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EFFECT OF AMINO ACIDS ON THE PRODUCTION OF BIOSURFACTANT BY *PEDIOCOCCUS ACIDILACTICI* F70

– Short communication –

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Abstract: In this research, the surface activity of bacterial supernatant and cell surface was measured by the method of oil drain ring. The influence of 19 kinds of amino acids (Histidine, Threonine, Valine, Isoleucine, Leucine, Phenylalanine, Arginine, Proline, Methionine, Tryptophan, Alanine, Glycine, Glutamine，Cysteine, Hydroxyproline, Valine, Asparagine, Proline, Glutamine, Serine, and Glutamic acid, Hydroxyproline, Tyrosine) on the production of biosurfactant by *Pediococcus acidilactici* F70 was studied by single factor experiment, and the main amino acids promoting the production of biosurfactant were selected by Plackett–Burman design. The results showed that the yield of biological surfactant with added amino acid increased, and the yield of Glutamine produced biosurfactant in the supernatant was the highest, which was two times higher than that of the control group (414.00mg/L); the yield of biosurfactant on the cell surface was the highest when Arginine was added, which was three times higher than that of the control group. In the end, 8 of the 19 amino acids (Glycine, Tryptophan, Proline, Methionine, Arginine, Leucine, Serine and Alanine) were selected to promote the production of biosurfactants of *Pediococcus acidilactici* F70. The results of Plackett–Burman design showed that Alanine, Proline and Leucine had significant effects on the production of biosurfactants.

Keywords: *Pediococcus acidilactici* F70, biosurfactant, amino acids, Plackett–Burman design

INTRODUCTION

Surfactant is a kind of amphiphilic substances with nonpolar hydrophobic groups and polarity hydrophilic group, can reduce the surface tension between the two phase emulsion formation, Because of its good amphiphilic properties, it has the functions of solubilization, foaming, dispersion, emulsification, wetting, etc. (Banat et al., 2010; Karapetsas et al., 2011; Meng et al., 2019; Baha et al., 2019) , so it is widely used in the fields of oil extraction, repair, food, daily chemical, medicine and so on in recent years (Befkadu et al., 2018; Takassi et al., 2016; Zhang et al., 2018; Fujii et al., 2017). At present, most surfactants are chemical synthesis, which inevitably brings pollution to the environment in the process of production and use. Nowadays, with increasing sensitivity to environmental problems, reducing and controlling pollution at the source has attracted wide attention. In this respect, biological surfactants will undoubtedly become the development trend of future surface activity (Banat et al., 2000; Mnif et al., 2015).

Biosurfactant (BS) is a kind of surfactant secreted by microorganisms, which not only has the function of surface activity, but also has the potential antibacterial activity (Sinha et al., 2008). Its non-pathogenic application in food, agriculture and other industries has been revealed, but it also needs economical and efficient products to meet the industrial demand (Singh et al., 2018). At present, there are two main methods to produce biosurfactants: microbial fermentation and exogenous enzyme fermentation. Microbial fermentation is the most promising method for the production of biological surfactants due to its abundant raw materials, mild production conditions, simple process, environmental friendliness and

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relatively low product cost. There have been many reports on the screening, fermentation conditions, structure and surface activity of BS producing bacteria. At present, the surfactant producing bacteria reported by scientific research institutions include *Enterococcus faecalis, Pseudomonas, Streptomyces, Bacillus licheniformis, Acinetobacter*, etc., and there are many related studies on the production of surfactant by *Bacillus licheniformis* and Pseudomonas (Abbasi et al., 2014; Bezza et al., 2015).

At present, due to the low yield and high production cost of biological surfactants, only a few products have entered the market, which hinders its industrialization. There are also a large number of domestic scholars to improve productivity by reducing the cost of raw materials and cultivation, using renewable materials, potentially reducing waste and waste reuse and recovery (Adekilekun et al., 2019), and identifying the best cultivation conditions for high-yielding strains. Behzadnia et al. (2019) studied the kinetic parameters, biomass and biosurfactant production of *Lactobacillus plantans* ATCC 8014 with ultrasonic frequency and modified MRS medium. Most of these studies are based on carbon and nitrogen sources and fermentation conditions to improve production, while biosurfactants produced by lactic acid bacteria exist in a variety of structures, such as fatty acids, neutral lipids, phospholipids, glycolipids and lipids(Yang et al., 2019; Cheng et al., 2019). In some studies, structural identification of surfactants was carried out, and GC-MS analysis of purified biological surfactants from *Bacillus licheniformis* confirmed the presence of n-hydroxyisoleucine ($C_6H_{13}O_3N$- retention time 23.84 min) and short-chain tetracanoic acid ($C_{14}H_{30}O$ - retention time 20.691 min). Chemical surfactant type amino acid surfactant is also existing, related research is more, but amino acid production of lactic acid bacteria on surfactant study is less (Zhang et al., 2016; Fisseha et al., 2015). In a previous work, the above liquid and the surface activity of the cell surface were used as indicators to screen out lactobacillus F70 from 69 strains of lactobacillus as the target bacteria for producing surface active substances, which were identified as *Pediococcus acidilactici* F70 by 16S rDNA (Dong et al., 2019). On the basis of these studies, the purpose of this study is to further screen 19 amino acids by Plackett Burman design to optimize the composition of the culture medium, so as to obtain higher surface activity through *Pediococcus acidilactici* F70 fermentation, providing a new idea for the production of surfactant by microorganisms.

MATERIALS AND METHODS

Bacterial strain and media

Pediococcus acidilactici F70 was provided by goat milk science and Technology Laboratory of Shaanxi University of science and technology. In order to reduce the effect of surface activity on the medium, tween was removed from MRS medium. Peptone 10.0g, beef powder 10.0g, yeast powder 5.0g, glucose 20.0g, magnesium sulfate 0.1g, sodium acetate 5.0g, ammonium citrate 2.0g, potassium diphosphate 2.0g, manganese sulfate 0.05g per 1L of No-Tween MRS. Experimental reagents were purchased at Aoboxing Bio-tech Co., Ltd. (Beijing, China) and Hongyan Chemical Reagent Factory (Tianjin, China). After autoclaving at 121°C for 15 minutes, the temperature was reduced to room temperature, and under sterile conditions, the prepared amino acid solution was added to the sterile medium through a 0.22 μl filter membrane according to the experimental requirements to obtain the medium used in the experiment (Hajfarajollah et al., 2018). *Pediococcus acidilactici* F70 was inoculated into No-Tween Medium at a 5% (v/v) inoculation volume and cultured at 37 °C for 72 h to obtain samples.

The preparation and measurement of biosurfactant on supernatant and on cell surface

The supernatant obtained by culturing and centrifugation is the supernatant to be tested.The supernatant was separated from the bacterial cells and added to a phosphate buffer solution (PBS: 10 mmol/L KH_2PO_4, 10 mmol/L K_2HPO_4, 150 mmol/L NaCl, pH 7.0), and resuspended by shaking at 25°C for 2 h, so that the cell surface active substance was released into the buffer solution. The supernatant obtained after centrifugation was the sample to be tested.

Culture pH and *Pediococcus acidilactici F70* growth analysis

A pH meter was used to measure the pH value of the culture. Each group of samples was measured three times in parallel. The average value was calculated to obtain the final pH value. (PHS-3C Shanghai Precision Scientific Instrument Co., Ltd, Shanghai).

Bacterial growth determination

By measuring the total weight of the bacteria and the tube after removing the supernatant from the centrifuge tube and centrifugation, the value obtained by subtracting the two indirectly gave the wet weight of the bacteria (Eq. 1). Centrifugation conditions were 10,000 r/min at 10°C for 10 minutes.

Wet weight=total weight - centrifuge tube weight, (g/L) (1)

Determination of surface activity

The diameter of the oil drain ring ws measured by the oil drain ring method and compared after quantification by the Eq. 2 (Dong et al., 2019):

$Y=34.82+(-1495.97)X_1+33.11X_2$ (2)

where Y: Biosurfactant concentration(mg/L); X_1: Half the film thickness(mm); X_2: Half the film thickness(mm)

The screening of main amino acids

Plackett–Burman design (PB design) is an efficient method, which can eliminate the influence of unimportant animo acid and obtain reliable and efficient composition of culture medium. According to the PB design, each factor is tested in two levels: a low level of "-1" and a high level of "+1". In this study, eight influencing factors were screened using the software Design-Expert 8.0.6 (Stat Ease Inc., Minneapolis, USA), with 11 variables (including the effects of three error terms I, J, K in order to estimate the standard deviation).This analysis software mainly applies analysis of variance for the significance test of the difference between the mean of two or more samples. The F value is used to indicate the significance of the entire fitted equation. The larger the F value, the more significant the equation. P value is used to measure the difference between the control group and the experimental group. $p<0.05$ indicates a significant difference, $p<0.01$ indicates a very significant difference. In 12 independent experiments, the two responses were supernatant BS concentration and cell surface BS concentration. A: Alanine, B: Valine, C: Methionine, D: Aarginine, E: Glycine, F: Leucine, G: Tryptophan, H: Serine. The amino acid concentration of "-1" level is 0.024g / L, the amino acid concentration of "+1" level is 0.030g / L, and the amount added in the medium is shown in the Table 1.

Table 1. The values of low (coded -1) and high (coded +1) of the factors in fractional factorial design

Symbols	Amino acid	Level	
		-1	+1
A	Alanine	0.024	0.030
B	Proline	0.024	0.030
C	Methionine	0.024	0.030
D	Arginine	0.024	0.030
E	Glycine	0.024	0.030
F	Leucine	0.024	0.030
G	Tryptophan	0.024	0.030
H	Serine	0.024	0.030

RESULTS AND DISCUSSIONS

Effect of Amino Acids on BS Production by *Pediococcus acidilactici* F70

Different amino acids were added to the No-Tween medium at a concentration of 0.03 g/L, and the culture conditions remained unchanged. Eight amino acids with great influence on the surface activity of the strain were screened out with the evaluation indexes of supernatant and cell surface activity. The effect of each amino acid on the surfactant produced by the bacteria is shown in Figure 1. The concentration of BS in supernatant is 463.3-878.64 mg/L, and adding Glutamine into culture medium can produce BS twice as much as that in control group (414.00 mg/L). In addition, there are four groups with BS concentration higher than 800 mg/L, which are supplemented with Alanine (878.15 mg/L) and Proline (861.20 mg/L), respectively. Methionine (853.95 mg/L), Arginine (800.65 mg/L); cell surface activity concentration ranged from 306.14 mg/L to 960.62 mg/L, adding Arginine to the culture medium produced BS most, which was three times higher than the control group. Most of the concentration of BS was between 500 mg/L and 700 mg/L. There were 10 groups of data in this range. Alanine, Proline, Methionine, Arginine, Glycine, Leucine, Tryptophan and Serine, were selected for PB test design.

Screening of major amino acids affecting BS production by *Pediococcus acidilactici* F70

According to the contribution of each amino acid, three amino acids were selected as significant factors for surface activity, as shown in Table 2 and Figures 2-3. It can be seen from Table 2 that the wet weight range of the bacteria is 15.67-19.95g/L, with a small difference.

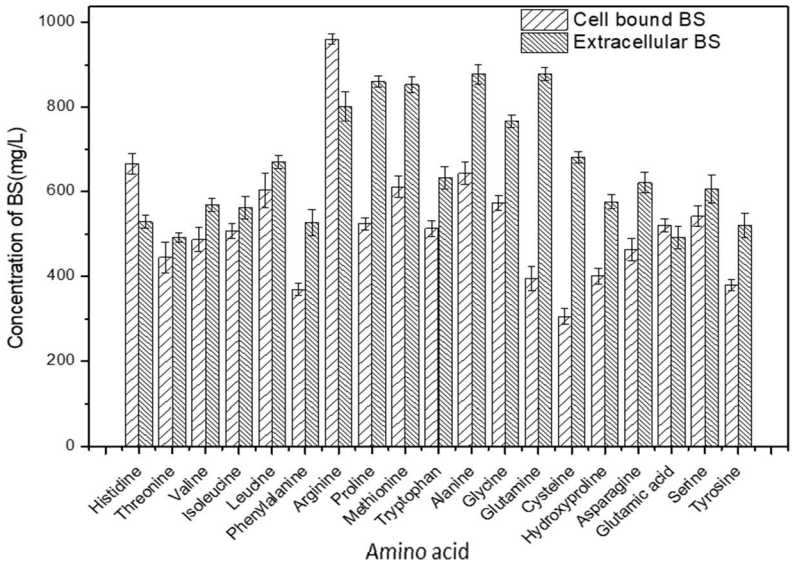

Figure 1. Effects of amino acids on, supernatant and cell surface activity of *Pediococcus acidilactici* F70

Table 2. Design and data result of PB Test for compound amino acids

RUN	A	B	C	D	E	F	G	H	I	J	K	R_1*	R_2*	R_3	R_4
1	-1	1	1	1	-1	-1	-1	1	-1	1	1	690.95	398.77	16.69	4.18
2	1	1	1	-1	-1	-1	1	-1	1	1	-1	577.02	501.44	15.86	4.18
3	1	1	-1	-1	-1	1	-1	1	1	-1	1	583.19	500.26	19.95	4.18
4	1	1	-1	1	1	1	-1	-1	-1	1	-1	701.56	572.61	18.26	4.17
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	600.07	553.67	15.67	4.17
6	-1	-1	-1	1	-1	1	1	-1	1	1	1	586.22	529.18	19.34	4.17
7	-1	1	-1	1	1	-1	1	1	1	-1	-1	582.93	521.20	18.77	4.18
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	518.45	568.80	18.36	4.18
9	-1	1	1	-1	1	1	1	-1	-1	-1	1	684.35	399.39	19.28	4.17
10	-1	-1	1	-1	1	1	-1	1	1	1	-1	597.78	541.89	17.90	4.17
11	1	-1	1	1	-1	1	1	1	-1	-1	-1	745.32	591.37	16.59	4.17
12	1	-1	-1	-1	1	-1	1	1	-1	1	1	550.64	657. 27	18.77	4.17

R_1:Extracellular BS concentration, mg/L,; R_2:Cell Bound BS concentration, mg/L,; R_3:Wet weight, g/L,; R_4:pH

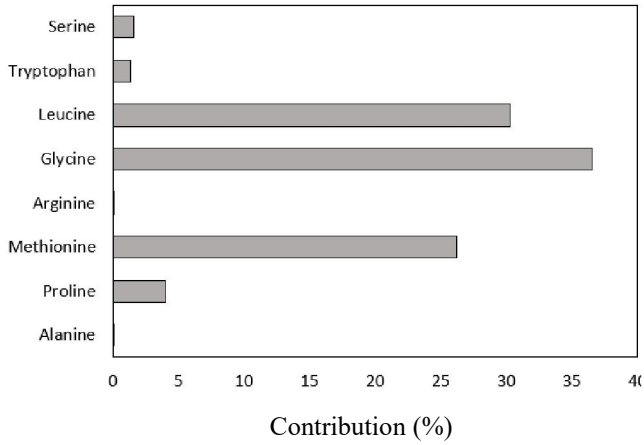

Figure 2. Contribution of amino acids to wet weight

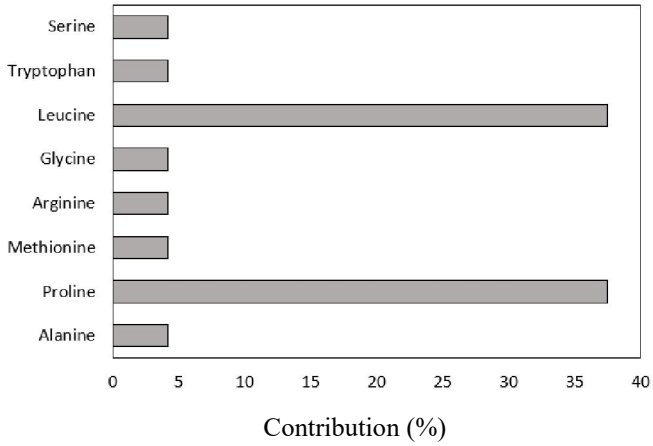

Figure 3. Contribution of amino acids to pH

According to Figure 2, E (Glycine), F (Leucine), C (Methionine), B (Proline) have a great influence on the growth of the bacteria. In Table 2, pH value of 4.17, 4.18, almost no difference, the reason may be due to join the amino acid is more, make its culture more acid, occurring in the process of growth of bacteria in the acid to produce large changes in acidity. The surface activity range of supernatant was 518.45~745.32mg/L, and the cell surface activity range was 398.77~657.27mg/L. However, it can be seen from the data that the growth of the strain is not directly proportional to the cell surface activity. The reason may be that the amino acids that promote the growth of the bacteria may not promote the growth of the bacteria and metabolize the surface-active substances.

Effect of amino acids on extracellular BS concentration of *Pediococcus acidilactici* F70

Table 3 presents the results of the analysis of variance of the effect of each amino acid on extracellular BS concentration of *Pediococcus acidilactici* F70. First, the model's p-value<0.05 (p=0.0389), indicating that this model has significant differences. The effects of these amino acids on extracellular BS concentration were found as follows: F> D> B> A> E> H> C> G. Results showed that Leucine (F) (p=0.0171), Arginine (D) (p=0.0278), Proline (B) (p=0.0291) and Alanine (A) (p=0.0308) were the most significant effect of extracellular BS concentration. The p value of the remaining amino acid is greater than 0.1, indicating that the amino acid has little effect on the surface activity. The effect of each amino acid on extracellular BS concentration can be expressed by a linear regression equation (Eq. 3).

Effect of amino acids on Cell Bound BS concentration of *Pediococcus acidilactici* F70

Table 4 shows the results of the analysis of variance of the effect of each amino acid on cell Bound BS concentration of *Pediococcus acidilactici* F70.The F-Value is 36.65, which indicates that the model has a good significance, and the p-Value is 0.0268, which indicates that the linear model has a good fitting degree, which indicates that the model is effective.The effect of each amino acid on cell surface activity is as follows: B > A > F > E > H > C > G > D. According to the p-Value, Alanine (A) (p=0.0111), Proline (B) (p=0.0074) and Leucine (F) (p=0.0199) have a significant difference in this model. The p values of other amino acids are greater than 0.01, which is not important for cell bound BS concentration. The effect of each amino acid on cell bound BS concentration can be expressed by a linear regression equation (Eq. 4).

The confidence intervals 95% showing the effect of Alanine, Proline, respectively Leucine on BS production are given in Figures 4, 5, respectively 6.

$$Extracellular\ BS\ concentration = 618.21 + 17.44*A + 18.46*B - 5.51*C + 19.37*D - 12.25*E + 31.53*F + 2.87*G + 6.93*H \quad (3)$$

$$Cell\ bound\ BS\ concentration = 527.99 + 37.30*A - 45.71*B - 5.54*C + 2.33*D + 15.54*E - 27.71*F + 5.32*G + 7.14*H \quad (4)$$

Table 3. ANOVA results of each animo acid on Extracellular BS concentration

Source	SS	df	MS	F-Value	p-Value(Prob>F)	significant
Model	27009.77	8	3376.22	394.04	0.0389	*
A-Alanine	3649.15	1	3649.15	425.89	0.0308	*
B-Proline	4089.26	1	4089.26	477.25	0.0291	*
C-Methionine	364.32	1	364.32	42.52	0.0969	
D-Arginine	4500.04	1	4500.04	525.20	0.0278	*
E-Glycine	1802.22	1	1802.22	210.34	0.0438	*
F-Leucine	11929.69	1	11929.69	1392.31	0.0171	*
G-Tryptophan	99.07	1	99.07	11.56	0.1821	
H-Serine	576.02	1	576.02	67.23	0.0773	

Table 4. ANOVA results of each animo acid on Cell Bound BS concentration

Source	SS	df	MS	F-Value	p-Value(Prob>F)	significant
Model	55268.11	8	6908.51	36.65	0.0268	*
A-Alanine	16699.21	1	16699.21	88.58	0.0111	*
B-Proline	25071.94	1	25071.94	132.99	0.0074	*
C-Methionine	367.97	1	367.97	1.95	0.2972	
D-Arginine	65.38	1	65.38	0.35	0.6156	
E-Glycine	2897.59	1	2897.59	15.37	0.0593	
F-Leucine	9214.68	1	9214.68	48.88	0.0199	*
G-Tryptophan	339.74	1	339.74	11.56	0.3115	
H-Serine	611.61	1	611.61	3.24	0.2135	

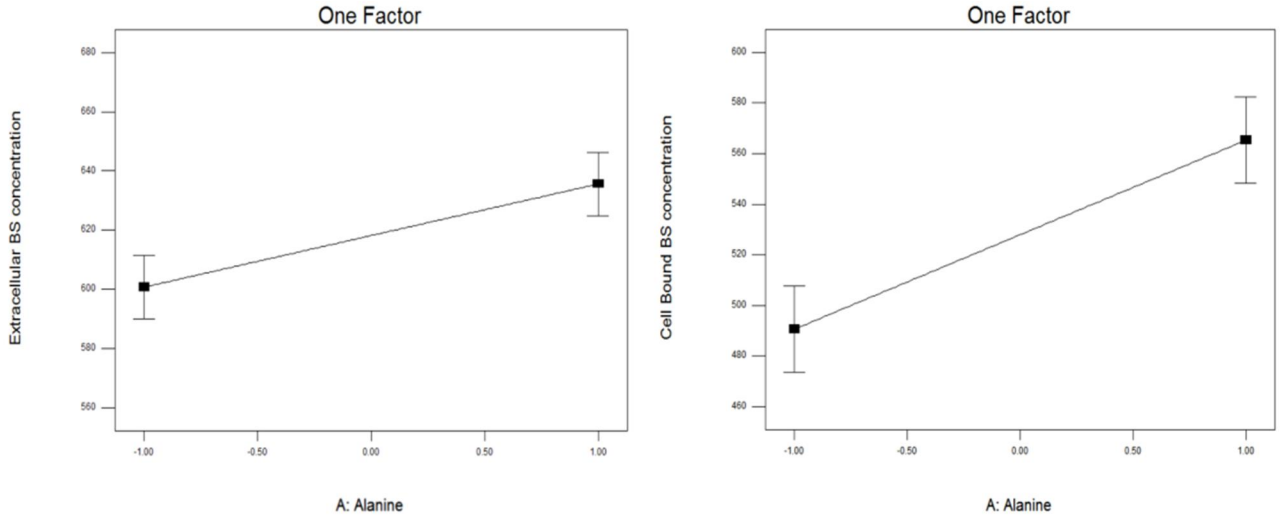

Figure 4. The confidence interval 95% shows the effect of Alanine on BS production

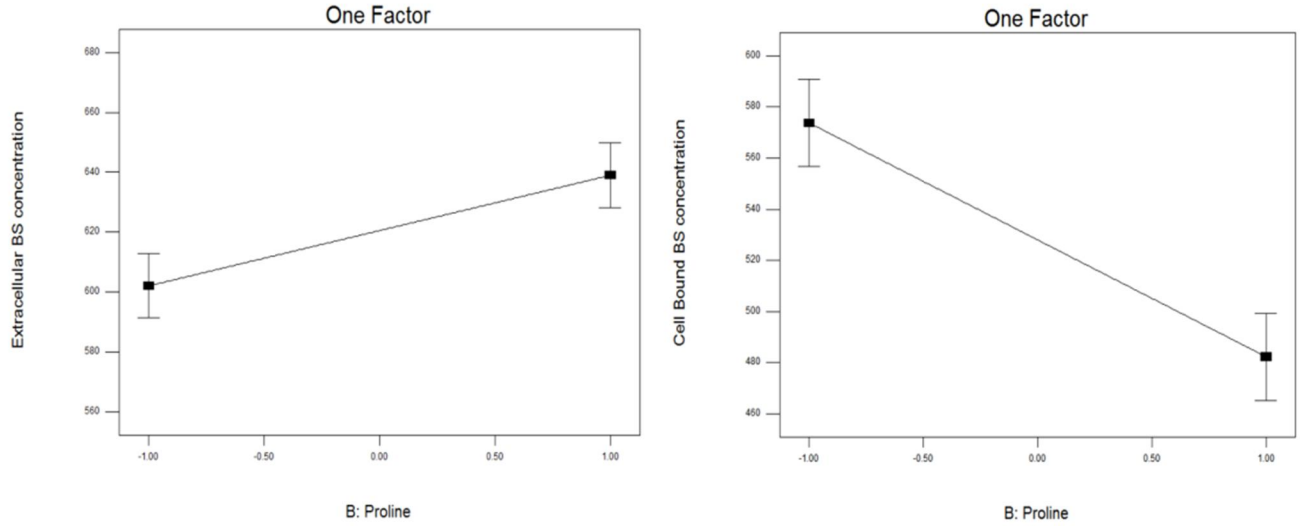

Figure 5. The confidence interval 95% shows the effect of Proline on BS production

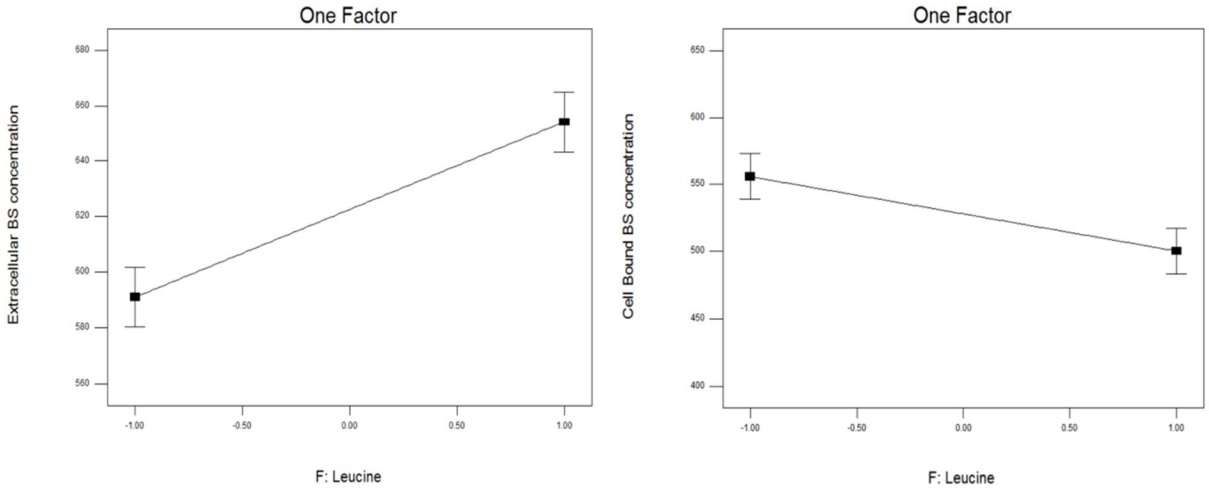

Figure 6. The confidence interval 95% shows the effect of Leucine on BS production

BS is a secondary metabolite in the process of microbial fermentation. In addition to the c source in the culture medium, which is an important factor in determining the yield and structure of surfactants, studies have shown that only a few sugars and amino acids can be used to produce surfactants. From experimental data, it was found that Glycine and Leucine can promote the growth of bacteria. Under the same conditions, metabolites will also increase, which may be one of the reasons for the high yield of biosurfactants. At home and abroad, more biosurfactants have been studied. Surfactin (srfA) is metabolized by *B. subtilis* during the growth stage (Arima et al., 1968). The substance consists of a heptapeptide cycled (Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu) and a fatty acid moiety (Galli et al,.1994). To synthesize surfactin, at least two genetic genes are needed. Among them, srfAORF1, srfAORF2, and srfAORF3, which srfA participates in as an operon, are essential for the synthesis of surfactants. The presence of Leucine and Glutamic acid stimulated the ATP/PPI exchange activity and promoted the transcription, which played a positive role in the synthesis of surfactant (Nakano et al,.1992). However, some studies have shown that changing the relative concentrations of Leucine and Valine in the reaction mixture can also increase the surface activity (Baumgart et al,.1991).

Different surface-active substances may have different synthetic pathways and different enzymes involved (Syldatk et al,.1985). Dams-Kozlowska et al, (2007) have found that the length of metabolites of ATCC 31012 (Zuckerberg et al,.1979) is regulated by the polysaccharide-copolymerase (PCP) protein, and the corresponding positions of the PCP protein involved in the regulation of polymer chain length have Proline. It can be speculated that Proline may affect the formation of polymers. Some scholars also discovered a new gene (MBSP1) related to BS production through metagenomics method. Comparing with 31 proteins in the uniprot database, it was found that the content of Alanine was the highest, reaching 13.09% (Araújo et al, 2020). It can be inferred that Alanine as a component of the medium may play a positive role in the synthesis of biosurfactants (Galli et al, 1994). As the structure types and synthetic pathways of the surface-active substances in this experiment are unknown, further experiments are needed to determine the mechanism of action of amino acids on the production of surface-active substances.

CONCLUSIONS

In this experiment, the growth of bacteria was used as the reference condition, and the concentration of extracellular BS and Cell Bound BS was used as the main screening conditions. The amino acid composition was optimized by adding amino acids to increase the yield of biosurfactants. The results show that all amino acids can increase their yield, and 8 kinds of amino acids (Alanine, Proline, Methionine, Arginine, Glycine, Leucine, Tryptophan and Serine) were selected from 19 amino acids through comparison. By using a PB design, Alanine, Proline and Leucine were selected as the main factors affecting the BS production of *Pediococcus acidilactici* F70.

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