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Optimization of fermentation conditions for human collagen a1(iii) chain production by pichia pastoris GS115 using response surface methodology

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ABSTRACT

The optimal fermentation conditions for recombinant human collagen a1 (III) chain (rhCOL3A1) production by *Pichia pastoris* GS115 in a shake-flask culture was studied using statistical experimental design and analysis. The response surface methodology (RSM) was effective in optimizing conditions using the limited number of experiments. The optimal fermentation conditions for maximum rhCOL3A1 yield, was determined on the basis of a three-level three-factor Box-Behnken design (BBD), obtained by RSM. The high correlation between the observed and predicted values indicated the validity of the model. A maximum rhCOL3A1 yield of 508.46 mg/L was obtained at 28.2 ^{o}C, initial pH 4.95 and 1.35%/24h of methanol, which was 18.9% higher than production of 427.63 mg/L before optimization.

KEYWORDS

Fermentation conditions; Optimization; Placket-burman design; Response surface methodology.

INTRODUCTION

The methylotropic yeast *Pichia pastoris* is widely used as a host for heterologous protein expression. *P. pastoris* can be grown to high cell densities in a defined mineral medium and it can also perform many of the post translational modifications, such as protein folding, proteolytic processing, disulfide bond formation and glycosylation[1].

Placket-Burman (PB) design is an efficient statistical tool to identify factors which have significant effects on the response value. Response surface methodology (RSM) is a commonly used method to find the optimal fermentation conditions, and also an efficient statistical method for optimization of multiple variables with minimum number of experiments[2,3]. It is a less expensive and faster method for gathering research result than the classical method[4,5]. In the process of methanol induction for heterologous protein expression by *P. pastoris*, fermentation conditions may affect the expression level of recombinant protein. Therefore, RSM can be used to predict the production of recombinant protein and study the interactions among the factors[6], which can help us to find the optimum fermentation conditions.

In the present study, the parameters of fermentation conditions for the rhCOL3A1 production were investigated by recombinant *P. pastoris* GS115, including temperature, pH, addition amount of methanol, inoculation quantity, broth content and rotation speed. PB design was employed to identify the significant parameters influence on rhCOL3A1 production by *P. pastoris* GS115. The significant parameters were then optimized by Box-Behnken design (BBD) according to RSM. Effects of these factors on the rhCOL3A1 production were statistically analyzed with response surfaces, and the fermentation conditions was optimized using mathematical equations and response surface plots.

METHODS

Strain and culture method

Recombinant *P. pastoris* strain GS115/pPIC9K-COL3A1, expressing human collagen a1(III) chain under the control of alcohol oxidase promoter was used for optimization studies. Recombinant plasmid pPIC9K carrying the gene coding for human collagen a1(III) chain was integrated into the *Pichia* genome. Cells from *P. pastoris* GS115/pPIC9K-COL3A1were cultivated on YPD (1% yeast extract, 2 % peptone, 2 % glucose, 1.5% agar) at 30 ^{o}C for 48 h. Single colony was grown in BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin) at 30 ^{o}C with vigorous shaking. The cells were harvested by centrifugation at 1, 500 g for 10 min until OD_{600} reached 2-6, and then the cell pellet was resuspended to BMMY medium (The same composition as BMMY, except for replacing glycerol with methanol) in 500 mL of shake-flask for rhCOL3A1 induction. Methanol was supplemented every 24 h for 96 h inducible expression.

Identifying the significant factors by plackett-burman design

The PB experimental design was employed to identify the parameters which had significant effect on the rhCOL3A1 production. Each parameter was represented at two levels (-1 and +1). The parameter code and their levels in the PB experimental design were shown in TABLE 1. As shown in TABLE 2, 12 experiments was generated by PB design and response values were measured by rhCOL3A1 production.

The effect of each parameter on rhCOL3A1 production was calculated by the following function:

$$\sum (x_i) = \frac{\sum M_{i+} - \sum M_{i-}}{N} \quad (1)$$

Where $\sum(x_i)$ represents the effect of tested parameters; M_{i+} and M_{i-} represents the rhCOL3A1 production in which the parameters were tested at their low and high levels respectively; N representsis the number of experiments.

TABLE 1 : Levels of parameters for Plackett-Burman design

Parameters	Parameters code	Low level (-1)	High level (+1)
Temperature (°C)	X_1	26	30
Initial pH	X_2	4.0	6.0
Addition amount of methanol (%)	X_3	0.5	2.0
Inoculation quantity (OD_{600})	X_4	2.0	6.0
Broth content (%)	X_5	2	10
Rotation speed (rpm)	X_6	150	250

TABLE 2 : Screening significant parameters for rhCOL3A1 production using Plackett-Burman design

Runs	X1	X2	X3	X4	X5	X6	rhCOL3A1 yield(g/L)
1	1	-1	1	-1	-1	-1	413.28
2	-1	1	-1	-1	-1	1	424.13
3	-1	-1	-1	-1	-1	-1	389.22
4	1	1	1	-1	1	1	392.34
5	-1	-1	-1	1	1	1	403.22
6	-1	-1	1	1	1	1	391.23
7	1	1	-1	-1	-1	-1	401.45
8	-1	1	1	-1	1	-1	425.34
9	1	1	-1	1	1	-1	372.35
10	1	-1	1	1	-1	1	398.13
11	-1	1	1	1	-1	1	423.34
12	1	-1	-1	-1	1	1	384.24

Response surface experimental design

The factors which had significant influence on the rhCOL3A1 yield were identified by PB design, including temperature, initial pH and addition amount of methanol. In order to optimize the three fermentation conditions for rhCOL3A1 production, a three-level (-1, 0, 1) three-factor Box-Behnken design was applied. A total of 17 experimental runs with different combinations of three factors were carried out (TABLE 3). For predicting the optimal level, a second-degree polynomial function was fitted to correlate the relationship between variables and response value. The second-degree polynomial equation for the three factors is:

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i Y_j \quad \textbf{(1)}$$

Where Y, β_0, β_i, β_{ii}, β_{ij}, represents the predicted response value in the form of rhCOL3A1 production, the constant process effect in total, the linear, quadratic effect of X_i and the interaction effect between X_i and X_j on rhCOL3A1 production, respectively.

Sample analysis

The fermentation supernatant was collected and analyzed by SDS-PAGE. The expression level of rhCOL3A1 was measured by quantifying the relevant band in the gel by software of Quantity One

4.62. The total protein in the fermentation supernatant was quantified by the BCA Protein Assay with analytical grade gelatin as a reference[7].

TABLE 3 : Box-Behnken design for the three significant factors and experimental values of responses

Run	X_1	X_2	X_3	rhCOL3A1 yield (g/L)
1	28	4.0	0.50	275.24
2	30	4.0	1.25	295.90
3	26	5.0	2.00	188.23
4	28	5.0	1.25	475.94
5	30	6.0	1.25	228.24
6	28	6.0	0.50	443.45
7	30	5.0	0.50	154.65
8	28	5.0	1.25	521.34
9	28	5.0	1.25	509.45
10	26	5.0	0.50	108.96
11	28	5.0	1.25	499.55
12	26	4.0	1.25	138.37
13	30	5.0	2.00	253.45
14	28	4.0	2.00	347.63
15	26	6.0	1.25	54.24
16	28	6.0	2.00	348.35
17	28	5.0	1.25	514.15

Software for experimental design

The software Minitab 16 was used for generation and evaluation of the statistical experimental design. The optimal fermentation conditions for rhCOL3A1 production was obtained by solving the regression equation and analyzing the response surface contour plots using the same software.

RESULTS AND DISCUSSION

Screening factors using plackett-burman design

Six factors, including temperature, initial pH, addition amount of methanol, inoculation quantity, broth content and rotation speed during the stage of methanol induction were chosen as variables for PB design. Statistical analysis on variables was performed using the PB design. The variables showing statistically significant effect were screened by analysis of variance (ANOVA) (TABLE 4). The variables with confidence level greater than 95% were considered to be significant. The temperature, initial pH and addition amount of methanol with confidence levels over than 95% showed significant effects on rhCOL3A1 production. Meanwhile, variables with confidence levels much lower than 95% were considered to be insignificant. Therefore, three significant factors were identified, including temperature, initial pH and addition amount of methanol.

TABLE 4 : Statistical analysis of Plackett-Burman design

Variables	Coefficient	F	P-value
Intercept	2.8643	119.429	0.000
X_1	0.1264	5.271	0.013
X_2	-0.1367	5.699	0.042
X_3	-0.1241	-5.178	0.031
X_4	-0.5978	-2.492	0.053
X_5	-0.0372	-1.551	0.132
X_6	-0.0603	-2.516	0.056

Optimization of significant factors by box-behnken design

In order to establish the culture conditions for the optimization of rhCOL3A1 production, several preliminary tests have been performed to evaluate the efficacy of temperature, initial pH and adding amount of methanol during the stage of induction in rhCOL3A1 production from *P. pastoris* GS115.

In this study, three culture conditions were studied to evaluate the approximate polynomial for all dependent variables, explaining their effects on rhCOL3A1 production. RSM and BBD were used to optimize rhCOL3A1 production by *P.pastoris* GS115 in shake-flask cultivation. The levels of the variables for the BBD experiments were selected according to the results of the previous experiments (data not shown).

The rhCOL3A1 production was measured with 17 runs (5 Center points) by BBD and response value was presented in TABLE 3. The center point of the corresponding fermentation condition was selected to be 28 ^{o}C, initial pH 5.0 and 1.25% of methanol during the stage of induction. Seventeen experiments were performed in triplicate.

The experimental results were fitted to a second-order polynomial equation by linear multiple regression using software Minitab 16. The corresponding second-order response model for Eq. (1) that was builded after regression analysis was:

$$Y_{(mg/L)} = -51537.2 + 3529.0X_1 + 752.1X_2 + 554.5X_3 + 2.1X_1X_2 + 3.3X_1X_3 - 55.8X_2X_3 - 62.8X_1^2 - 73.8X_2^2 - 136.3X_3^2 \quad (2)$$

The regression analysis of the results showed that coefficient R^2 value was 93.62% and adjusted R^2 value was 85.42%. The results suggested the proposed model could explained 93.62% variability in response, and only 6.38% of the total variance could not be explained (TABLE 5). The adjusted coefficient (Adj R^2= 85.42%) indicated that Eq. (2) was an applicable model to describe the response values of rhCOL3A1 production.

TABLE 5 : Model coefficient estimated by polynomial linear regression

Variables	Coefficient	Standard error	t-value	p-value
Intercept	-51537.2	6150.23	-8.380	0.000
X_1	3529.0	417.24	8.458	0.000
X_2	752.1	514.85	1.461	0.187
X_3	554.5	609.59	0.910	0.393
X_1X_2	2.1	15.01	0.137	0.895
X_1 X_3	3.3	20.01	0.163	0.875
X_2 X_3	-55.8	40.02	-1.395	0.206
X_1^2	-62.8	7.31	-8.585	0.000
X_2^2	-73.8	29.25	-2.522	0.040
X_3^2	-136.3	52.00	-2.620	0.034

R2=93.62%, Adjusted R^2= 85.42%

To determine the optimal condition of rhCOL3A1 production and the relationship between the response and the significant variables, analysis of variance (ANOVA) was performed through a joint test of three parameters (TABLE 6). As shown in TABLE 6, ANOVA for rhCOL3A1 production indicates that fitted second-order response surface model is highly significant with F-test = 11.41 (p=0.002). A p-value is the indicator of the significance of the test, whose value below 0.05 indicates that test parameter is significant at 5% level of significance. The p-value is used for the evaluation of model significance. The F value (11.41) and lack of fit (0.004) show that the quadratic model founded by BBD experiments was successfully built and has good validity.

TABLE 6 : Analysis of variance (ANOVA) for response surface quadratic model

Source	DF	Seq SS	Adi MS	F	p
Regression	9	370153	41128	11.41	0.002
Linear	3	27523	86518	24.01	0.000
Square	3	335454	111818	31.03	0.000
Interactions	3	7176	2392	0.66	0.600
Residual error	7	25221	3603	——	——
Lack of fit	3	23981	7994	25.77	0.004
Pure error	4	1241	310	——	——

The three-dimensional response (3D) and two-dimensional (2D) plots were generated to study the interaction among the three factors[8,9]. Figure 1A,B show that there is no significant interaction between temperature and pH. Meanwhile, temperature and addition amount of methanol also shows less significant interaction (Figure 1C,D), the same as the interaction between pH and addition amount of methanol (Figure 1E,F). Thus the plots in the Figure 1 indicate no significant interaction between the three factors.

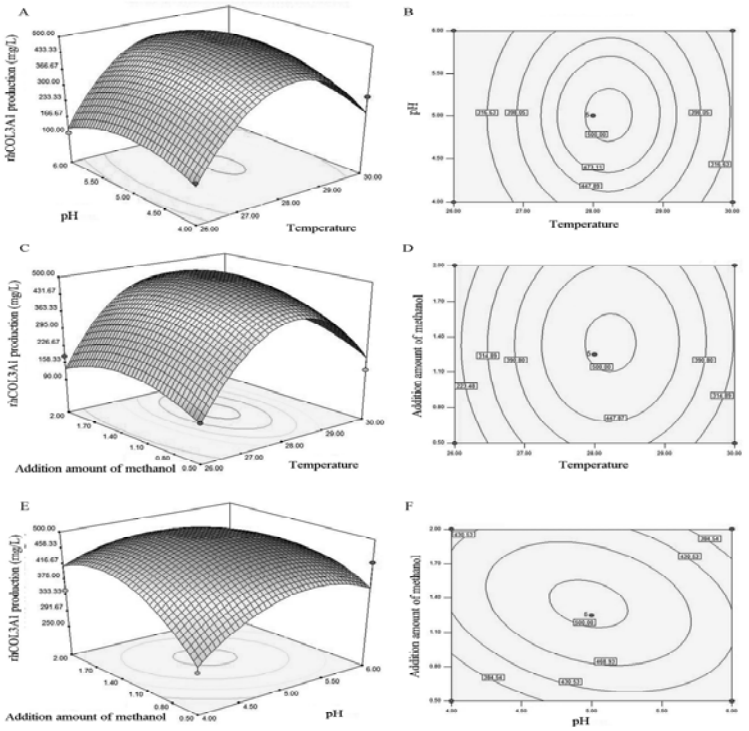

Figure 1 : Response surface plot and contour plot of the effects of three factors on the rhCOL3A1 production

A, C, E represent response surface plot of temperature and pH, temperature and addition amount of methanol, pH and addition amount of methanol, respectively; B, D, F represent contour plot of temperature and pH, temperature and addition amount of methanol, pH and addition amount of methanol, respectively.

The optimal fermentation conditions of the three factors for the rhCOL3A1 production were predicted using the optimization function of the Minitab 16 software. Culture conditions of 28.2^{o}C, initial pH 4.95 and 1.35%/24h of methanol during the stage of methanol induction were chosen as the optimal condition. A verification experiment was performed using the predicted optimum fermentation

conditions, and the highest rhCOL3A1 production of 508.46 mg/L was achieved, which reached 99.2% of the predicated value (504.39 mg/L) by the software.

CONCLUSION

The optimum fermentation conditions are 28.2°C, initial pH 4.95 and 1.35%/24h of methanol during the stage of methanol induction. The results of the study have indicated that RSM is an effective method for optimization of rhCOL3A1 production by *P. pastoris* GS115. The rhCOL3A1 production was increased by about 18.9% from 427.63 mg/L to 508.46 g/L when the strain was cultivated in the optimized fermentation conditions that developed by RSM, compared to the original fermentation conditions. Therefore, RSM was proved to be powerful and useful tool for enhancing rhCOL3A1 production by *P. pastoris* GS115. As far as known, there are no reports about fermentation conditions optimization for rhCOL3A1 production by *P. pastoris* GS115, and this study will be beneficial for the effective production of rhCOL3A1.

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