinal diseases and protect the health of the host. (Tatsuya et al., 2015; Shu et al., 2016; Ren,2020). *Lactobacillus plantarum* is a common type of probiotics, which has the ability to tolerate acid and bile salts. (Ding et al.,2021). Because of its probiotic properties and health functions, *Lactobacillus plantarum* has a wide range of applications and can be used in many fields such as the environment, food, and human health (Wu et al., 2019).

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Lactic acid bacteria cell-envelope protease (CEPs) plays a very important role in the protease hydrolysis system and is the key enzyme for the hydrolysis of milk protein. It hydrolyzes proteins into short peptides, transports them into the cell, and is further hydrolyzed into amino acids or smaller peptides. (Sadat-Mekmene et al., 2011; Griffiths et al., 2013; Miyamoto et al., 2015; Borsting et al., 2015) The hydrolyzed peptide has a variety of physiological activities, such as antibacterial effect, antioxidation, decreasing blood pressure and some other immune functions (Tsai et al., 2008). Our laboratory has previously confirmed that *Lactobacillus plantarum* LP69 can ferment to produce angiotensin-I-converting enzyme (ACE) inhibitory peptides (Chen et al., 2018). However, the low yield, low recycling rate and high price of CEPs restrict their development and utilization (Agyei et al., 2014).

CEPs have attracted the attention of many people because of their huge development prospects. Current researches mostly focus on the separation and purification of cell-envelope proteases and the study of hydrolysis characteristics (Wu et al., 2011;

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Ren et al., 2013; Huang et al., 2019). Others focus on the induction of gene expression by cell-envelope proteinases (Zhang., 2021).Bacteria have very high requirements for the composition of the culture medium. They not only need basic nutrients such as carbon sources and nitrogen sources, but also other nutrients such as inorganic salts, vitamins, and amino acids (Hebert et al., 2004). Some studies have also investigated the effects of fermentation conditions on bacterial growth and CEP production, such as temperature, pH, time, and dissolved oxygen (Agyei et al., 2012a; Agyei et al., 2012b). Ren et al. (2015) found that the yield of ACE inhibitory peptides was the highest, reaching 11.75 mg/mL^{-1} at 38.8 °C, pH 8.00 and [E]/[S] ratio (w/w) of 0.132. Agyei et al. (2012c) optimized the effects of different sugars on the production of CEP by *Lactobacillus delbrueckii subsp. lactis* 313, and concluded that maltose had the greatest effect on the contrast activity. Ren (2014) conducted orthogonal experiments on the carbon source and nitrogen source of the cell-envelope proteinase produced by *Lactobacillus acidophilus* JQ-1, and found that the best combination of carbon source and nitrogen source was 2% glucose + 1% beef peptone.

In the previous work, our laboratory screened out that Na_2HPO_4, inulin, casein peptone and leucine had a significant effect on the CEP production by *Lactobacillus plantarum* LP69 through the Plackett-Burman experiment (Cheng et al., 2019a; Cheng et al., 2019b; Cheng et al., 2019 c) and optimized the fermentation condition (Zhang et al., 2021). On this basis, this experiment further screened the optimal medium formula for CEP production by *Lactobacillus plantarum* LP69 through Box-Behnken design and response surface optimization, and provided some references for subsequent industrial applications.

MATERIAL AND METHODS

Strains and cultivation

Lactobacillus plantarum LP69 was from the School of Food and Biological Engineering, Shaanxi University of Science and Technology.The strains were inoculated on the MRS broth medium, placed in a 37°C incubator for 22 hours, and transferred to the next generation at 5%. The above steps were repeated three times. All utensils were disinfected under 121°C for 15 minutes.

Obtaining of CEP fractions

After Centrifugation at 4°C and 4500r/min for 20min the pellet obtained was the bacterial cell. The cells were washed three times with 50mM Tris-HCL (pH 7.8, added with 30mM Ca^{2+}) buffer solution, and centrifuged for 20 min in a centrifuge at 4°C and 4500r/min for each wash. The supernatant was discarded, and the bacterial pellet was collected. We upended the mixed buffer (pH 7.0) including 50 mM EDTA-Na_2 and 50 mM Tris-HCL into the bacterial pellet, stired evenly on a mixer, and incubated at 37°C for 1 hour. Afterwards, the centrifuge was set to 4°C and 4500 r/min, and the supernatant obtained after centrifugation for 20 minutes was the crude CEPs.

Box-Behnken design

Box-Behnken design optimizes the optimal ratio of nutrients through experiments with enzyme activity, protein content and specific activity as response values. In the previous work, our laboratory screened out that Na_2HPO_4, inulin, casein peptone and leucine had a significant effect on the CEP production by *Lactobacillus plantarum* LP69 through the Plackett-Burman experiment (Cheng et al., 2019a; Cheng et al., 2019b; Cheng et al., 2019 c). Based on it, with inulin 0.3%, casein peptone 0.6%, Na_2HPO_4 0.52%, and leucine 16 mg/L as the center, two levels were selected to establish a four factors and three levels Box-Behnken test matrix (Table 1).

Table 1. The factor level of Box-Behnken experiment of LP69 producing CEP culture medium

Factor level	A Inulin （%）	B Casein Peptone （%）	C Na_2HP0_4 （%）	D Leucine （mg/L）
-1	0.2	0.4	0.50	14
0	0.3	0.6	0.52	16
1	0.4	0.8	0.54	18

Determination of pH and development of strain LP69

The pH of the solution was measured with a pH meter.The growth of strain LP69 was determined by turbidimetric method. The growth of the strain was obtained by measuring the absorbance (OD_{600} value) under the condition of 600 nm wavelength by SP-756PC UV spectrophotometer.

Protease activity assay

The activity of the protease was measured by the Folin method (Chen et al., 2018). The protease activity is defined as: at a pH of 7.0 and 40°C, 1μg of tyrosine produced by hydrolyzing casein per hour is a unit of protease activity.

Determination of protein content

With bovine serum albumin as the criterion, the protein amount was estimated by the Bradford means (Bradford, 1976). The absorbance at 595nm is measured and is proportional to the protein concentration within a certain range.

Calculation of specific activity

The specific activity can be calculated by the enzyme activity per unit protein content, and the formula is shown below: (Ngo et al., 2008)

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

Data processing

The experimental data is the average of three times. Through Design Expert（Version, 8.0.6）and Analysis of variance (ANOVA) to process the data, the significant difference of the value and correlation factor are obtained.

RESULTS

Box-Behnken experiment design to screen the best medium composition

Taking enzyme activity, protein content, and specific activity as the response values, referring to OD_{600} and pH values, the results are shown in Table 2.

Establishment of regression model

Utilizing the statistical software Design-Expert 8.0.6 to analyze the data in Table 2, the quadratic regression equations of the four substances on the enzyme activity、protein content and specific activity can be obtained as indicated in eqs. (2) to (6).

Variance analysis of the data

The regression equation was analyzed by variance analysis so as to verify the validity of the model.The analysis results are shown in Table 3. The results show that the p values of the regression equation model are all <0.001, indicating that the model is very significant. The p figure of the lack-of-fit item are all >0.1, which is not notable, indicating that the difference between the used model and the experiment is small.

The coefficient of determination R_1=0.9206 for enzyme activity indicates that the equation can be used to explain 92.06% of the test results, and further shows that the test results fit the equation well. It can also be seen from the table 3 that the primary term B ($p<0.0001$) is a very significant factor, which has a great contribution to the model. The first-order term A ($p=0.0046$), C ($p=0.0016$), the interactive phase AB ($p=0.0017$), the quadratic term C^2 ($p=0.0059$) and D^2 ($p=0.0047$) have their p figure less than 0.01, which is extremely important factors and have a greater contribution to the model. The correlation coefficient R_2=0.8891 for enzyme content, indicating that the equation can be used to explain 88.91% of the test results. It shows that it is feasible to use the equation to fit the impact of the four elements on the protein content, and the test error is small. Therefore, the regression equation can be used to replace the real points of the test for result analysis. It can be perceived from the table that the p value of the primary term A of the equation is less than 0.0001, indicating that the factor is extremely significant; the p figure of the primary term D is less than 0.01, showing that it has a significant impact on the protein content and has a greater effect on the model.

$$R_1=24.39+1.39A-2.96B-1.62C+0.23D+2.80AB-1.01AC+0.18AD-0.85BC+1.49BD+0.44CD+0.96 \quad (2)$$

$$A^2-0.99B^2-2.01C^2-2.08D^2 \quad (3)$$

$$R_2=21.21+11.03A-0.17B-2.09C-4.25D-5.08AB-1.89AC-3.48AD+3.37BC+2.53BD-1.94CD+3.35 \quad (4)$$

$$A^2-0.18B^2+1.02C^2-3.15D^2 \quad (5)$$

$$R_3=1.19-0.82A-0.23B+0.24C+0.34D+0.55AB-0.37AC-0.082AD-0.17BC-0.09BD+0.40CD+$$
$$+ 0.36\ A^2\ -0.20B^2-0.10C^2+0.32D^2 \quad (6)$$

where: A, B, C, and D represent inulin, casein peptone, Na_2HPO_4, and leucine, respectively,and R_1, R_2, R_3 are enzyme activity, enzyme content, and specific activity, respectively.

Table 2. The Box-Behnken experiment program and data of the medium composition of strain LP69 producing CEP

Run	A	B	C	D	OD	pH	R_1*	R_2	R_3
1	0	0	0	0	2.219	3.66	23.78	27	0.88
2	0	0	-1	-1	2.597	3.85	21.78	26.44	0.82
3	0	-1	0	-1	2.046	3.74	26.39	25.13	1.05
4	-1	-1	0	0	2.022	3.74	28.21	10.06	2.8
5	-1	0	-1	0	2.081	3.74	24.12	17.25	1.4
6	0	0	-1	1	2.174	3.7	20.14	18.76	1.07
7	1	0	0	1	2.097	3.74	26.19	29.95	0.88
8	0	0	0	0	2.212	3.65	25.58	19.31	1.32
9	-1	0	1	0	2.074	3.89	21.28	14.28	2.89
10	0	0	0	0	2.281	3.65	23.8	17.32	1.37
11	0	1	0	-1	2.215	3.68	17.07	16.26	1.05
12	0	1	0	1	1.989	3.69	20.59	15.31	1.34
13	1	0	-1	0	2.237	3.62	28.8	40.28	0.71
14	0	0	1	-1	2.226	3.65	18.67	22.49	0.83
15	-1	0	0	1	2.259	3.64	21.5	6.11	3.52
16	0	0	1	1	2.479	3.66	18.79	7.03	2.67
17	-1	0	0	-1	2.271	3.65	20.24	7.09	2.85
18	0	-1	0	1	2.233	3.61	23.94	14.08	1.7
19	0	1	-1	0	2.334	3.65	19.79	18.87	1.05
20	1	0	0	-1	2.295	3.63	24.22	44.85	0.54
21	1	1	0	0	2.291	3.66	25.19	27.73	0.9
22	0	-1	1	0	2.072	3.74	24.22	19.69	1.23
23	0	1	1	0	1.996	3.89	15.48	27.64	0.56
24	-1	1	0	0	2.084	3.88	18.23	21.78	0.84
25	0	-1	-1	0	1.925	3.64	25.14	24.39	1.03
26	1	0	1	0	2.212	3.61	21.9	29.76	0.73
27	1	-1	0	0	2.201	3.66	23.97	36.32	0.65

A, Inulin (%); B, Casein Peptone (%); C, Na_2HPO_4 (%); D, Leucine (%);*Enzyme activity, U/mL; Protein content, mg/mL; Specific activity, U/mg

Table 3. Analysis of variance (ANOVA) table

Source	DF	Enzyme activity		Enzyme content		Specific activity	
		MS	*p*-Value (Prob > F)	MS	*p*-Value (Prob > F)	MS	*p*-Value (Prob > F)
Model	14	19.15	0.0002	154.05	0.0009	1.10	0.0024
A-Inulin	1	23.21	0.0046	1459.05	< 0.0001	8.15	< 0.0001
B- Casein Peptone	1	105.14	< 0.0001	0.36	0.9012	0.62	0.1015
C- Na_2HPO_4	1	31.46	0.0016	52.50	0.1519	0.67	0.0898
D- Leucine	1	0.64	0.5738	216.92	0.0090	1.36	0.0218
AB	1	31.36	0.0017	103.12	0.0532	1.22	0.0281
AC	1	4.12	0.1692	14.25	0.4409	0.54	0.1228
AD	1	0.13	0.8024	48.44	0.1674	0.027	0.7159
BC	1	2.87	0.2454	45.36	0.1805	0.12	0.4510
BD	1	8.91	0.0526	25.50	0.3073	0.032	0.6915
CD	1	0.77	0.5379	15.13	0.4274	0.63	0.0978
A^2	1	4.92	0.1358	59.72	0.1287	0.71	0.0818
B^2	1	5.26	0.1244	0.17	0.9320	0.21	0.3184
C^2	1	21.48	0.0059	5.59	0.6266	0.054	0.6085
D^2	1	23.08	0.0047	52.88	0.1506	0.53	0.1259
Lack of fit	10	2.10	0.3843	21.69	0.6603	0.22	0.2731
Pure error	2	1.07		26.13		0.073	
R^2			0.9206		0.8891		0.8677

The coefficient of determination R_3=0.8677 for specific activity, indicating that the equation can be used to explain 86.77% of the test results, with small errors. Therefore, it is feasible to use the regression equation to fit the effect of the four elements on the specific activity. It can be seen from the table that the first order term A ($p<0.0001$) of the equation is a very significant factor, and the effect of specific activity is extremely significant. The p-values of the primary term D (p= 0.02 18) and the interaction term AB (p=0.0218) are both less than 0.05 ,indicating that they also have a significant effect on the specific activity.

Use the software Design-Expert to perform the response surface analysis of the regression model, draw contour maps and response surface maps, as shown in Figures 1 to 18.

Figures 1 to 6 are contour diagrams and 3D surface diagrams of the influence of various factors on enzyme activity according to the model equations. The contour maps in Figures 1, 2 and 3 are irregular graphs, indicating that there is no interaction between inulin and casein peptone, inulin and Na_2HPO_4, and inulin and leucine. In addition, it can be seen from the 3D surface graph that if the addition amount of casein peptone, Na_2HPO_4 and leucine remains unchanged, when the addition amount of inulin increases from 0.2 % to 0.4 %, the enzyme activity increases accordingly.The contour maps of Figures 4, 5 and 6 are all oval, indicating that there are significant interactions between casein peptone and Na_2HPO_4, casein peptone and leucine, and Na_2HPO_4 and leucine. This shows that they work together to improve enzyme activity.Their 3D images are approximately convex round, indicating that as the substances of casein peptone and Na_2HPO_4, casein peptone and leucine, Na_2HPO_4 and leucine respectively approach their center points, the enzyme activity gradually reach the maximum.

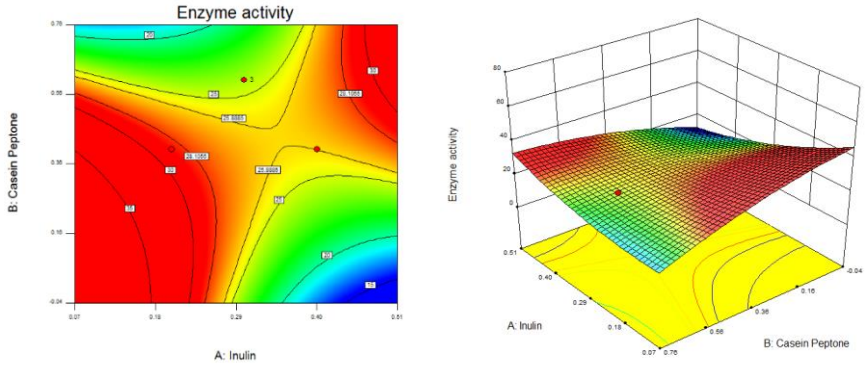

Figure 1. The 2D contour and 3D response of inulin and casein peptone on enzyme activity

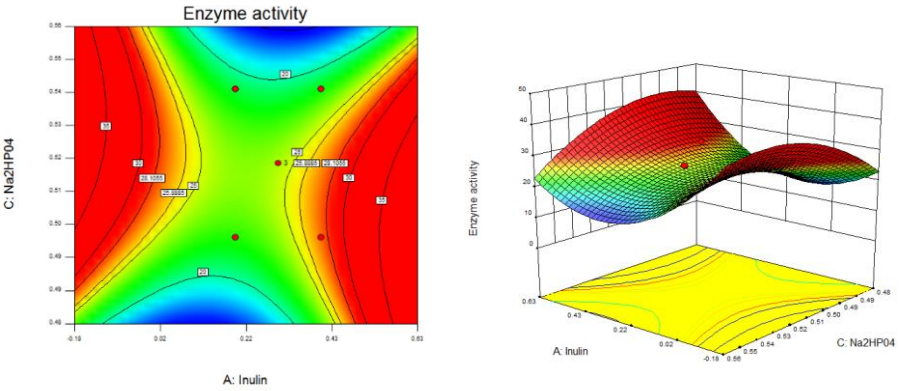

Figure 2. The 2D contour and 3D response of inulin and Na_2HPO_4 on enzyme activity

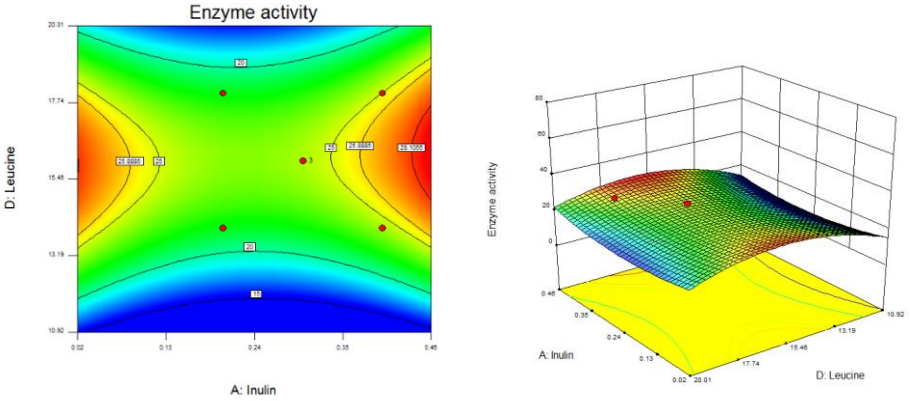

Figure 3. The 2D contour and 3D response of inulin and leucine on enzyme activity

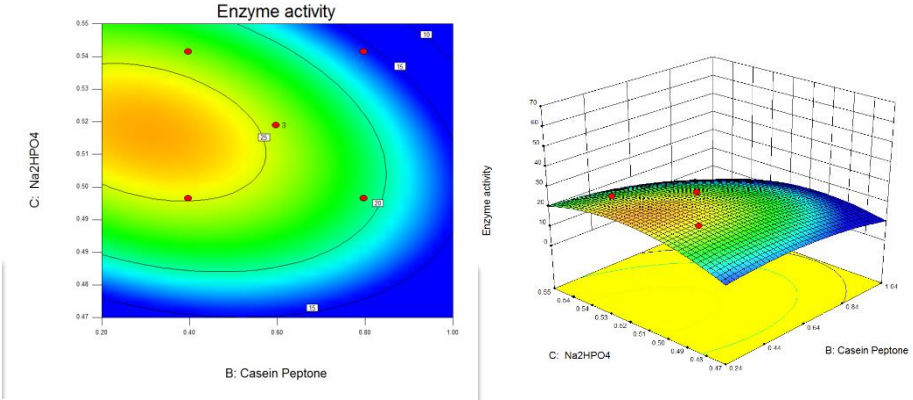

Figure 4. The 2D contour and 3D response of casein peptone and Na_2HPO_4 on enzyme activity

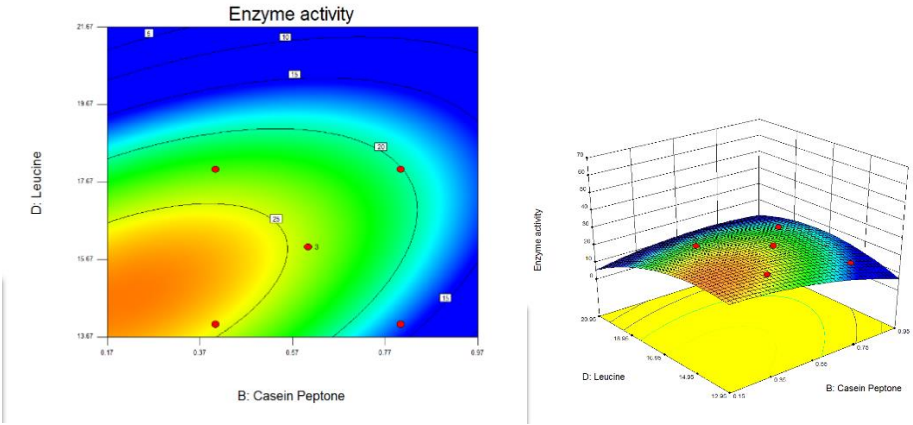

Figure 5. The 2D contour and 3D response of casein peptone and leucine on enzyme activity

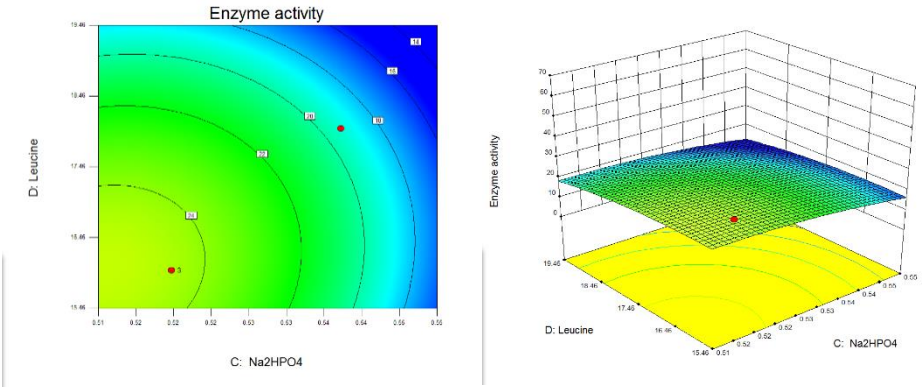

Figure 6 The 2D contour and 3D response of Na_2HPO_4 and leucine on enzyme activity

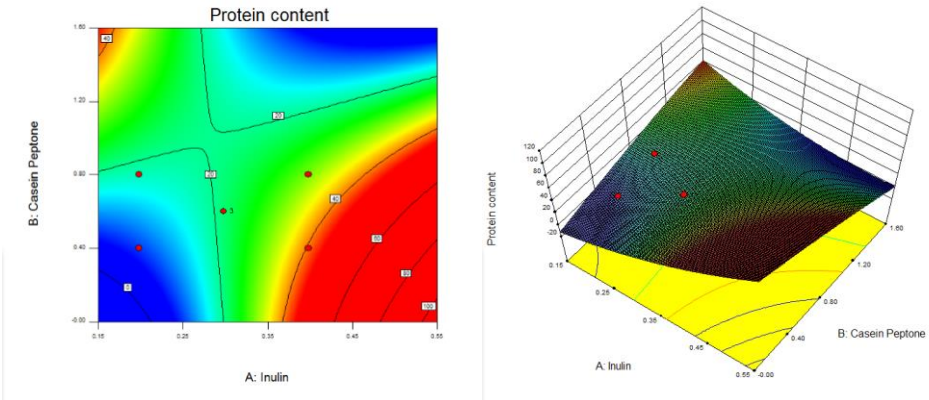

Figure 7. The 2D contour and 3D response of inulin and casein peptone on protein content

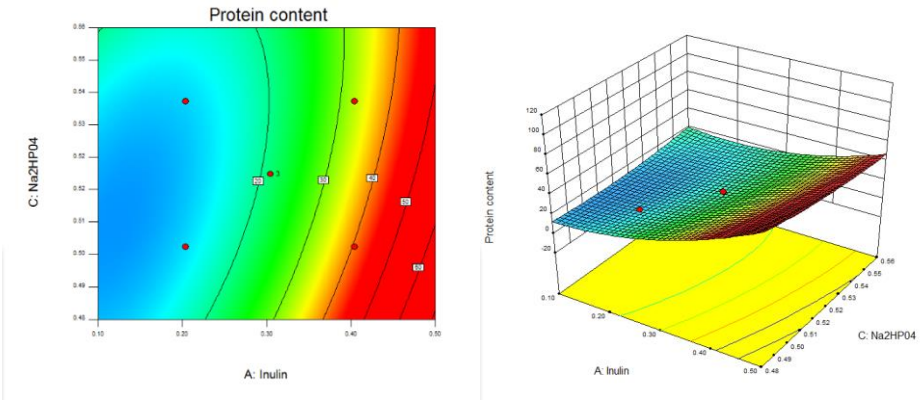

Figure 8. Th e 2D contour and 3D response of nulin and Na_2HPO_4 on protein content

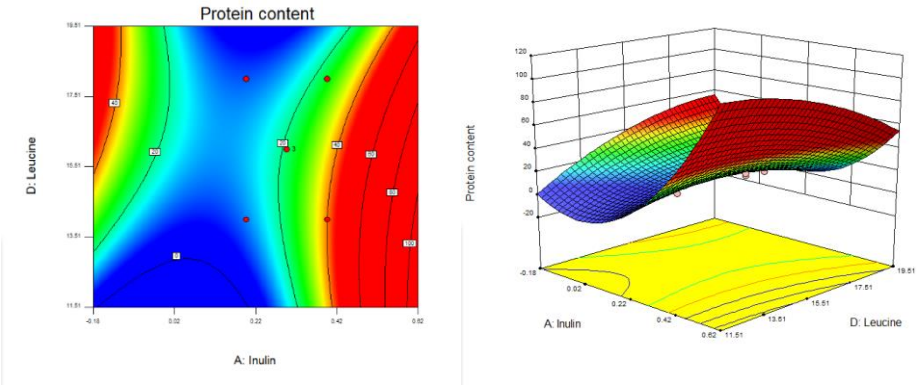

Figure 9. Th e 2D contour and 3D response of nulin and leucine on protein content

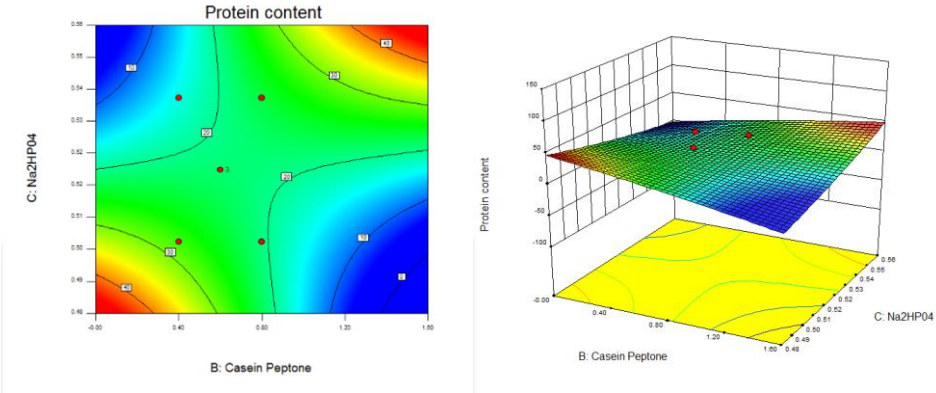

Figure 10. The e 2D contour and 3D response of casein peptone and Na_2HPO_4 on protein content

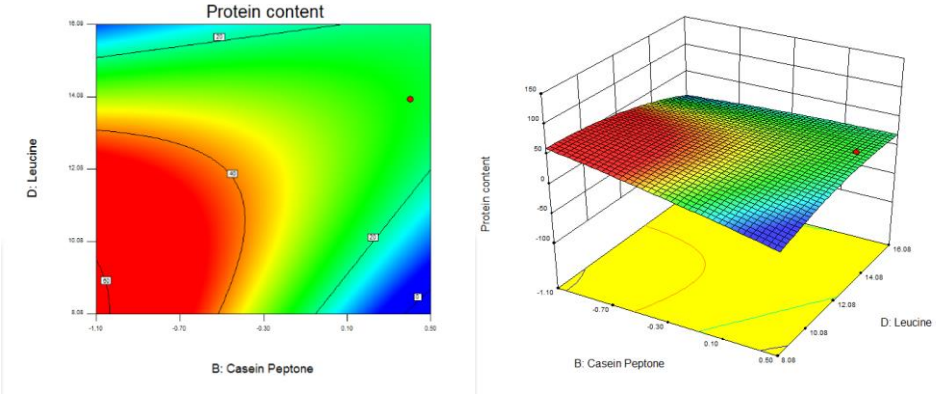

Figure 11. The e 2D contour and 3D response of casein peptone and leucine on protein content

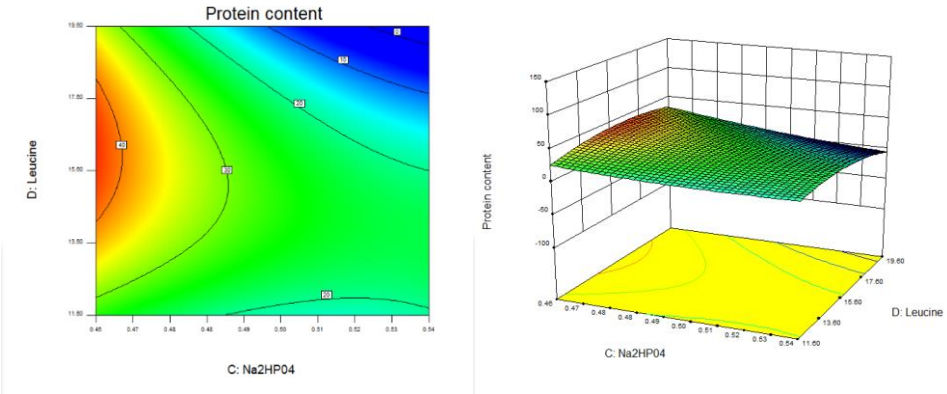

Figure 12. The 2D contour and 3D response of of Na_2HPO_4 and leucine on protein content

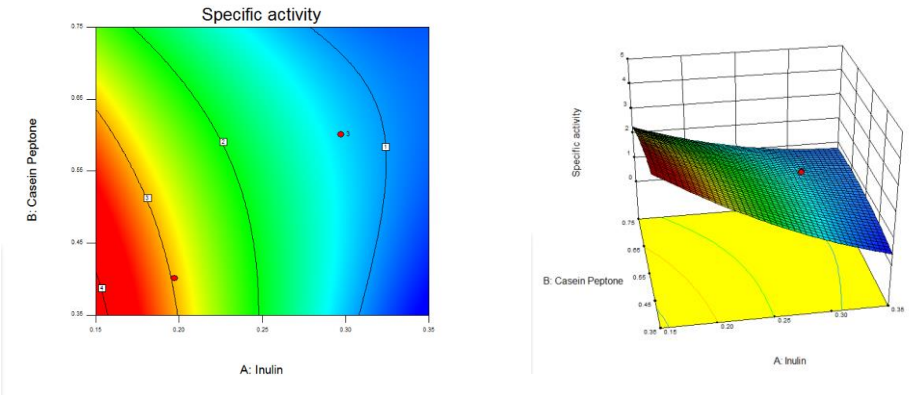

Figure 13. The 2D contour and 3D response of inulin and casein peptone on specific activity

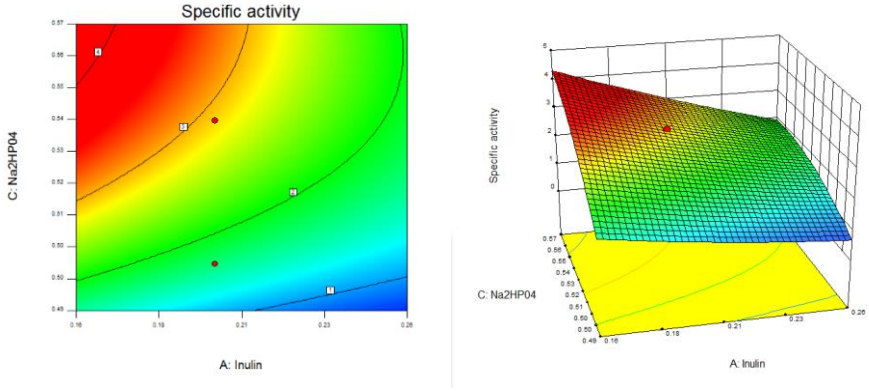

Figure 14. The 2D contour and 3D response of inulin and Na_2HPO_4 on specific activity

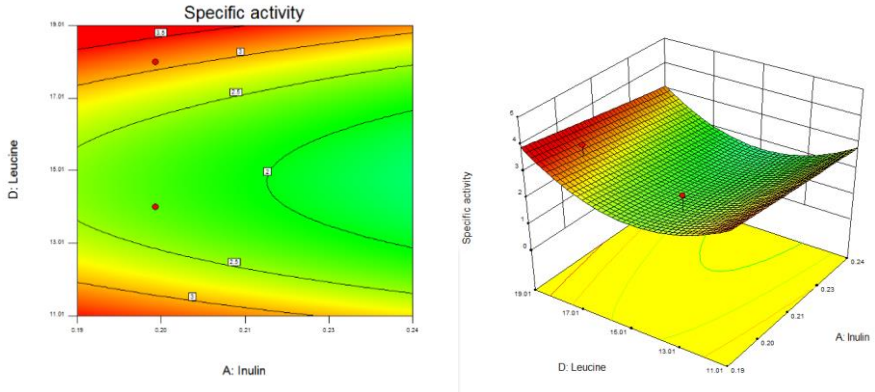

Figure 15. The 2D contour and 3D response of inulin and leucine on specific activity

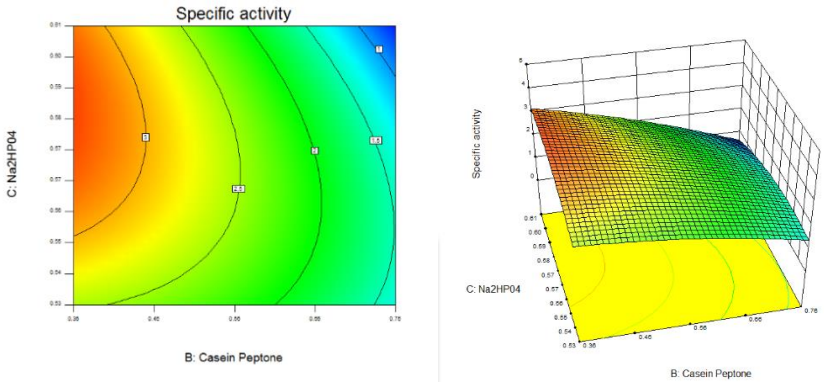

Figure 16. The 2D contour and 3D response of casein peptone and Na_2HPO_4 on specific activity

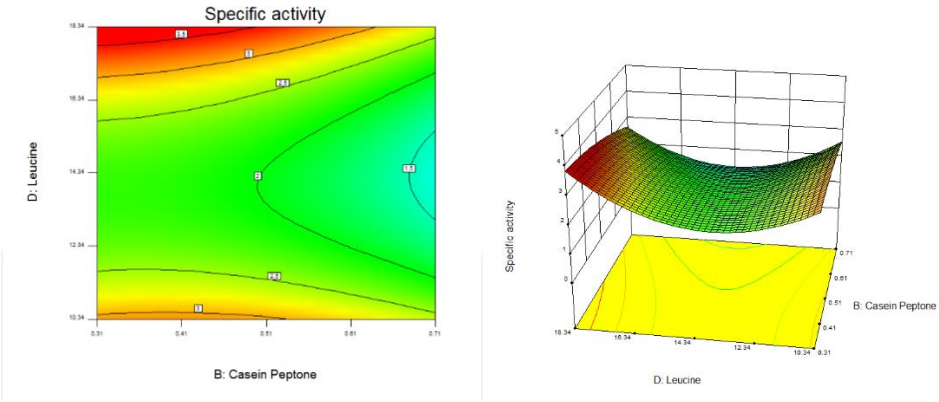

Figure 17. The 2D contour and 3D response of casein peptone and leucine on specific activity

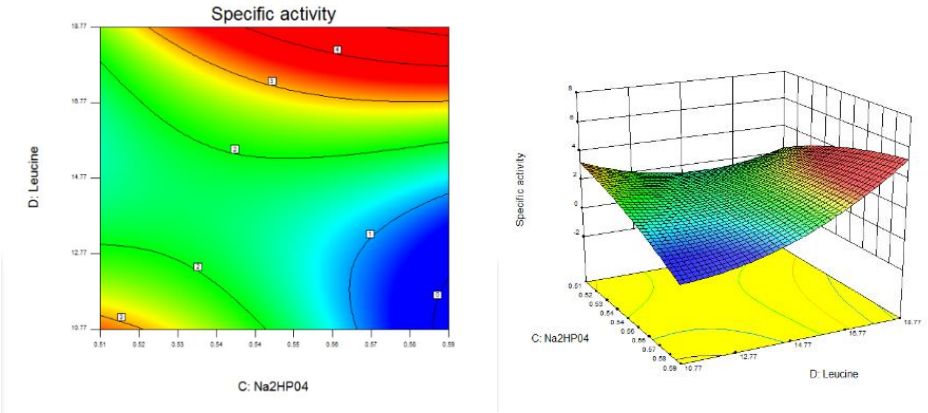

Figure 18. The 2D contour and 3D response of Na_2HPO_4 and leucine on specific activity

Figures 7 to 12 are the response surface plots and contour plots of inulin, casein peptone, Na_2HPO_4 and leucine to protein content, which can intuitively reflect the interaction between each two factors and the impact on protein content. The contour plot in Figure 8 is oval, indicating that there is an interaction between inulin and Na_2HPO_4, which co-influence the value of protein content. The contour maps of 7, 9, 10, 11, and 12 are irregular graphs, indicating that there is no interaction between inulin and casein peptone, inulin and leucine, casein peptone and Na_2HPO_4, casein peptone and leucine, and between Na_2HPO_4 and leucine. From the 3D diagrams in Figure 7 to Figure 9, when the concentration of casein peptone, Na_2HPO_4, and leucine remain unchanged, and the concentration of inulin increases from 0.2 % to 0.4 %, the protein content also increases. It can be seen from the 3D response graph in Figure 10 that keeping the concentration of casein peptone constant, as the concentration of Na_2HPO_4 increases, the protein content also increases. It can be seen from the 3D response surface graph in Figure 11 that the protein content increases with the growing of the leucine concentration when the concentration of casein peptone is fixed. The 3D response graph in Figure 12 can be obtained, keeping the concentration of Na_2HPO_4 unchanged, taking the middle value of the leucine concentration, and having the highest protein content.

Figures 13 to 18 are contour plots and response surface plots of the contrast activity between the four factors. The contour plots of figures 15 and 17 are oval, indicating that there are interactions between inulin and leucine, and between casein peptone and leucine, respectively. In 3D response surface plots 15 and 17, it can be found that when the concentration of inulin and casein peptone is constant, the concentration of leucine is in the range of 15-18, and the specific activity increases with it increases. The contour map in figure 16 is nearly circular, indicating that the interaction between casein peptone and Na_2HPO_4 is not obvious. It can be seen in 3D figure 16 that keeping the concentration of Na_2HPO_4 unchanged, the concentration of casein peptone increases, but specific activity decreases. The contour maps of figures 13, 14, and 18 are irregular graphs, indicating that there is no interaction between inulin and casein peptone, between inulin and Na_2HPO_4, or between Na_2HPO_4 and leucine. It can be seen in 3D figure 13 that when inulin and casein peptone are taken at low concentrations, the specific activity is greater. It can be seen in 3D figure 14 that keeping the concentration of inulin unchanged, the concentration of Na_2HPO_4 increases, and the

specific activity increases accordingly. It can be perceived from the 3D figure 18 that when the amount of Na_2HPO_4 remains unchanged, the specific activity increases with the increase of the leucine.

The regression equations of the three response values of enzyme activity, protein content and specific activity were analyzed by Design-expert software, and the best combination predicted was: inulin 0.4 %, casein peptone 0.66 %, Na_2HPO_4 0.5 % and leucine 14.03 mg/L.The predicted values of each result are: enzyme activity is 25.04, protein content is 39.74, and specific activity is 1.37. According to the predicted optimal combination, three repeated verification tests were carried out. The results of the verification test were that the enzyme activity was (24.46±0.81) U/mL, the protein content was (39.25±0.97) mg/mL, and the specific activity was (1.41± 0.46) U/mg. The error between the predicted value and the verified value is small, indicating that the best combination of predictions is more reliable.

DISCUSSION

The composition of the medium has a vital influence on the growth of the bacteria and the fermentation products. In addition to the indispensable carbon and nitrogen sources, a culture medium usually adds inorganic salts, amino acids, vitamins and other substances to meet the needs of the bacteria. The carbon source is generally glucose, which is easy to use by bacteria. In the early stage of our experiment, we have found that inulin is more beneficial to the production of cell-envelope protease by bacteria. Savedboworn et al. (2018) observed that adding a certain amount of inulin can provide a higher number of viable cells, which is beneficial to increase the storage stability of probiotic strains. Nitrogen sources can affect the expression of key enzyme genes in the proteolytic system of lactic acid bacteria (Griffiths et al.,2013). Casein peptone is rich in various amino acids, oligopeptides and growth factors needed for the growth of lactic acid bacteria. The bacteria can use these nitrogen sources to multiply in the early stage of growth in the medium, so that free amino acids and short peptides can accumulate, shorten the time for the bacteria to enter the logarithmic phase, and enter the stable phase ahead of schedule (Pang et al.,2018). Liu et al. (2020) found that *Streptococcus thermophilus IMAU20539* produced the largest molecular mass of EPS in M17 medium supplemented with casein peptone as the sole nitrogen source. When phosphate is added to the medium, the pH of the medium can be adjusted. Adding Na_2HPO_4 and K_2HPO_4 can promote protease production (Fang et al.,2008). Lactic acid bacteria themselves cannot directly use exogenous protein to provide nutrition for the bacteria, but can only use small peptides and free amino acids in the environment for growth and metabolism.

Gu (2000) studied a new synthetic medium (named LCX) for EPS production by *Streptococcus salivarius subsp. thermophilus*. The composition of the medium is: peptone 1.5%; tryptone 1.0%; glucose or lactose 2.0 %; K_2HPO_4 0.2 %; $MgSO_4$ 0.02 %; $MnSO_4$ 50 mg/L; Tween 80 1mL/L; sodium acetate 0.5 %; sodium glycerophosphate 1.9 %.Zhang et al. (2016) studied the medium composition of the cell-envelope protease produced by *Lactobacillus helveticus* ATCC15019, kept the other components in the MRS medium unchanged, and optimized the carbon source (glucose, lactose, sucrose) and nitrogen source (common peptone, beef peptone, soy peptone)by using an orthogonal experiment design. It was finally found that 0.5 % sucrose + 1 % beef peptone was the best combination.Wu and Pan(2013)used Plackett-Burman and Box-Behnken schemes to screen the MRS medium components that affect the production of CEP by *Lactobacillus casei* DI-1. The results showed that when 2.78 g/L of dipotassium hydrogen phosphate, 2.92 g/L of diammonium citrate and 4.36 g/L of sodium acetate were added to the MRS medium, the CEP activity was significantly increased, and the enzyme activity was 11.36 U/mL at this time, which is 63.68 % higher than the basic MRS medium. The reason for the difference between the results of this experiment and some of them may be that the selected strains are different, so the nutrient composition requirements of the medium are slightly different.

CONCLUSIONS

The experiment carried out Box-Behnken design and response surface optimization on the CEP medium formula of *Lactobacillus plantarum* LP69, seeking the best ratio of inulin, casein peptone, Na_2HPO_4, and leucine. At the same time, the effects of inulin, casein peptone, Na_2HPO_4, and leucine on enzyme activity, protease content and specific activity were also studied.In the end, the best ratio of 0.4 % inulin, 0.66 % casein peptone, 0.5 % Na_2HPO_4 and 14.04 mg/L leucine was obtained.

The final enzyme activity reached (24.46±0.81 U/Ml) and the specific activity reached (1.41±0.46) U/mg. Compared with before optimization, the enzyme activity increased by 49.2 % and the specific activity increased by 120 %.This provides some references for the development and utilization of CEP in the future.

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