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Optimization of Fermentation Medium for Cell Yield of Recombinant *Pichia pastoris* during Growth Stage using Response Surface Methodology

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In order to improve the cell yeild of recombinant *Pichia pastoris* during fermentation of glycerol growth stage, the Placket-Burman and Box-Behnken design were applied to optimize the fermentation medium (BMGY). Three variables (yeast extra, peptone and glycerol) which were proved to have significant effects on dry cell weight (DCW) were selected from six variables by Placket-Burman design. Through Box-Behnken design Regression coefficients analysis, a secondary degree polynomial equation was established, and the optimum levels of the three variables were as following: yeast extra 1.13%, peptone 1.61% and glycerol 0.86%. From the 3D response surface plots and 2D contour plots created by the Box-Behnken design, we can also observed the interaction between yeast extra and peptone or yeast extra and glycerol are significant. An average 4.41 g/l DCW after 12 h cultivation in the optimized BMGY media could be attained by the validation experiments, which was 20.6% higher than the formal yield of 3.50 g/l.

Key words: Fermentation, Optimization, Placket-Burman design, Response surface methodology.

Pichia pastoris is a methylotrophic yeast that can be genetically engineered to express proteins for both basic research and industrial use[1]. Growing the *Pichia pastoris* to high density and improving the volumetric productivity is the major objective of any *Pichia pastoris*-based process[2]. This objective needs the well optimized growth media, process parameters, controlled fermentation systems and culturing strategies. Placket-Burman design founded by R.L. Plackett and J.P. Burman is an efficient statistical tool to screen factors which have significant effects on the production[3]. Response surface methodology (RSM) is extensively used in recent years[4]. This method is time-savingÿbeing able to predict the response under untested sets of variables and study the interactions amongst those factors, which can help us to find the optimum values of the related factors. Some early researches have been accomplished to optimize parameters of fermentation process by response surface methodology (RSM) successfully[5].

In this study, the parameters of fermentation growth media (buffered BMGY medium) including yeast extract, peptone, glycerol, ammonia sulfate, yeast nitrogen base (YNB), biotin

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for the cell yield by recombinant *Pichia pastoris* were investigated, as previous works have not been researched and reported on this area. Plackett-Burman design was employed initially for identifying the significant process parameters imposing major influence on cell yield of *Pichia pastoris*. Those parameters were then optimized by RSM. The whole work was under the aid of Statistical software Minitab 16.0, release of Minitab Inc.

MARERIALS AND METHODS

Microorganism and medium

The expression vector pPIC9k and host cells of *P. pastoris* GS115 containing the *AOX1* promoter, which allows rapid growth on methanol as the carbon source, were used for heterologous protein expression. The 3.3-kb coding region for the *CO3A1* gene from *Human* genomic DNA was amplified, using the primers 5'-CGGAATTCATGTTTCCCTCTCTC-3'and 5'-CCCTCGAGTCAGTGGTGGTGGTGGTGGTGT-3' cloned into the pPIC9k vector at the *Eco*RI and *NoT*I sites for extracellular CO3A1 expression. The constructs were linearized at the AOX1 promoter with SacI and used to transform competent GS115 cells by electroporation. Ten transformants were selected on yeast extract-peptone-dextrose (YPD) medium containing 4 mg/mL of G418 for the extracellular production of recombinant CO3A1.

Conditions for cell growth

Cells from *Pichia pastoris* were cultived on YPD (1% yeast extract, 2 % peptone, 2 % glucose, 2 % agar) at 30!for 24 h. Operating at 220 rpm in 25 mL of buffered BMGY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate buffer at pH=6.0, 1.34 % yeast nitrogen base (YNB) without amino acids, $4{\cdot}10^{-5}$ % biotin, 1 % glycerol).

Analysis methods

The cell density (OD) was measured turbidimetrically at 600nm with a spectroglycerolotometer (Model 2802PCS UNICO) and converted to the dry cell weight (DCW) by an appropriate calibration. [6]

Optimization of process parameters

Identifying the significant variables using Plackett-Burman design

The first step was to identify the parameters which had significant effects on the DCW of *P. pastoris*, and then the Plackett-Burman statistical design was employed, in which each variable was represented at two levels: -1 for a low level and +1 for a high one. Table 1 illustrates the factors under investigation and their levels employed in the experimental design. A design of 12 experiments was generated and response values were measured by DCW of *P. pastoris* in Table 2. The effect of each variable on DCW of *P. pastoris* was calculated by the following equation:

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

where $E_{(x_i)}$ is the effect of tested parameters, M_{i+} and M_{i-} are the DCW of *P. pastoris* from the experimental runs in which the variables were tested at their maximum and minimum levels respectively. *N* is the number of experiments.

Response surface methodology

The variables which had significant influences on the response value were identified, Box-Behnken design (BBD) was applied to find the optimal levels of these variables. Each significant factors was coded respectively by three levels, low (-1), medium (0), high (+1). The following equation was used for coding the variables, Eq. (2)

$$x_i = \frac{X_i - X_0}{\Delta_i} \quad ...(2)$$

where x_i is the dimensionless value of an independent variable, and X_i is the real value of an independent variable, X_0 is the value of X_i at the average point, and the Δ_i is the step change. The corresponding design and results of experiments carried out with the Box-Behnken design were

Table 1. Levels of Process parameters for Plackett-Burman design experiment

Variable	Variable code	Low level(-1)	High level(+1)
Yeast extra (%)	X_1	0.8	1.2
Peptone (%)	X_2	1.5	2.25
Glycerol(%)	X_3	0.8	1.2
Ammonia sulfate (%)	X_4	8	12
Biotin (%)	X_5	3×10^{-5}	4.5×10^{-5}
YNB (%)	X_6	3	4.5

Table 2. Plackett-Burman design for the screening of significant process parameters influencing the DCW of *P. pastoris* production

Runs	Yeast extra	Peptone	Glycerol	Ammonia sulfate	Biotin	YNB	DCW(g/l)
1	-1	1	1	1	-1	-1	2.93102
2	1	-1	-1	-1	1	-1	3.12982
3	1	1	-1	-1	-1	1	2.92426
4	1	1	1	-1	-1	-1	2.69284
5	1	-1	1	1	1	-1	2.98374
6	1	1	-1	1	1	1	2.76337
7	1	-1	1	1	-1	1	2.99406
8	-1	-1	-1	1	-1	1	3.33438
9	-1	1	-1	1	1	-1	2.45917
10	-1	-1	-1	-1	-1	-1	2.94497
11	-1	-1	1	-1	1	1	2.78904
12	-1	1	1	-1	1	1	2.42592

given in the Table 3. The second degree polynomial equation is

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i Y_j \quad ...(3)$$

Y_i is the predicted response, X_i and X_j are the independent variables; β_0 is the offset term; β_i is the *i*th linear coefficient; β_{ii} is the *i*th quadratic coefficient; and β_{ij} is the *ij*th interaction coefficient.

Table. 3. Box-Behnken design plan in coded value and the observed response

RUN	X_1	X_2	X_3	Y
1	1.2	1.875	1.2	4.534
2	1.0	1.500	1.2	3.634
3	0.8	1.875	1.2	4.225
4	1.0	2.250	0.8	4.834
5	1.0	1.500	0.8	1.890
6	1.0	1.875	1.0	2.941
7	1.2	1.500	1.0	4.356
8	1.0	1.875	1.0	2.222
9	1.0	1.875	1.0	4.281
10	1.0	1.875	1.0	1.791
11	1.0	2.250	1.2	3.869
12	0.8	2.250	1.0	3.603
13	0.8	1.500	1.0	2.894
14	0.8	1.875	0.8	1.751
15	1.2	1.875	0.8	3.782
16	1.0	1.875	1.0	4.628
17	1.2	2.25	1.0	3.569

RESULTS AND DISCUSSION

Screening of parameters using Plackett-Burman design

12 runs were arrayed in the experiment to study the effects of the selected variables on the DCW of *P. pastoris*. Analysis on variables listed in the PB design was performed and represented in Table 4. The variables exerting statistically significant effects were screened via *t*-test for analysis of variance (ANOVA) (Table 4). The variables with confidence level greater than 95% were considered to be significant. The peptone, yeast extra and glycerol with confidence levels over than 95% showed significant effects on the DCW of *P. pastoris* and were chosen for the further optimization. The other variables, *i.e*. ammonia sulfate, biotin, yeast nitrogen base with confidence levels much lower than 95% were considered to be insignificant.

Table 4. Statistical analysis of Plackett-Burman design

Variables	Coefficient	t-value	P-value
Intercept	2.8643	119.429	0.000
X_1	0.1264	5.271	0.003
X_2	-0.1367	5.699	0.002
X_3	-0.1241	-5.178	0.004
X_4	-0.5978	-2.492	0.055
X_5	-0.0372	-1.551	0.182
X_6	-0.0603	-2.516	0.053

R^2= 95.33% R^2(adj) = 89.72%

Optimization of significant variables using response surface methodology

With the significant factors selected, Box-Behnken design experiment with 17 runs (5 Center points) was employed to study the interaction between each other among the three significant factors selected above and their optimal levels. The other variables in this research maintained at a constant level which led to the highest DCW yield in the Plackett- Burman design experiments. The significance of every coefficient was determined by Student's t-test and P value which were listed in Table 5.

Table 5. Regression coefficients and their significance for response surface quadratic model

Model term	Parameter estimate	Standarderror	t	P
intercept	4.43	0.138403	26.614	0.000
X_1	-0.077	0.109417	-0.662	0.533
X_2	0.059	0.109417	0.505	0.632
X_3	0.92	0.109417	7.889	0.000
X_1^2	-0.58	0.154739	-1.934	0.101
X_2^2	-0.26	0.154739	-3.535	0.012
X_3^2	-0.044	0.154739	-6.730	0.001
X_2X_3	-0.33	0.150821	-3.501	0.013
X_2X_3	-0.6	0.150821	-1.545	0.173
X_2X_3	-1.13	0.150821	-0.065	0.800

R^2 = 95.87% R^2(adj) = 89.67%

As is shown in Table 5, by applying multiple regression analysis on the experimental data, the following second-order polynomial equation was found to represent the DCW

$$Y_{DCW} = 4.43 - 0.077X_1 + 0.059X_2 + 0.92X_3 - 0.33X_1^2 - 0.60X_2^2 - 1.13X_3^2 - 0.58X_1X_2 - 0.26X_1X_3 - 0.044X_2X_3 \quad ...(4)$$

where Y_{DCW} is the predicted response, variable X_1, X_2 and X_3 are the coded values of the test variables of yeast extra, peptone and glycerol, respectively. The fit of the model equation can be tested by the determination coefficient R^2, the adjusted R^2=95.87% suggested that the model could explain 95.87% of the total variation in response, only 4.13% of the total variation cannot be explained by the model. Meanwhile, the analysis of variance(ANOVA) for response surface quadratic model is summarized in Table 6. The 15.46 Model F-value implies the model is significant and adequateÿand the F-value for lack of fit is 0.05. The high F-value and non-significant lack of fit indicate that the model is a good fit. The P-value for the model (0.002) and for lack o fit (0.982) also suggests that the obtained experimental data is well fit to the model.

Table. 6. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of the optimization of DCW

Source	DF	Seq SS	Adj MS	F	P
Regression	9	15.2171	1.6908	15.46	0.002
Linear	3	6.8803	2.2934	20.97	0.001
Square	3	6.7275	2.2425	20.51	0.001
Interaction	3	1.6092	0.5361	4.91	0.047
Residual error	7	0.6561	0.1093	%%	%%
Lack of fit	3	0.032	0.0107	0.051	0.982
Pure error	4	0.6239	0.2080	%%	%%
Total	16	15.8731	%%	%%	%%

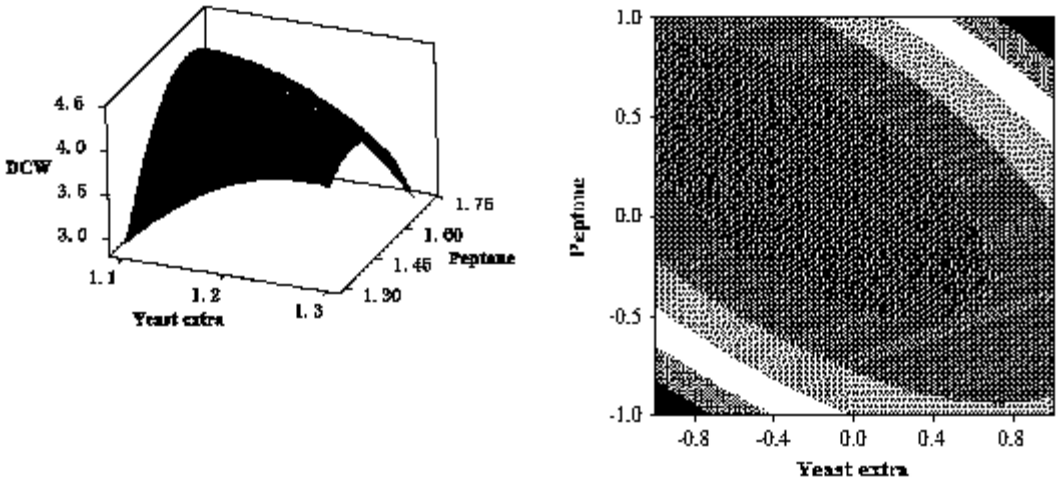

Fig. 1. Response surface plot and contour plot of the effects of yeast extra and peptone

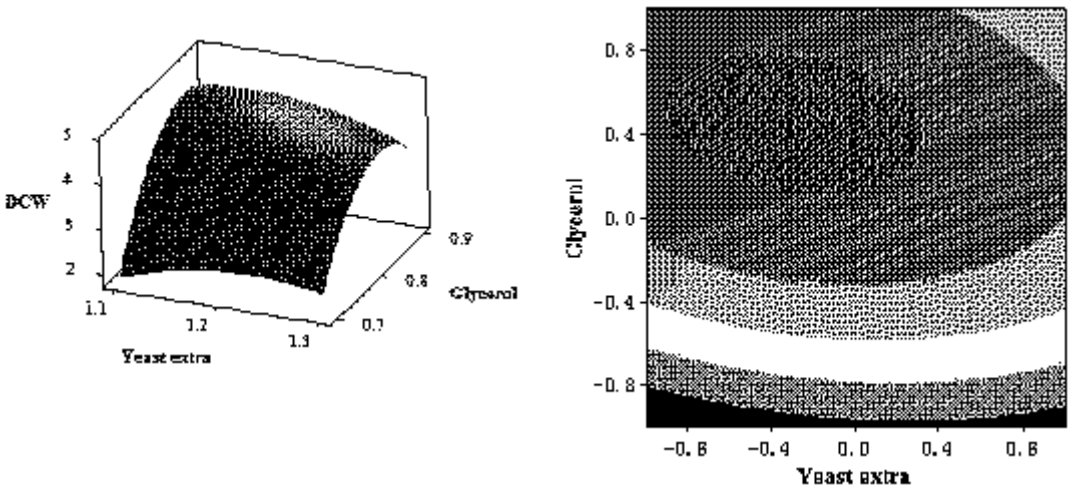

Fig. 2. Response surface plot and contour plot of the effects of yeast extra and glycerol

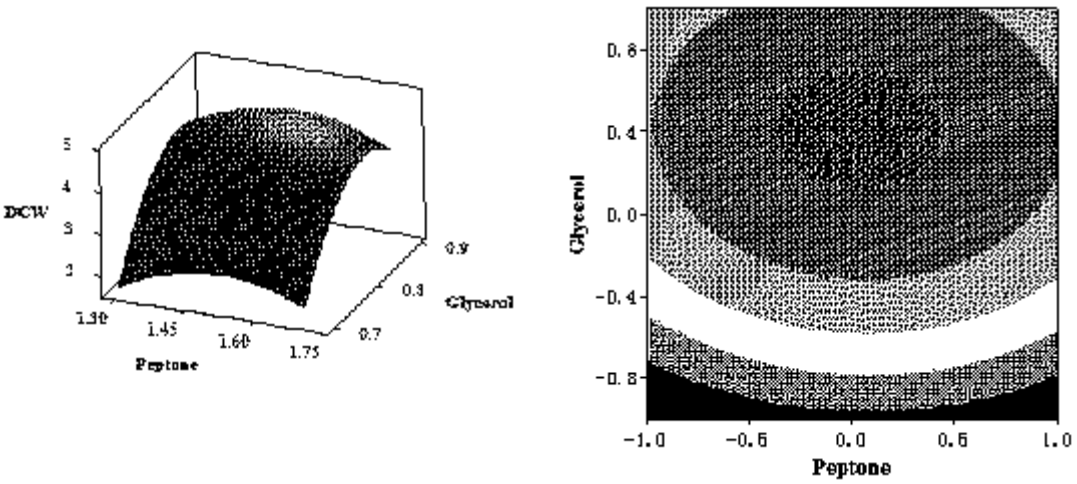

Fig. 3. Response surface plot and contour plot of the effects of peptone and glycerol

3D response surface plots and 2D contour plots were constructed (Fig 1-3). These 3D and 2D plots are the graglycerolical representation of regression equation generally used to visualize the relationship between the response, experimental levels of each variable and type of interaction between the variables to deduce the optimum conditions[7-8].

Fig.1 shows that there is significant interaction between yeast extra and peptone. Meanwhile, the interaction between peptone and glycerol is less significant than the former (Fig.2), as peptone could affect enzyme activity, substrate (glycerol) consumption rate and cell structure, which finally exerted influences on the cell growth numbers and synthesis of the product [1]. The plots in the Fig.3 indicate lower significant interaction between the peptone and glycerol.

By solving the regression equation and analyzing the response surface plots, the optimal values of the test variables in coded unit were as follows: X_1=-0.7389, X_2=0.4367, X_3=0.6443, and the corresponding real values were yeast extra 1.13%, peptone 1.61% and glycerol 0.86% respectively. Based on these optimal variable levels, the predicted maximum DCW of *P. pastoris* is 4.44g/l. Validation experiment was carried out, and the final DCW reached 4.41 g/l. This shows an excellent correlation between the experimental and predicted values, which was 20.6% yield higher in comparison to the yield before optimization.

CONCLUSION

The optimum levels of the three factors are yeast extra 1.13%, peptone 1.61% and glycerol 0.86%. The improvement of cell weight (1.21 fold) showed this optimization work was successful for DCW of *P. pastoris.* Statistical analysis has been proved to be a useful and powerful tool in developing optimum fermentation medium. As far as known, there are no reports about BMGY fermentation medium optimization for DCW of *P. pastoris*, the present study provides a valuable reference for both laboratory and factory approach.

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