Annals of Microbiology, **58 (4)** 677-681 (2008)

Optimisation of riboflavin production by the marine yeast *Candida membranifaciens* subsp. *flavinogenie* W14-3 using response surface methodology

Zhe CHI, Lin WANG, Liang JU, Zhenming CHI*

Unesco Chinese Center of Marine Biotechnology, Ocean University of China, Yushan Road, No.5, Qingdao, China

Received 23 May 2008 / Accepted 22 September 2008

Abstract - In this study, the optimisation of the process parameters for riboflavin production by the marine yeast strain *Candida membranifaciens* subsp. *flavinogenie* W14-3 was carried out using response surface methodology (RSM) based on Central Composite Designs (CCD). We found that the amount of xylose, pH, temperature and shaking speed had great influence on riboflavin production by strain W14-3. Therefore, a response surface design was used to evaluate the influence of the four factors on the riboflavin production. Then, five levels of the four factors above were further optimised using a Central Composite design. Finally, the optimal parameters for the riboflavin production were obtained with RSM. Under the optimised conditions (the production medium containing 2.0% xylose, pH 4.5, temperature 28 °C and shaking speed 170 rpm), 22 μg ml^{-1} of riboflavin was reached in the culture of strain W14-3 within 54 h of fermentation whereas the predicted riboflavin yield of 22 μg ml^{-1} was derived from RSM regression.

Key words: riboflavin, marine yeasts, *Candida membranifaciens*, response surface methodology.

INTRODUCTION

In recent years, riboflavin production by fermentation has received increasing attention because riboflavin has many physiological roles in human and animals. Riboflavin is an essential vitamin that is required by all bacteria, animals and plants; it is a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Riboflavin is synthesized by plants and microorganisms, but it is not produced by higher animals, which must acquire it from their diet. Riboflavin is produced commercially for use as a food and feed additive (Wang *et al*., 2008). Major portion of riboflavin is used in pharmaceutical formulations. Chemical production still accounts for the major part of industrial riboflavin synthesis. However, the disadvantages of the chemical synthesis are that the process generates a lot of waste and requires organic solvents and 25% more energy in comparison to fermentation process (Stahmann *et al*., 2000). In recent years, commercial fermentations for riboflavin production have been established and it was found that riboflavin production by fermentation has many merits over the chemical process. For example, mild conditions are used, less energy is needed, riboflavin is easier recovered, and less waste is produced during the fermentation. So far the microorganisms which have been used for riboflavin biosynthesis on large scale include the hemiascomycetes *Ashbya gossypii* (Stahmann *et al*., 2000; Wendland and Walther, 2005), a filamentous fungus, *Candida famata* (Stahmann *et al*., 2000), a yeast, *Pichia guilliermondii* (Fayura *et al*., 2007) and the genetically engineered *Bacillus subtilis* which requires at least deregulation of purine synthesis and a mutation in a flavokinase/FAD synthetase (Stahmann *et al*., 2000). It has been shown that many yeasts, such as *P. guilliermondii* and *C. famata* are able to overproduce riboflavin (vitamin B2) only in iron-deficient media as iron represses synthesis of riboflavin (Boretsky *et al*., 2005).

In our previous study (Wang *et al*., 2008), we found that the marine yeast strain W14-3 isolated from seawater of China Eastern Sea and identified to be *Candida membranifaciens* subsp. *flavinogenie* W14-3 could produce riboflavin when it was grown in the medium containing xylose at 25 °C for 24 h. Therefore, the main purposes of the present study are to optimise riboflavin production by the marine yeast *C. membranifaciens* subsp. *flavinogenie* W14-3 using response surface methodology in order to genetically modify the yeast strain for enhanced riboflavin production in the future. This is the first report that riboflavin production by marine yeasts was optimised using response surface methodology.

MATERIALS AND METHODS

Yeast strain and medium. The marine yeast strain W14-3 was isolated from seawater of China Eastern Sea and identified to be *Candida membranifaciens* subsp. *flavinogenie* W14-3 (Wang *et al*., 2008). This yeast strain was maintained at 4 ºC in YPD medium (prepared with seawater) containing (%, w/v) 2.0 glucose, 1.0 yeast extract and 2.0 polypeptone.

* Corresponding author. Phone and Fax: 0086-532-82032266; E-mail: zhenming@sdu.edu.cn

TABLE 1 - Range of the factors investigated in the experimental design

Variables	Code	Levels				
		−2	−1	0	+1	+2
Xylose	A	0%	1.0%	2.0%	3.0%	4.0%
pH	B	2.5	3.5	4.5	5.5	6.5
Temperature	C	26	27	28	29	30
Shaking speed	D	150	160	170	180	190

Screening of physical and chemical parameters using Central Composite Designs. The Central Composite Design for 4 variables which includes physical and chemical parameters at five levels (+2, +1, 0, −1, −2) (Plackett and Burman, 1944) was used for screening. Among the physical and chemical parameters, the amount of xylose, pH, temperature and shaking speed were tested for their significance in the riboflavin production by the marine yeast strain W14-3.

Riboflavin production. One loop of the cells of the yeast strain was transferred to 50 ml of YPD medium prepared with seawater in 250 ml flask and aerobically cultivated at 25 °C for 24 h. When the culture reached a high cell density (OD_{600nm} = 20.0), 0.2 ml of the culture was transferred to 50 ml of the production medium which contained (% w/v): 0.5 of $(NH_4)_2SO_4$, 0.1 of KH_2PO_4, 0.05 of $MgSO_4 \cdot 7H_2O$, 0.01 of $CaCl_2 \cdot 2H_2O$, 0.01 of NaCl, 0.2 of yeast extract, and different amount of xylose, at different pH and grown by shaking at different speed and different temperatures for 3 days. The culture was centrifuged at 12000 rpm and 4 °C for 10 min and the supernatant obtained was used as the crude riboflavin solution for quantitative determination of riboflavin.

A 2^4 factorial design was performed to assess the effect of the amount of xylose, pH, temperature and shaking speed on the riboflavin production by the marine yeast strain W14-3. A central point was carried out in triplicate plus two axial points for each independent factor for experimental error evaluation and second-order effects estimation, respectively. Table 1 shows the range of the studied factors and the corresponding coded levels.

Quantitative determination of riboflavin. The amount of riboflavin in the supernatant was measured quantitatively at 440 nm by using spectrophotometer and riboflavin from Sigma served as standard (Wang *et al*., 2008).

TABLE 2 - Experiments designs used in RSM studies by using four independent variables each at four levels showing observed values of riboflavin production

Runs	A	B	C	D	Riboflavin (μg mL^{-1})
1	−1	−1	−1	−1	6
2	1	−1	−1	−1	15
3	−1	1	−1	−1	10
4	1	1	−1	−1	27
5	−1	−1	1	−1	5
6	1	−1	1	−1	16
7	−1	1	1	−1	11
8	1	1	1	−1	15
9	−1	−1	−1	1	7
10	1	−1	−1	1	15
11	−1	1	−1	1	13
12	1	1	−1	1	24
13	−1	−1	1	1	5
14	1	−1	1	1	15
15	−1	1	1	1	9
16	1	1	1	1	13
17	−2	0	0	0	0
18	2	0	0	0	25
19	0	-2	0	0	5
20	0	2	0	0	10
21	0	0	−2	0	7
22	0	0	2	0	1
23	0	0	0	−2	22
24	0	0	0	2	23
25	0	0	0	0	22
26	0	0	0	0	22
27	0	0	0	0	22
28	0	0	0	0	22
29	0	0	0	0	22
30	0	0	0	0	22

TABLE 3 - Analysis of variance (ANOVA) for regression

Source	Sum of squares	DF	Mean square	F-value	Prob > F
Model	1715.6	14	122.54	28.43	< 0.0001
A-xylose	642.02	1	642.02	148.95	< 0.0001
B-pH	105.11	1	105.11	24.39	0.002
C-Temperature	72.08	1	72.08	16.72	0.001
D-Shaking speed	7.02	1	7.02	1.63	0.99
AB	0.09	1	0.094	0.02	0.88
AC	18.54	1	18.54	4.30	0.06
AD	4.33	1	4.33	1.01	0.33
BC	2.56	1	2.56	0.65	0.43
BD	1.28	1	1.28	0.30	0.59
CD	2.63	1	2.63	0.61	0.45
A2	134.15	1	134.15	31.12	< 0.0001
B2	320.15	1	320.15	74.28	< 0.0001
C2	504.82	1	504.82	117.12	< 0.0001
D2	2.94	1	2.94	0.68	0.42
Residual	64.65	15	4.31		
Lack of Fit	64.65	10	6.47		
Pure Error	0.000	5	0.000		
Cor total	1780.26	19			

Partial purification of riboflavin. Partial purification of riboflavin in the culture was carried out by using the methods described by Wang *et al*. (2008). Fifty millilitres of 0.1 mM hydrochloric acid was added to the crude riboflavin solution (50 ml) in a 250 ml conical flask. The solution was placed in a water bath at 100 °C for 30 min. After being allowed to cool, it was adjusted to pH 4.5 with 2.5 M sodium acetate and filtered through filter paper. The filtrate obtained was applied to an MT cleanup and concentration column (TEDA FUJI Chromatogram Developing Corp). The riboflavin absorbed on the column was eluted by using pure methanol. The eluate was filtered through a MILLEX®HV filter (0.45 μm). The filtrate was used for high-performance liquid chromatography (HPLC) analysis of riboflavin.

Chromatographic analysis. The riboflavin in the filtrate was analysed by using HPLC system (Waters, USA) according to the methods described by Wang *et al*. (2008).

Statistical analysis of the data. The statistically planned experimentation is to identify the significant variables and their corresponding coefficients, so that the levels of variables can be managed to obtain a desired output. Hence, the coefficients, sum of squares in percentage (SS%) and coefficient of variation (CV) were analysed using the experimental results of the riboflavin yields produced. Using Design Expert v. 7.0.0 (Statease, Minneapolis, USA), the experimental plan, the analysis and the results were obtained.

RESULTS AND DISCUSSION

Optimisation of the screened variables

Response surface methodology (RSM) is a model, consisting of mathematical and statistical techniques, widely used to study the effect of several variables and to seek the optimum conditions for a multivariable system. In RSM, the number of experimental runs required is very few, leading to saving of time, chemicals, glassware and manpower. Experimental design and data analysis using appropriate software make the analysis easier as observed in the present study (Kalil *et al*., 2000; Sunitha *et al*., 2000; Volhra and Satyanarayana, 2002; Li *et al*., 2008).

The amount of xylose, pH, temperature and shaking speed were identified as the most influential among physical parameters for production of riboflavin by the marine yeast strain W14-3 on single variable optimisation methods. A Central Composite Designs were employed to analyse the interactive effect of these parameters and to arrive at an optimum. The base points for the design were selected from a single-parameter study (data not shown). A summary of the variables and their variation levels is given in Table 1.

The liquid fermentation was carried out according to the design (Table 2) for 72 h. The fermented samples were assayed for riboflavin yields. The results were analysed on a PC running under Windows OS, using Design Expert v. 7.0.0 (Statease) and the response surface was generated. The design and results (riboflavin yields) of experiments carried out with the Central Composite Designs are given in Table 2.

The analysis of variance (ANOVA) was employed (Table 3) for determination of significant parameters. ANOVA consists of classifying and cross classifying statistical results and tests whether the means of a specified classification differ significantly. This was carried by Fisher's statistical test for square due to regression to the mean square due to error and indicated the influence (significance) of each controlled factor on the tested model.

The results obtained were submitted to ANOVA and the regression model was given as Eq. (1):

$$Y = 22.02+5.17^{*} A+2.09^{*} B-1.73^{*} C-1.71E-1.08^{*} A^{*}C- 1.61^{*}B^{*}C -0.28^{*}B^{*}E -2.21^{*}A^{2}-3.42^{*}B^{2}-4.29^{*}C^{2}$$

where Y was riboflavin yield, A was the amount of xylose, B was pH, and C was temperature.

The ANOVA of the quadratic regression model demonstrates that Eq. (1) was a highly significant model, as was evident from the Fisher's F-test with a very low probability value (F-value = 28.43) (Table 3). Values of "Prob > F" less than 0.001 (Table 3) indicate model terms were significant. The Model F-value of 28.43 implies that the model was significant. There was only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate that model terms were significant.

TABLE 4 - Test of significance for regression square

Std. Dev.	2.08	R-Squared	0.96
Mean	14.35	Adj R-Squared	0.93
C.V. %	14.17	Pred R-Squared	0.79
PRESS	372.40	Adeq Precision	15.70

The goodness of fit of the model was checked by determination coefficient (R^2) (Table 4). In this case, the value of the determination coefficient (R^2= 0.96) (Table 3) indicates that only 3.4% of the total variations was not explained by the model. The value of the adjusted determination coefficient (AdjR^2 = 0.93) was also very high to advocate for a high significance of the model. The 'predicted R^2- value of 0.79 for the riboflavin production was a reasonable agreement with the 'adjusted R^2-values of 0.93. At the same time, a relatively lower value of the coefficient of variation (CV = 14.17%) indicates a better precision and reliability of the experiments carried out.

The fitted response for the above regression model was plotted in Fig. 1. Three-dimensional graphs were generated for the pair-wise combination of the five variables, while keeping the other one at their optimum levels for the riboflavin production by strain W14-3. Graphs were given here to highlight the roles played by the various variables.

The predicted maximum riboflavin yield (22 μg ml^{-1}) derived from RSM regression was obtained when the amount of xylose in the medium was 2.0% (w/v), pH was 4.5, cultivation temperature was 28 °C and shaking speed was 170 rpm (Fig. 1).

Validation of the experimental model

The time course of the riboflavin production by yeast strain W14-3 was examined during the liquid fermentation under the optimal conditions obtained from RSM. The results in Fig. 2 show that the highest riboflavin yield (22 μg ml^{-1}) was reached within 54 h of the liquid fermentation when cell growth reached the late stationary phase. The results also suggest that the actual riboflavin yield (22 μg ml^{-1}) in the optimised medium from three replications was very close to the predicted value and the model was proven again to be adequate (Figs. 1 and 2). This means that the wild type of *C. membranifaciens* subsp. *flavinogenie* W14-3 could produce very high level of riboflavin. For example, when different *P. guilliermondii* strains were cultured in a complex medium containing 2.0% xylose, only *P. guilliermondii* NRRL Y-2076 could produce 14.4 ± 1.2 μg ml^{-1} of riboflavin (El-Refal and Gamati, 1989). It has been shown that many yeasts, such as *P. guilliermondii and C. famata* are able to overproduce riboflavin (vitamin B2) only in iron-deficient media as iron represses synthesis of riboflavin (Boretsky *et al*., 2005). However, it is interesting to note that *C. membranifaciens* subsp. *flavinogenie* W14-3 used in this study could produce riboflavin in the production medium in which iron was not removed before the liquid fermentation.

In our previous study (Wang *et al*., 2008), it was found that *C. membranifaciens* subsp. *flavinogenie* W14-3 could produce more riboflavin in the medium containing xylose than in that containing other carbon sources. That is why xylose was used as the main carbon source in this study. It was also found that strains of *P. guilliermondii* that produced more than 9 μg ml^{-1} riboflavin on xylose produced half that amount on glucose (Leathers and Gupta, 1997). The production of riboflavin (vitamin B2) by *Candida guilliermondii* Wickerham was tested by using different media and 11 mg 100 ml^{-1} of riboflavin was achieved (El-Refal and Gamati, 1989). However, the mechanisms of enhanced riboflavin production in the presence of xylose are still completely unknown. Xylose can be easily obtained from hydrolysate of xylan in wood and grass. Therefore, xylose is an easily obtained carbon source.

Confirmation of riboflavin

In order to confirm if yellow substance in the supernatant of the culture of the marine yeast is riboflavin, the supernatant from the culture with yellow colour was treated as described in Materials and methods. The treated supernatant was applied to the column and the yellow substance in the eluate was analysed by using HPLC. Our results show that the spectra of the partially purified yellow substance were completely identical to those of riboflavin standard (data not shown). This means that the partially purified yellow substance was riboflavin. The results were identical to those obtained by Wang *et al*. (2008).

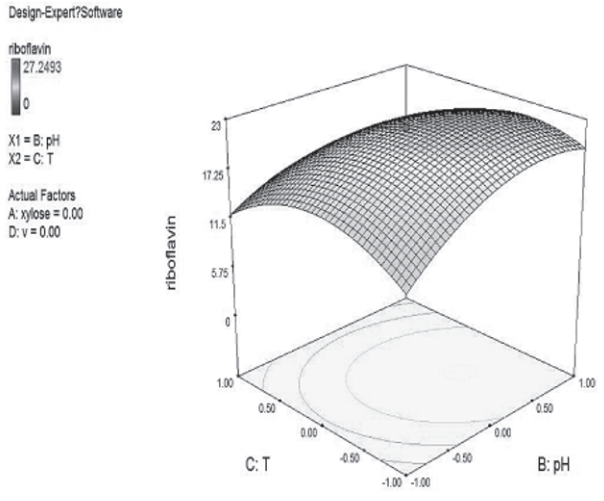

FIG. 1 - Response surface for riboflavin yield.

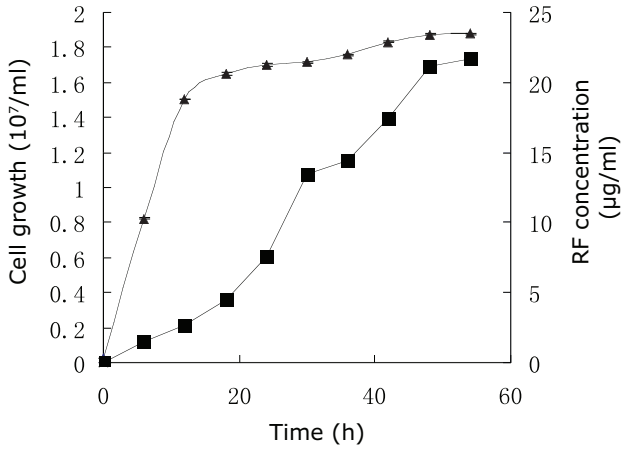

FIG. 2 - Time course of cell growth (▲) and riboflavin production (■) during the fermentation in the production medium containing 2.0% (w/v) xylose, pH 4.5 by cultivation at 28 °C and 170 rpm. RF: Riboflavin.

CONCLUSIONS

In our previous study (Wang *et al*., 2008), it was found that *C. membranifaciens* subsp. *flavinogenie* W14-3 could secrete riboflavin into medium. After riboflavin production by the marine yeast *Candida membranifaciens* subsp. *flavinogenie* W14-3 was optimised using response surface methodology, the highest riboflavin yield (22 μg ml^{-1}) was reached within 54 h of the liquid fermentation when cell growth reached the late stationary phase and the actual riboflavin yield (22 μg ml^{-1}) was very close to the predicted value and the model was proven again to be adequate. The partially purified yellow substance from the supernatant was proved to be riboflavin.

Acknowledgements

This work was supported by National Infrastructure of Natural Resources for Science and Technology Program of China (No. 2005DKA21209) and Student Research Training Programme of Ocean University of China (No. 0711010403).

REFERENCES

Boretsky Y.R., Kapustyak K.Y., Fayura L.R., Stasyk O.V., Stenchuk M.M., Bobak Y.P., Drobot L.B., Sibirny A.A. (2005). Positive selection of mutants defective in transcriptional repression of riboflavin synthesis by iron in the flavinogenic yeast *Pichia guilliermondii*. FEMS Yeast Res., 5: 829-837.

El-Refal S.S.A.H., Gamati S.Y. (1989). Physiological study on riboflavin production by a hydrocarbon-utilizing culture of *Candida guilliermondii* Wickerham. J. Islamic Acad. Sci., 2: 27-30.

Fayura L.R., Fedorovych D.V., Prokopiv T.M., Boretsky Y.R., Sibirny A.A. (2007). The pleiotropic nature of *rib80, hit1*, and *red6* mutations affecting riboflavin biosynthesis in the yeast *Pichia guilliermondii.* Microbiology, 76: 55-59.

Kalil S.J., Maugeri F., Rodrigues M.I. (2000). Response surface analysis and stimulation as a tool for bioprocess design and optimization. Process Biochem., 35: 539-550.

Leathers T.D., Gupta S.C. (1997). Xylitol and riboflavin accumulation in xylose-grown cultures of *Pichia guilliermondii*. Appl. Microbiol. Biotechnol., 47: 58-61.

Li X.Y., Liu Z.Q., Chi Z.M. (2008). Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: Optimization by response surface methodology. Bioresour. Technol., 99: 6386-6390.

Plackett R.L., Burman J.P. (1944). The design of optimum multifactorial experiments. Biometrica, 33: 305-325.

Stahmann P., Revuelta J.L., Seulberge H. (2000). Three biotechnical processes *Ashbya gossypii*, *Candida famata* or *Bacillus subtilis* compete with using chemical riboflavin production. Appl. Microbiol. Biotechnol., 53: 509-516.

Sunitha K., Kim Y.O., Lee J.K., Oh T.K. (2000). Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli*. Biochem. Eng. J., 5: 51-56.

Vohra A., Satyanarayana T. (2002). Statistical optimization of the media components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochem., 37: 999-1004.

Wang L., Chi Z.M., Wang X.H., Ju L., Chi Z., Guo N. (2008). Isolation and characterization of *Candida membranifaciens* subsp. *flavinogenie* W14-3, a novel riboflavin-producing marine yeast. Microbiol. Res., 163: 255-266.

Wendland J., Walther A. (2005). *Ashbya gossypii*: A model for fungal development biology. Nat. Rev. Microbiol., 5: 421-429.

Wu Q.L., Chen T., Gan Y., Chen X., Zhao X.M. (2007). Optimization of riboflavin production by recombinant *Bacillus subtilis* RH44 using statistical designs. Appl. Microbiol. Biotechnol., 76: 783-794.