Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(7):1802-1809

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Optimization of fermentation medium for collagen production of recombinant *Pichia pastoris* during induction phase

Xiao X. Ma[1,2], Dai D. Fan[1,2*], Chen H. Zhu[1,2], Zi F. Shang[1,2] and Yu Mi[1,2]

[1]*Shaanxi Key Laboratory of Degradable Biomedical Materials, Northwest University, Tai Bai North Road 229 Xi'an, Shaanxi, China*
[2]*Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi'an, China*

ABSTRACT

To improve the collagen production of recombinant Pichia pastoris during fermentation of induction phase, in this paper, the Placket-Burman and Box-Behnken design were applied to optimize the fermentation medium (BMMY). Three variables (the concentration of yeast extra, methanol and ammonia sulfate) which were proved to have significant effects on the production of collagen were selected from six variables by Placket-Burman design. Through Box-Behnken design (BBD) regression coefficients analysis, a secondary degree polynomial equation was established, and the optimum levels of the three variables were as following: yeast extra 1.19%, peptone 1.18% and glycerol 0.77%. From the 3D response surface plots and 2D contour plots created by the BBD, it is significantly observed the interaction between yeast extra and methanol or yeast extra and ammonia sulfate. An average production of 5.02 g/L after 84 h cultivation in the optimized BMMY media could be attained by the validation experiments, which was 19.3% higher than the formal yield of 4.05 g/L.

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

---

INTRODUCTION

Collagen is the most abundant of all body proteins [1]. It is a major structural protein, forming molecular cables that strengthen the tendons and vast, resilient sheets that support the skin and internal organs [2].The role of collagen in utilization has been known for a long time. Therefore, multifarious applications have been developed in the food and cosmetic as well as pharmaceutical industries [3-5].

Pichia pastoris is methyltrophic yeast that can be genetically engineered to express proteins for both basic research and industrial use [6, 7]. Compared with mammalian cells,*Pichia* does not require a complex growth medium or culture conditions, is genetically relatively easy to manipulate, and has a eukaryotic protein synthesis pathway. Because of these characteristics, some proteins, such as G protein-coupled receptors, that cannot be expressed efficiently in bacteria, *Saccharomyces cerevisiae* or the insect cell/baculovirus system, have been successfully produced in functionally active form in *P. pastoris* [8].As a rule of thumb, growing the *Pichia pastoris*to high density and improving the volumetric productivity is the major objective of any *Pichia pastoris*-based process [9]. This objective needs the well optimized growth media, process parameters, controlled fermentation systems and culturing strategies. Among these major complications, the optimization of growth media should be the primary task that needs to accomplish. The 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 [10]. Response surface methodology (RSM) is extensively used in recent years [11-13]. This method is time-saving , being 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 medium by response surface methodology (RSM) successfully [14-17].

In this study, the parameters of fermentation media (buffered BMMY medium) including yeast extract, peptone, methanol, ammonia sulfate, yeast nitrogen base (YNB) and biotin for the collagen production 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.

EXPERIMENTAL SECTION

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 *CO3*A*1* 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 CO3A*1* 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 g/L of G418 for the extracellular production of recombinant CO3A*1*.

Conditions for cell growth

At the stage of 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, 4×10^{-5}% biotin, 1% glycerol). After 18 h cultivation, harvest the cells by centrifuging at 1,500-3,000 g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD600 of 1.0 in BMMY medium to induce expression.

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

Variables	Variable code	Low level (-1)	High level (+1)	Variables	Variable code	Low level (-1)	High level (+1)
Yeast extra (%)	X_1	0.8	1.2	YNB (%)	X_4	3	4.5
Peptone (%)	X_2	1.4	2.1	Ammonia sulfate (%)	X_5	8	12
Methanol (%)	X_3	1.2	1.8	Biotin (%)	X_6	3×10^{-5}	4.5×10^{-5}

Table 2. Plackett-Burman design for the screening of significant process parameters influencing the collagen production

Runs	Yeast extra	Peptone	Methanol	YNB	Ammonia sulf	Biot	Collagen (g/L) Experimen	Predict
1	-1	1	1	1	-1	-1	2.735	2.692
2	-1	-1	1	-1	1	1	2.346	2.108
3	1	-1	-1	-1	1	-1	3.456	3.332
4	1	-1	1	1	-1	1	3.262	3.288
5	1	1	1	-1	-1	-1	3.936	3.812
6	-1	-1	-1	1	-1	1	3.252	3.128
7	1	-1	1	1	1	-1	2.233	2.372
8	-1	1	-1	1	1	-1	2.658	2.512
9	-1	1	1	-1	1	1	2.160	2.408
10	1	1	-1	1	1	1	3.243	3.408
11	-1	-1	-1	-1	-1	-1	3.038	3.352
12	1	1	-1	-1	-1	1	4.612	4.548

Analysis methods

The Cell density was measured turbidimetrically at 600 nm with spectrophotometer (UNICO Model 2082PCS, USA). Collagen level was calculated by the analysis of the corresponding band of SDS-PAGE using BandScan 5.0 software.

Optimization of process parameters

Identifying the significant variables using Plackett-Burman design

For the identification of significant variables for collagen production, a variety of fermentation mediums (yeast extra,

peptone, methanol, YNB, ammonia sulfate and biotin) were tested and identified via the Plackett-Burman design experiment. A total of six parameters were included for identification, with each variable 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 production in Table 2.

The effect of each variable on the production of collagen was calculated by the following equation:

$$E_{xi}=(\sum M_{i+}-\sum M_{i-})/N \quad (1)$$

where E_{xi}effect of tested parameters, $M_{i+}$and$M_{i-}$are the production of collagen 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 next step in the formulation of the medium was to determine the optimum levels of significant variables for collagen production. For this purpose, the response surface methodology (RSM), using a Box-Behnken design (BBD), was adopted for the augmentation of total collagen production. The significant variables utilized were as follows: yeast extra (X_1), methanol (X_3) and ammonia sulfate (X_5), each of which was assessed at three coded levels (low (-1), medium (0), high (+1)), as is shown in Table 3. A total of 17 experiments were conducted. All variables were taken at a central coded value, which was considered as zero. The minimum and maximum ranges of the variables were used, and the full experimental plan with regard to their values in coded form is provided in Table 4. The response values (Y) in each trial were the average of the duplicates.

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

RUN orde	Coded levels			Collagen production (g/L)	
	X_1	X_3	X_5	Experimental	Predicted
1	1	0	-1	3.23131	3.275
2	0	1	-1	1.870	1.920
3	-1	1	0	1.628	1.632
4	-1	0	1	2.916	2.865
5	0	-1	-1	3.384	3.360
6	1	-1	0	2.286	2.282
7	1	0	1	3.588	3.635
8	0	1	1	2.128	2.160
9	-1	-1	0	2.359	2.458
10	0	-1	1	2.220	2.160
11	0	0	0	4.546	4.900
12	1	1	0	1.768	1.668
13	0	0	0	4.797	4.900
14	0	0	0	5.43835	4.900
15	0	0	0	4.8882	4.900
16	-1	0	-1	4.25183	4.185
17	0	0	0	4.82248	4.900

Statistical analysis and modeling

The data obtained from RSM on collagen production were subjected analysis of variance (ANOVA). The experimental results of RSM were fitted via the response surface regression procedure. The following equation was used for coding the variables, Eq. (2)

$$x_i=(X_i-X_0)/\Delta_i \quad (2)$$

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 isthe step change. The corresponding design and results of experiments carried out with the Box-Behnken design were given in the Table 3. The second degree polynomial equation is:

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

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 ith linear coefficient; β_{ii} is the ith quadratic coefficient; and β_{ij} is the ijth interaction coefficient. In this study, however, the independent variables were coded as X_1, X_3, and X_5. Thus, the second order polynomial equation can be presented as follows:

$$Y=\beta_0+\beta_1X_1+\beta_3X_3+\beta_5X_5+\beta_{11}X_1^2+\beta_{33}X_3^2+\beta_{55}X_5^2+\beta_{13}X_1X_3+\beta_{15}X_1X_5+\beta_{35}X_3X_5 \quad (4)$$

The statistical software package, Design-Expert 8 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for the

regression analysis of the experimental data. The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2) and the adjusted R^2. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

RESULTS AND DISCUSSION

Screening of parameters using Plackett-Burman design

A total of six variables were analyzed with regard to their effects on collagen production using a Plackett-Burman design (Table 1). 12 runs arrayed in the experiment to study the effects of the selected variables on the production of collagen shown in Table 2.Variations ranging from 2.16 to 4.612 g/L in the production of collagen in the 12 trials were observed by Plackett-Burman design. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's t-test for ANOVA (Table 4). Factors evidencing P-values of less than 0.05 were considered to have significant effects on the response, and were therefore selected for further optimization studies. Ammonia sulfate, with a probability value of 0.003, was determined to be the most significant factor, followed by yeast extract (0.004), and methanol (0.01). The lower probability values indicate the more significant factors on the collagen production. One of these three significant variables screened, yeast extra, exerted a positive effect, whereas the other two variables, methanol and ammonia sulfate, exerted negative effects on the collagen production. Meanwhile, the other three variables, i.e. peptone, biotin and YNB with confidence levels much lower than 95% were considered to be insignificant.

Table 4. Statistical analysis of Plackett-Burman design for collagen production in six variables

Variables	Coefficient	Std. Dev.	t-value	P-value
Intercept	3.07777	0.07494	41.072	0.000
X_1	0.37950	0.07494	5.064	0.004[b]
X_2	0.14638	0.07494	1.953	0.108[a]
X_3	-0.29891	0.07494	-3.989	0.010[c]
X_4	-0.18049	0.07494	-2.409	0.061[a]
X_5	-0.39482	0.07494	-5.269	0.003[c]
X_6	0.06839	0.07494	0.913	0.403[a]

R^2= 94.10% R^2 (adj) = 87.02%; [a] Non-significant at P < 0.05.; [b] Significant positive effect.; [c] Significant negative effect.

Optimization of significant variables using response surface methodology

With the significant factors selected, a three level 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 and to fit the second order polynomial model. The other variables in this research maintained at a constant level which led to the highest collagen production in the Plackett-Burman design experiments. The statistical combinations of the critical parameters along with the maximum observed and predicted collagen production are listed in Table 3. These predicated values were very close to the observed ones in all set of experiments. The highest collagen production of 5.438 g/L and the lowest production of 1.57 g/L were observed. The significance of every coefficient was determined by Student's t-test and P-value which were listed in Table 5. The Student's t-test is used to determine the significance of the regression coefficients of the parameters. The P-values are used as a tool to check the significance of each of the coefficients, which, in turn are necessary to understand the pattern of the mutual interaction between the test variables. The larger the magnitude of the t-value and smaller the P-value, the more significant is the corresponding coefficient. The parameters estimate and the corresponding P-values suggest that among the test variables the collagen production is significantly affected by the antagonistic effect of the quadratic term ofX_1^2, X_3^2 and X_5^2.

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

Variables	Coefficient estimated	Standard Error	t-value	P-value	F-value
intercept	4.898518	28.282	-7.493	0.0000	< 0.0001
X_1	-0.03533	33.669	5.246	0.001	0.7112
X_3	-0.35682	18.352	10.184	0.000	0.0059
X_5	-0.23571	27.528	-0.023	0.982	0.0368
X_1X_3	0.053238	12.625	-7.009	0.000	0.6933
X_1X_5	0.422868	5.611	-15.781	0.000	0.0138
X_3X_5	0.355528	12.625	-4.005	0.005	0.0287
X_1^2	-0.89619	8.635	0.411	0.693	0.0002
X_3^2	-1.99223	12.953	3.265	0.014	< 0.0001
X_5^2	-0.50564	8.635	2.745	0.029	0.0052

R-Sq = 97.37% Adj R-Sq= 93.98%

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 collagen production (Y_C):

Y_C=4.90-0.035X_1-0.36X_3-0.24X_5+0.053X_1X_3+0.42X_1X_5+0.36X_3X_5-0.90X_1^2-1.99X_3^2-0.51X_5^2 (5) where $Y_C$is the predicted response, variable X_1, $X_3$and X_5 are the coded values of the test variables of yeast extra, methanol and ammonia sulfate, respectively.

The fit of the model equation can be tested by the determination coefficient R^2 which provide a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. In this experiment, the R^2 value, 0.9819, indicates that 98.19% of the variability in the response could be explained by the model. As well, the adjusted determination coefficient (adjusted R^2=0.9587) is also very high to advocate for a high significance of the model. These ensured a satisfactory adjustment of the polynomial model to the experimental data. The adjusted R^2 corrects the R^2 value for the sample size and the number of the terms in the model. If there are many terms in the model and the sample size is not large the adjusted R^2 may be noticeably smaller than the R^2. Here the adjusted R^2 was smaller than the R^2 value [18, 19].

Meanwhile, the analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 6. The 28.75 Model F-value implies the model is significant and adequate，and 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 in good fit. The P-value for the model (0.000) and for lack o fit (0.983) also suggests that the obtained experimental data is considered to be statistically significant [20].

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

Source	DF	Seq SS	Adj MS	F	P
Regression	9	25.5269	2.8363	42.27	0.000
Linear	3	1.4730	2.7182	40.51	0.000
Square	3	22.8217	7.6072	113.36	0.000
Interaction	3	1.2322	0.4107	6.12	0.023
Residual error	7	0.4697	0.0671	—	—
Lack of fit	3	0.0382	0.0127	0.12	0.945
Pure error	4	0.4316	0.1079	—	—
Total	16	25.9967	—	—	—

Interactions between the operational variables

Response surface plots offer ways to predict the collagen production for different values of the tested variables and the contours of the plots help in identification of the type of interactions between these variables. Each contour curve represents an infinite number of combinations of two tested variables with the other two maintained at their respective zero level. A circular contour of response surface indicates that the interaction between the corresponding variables is negligible. In contrast, an elliptical or saddle nature of the contour plots indicates that the interaction between the corresponding variables is significant. The 3D response surface plots and 2D contour plots are the graphical 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 [21]. The response surface contour plots for the effect of each pair of variables are shown in Figs.1-3.

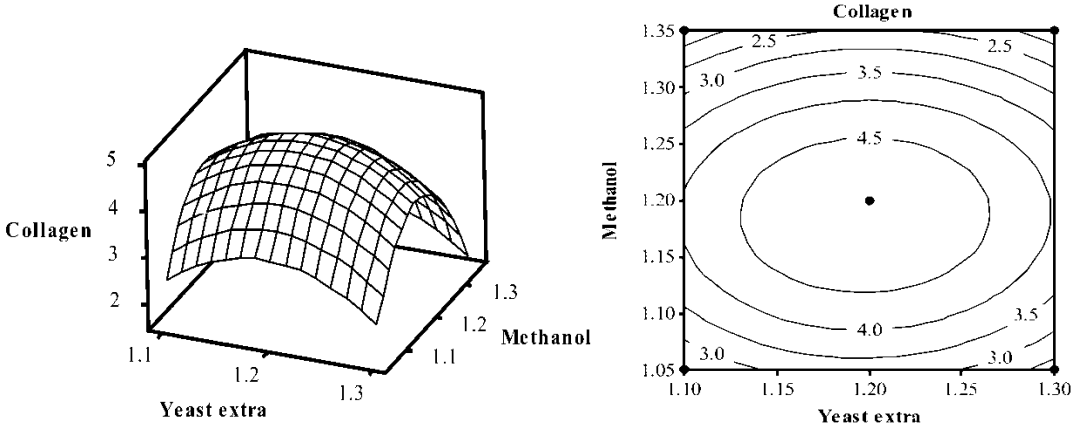

Fig.1 Response surface plot and contour plot of the effects of yeast extra and methanol

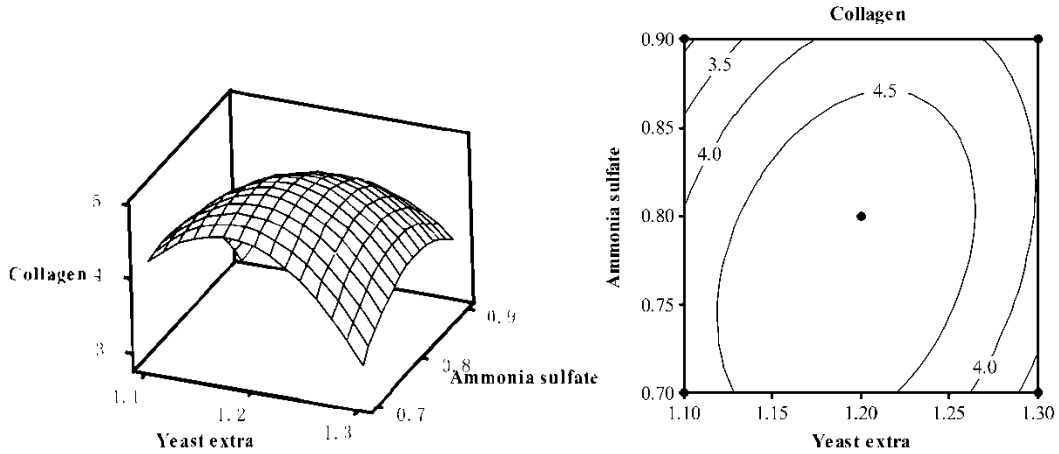

Fig.2 Response surface plot and contour plot of the effects of yeast extra and ammonia sulfate

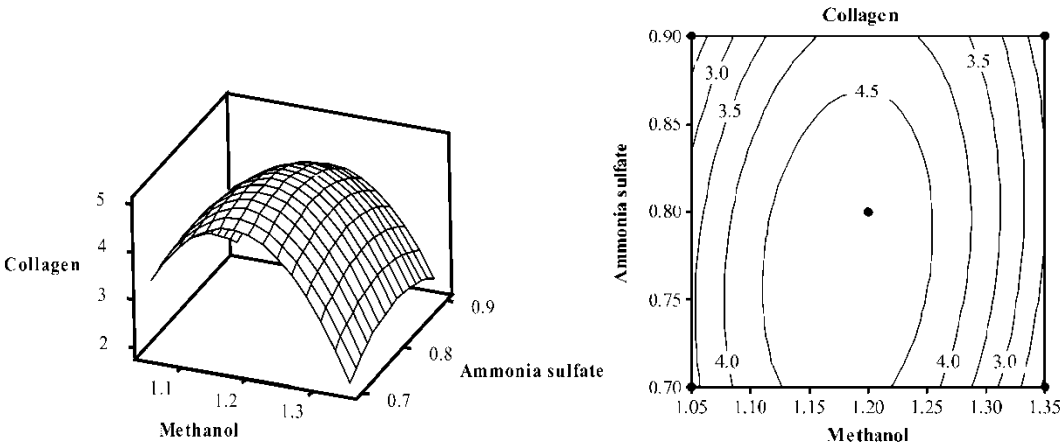

Fig.3 Response surface plot and contour plot of the effects of methanol and ammonia sulfate

Fig.1 shows that there is no significant interaction between yeast extra and peptone, which is evident from the relatively circular nature of the contour curves. With the increase of the concentration of methanol from 1.05% to 1.2%, the collagen production significantly increased from 1.628 to 4.251 g/L at a low concentration of yeast extra, and increased from 1.768 to 3.578 g/L at a high level of yeast extra. When the concentration of yeast extra was near 1.2%, increasing the concentration of methanol from 1.05% to 1.2% to some extent favored the accumulation of collagen, and any further increase in its values resulted in decreased collagen production. The 3D plot and its respective contour plot facilitated the identification of the optimum levels of yeast extra and methanol. The analysis of Figure 1 reveals that the optimal concentration of yeast extra was around 1.2%, and the concentration of methanol was around 1.2%.

Figure 2 depicts the 3D plot and its corresponding contour plot showing the effects of yeast extra and ammonia sulfate on the collagen production, while the methanol were fixed at its middle level. There was significant mutual interaction between yeast extra and ammonia sulfate, which is also evident from the relatively elliptical nature of the contour curves. When the concentration of yeast extra was near 1.2%, increasing the concentration of ammonia sulfate from 0.7 to 0.8% to some extent favored the accumulation of collagen, the collagen production increased from 3.231 to 5.438 g/L, and any further increase in its values resulted in decreased collagen production. With the increase of the concentration of ammonia sulfate from 0.7 to 0.8%, the collagen production significantly increased from 2.916 to 4.252 g/L at a low concentration of yeast extra, and increased from 3.231 to 3.587 g/L at a high level of yeast extra. The analysis of Figure 2 reveals that the optimal concentration of ammonia sulfate was around 0.8%.
Figure 3 presents 3D plot and its corresponding contour plot showing the effects of methanol and ammonia sulfate on the collagen production, while the concentration of yeast extra was fixed at its middle level. There was a significant mutual interaction between methanol and ammonia sulfate. Under the moderate concentration of ammonia sulfate, the collagen production increased with increasing the concentration of methanol from 1.05 to 1.2%, and any further increase in its values resulted in decreased collagen production.

The significance of the interaction between yeast extra and methanol and between yeast extra and ammonia sulfate could partly illustrate the fact that yeast extract contains complex nutrients such as vitamin, nucleic acid, lipid and

other substances, and it might be necessary for growth and production of secondary metabolites from microbes and thus it is the key nutrient material which controls the biosynthesis of this protein.[22]This fact has also been suggested previously during other enzyme production experiments on nitrogen repression effects.

Validation of the optimized results

By solving the regression equation through MATLAB 7.0 (The MathWorks, Inc., Natick, MA, USA) and analyzing the response surface plots, the optimal values of the test variables in coded unit were as follows: X_1=-0.0972, X_3=-0.1205, X_5=-0.3178, and the corresponding real values were yeast extra 1.19%, peptone 1.18% and glycerol 0.77%, respectively. Based on these optimal variable levels, the predicted maximum production of collagen of *P. pastoris* is 4.96 g/L. Validation experiment was carried out, and the final DCW reached 5.02 g/L. This shows an excellent correlation between the experimental and predicted values, which was 19.3% yield higher in comparison to the yield before optimization.

CONCLUSION

The improvement of production (1.19 fold) showed this optimization work was successful for the production of collagen 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 BMMY fermentation medium optimization for the production of collagen of *P. pastoris*, the present study provides a valuable reference for both laboratory and factory approach. This knowledge is very important, particularly for optimizing the performance and minimizing the operation cost for an industrial fermentation processes.

Acknoledgement

This study was financially supported by the National Natural Science Foundation of China (21276210, 21106112, 21106111, 21106114, 31000019, 21206135 and 21376190); the National High Technology Research and Development Program of China (863 Program, 2014AA02108)

REFERENCES

[1] Di Lullo, G. A., Sweeney, S. M., Körkkö, J., Ala-Kokko, L., and San Antonio, J. D, "Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen",*J. Biol. Chem.*, *277*, 4223-4231 (**2002**).

[2] Mhaske, A., Raut, D., Singh, R., Mani, A., Patel, J., and Quereshi, S, "Extraction and Partial Characterization of Collagen from Different Animal Skins",*RecentRes. Sci. Technol*., *2*(9) (**2010**).

[3] Aiqing, L., Haiyan, W., and Fengjuan, D, "Application of Hydrolyzed Collagen in Dairy Products", *Agric. Eng.*, *9*, 016 (**2012**).

[4] Wang, X., Ghasri, P., Amir, M., Hwang, B., Hou, Y., Khilili, M.and Chen, M, "Topical Application of Recombinant Type VII Collagen Incorporates Into the Dermal–Epidermal Junction and Promotes Wound Closure" *Mol. Ther* (**2013**).

[5] Yorgancioglu, A., and Bayramoglu, E. E, "Production of cosmetic purpose collagen containing antimicrobial emulsion with certain essential oils",*Ind. Crops Prod.*, *44*, 378-382 (**2013**).

[6] Cereghino, J. L., and Cregg, J. M., "Heterologous protein expression in the methylotrophic yeast Pichia pastoris",*FEMS Microbiol. Rev.*, *24*, 45-66 (**2000**).

[7] de Jong, L. A., Grünewald, S., Piet Franke, J., Uges, D. R., and Bischoff, R, "Purification and characterization of the recombinant human dopamine D2S receptor from *Pichia pastoris*. *Protein Expression Purif.*, *33*, 176-184 (**2004**).

[8] Singh, S., Gras, A., Fiez-Vandal, C., Martinez, M., Wagner, R., and Byrne, B, "Large-scale production of membrane proteins in Pichia pastoris: the production of G protein-coupled receptors as a case study", In *Recombinant Protein Production in Yeast*, pp. 197-207 (**2012**).

[9] Stratton, J., Chiruvolu, V., and Meagher, M High cell-density fermentation, "In *Pichia protocols*, pp. 107-120 (**1998**).

[10] Plackett, R. L., and Burman, J. P, "The design of optimum multifactorial experiments",*Biometrika*, *33*, 305-325 (**1946**).

[11] Baş, D., and Boyacı, İ. H, "Modeling and optimization I: Usability of response surface methodology", *J. Food Eng.*, *78*, 836-845 (**2007**).

[12] Khuri, A. I., and Mukhopadhyay, S, "Response surface methodology",*Wiley Interdiscip. Rev. Comput. Stat.*, *2*, 128-149 (**2010**).

[13] Soni, S. K., Goyal, N., Gupta, J. K., and Soni, R, "Enhanced production of α-amylase from Bacillus subtilis subsp. spizizenii in solid state fermentation by response surface methodology and its evaluation in the hydrolysis of raw potato starch",*Starch.*, *64*, 64-77 (**2012**).

[14] Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Babu, S. V., Senthilkumar, S. R., and Sangiliyandi, G, "Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology",*Bioresour. Technol., 99*, 8170-8174 (**2008**).
[15] Pansuriya, R. C., and Singhal, R. S, "Response surface methodology for optimization of production of lovastatin by solid state fermentation",*Braz. J. Microbiol., 41*, 164-172 (**2010**).
[16] Singh, S., Pakshirajan, K., and Daverey, A, "Screening and optimization of media constituents for decolourization of Mordant Blue-9 dye by Phanerochaete chrysosporium",*Clean Technol. Environ. Policy*, *12*, 313-323 (**2010**).
[17] Fernandez, J., Kiwi, J., Lizama, C., Freer, J., Baeza, J., and Mansilla, H. D, "Factorial experimental design of Orange II photocatalytic discolouration",*J. Photochem. Photobiol. A*, *151*, 213-219(**2002**).
[18] Fu, J., Zhao, Y., and Wu, Q, "Optimising photoelectrocatalytic oxidation of fulvic acid using response surface methodology",*J. Hazard. Mater.144*, 499-505 (**2007**).
[19] Kim, H. K., Kim, J. G., Cho, J. D., and Hong, J. W. "Optimization and characterization of UV-curable adhesives for optical communications by response surface methodology",*Polym. Test., 22*, 899-906 (**2003**).
[20] Majumder, A., Bhandari, S., Purama, R. K., Patel, S., and Goyal, A, "Enhanced production of a novel dextran fromLeuconostoc mesenteroides NRRL B-640 by Response Surface Methodology", *Ann. Microbial., 59*, 309-315 (**2009**).
[21] Hakobyan, L., Gabrielyan, L., and Trchounian, A, "Yeast extract as an effective nitrogen source stimulating cell growth and enhancing hydrogen photoproduction by *Rhodobacter sphaeroides* strains from mineral springs",*Int. J. Hydrogen Energy*, *37*, 6519-6526 (**2012**).
[22] Venil, C. K., and Lakshmanaperumalsamy, P., "Application of Response Surface Methodology in Medium Optimization for Protease Production by the New Strain of Serratia marcescens SB 08",*Pol. J. Microbiol., 58*, 117-124 (**2009**).