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ORIGINAL RESEARCH PAPER

SCREENING OF THE MAIN NUTRIENTS FOR CELL ENVELOPE PROTEINASES PRODUCTION BY *Lactobacillus plantarum* LP69 USING PLACKETT-BURMAN DESIGN

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In order to select the most important nutrients that influence the production of cell-envelope proteinase (CEP), Plackett–Burman design was employed to assess the effects of glucose, maltose, peptone, casein peptone, inulin, isomaltooligosaccharide, Na_2HPO_4, CH_3COONa, leucine, and serine on the activity of CEP, protein content, specific activity, optical density (OD_{600}) value and pH in MRS broth fermented by *Lactobacillus plantarum* LP69. The results revealed that Na_2HPO_4, inulin, casein peptone and leucine significantly affect ($P<0.05$) the production of CEP. Additionally, Na_2HPO_4, inulin and leucine were negatively correlated with the enzyme activity and the specific activity, while casein peptone was positively correlated with the enzyme activity and the specific activity.

Keywords: Lactobacillus plantarum LP69, cell-envelope proteinases, medium optimization, Plackett–Burman design

Introduction

For decades, lactic acid bacteria (LAB) have been used safely in the enzymes production for proteolysis in order to generate ideal flavour and texture in the fermented dairy products. Cell-envelope proteinases (CEP) are a type of extracellular proteases essential produced by LAB and has the ability to degrade different parts of casein into peptides (Borsting *et al.,* 2015). These peptides, as particular protein fragments, express multiple physiological activities such as immunomodulatory, antioxidative, antihypertensive, antibacterial and antithrombus, with therapeutic roles in body systems (Tsai *et al.,* 2008). In the recent years, the application of CEPs in releasing bioactive peptides has gain considerable interests in the scientific community because of the functional roles.

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However, the lower yield and enzyme activity, poor stability, lack of recycling and high costs (Agyei *et al.,* 2014) of CEP hinder its deep utilization.

The CEPs exhibit numerous development prospects in exploiting and manufacturing health products and special functional food. Such developments will rely on optimizing of the process conditions crucial for the CEP production. The isolation and characterization of CEP from some lactobacilli species have been described in *Lactobacillus delbrueckii* (Tsakalidou *et al.,* 1999), *Lactobacillus rhamnosus* (Sánchez *et al.,* 2009) and *Lactobacillus casei* (Xing *et al.,* 2012). Some studies reported that culture conditions such as pH, temperature, carbon/nitrogen ratio and dissolved oxygen affect the microbial growth and CEP production for some lactobacilli (Agyei *et al.,* 2012a; Agyei *et al.,* 2012b). Agyei and Danquah (2012c) investigated the effect of different sugars on CEP production by *L. delbrueckii* subsp. *lactis* 313, and found that maltose showed the highest specific proteinase yield of 12.59 U/mg. By exploring the effect of carbohydrates, nitrogen sources and amino acids on CEP activity of *L. delbrueckii* subsp. *lactis* CRL 581, Hebert *et al.* (2008) observed the maximum activity in a basal minimal defined medium. Wu and Pan (2013) analysed the effects of basal MRS medium ingredients on CEP production of *Lactobacillus casei* DI-1 by Plackett-Burman and Box-Behnken design, and concluded that K_2HPO_4, CH_3COONa, L-serine and L-isoleucine significantly increased CEP activity. However, scientific information is limited on culture conditions optimization of *L. plantarum* to aid CEP production and improve their activity.

Lactobacillus plantarum is considered as a versatile LAB, which participates in food fermentation and can survive in the harsh conditions of human intestinal tract. Some advances have been made in the genomics and functional probiotic attributes of *L. plantarum* (Guidone *et al.,* 2014; Behera *et al.,* 2018). At present, many studies have described that *L. plantarum* has multiple physiological characters including immune adjustment and gastrointestinal disease regulation, and thus have a widespread use in food, feed, medical care and other fields (Wu *et al.,* 2019). Previously, our laboratory had elucidated that *L. plantarum* LP69 had great potential to produce angiotensin-I-converting enzyme (ACE, peptidyldipeptide hydrolase)-inhibitory peptides during goat milk fermentation (Chen *et al.,* 2018a), and also CEPs (Chen *et al.,* 2018b).

In previously work, our lab screened substances that have important impact on CEP production of *L. plantarum* LP69 from carbon sources, nitrogen sources, prebiotics, inorganic salts, amino acids, and determined the optimal concentration of these substances by single-factor experiments. Based on these studies, the objective of this project was to further screen the important nutrients from different substances (glucose, maltose, peptone, casein peptone, inulin, isomaltooligosaccharide, Na_2HPO_4, CH_3COONa, leucine, serine) using Plackett-Burman design to achieve higher CEP activity by fermentation with *L. plantarum* LP69.

Materials and methods

Microbial strains

Lactobacillus plantarum LP69, isolated from fermented bovine milk, was provided by the School of Food and Biological Engineering, Shaanxi University of Science & Technology. Before fermentation, the strain was activated in de Man, Rogosa and Sharpe (MRS media) with 5% inoculum and incubated at 37°C for 22 h, under anaerobic conditions.

Harvesting of CEP fractions

Based on the ingredients of a standard MRS broth, the medium composition was varied according to the Plackett-Burman experiment design. The 5% (v/v) of the active strain, *L. plantarum* LP69, was transfered for into a modified MRS medium in anaerobic tube and cultured at 37°C for 22 h. Fresh cells were harvested by centrifugation (4500 rpm, 20 min, 4°C), and then rinsed three times with 50 mM Tris-HCL buffer solution (pH 7.8) supplemented with 30 mM $CaCl_2$. Washed sediments were resuspended in 50 mM Tris-HCl buffer (pH 7.0) in the presence of 50 mM EDTA-Na_2 and incubated for 1 h at 37°C. The clear supernatant obtained after centrifugation at 4500 rpm, for 15 min, at 4°C, was designated as the CEP fractions and used for further analysis, such as enzyme activity, protein contents and specific activity.

Culture pH and strain LP69 growth assay

The pH was determined with a PHS-3C pH meter. The growth of *L. plantarum* LP69 was analysed by measuring the optical density at 600 nm with SP-756PC ultraviolet spectrophotometer (Shanghai Spectrum Instruments Co., Ltd, Shanghai).

Assay of proteinase activity

Proteinase activity was measured according to Folin method with modifications (Agyei *et al.,* 2015). One mL of crude CEPs was mixed with 1 mL of substrate solution (50 mM sodium phosphate buffer with 2 mg/mL casein; pH 7.0) and incubated 10 min at 40℃, after which the reaction was stopped by adding 2 mL of 65.4 g/L trichloroacetic acid (TCA). After centrifugation (6000 rpm, 5 min), 1 mL of supernatant, mixed with 1 mL of 2.0 N Folin-Ciocalteu's reagent (Shanghai Yubo biotech Co., Ltd, Shanghai) and 5 mL of 42.4 g/L Na_2CO_3 were heated in water 40°C for 20 min. The content of tyrosine was evaluated by measuring the absorbance at 680 nm. One unit of enzyme activity is defined as the produced of 1.0 micrograms of tyrosine after 60 min of casein hydrolysis at pH 7.0 and 40°C.

Protein assay

Protein contents were measured by Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Measurement of specific activity

The formula for determining the specific activity is as follows (Ngo *et al.,* 2008).

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

Plackett-Burman design

Plackett-Burman design was used to identify the nutrients which influence CEP production by fermentation with *L. plantarum* LP69 in MRS broth, while the enzyme activity, protein content and specific activity were analysed. Based on the results of our previous research, all ten factors (glucose, maltose, casein peptone, peptone, inulin, isomaltooligosaccharide, Na_2HPO_4, CH_3COONa, leucine, serine) were tested at two levels (Table 1). As shown in Table 2, the eleven independent variables (including a dummy term (E) for estimating standard deviation) were screened in twelve trials. All experiments were carried out in triplicate and the averages were taken as results.

Table 1. The variables and levels used in Plackett-Burman design for screening medium ingredients influencing the CEP production

Variables	Medium ingredients	Lower level (-1)	Higher level (+1)
A	Glucose (%)	2	2.4
B	Maltose (%)	2	2.4
C	Peptone (%)	1.0	1.2
D	Casein Peptone (%)	1.0	1.2
F	Inulin (%)	0.5	0.6
G	Isomaltooligosaccharide (%)	0.5	0.6
H	Na_2HPO_4 (%)	0.40	0.48
J	CH_3COONa (%)	0.40	0.48
K	Leucine (mg/L)	20	24
L	Serine (mg/L)	20	24

Statistical Analysis

The experimental data was analysed using Design-Expert (Version, 8.0.6) to determine the significant variables and corresponding coefficients. Data from three replicated trials for each treatment are expressed as means with standard deviation (Mean±SD). The Analysis of variance (ANOVA) was used to evaluate the significant differences among the values ($P < 0.05$).

Results and discussion

The experimental design for screening of the significant factors

The experimental design matrix and the results were showed in Table 2 and Table 3. The enzyme activity, protein content and the specific activity were used as the responses, while the OD_{600} and pH values were used as reference items.

Effect of the main factors on CEP activity of L. plantarum LP69

ANOVA was conducted to evaluate the effect of each factor on CEP activity of *L. plantarum* LP69 (Table 4). It can be seen from Table 4 that the *p*-value of 0.0458 showed that the model was significant. The relative importance of the variables on

enzyme activity was found as follows: F> B> K> L> J> C> D= G> A> H. Results of ANOVA test showed that inulin (F) (p=0.0197), maltose (B) (p=0.0282), leucine (K) (p=0.0327) and serine (L) (p=0.0439) were the most significant variables affecting the enzyme activity. Among the remaining factors, CH_3COONa (J) (p=0.0481) and peptone (C) (p=0.0486) were significant factors, while casein peptone (D) (p=0.0577), isomaltooligosaccharide (G) (p=0.0577), glucose (A) (p=0.0656) and Na_2HPO_4 (H) (p=0.0846) were important factors.

Table 2. The design matrix and experimental results of Plackett-Burman for CEP production with *L. plantarum* LP69

Run	A	B	C	D	E	F	G	H	J	K	L	R1*	R2	R3
1	1	-1	-1	-1	1	-1	1	1	-1	1	1	16.39	15.79	0.98
2	1	-1	1	1	-1	1	1	1	-1	-1	-1	12.78	15.22	0.82
3	-1	-1	-1	1	-1	1	1	-1	1	1	1	14.21	14.68	1.17
4	-1	-1	1	-1	1	1	-1	1	1	1	-1	10.18	16.81	0.87
5	1	1	-1	-1	-1	1	-1	1	1	-1	1	17.15	18.64	0.85
6	-1	1	1	-1	1	1	1	-1	-1	-1	1	18.01	17.75	0.95
7	1	1	-1	1	1	1	-1	-1	-1	1	-1	17.00	15.29	1.12
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	19.07	19.09	1.10
9	-1	1	-1	1	1	-1	1	1	1	-1	-1	20.53	18.00	0.90
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	20.19	19.13	0.97
11	1	1	1	-1	-1	-1	1	-1	1	1	-1	15.00	15.85	1.13
12	-1	1	1	1	-1	-1	-1	1	-1	1	1	21.14	19.00	0.99

A, Glucose (%); B, Maltose (%); C, Peptone (%); D, Casein Peptone (%); E: dummy factor; F, Inulin (%); G, Isomaltooligosaccharide (%); H, Na_2HPO_4 (%); J, CH_3COONa (%); K, Leucine (%); L, Serine (%);*Enzyme activity, U/mL; Protein content, mg/mL; Specific activity, U/mg

Table 3. The design matrix and experimental results of Plackett-Burman for growth of *L. plantarum* LP69

Run	A	B	C	D	E	F	G	H	J	K	L	OD	PH
1	1	-1	-1	-1	1	-1	1	1	-1	1	1	2.161	3.59
2	1	-1	1	1	-1	1	1	1	-1	-1	-1	2.179	3.59
3	-1	-1	-1	1	-1	1	1	-1	1	1	1	2.303	3.68
4	-1	-1	1	-1	1	1	-1	1	1	1	-1	2.337	3.68
5	1	1	-1	-1	-1	1	-1	1	1	-1	1	2.154	3.66
6	-1	1	1	-1	1	1	1	-1	-1	-1	1	2.178	3.62
7	1	1	-1	1	1	1	-1	-1	-1	1	-1	2.283	3.70
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	2.148	3.72
9	-1	1	-1	1	1	-1	1	1	1	-1	-1	2.162	3.68
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.250	3.69
11	1	1	1	-1	-1	-1	1	-1	1	1	-1	2.425	3.71
12	-1	1	1	1	-1	-1	-1	1	-1	1	1	2.194	3.67

A, Glucose (%); B, Maltose (%); C, Peptone (%); D, Casein Peptone (%); E: dummy factor; F, Inulin (%); G, Isomaltooligosaccharide (%); H, Na_2HPO_4 (%); J, CH_3COONa (%); K, Leucine (%); L, Serine (%)

Table 4. ANOVA table for the effect of various factors on R_1 (enzyme activity)

Source	SS	DF	MS	F-Value	*p*-Value	Significance
Model	121.08	10	12.11	288.23	0.0458	*
A- Glucose	3.93	1	3.93	93.63	0.0656	
B- Maltose	21.36	1	21.36	508.47	0.0282	*
C- Peptone	7.19	1	7.19	171.20	0.0486	*
D- Casein Peptone	5.08	1	5.08	121.00	0.0577	
F- Inulin	44.05	1	44.05	1048.48	0.0197	*
G-Isomaltooligosaccharide	5.08	1	5.08	121.00	0.0577	
H- Na_2HPO_4	2.35	1	2.35	55.93	0.0846	
J- CH_3COONa	7.32	1	7.32	174.17	0.0481	*
K- Leucine	15.89	1	15.89	378.33	0.0327	*
L- Serine	8.82	1	8.82	210.05	0.0439	*

**p<0.05, significant*

Effect of the main factors on protein content of L. plantarum LP69

The Table 5 displayed the ANOVA of the nutrients for the amount of CEP produced by *L. plantarum* LP69. The *p*-value of 0.0267 revealed that the model was significant. The relative importance of the variables on protein content was found as follows: G> K> F> A> L> B> D> C> H> J. Among these factors, isomaltooligosaccharide (G) (p=0.0125), leucine (K) (p=0.0128), inulin (F) (p=0.0158) and glucose (A) (p=0.0243) exhibited huge influence on protein concentration, which were all significant factors. In addition, serine (L) (p=0.0287), maltose (B) (p=0.0351) and casein peptone (D) (p=0.0496) were also significant factors in this test, while peptone (C) (p=0.0609) and Na_2HPO_4 (H) (p=0.0796) were important factors. The CH_3COONa was not a significant factor for the model since the *p*-value was greater than 0.1000

Table 5. ANOVA table for the effect of various factors on protein content

Source	SS	DF	MS	F-Value	*p*-Value	Significance
Model	31.32	10	3.13	852.27	0.0267	*
A- Glucose	2.51	1	2.51	683.45	0.0243	*
B- Maltose	1.21	1	1.21	329.16	0.0351	*
C- Peptone	0.40	1	0.40	108 .76	0.0609	
D- Casein Peptone	0.60	1	0.60	164.08	0.0496	*
F- Inulin	5.98	1	5.98	1626.78	0.0158	*
G-Isomaltooligosaccharide	9.49	1	9.49	2581.61	0.0125	*
H- Na_2HPO_4	0.23	1	0.23	63.24	0.0796	
J- CH_3COONa	0.066	1	0.066	17.96	0.1475	
K- Leucine	9.03	1	9.03	2457.33	0.0128	*
L- Serine	1.80	1	1.80	490.31	0.0287	*

**p<0.05, significant*

Effect of the main factors on specific activity of L. plantarum LP69

Table 6 showed the variance analysis of various factors on specific activity. The model *p*-value of 0.0181 indicated that the experimental model is remarkable. Through analysing these factors, the relative importance of the variables was as follows: H> K> D> F> L> J> A> C> G> B. Therefore, Na_2HPO_4 (H) (p=0.0062), leucine (K) (p=0.0095), casein peptone (D) (p=0.0182) and inulin (F) (p=0.0219) all were significant factors, which exerted more significant influence on specific activity. Moreover, the *p*-values of serine (L) (p=0.0277), CH_3COONa (J) (p=0.0335), Glucose (A) (p=0.0424) and peptone (C) (p=0.0489) were all between 0.01 and 0.05, which showed that they were all significant factors. The isomaltooligosaccharide and maltose were not significant variables.

Table 6. ANOVA table for the effect of various factors on the specific activity

Source	SS	DF	MS	F-Value	*p*-Value	Significance
Model	0.15	10	0.015	1848.2	0.0181	*
A- Glucose	0.001875	1	0.001875	225	0.0424	*
B- Maltose	0.000075	1	0.000075	9	0.2048	
C- Peptone	0.0014083	1	0.001408	169	0.0489	*
D- Casein Peptone	0.0102083	1	0.01	1225	0.0182	*
F- Inulin	0.0070083	1	0.007008	841	0.0219	*
G- Isomaltooligosaccharide	0.0002083	1	0.000208	25	0.1257	
H- Na_2HPO_4	0.0884083	1	0.088	10609	0.0062	**
J- CH_3COONa	0.0030083	1	0.003008	361	0.0335	*
K- Leucine	0.0374083	1	0.037	4489	0.0095	**
L- Serine	0.0044083	1	0.004408	529	0.0277	*

***p<0.01, very significant; *p<0.05, significant*

By analysing the variances of different factors on the three response values of enzyme activity, protein content and specific activity, it is observed that the four major factors affecting enzyme activity are inulin, maltose, leucine and serine. Isomaltooligosaccharide, leucine, inulin and glucose exert the most significant effects on protein content. For specific activity, Na_2HPO_4, leucine, casein peptone and inulin are the most remarkable factors. Furthermore, the four factors of Na_2HPO_4, leucine, casein peptone and inulin, which significantly affected the specific activity, were also significant or important factors for enzyme activity and protein content. Therefore, the Na_2HPO_4, leucine, casein peptone and inulin were selected as the key factors for subsequent testing.

Effect of main nutrition factors on CEP production by L. plantarum LP69

Figures 1, 2 and 3 showed the effects of Na_2HPO_4, leucine, casein peptone and inulin on enzyme activity, protein content and specific activity, respectively. It can be observed that Na_2HPO_4 has a positive effect on protein content, but has a negative effect on enzyme activity and specific activity. Casein peptone showed a

negative effect on protein content and positive effect on enzyme activity and specific activity. Inulin and leucine were all negatively correlated with enzyme activity, protein content and specific activity. Based on the results of the Plackett-Burman test and the key factor analysis, Na_2HPO_4, leucine, casein peptone and inulin were further approximated to the corresponding response levels by steepest ascent experiment.

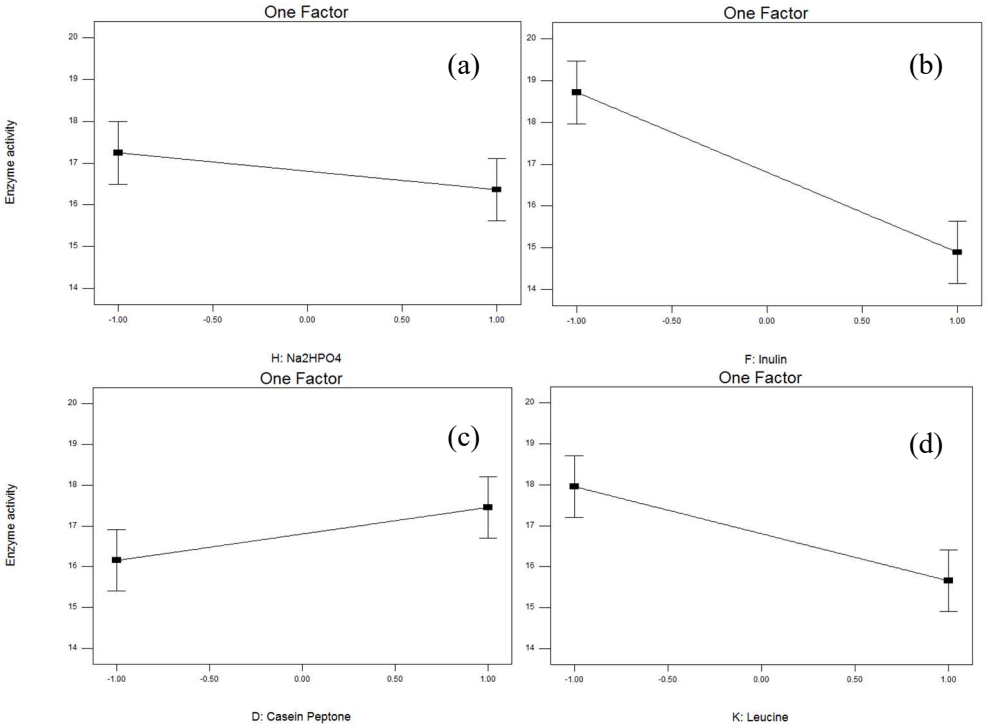

Figure 1. The 95% confidence interval showing the effect of Na_2HPO_4 (a), inulin (b), casein peptone (c) and Leucine (d) of CEP-producing medium components on enzyme activity

Medium compositions appreciably influence microbial proliferation and products synthesis. The complex nutrients available in media, such as carbohydrates, nitrogen sources, minerals and amino acid, can effectively regulate cell growth and fermentation production by changing the content and type of these components (Danquah *et al.,* 2007). Fang *et al.* (2008) reported that Na_2HPO_4 can remarkably improve the activity of thermoduric protease produced by lactic acid bacteria, which is in agreement with the result observed for CEP production. This may be that pH after fermentation affects proteinase activity of lactobacilli, and phosphates play a role in regulating pH in the medium. In addition, phosphates could affect the size of cells, which affect the growth rate of microorganisms. Excessive phosphate content in the culture medium would increase cells osmotic pressure, suppress cell growth and reduce enzyme activity of bacteria.

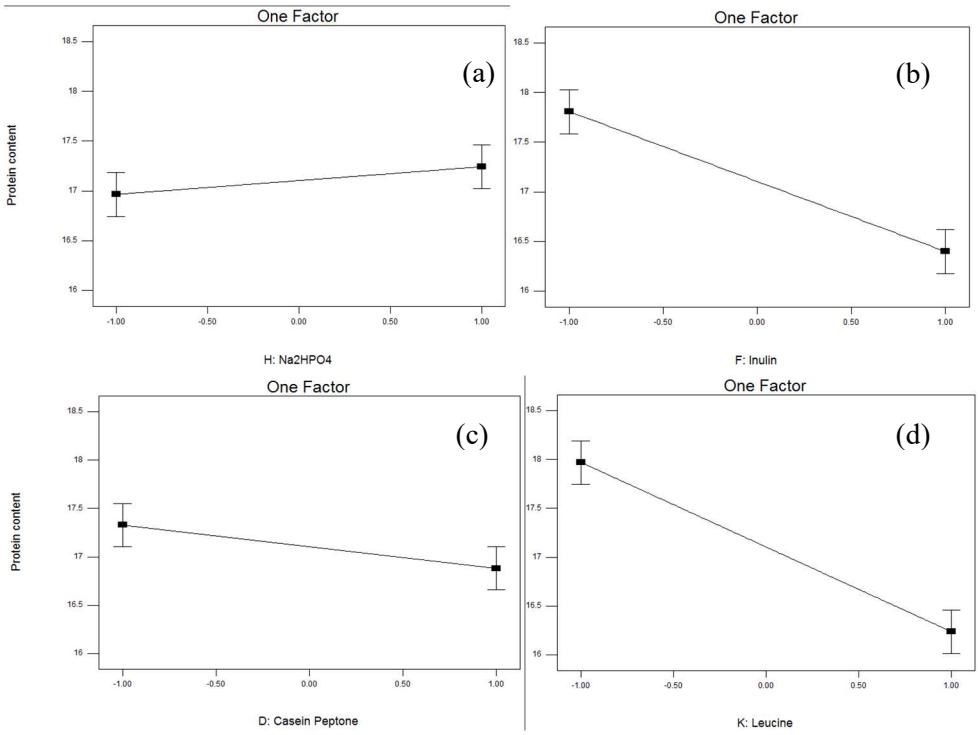

Figure 2. The 95% confidence interval showing the effect of Na_2HPO_4 (a), inulin (b), casein peptone (c) and Leucine (d) of CEP-producing medium components on protein content

Inulin served as prebiotic to stimulate the growth and vitality of *L. plantarum*, and the addition of inulin enhanced the levels of all main metabolic end-products (Oliveira *et al*., 2012). Da Silva Sabo *et al*. (2015) suggested that the presence of 1% inulin in MRS medium increased the maximum specific growth rate of *L.plantarum* ST16Pa, while Savedboworn *et al*. (2018) observed the addition of 2% inulin obtained the highest viable count of *L. plantarum* TISTR 2075. This indicated that the content of inulin added to the medium depending on the strain of *L. plantarum* (Huebner *et al*., 2007). A study also reported that inulin had ability to protect probiotics from the damaging effects of bile acids/salts, and thus achieved growth of the probiotic cultures (Adebola *et al.,* 2014). Inulin is used as an effective protecting agent for cell membranes, and the high enzyme activity observed for inulin could be accounted for the action of inulin in maintaining the morphological integrity of the bacterial cell envelope. In our results, inulin showed significant impact on the growth and CEP production of *L. plantarum* LP69, but the CEP activity was reduced and the inhibitory effect was concentration dependent. This could be because inulin in high concentrations inhibited the growth of *L. plantarum* LP69, and fructooligosaccharides released from partial inulin hydrolysis likely induced upregulation of genes involved in primary

metabolism and regulated negatively genes for protein formation and cell wall production (Saulnier *et al*., 2007).

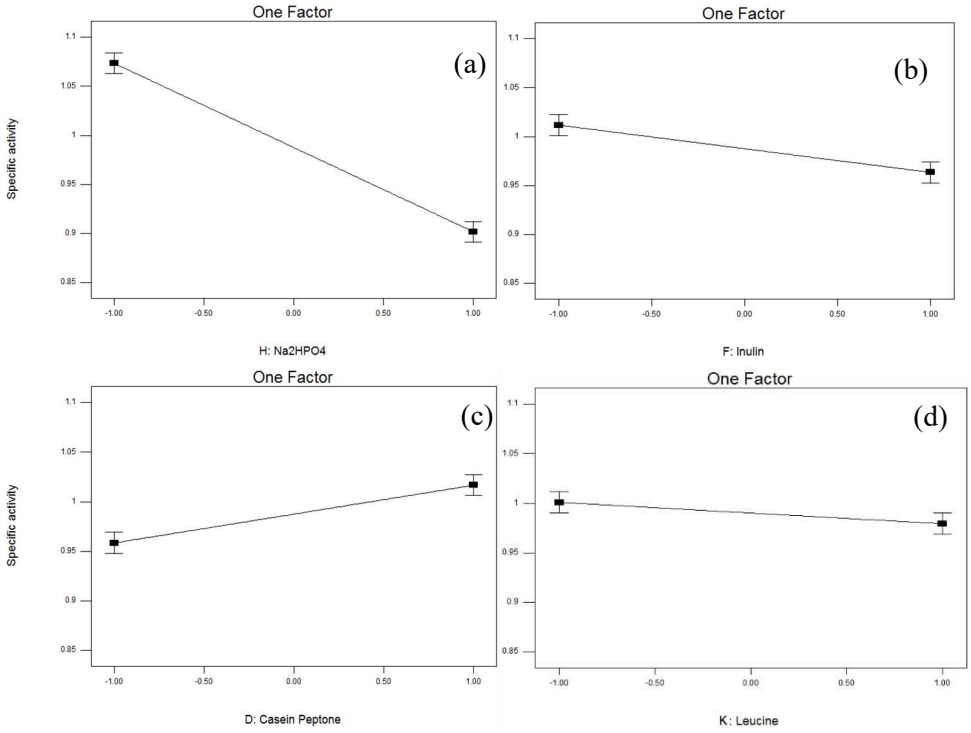

Figure 3. The 95% confidence interval showing the effect of Na_2HPO_4 (a), inulin (b), casein peptone (c) and Leucine (d) of CEP-producing medium components on specific activity

Casein peptone is commonly used as components in fermentation medium for production of starter cultures in dairy industry. Because these peptone hydrolysate contain abundant free amino acids and small peptides (Kevvai *et al.,* 2014), which could improve the cell growth and protein expression. Upon addition of a soy peptone to the cultivation medium of CHO cells, an increased protein specific productivity was observed. It was found that the peptone increased overall protein translation and recombinant protein secretion at the cellular and molecular levels (Michiels *et al.,* 2011). The peptide content in culture medium can regulate CEP activity of lactobacilli (Hebert *et al.,* 2002). A study reported that the modified MRS medium containing 1% beef peptone can appreciably increase CEP activity of *Lactobacillus acidophilus* (Ren *et al.,* 2014). In this study, casein peptone had remarkable effects on the CEP activity of *L. plantarum* LP69 as an important nitrogen source, which may be that nitrogen-derived substances have essential roles in promoting microbial growth and constituting the bacterial cell wall.

The provision of amino acids from growth medium satisfies the nitrogen requirements for biomass synthesis. Shu *et al.* (2017) reported that leucine had

remarkable influence on the growth and survival rate of *Lactobacillus bulgaricus* LB6, and leucine was positively correlated with growth of this strain. Another research showed that the selected lactobacillus strains had no growth when removed leucine from the medium, which suggested leucine was an essential factor for microbial growth (Hebert *et al.,* 2004). This may have relation to the deletion of genes for the synthesis of amino acids in some bacteria (Kleerebezem *et al.,* 2003). As with other lactic acid bacteria, *L. plantarum* LP69 has limited capacity to synthesize amino acids and therefore relies on the utilization of exogenous nitrogen sources for better growth and CEP production. In this study, leucine as an additional nitrogen sources showed a significant reduction in CEP activity of *L. plantarum* LP69, and the inhibitory effect was concentration dependent. This may be due to the high concentration of leucine selected in this experiment, which repressed the cell growth and decreased CEP production by *L. plantarum* LP69. The result agreed with this reported by Rahman *et al.* (2003) that excess accumulation of amino acids in turn reduced the protease synthesis, which failed to improve CEP activity. Some studies on gene expression level also have confirmed that production of proteinase was medium dependent.

Conclusions

The evaluation of the medium ingredients for CEP production was done by Plackett-Burman statistical method. Ten nutrients were investigated and among them Na_2HPO_4, inulin, casein peptone and leucine were found to have significant impact ($P<0.05$) on the production of CEP by *L. plantarum* LP69. Furthermore, the effect of casein peptone on the enzyme activity and the specific activity were positive. Na_2HPO_4, inulin and leucine showed a negative effect on the enzyme activity and the specific activity. These significant factors were considered for the further medium optimization by response surface methodology.

Acknowledgements

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