Response surface optimization of the nitrogen source for pullulan production by *Aureobasidium pullulans* CGMCC3945 with corn steep liquor

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Abstract:【Object】 Pullulan is a linear homopolymer composed of maltotriose subunits interconnected with a-1, 6 glucosidic linkages and one of such commercially emerging biopolymers and synthesized by a yeast-like fungus *A. pullulans*. Pullulan's solubility can be controlled or provided with reactive groups by chemical derivatization. It is moldable and spinnable, being a good adhesive and binder. It is also non-toxic, edible, and biodegradable. Consequently, pullulan and its derivatives have numerous potential for food, pharmaceutical and other industrial applications. The major constraint prevailing on the use of pullulan is its higher cost. In order to realize large-scale pullulan production by *A. pullulans*, an attempt was made to optimize medium components for maximizing the production of the strain from corn steep liquor.

【**Methods**】The optimal condition based on the single factor experiment was made in fermentation medium. We aimed to research effect of the ingredient of medium on growth and production of *A. pullulans*. Corn steep liquor (CSL), NH_4NO_3 and K_2HPO_4 which were identified to significantly affect pullulan production by Plackett–Burman design were further optimized using response surface methodology of central composite design (CCD).

【Results】An inexpensive medium for pullulan production was achieved in shake flasks. The experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis. By solving the regression equation and analyzing the response surface contour plots, the optimum variables that supported maximum pullulan production were 0.72 g/L NH_4NO_3, 1.38 g/L K_2HPO_4 and 0.70g/L CSL. The average productivity of pullulan is 23.22g/L under the desired fermentation conditions.

【Conclusion】CSL is an inexpensive and abundantly available raw material in comparison with yeast extract, and the cost of yeast extract is many times higher than that of CSL. It has further scope for improving pullulan production by optimizing the most significant variables and culture conditions from the present analysis. It was verified that CSL supplemented with yeast extract appeared to be an economical alternative to yeast extract alone.

Pullulan is a kind of extracellular water-soluble polysaccharide which secreted by accreobasidium pullulans. [1]Its molecule is composed of maltotriose subunits interconnected with α-1,6 glacosidic linkages[1]. It has non-toxic, low visicosity, high plasticity, good film-forming, ect, which can be used in food industry, pharmacy, cosmetics, industry and waste water treatment[2-4]. The pullulan

products in our country have a higher cost and the market price is 300000 RMB/ton. It caused that we had to mainly rely on imports at present[4]. The major reason is that pullulan has a lower yield but a higher cost and it had not realized industrialized production. Consequently, reducing the production cost of pullulan is a bottleneck of solving the industrial production of pullulans. Nitrogen source (Yeast extract) is one of the main cost of producing the pullulan and the market price of the yeast is 100 RMB per kilogram[4-6]. Putting the cheap castoff of agricultural production such as corn steep liquor as the nitrogen source to take place of the yeast extract can effectively reduce the production cost of the pullulan[4-6].

Plackeet-Burman(PB)experimental design is based on the balanced incomplete blocks principle which can fast screen the most important factor that is the most remarkable factor in a large number of variables by the method of test. (RSM) is a mathematical statistical methods of optimal experiment that can put the experimental design and mathematical model combined to get an accurate and effective experimental conclusion by the global function relationship between the factor and the conclusion of local experimental regression fitting[6-8]. By means of the test principle, the fermentation medium by using response surface method to optimize the pullulan can make up plenty of shortages in the microbial optimal culture before. A that it took the more reasonable experimental design, the most economic way and less experimental number and time to make comprehensive researches, provided the relationship between partial and whole scientifically and received the more specific and purposive theory[7]. Hence, the response surface methodology is more and more valued, at the meantime, it' s applied widely in microculture optimization.

The study was based on the previous work and single factor experiment for the component of medium and used the mathematical statistics method that Plackeet-Burman experiment, steepest ascent design and response surface analysis combined. Inspected and evaluated each factor of medium which influenced the fermentation of *A. pullulans* CGMCC3945, optimized the fermentation medium of the stain with the corn steep liquor as the main nitrogen source, and offered the enough theory evidence for next study.

Materials and Methods

Strain

Aureobasidium pullulans CGMCC3945

Medium

Solid Medium(g/L):PDA solid medium(g/L):potato 200.0,glucose 20.0,agar 20.0,115℃sterilization 30min.

Seed Medium(g/L):sucrose 50.0,yeast extract power 2.0,NaCl 1.0,$MgSO_4 \cdot 7H_2O$ 0.2,K_2HPO_4 2.0,$(NH_4)_2SO_4$ 0.4,pH 6.5,115℃sterilization 30min.

Fermentation Medium(g/L):sucrose 100.0,yeast extract powe2.0,NaCl 2.0,$MgSO_4 \cdot 7H_2O$ 0.2,K_2HPO_4 2.0,$(NH_4)_2SO_4$ 0.4,pH 6.5,115℃sterilization 30min.

Culture Method

Seed cultivation: The strain was cultivated at 28℃ for 3d on the PDA solid plate. And then colon was inoculated into 20cm tube including 5mL seed medium, and cultured at 28℃ for 36h, rotate speed was 180 rpm.

Fermentation cultivation: 3% seed fermentation liquor was inoculated into 250mL flask of 100mL fermentation medium, put it into the shake culture tank, rotate speed 180rpm, 28℃ for 96h.

The method of analysis

pH analysis pH was measured by precision acidity meter

Sucrose analysis The method of 3, 5-dinitrosalicylic acid colorimetry was used to measure the standard curve of glucose, sucrose hydrolyzed to reducing sugar by HCl, measured the value R1 before hydrolyzation and the value R2 after hydrolyzation.

Sucrose content(%)=(R2-R1)×0.95×100%

The analysis of the pullulan yield: Took 10mL fermentation solution into 50mL centrifugal tube, 6000r/min centrifuged 15min, got the supernate, put in the two times volume of freezing absolute ethyl alcohol, shook, 4℃static over night, let the pullulan to precipitate fully, 4500r/min centrifuged 30min, the precipitation was used 95% alcohol to clean three times, 70℃ vacuum dry till constant weight, weight, count the crude pullulan yield[9].

Crude pullulan yield (g/L)=dry weight of polysaccharide (g)/the volume of fermentation solution (L)

The conversion ratio of polysaccharide (%)= crude polysaccharide yield of fermentation solution (g/L)/ Sucrose content in the medium (g/L)×100%

Optimization method of fermentation conditions

single factor test It's based on fermentation cultivation , took the different concentrations of the sucrose ,the corn steep liquor, yeast extract power, NH_4NO_3 and $(NH_4)_2SO_4$, K_2HPO_4 and KH_2PO_4, NaCl and $MgSO_4 \cdot 7H_2O$ as the metabolic single factor to ferment , observed the change of the pullulan yield.

Plackett-Burman experimental design Plackett-Burman experimental design is a kind of fractional factorial design of two levels. It's based on the balanced incomplete blocks which could use the least experimental times to estimate the main effect of factors and fast screen the most significant factors to do the study from the numerous study factors. The experiment was performed and the results were analyzed by Minitab software.

Steepest ascent design fitted equation of the response surface could abundantly approximate the real situations only on the near area of investigation, but it isn't similar to other area fitted equation and the similar functional equation, has no sense. Hence, only approximates the near area of investigation can abundantly approximate the real situations. Steepest ascent design puts the gradient direction of the experimental values as ascent direction. According to the size of effect value of each factor confirms the change step and fastly approximates the best area.

Response surface analysis Response surface analysis is a method of mathematic statistics which studied the best condition when the factor interaction arrived the maximum impact in the system of multiple factor. The central composition design, data analysis and modeling of the experiment all applied the SAS software.

Results and Analysis

Experiment design and results of Plackett-Burman

Put the different concentrations of molasses and sucrose, corn steep liquor and yeast extract, NH_4NO_3 and $(NH4)_2SO_4$, K_2HPO_4 and KH_2PO_4, NaCl and $MgSO_4 \cdot 7H_2O$ into single-factor conditional optimization respectively. According to the results of single-factor experiment, selected the four factors to do the next study of optimized medium, the sucrose 110g/L, the corn steep liquor 4 g/L, NH_4NO_3 0.4 g/L, K_2HPO_4 2 g/L, NaCl 1.5 g/L and $MgSO_4 \cdot 7H_2O$ 0.15 g/L.

The study selected N=12 Plackett-Burman experimental design, studied on the single factor of six high yield in the medium. Take two levels of +1 and -1 per factor, but the difference value of two levels can't be too large to cower the importance of other factors. The +1 level is 1.25 times more than the -1 level, the response value is the yield of pullulan (g/L), experimental design and results of

Plackett-Burman analysis referred to table 1, levels of variables and analysis of the main effect for Plackett-Burman referred to table 2.\

Table1. Experimental design and results of Plackeet-Burman

Num	X_1	X_2	X_3	X_4	X_5	X_6	The yield of polysaccharide(g/L)
1	-1	1	-1	-1	-1	1	13.95
2	1	-1	1	1	-1	1	15.67
3	1	1	1	-1	1	1	15.43
4	-1	-1	-1	1	1	1	10.94
5	-1	-1	1	1	1	-1	16.18
6	1	-1	1	-1	-1	-1	21.45
7	1	1	-1	1	-1	-1	15.36
8	-1	-1	-1	-1	-1	-1	15.63
9	1	-1	-1	-1	1	1	10.02
10	1	1	-1	1	1	-1	17.32
11	-1	1	1	-1	1	-1	18.92
12	-1	1	1	1	-1	1	13.94

Table 2 Levels of variables and analysis of the main effect for Plackett-Burman

Factor		Level		Significance		
Num	Name	-1	+1	T text	Pr > | t |	Importance yank
X_1	sucrose	10	12.5	0.96	0.380	5
X_2	corn steep liquor	0.4	0.5	0.85	0.434	4
X_3	NH_4NO_3	0.04	0.05	3.11	0.027	2
X_4	NaCl	0.1	0.2	-1.01	0.357	6
X_5	$MgSO_4$-$7H_2O$	0.01	0.02	-1.22	0.278	3
X_6	K_2HPO_4	0.2	0.3	-4.22	0.008	1

From table 2, t test brought the factor of positive effect:sucrose, corn steep liquor, NH_4NO_3. The factor which came about negative effect is:NaCl, $MgSO_4$-$7H_2O$, K_2HPO_4. On the level of 95% the difference between NH_4NO_3, K_2HPO_4 are fairly significant. On this level, other factors don't have significant difference. Therefore we should select NH_4NO_3 and K_2HPO_4 this two factors as study object of steepest ascent design.

Experimental design and results of steepest ascent

According to result of Plackett-Burman experimental analysis confirms steepest ascent route of the next fest. From table 2, NH_4NO_3 has significant positive effect, should be increased. . K_2HPO_4 has significant negative effect, should be increased. The factor of negative effect fixed on the -1 level and the factor of positive effect, on the +1 level. On the basis of the ratio of the two factors set their movement directions and step (table 3).

Table 3 Experimental design and results of steepest ascent

Num	NH_4NO_3(g/L)	K_2HPO_4(g/L)	The yield of polysaccharide (g/L)
1	0. 5	2	22.81
2	0. 6	1.6	22.86
3	0. 7	1.2	22.56
4	0. 8	0.8	23.50
5	0. 9	0.4	22.37
6	1.0	0	21.75

Group 3 has the highest polysaccharide yield, when the dosage additive volune of NH_4NO_3 is 0.7g/L, and the dosage of K_2HPO_4 is 1.2g/L, the highest polysaccharide yield is 26.56 g/L. So took the experimental condition of Group 3 as central point to the response surface analysis in the next experiment.

Optimize pullulan fermentation medium by surface response analysis

Second regression models fitting and cariance

We confirmed the main factor which affected the pullulan yield by Plackett-Burman experiment and then confirmed the concentration of important factors near the area of response value by steepest ascent experiment. Because of the experimental objective was decreasing the pullulan production cost, when designed the central composite design and response surface analysis, we joined corns and put this variable to analyze together. When analyzed the experimental results of Plackett-Burman, the corn steep liquor had positive effect and the volume of addition should be increased, meantime took the +1 level 5g/L. According to this experimental results, reponse surface analysis is made. Factor level saw Table 4, experimental design and results saw Table 5:

Table 4 Factors and levels of Central composite design

Factor		Level(g/L)		
Num	Name	-1	0	+1
1	Corn steep liquor	3	5	7
2	NH_4NO_3	0.5	0.7	0.9
3	K_2HPO_4	1.0	1.2	0.4

Table 5 Experimental design and results of Central composite design

Group	X1 (%)	X2 (%)	X3 (%)	Y1 (the yield of polysaccharide g/L)
1	-1	-1	1	22.46
2	-1	1	-1	22.49
3	1	-1	-1	21.75
4	1	1	1	20.98
5	-1.41421	0	0	23.19
6	1.414214	0	0	21.17
7	0	-1.41421	0	21.00
8	0	1.414214	0	20.23
9	0	0	-1.41421	22.67
10	0	0	1.414214	22.43
11	0	0	0	22.62.
12	0	0	0	23.00
13	0	0	0	22.59
14	0	0	0	23.22
15	0	0	0	22.33

Put the pullulan yield as the response value, took the central composite design by using SAS software and took two regression analysis of the results. The predictive model for Y was Y_1 = 23.21518 - 0.714178*X_1- 0.272232*X_2 - 0.084853*X_3 - 0.346573*X_1*X_1- 0.115147*X_1*X_2 + 0.087232*X_1*X_3 - 1.129075*X_2*X_2 + 0.159178*X_2*X_3- 0.161573*X_3*X_3 . Regression of the model is significant on the level of α=0. 01, so the model selected right. R^2=0.9046 declared that the model could explain the variation of the pullulan yield in 90.46 percent of the experiment and indicated the equation fitting well. So, the regression equation offered a suitable model for the production of pullulan by *A. pullulans*.

Response surface analysis and determination of optimum medium conposition

Plotted analysis chat by regression equation and studied the shape of the corresponding surface fitting, response surface plot saw picture 1-3.

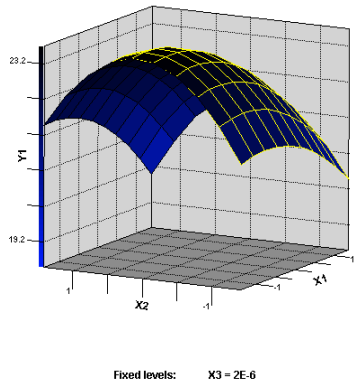

Fig. 1 Response surface polt for the function Y=*f*(X_1,X_2)

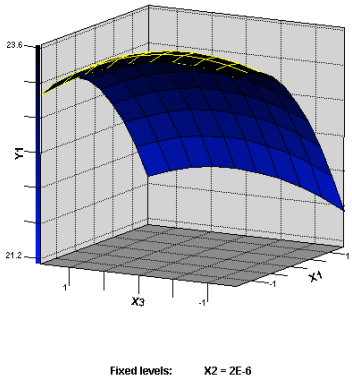

Fig. 2 Response surface polt for the function Y=*f*(X_1,X_3)

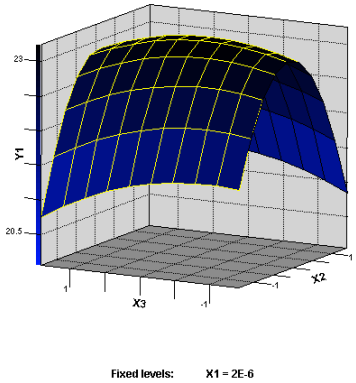

Fig. 3 Response surface polt for the function Y=*f*(X_2,X_3)

From the picture and software analysis, the regression equations existed stable point which was the maximum point, the code of each main factor(X_1,X_2,X_3) which got the maximum by analyzing were 0.701782, 0.07216, 0.13829, the optimal concentration of the corn steep liquor, NH_4NO_3, K_2HPO_4 7.02g/L, 0.72 g/L,1.38g/L, meantime the yield of pullulan was the highest, the highest yield was 23.22 g/L.

Experimental verification

Deployed the fermentation medium with the main factor concentration was confirmed in which 2.3.2. Fermented in the batch (three times) , the yield of pullulan were 23.60 g/L, 22.98g/L, 23.17 g/L, mean value 23.58 g/L, it's extremely close to the predicted value 23.22 g/L. Meantime, Compared with the highest yield which applied the corn steep liquor as nitrogen source of fermentation production with the original factor experiment, it increased 9.1%. The former yield was 21.62g/L.

Discussion

Pullulan yield is greatly affected by the medium composition and the amount. In the medium of this study, the application of corn industrial waste will be replaced the yeast extract to product pullulan and it will greatly reduce the pullulan production cost[6]. Among them, the market price of corn steep liquor is 200~400RMB/ton and the yeast extract powder is 100 RMB/kg. After calculation, if the amount of corn steep liquor in medium is 7g/L, the yeast extract powder is 2g/L, and the yield of pullulan is 20g/L, producing pullulan with corn steep liquor can save 144 RMB/kg than with yeast extract. Therefore, applying this method to product pullulan effectively reduces the production cost, production feasibility.

Conclusion

The optimal medium of pullulan which is produced by *A. pullulans* is the corn steep liquor 7.0g/L, $NH_4NO_3$0. 72 g/L and $K_2HPO_4$1.38 g/L. Meanwhile, the pullulan yield is 23.22g/L, compared with the most yield which applied the corn steep liquor as nitrogen source to product the pullulan in the original single factor experiment. It's improve 9.1%. It decreased the cost of production of pullulan effectively and based on the firm foundation of production.

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