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Determination of optimum fermentation conditions for carotenoid production by *Rhodotorula aurantiaca* K-505

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Abstract−Carotenoid production by *Rhodotorula aurantiaca* K-505 was optimized in shake-flask cultures using a central composite design. Quadratic polynomial models were used to correlate the relationships between six fermentation factors (pH, temperature, and the concentrations of glucose, yeast extract, peptone extract, and ammonium sulfate) and three response variables (cell mass yield, carotenoid content, and carotenoid production). Different optimum culture conditions were predicted by the models for maximizing the three response variables, indicating that there is no direct correlation between cell growth and carotenoid production. The maximum carotenoid production of 4.9 mg/L predicted by the relevant model under optimum culture conditions agreed well with the experimentally measured value of 5.3 mg/L under the same culture conditions.

Key words: Carotenoid, *Rhodotorula aurantiaca*, Response Surface Methodology

INTRODUCTION

Carotenoids are probably the most widespread natural pigments occurring in animals, plants, and microorganisms. They are used commercially as colorants and antioxidants. Wider use of carotenoids as pharmaceuticals is expected due to the recent discovery of their anticarcinogenic effect [1]. Consequently, large-scale production of carotenoids from microorganisms has attracted considerable interest. Recent research efforts have focused on the optimization of culture conditions for carotenoid production in order to minimize production costs and maximize volumetric productivity. It is well known that the effects of culture conditions on yield and productivity are very complex with possible interactions among the various variables. To account for the interactive influences of fermentation variables and to reduce the number of laborious experiments, response surface methodology (RSM) has been successfully used to elucidate the effects of these variables on carotenoid production [2-6].

RSM is a powerful method for accumulating and analyzing information about a process rapidly and efficiently from a small number of experiments. It combines statistical experimental designs and empirical model building by regression for the purpose of process or product optimization [7,8]. RSM usually involves three distinct stages. In the first stage, information on the effects of some independent variables (e.g., culture conditions) on dependent variables (e.g., product yield) is generated from statistically designed experiments. Empirical models are then used to relate the dependent variables to the independent variables. Once a suitable model is obtained, it can be used for optimization, i.e., finding the values of the independent variables that maximize or minimize the dependent variables.

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Different combinations of culture factors were investigated in recent studies employing the RSM approach to optimize carotenoid production by different species of *Rhodotorula*, e.g., concentrations of five trace elements: Fe^{3+}, Co^{2+}, Mn^{2+}, Al^{2+} and Zn^{2+} [9]; concentration of mung bean waste flour, concentration of sweet potato extract, pH, temperature, agitation rate and cultivation time [10]; concentrations of yeast extract, glucose and $(NH_4)_2SO_4$ and volumes of medium, inoculum, tomato extract and peanut oil [11]; temperature, pH and dissolved oxygen [12]; and concentrations of yeast extract, malt extract, peptone and glucose [13]. This study is primarily aimed at evaluating the effects of two fermentation factors (pH and temperature) and four nutrient factors (concentrations of glucose, yeast extract, peptone extract, and ammonium sulfate) on cell mass yield, carotenoid content, and carotenoid production in the yeast *Rhodotorula aurantiaca* K-505. RSM was then employed to optimize the six factors to enhance carotenoid production.

MATERIALS AND METHODS

1. Microorganism

The microorganism used was *Rhodotorula aurantiaca* K-505 isolated from soil and identified by the Korea Research Institute of Bioscience & Biotechnology. The cells were stored on yeast malt agar plates at 4 °C and transferred every month.

2. Media

The culture medium contained glucose, yeast extract, peptone extract, and ammonium sulfate. The concentration levels of these components were varied according to the experimental design described below. Erlenmeyer flasks containing 100 mL of the culture medium were inoculated with 3% of a seed culture medium and incubated on a reciprocating shaker at 130 rpm. The seed culture medium had the following composition (in g/L): glucose 10, yeast

extract 3, malt extract 3, and peptone extract 5.

3. Isolation and Extraction of Carotenoids

The DMSO (dimethylsulfoxide) method was used to rupture cells prior to extraction of carotenoids [14]. Great care was taken to protect samples from light. Butylated hydroxytoluene (0.01% w/v) added to acetone was used for carotenoid extraction. Each carotenoid was separated by thin layer chromatography on silica gel plates. After development, each band was scraped and dissolved in acetone and ethanol. The resulting absorption spectra were used for identification of individual carotenoids.

4. Analytical Methods

Cell density was determined by turbidity measurements using a UV/VIS spectrophotometer (HP 8452A) at 660 nm and correlated to dry cell weight. The residual glucose concentration in broth was determined by the dinitrosalicylic acid method [15]. Quantitative determination of carotenoids was carried out spectrophotometrically at the maximum absorption wavelength (478 nm) using an appropriate solvent. In this study, the total amount of carotenoids was calculated by the following equation:

$$m = \frac{An}{100E^{1\%}_{1\,cm}}$$

where m is the amount of carotenoids in g present in n mL of sol-

Table 1. Experimental ranges and levels of the six factors tested in the central composite design

Factor	Symbol	Ranges and levels				
		−2	−1	0	1	2
Glucose (g/L)	x_1	5	10	15	20	25
Yeast extract (g/L)	x_2	0	2	4	6	8
Peptone extract (g/L)	x_3	0	2	4	6	8
Ammonium sulfate (g/L)	x_4	0	0.5	1	1.5	2
pH	x_5	1	4	7	10	13
Temperature (°C)	x_6	15	20	25	30	35

Table 2. Experimental design matrix and experimental results for the central composite design

Run	Design matrix						Experimental results		
	x_1	x_2	x_3	x_4	x_5	x_6	Cell mass yield (g/L)	Carotenoid content (mg/g)	Carotenoid production (mg/L)
1	−1	−1	−1	−1	−1	−1	6.9	404.8	2.79
2	1	−1	−1	−1	1	−1	7.9	328.9	2.60
3	−1	1	−1	−1	1	1	6.6	255.5	1.69
4	1	1	−1	−1	−1	1	7.5	250.9	1.88
5	−1	−1	1	−1	1	1	5.8	236.3	1.37
6	1	−1	1	−1	−1	1	7.2	328.7	2.37
7	−1	1	1	−1	−1	−1	7.8	388.6	3.03
8	1	1	1	−1	1	−1	12.6	371.6	4.68
9	−1	−1	−1	1	−1	1	5.4	337.7	1.82
10	1	−1	−1	1	1	1	5.7	324.4	1.85
11	−1	1	−1	1	1	−1	7.5	366.2	2.75
12	1	1	−1	1	−1	−1	12.9	374.9	4.84
13	−1	−1	1	1	1	−1	6.8	379.3	2.58
14	1	−1	1	1	−1	−1	9.4	366.5	3.45
15	−1	1	1	1	−1	1	6.9	303.1	2.09
16	1	1	1	1	1	1	9.2	241.6	2.22
17	−2	0	0	0	0	0	4.1	377.2	1.55
18	2	0	0	0	0	0	13.5	342.4	4.62
19	0	−2	0	0	0	0	1.7	330.3	0.56
20	0	2	0	0	0	0	10.0	373.3	3.73
21	0	0	−2	0	0	0	8.9	374.5	3.33
22	0	0	2	0	0	0	9.0	417.4	3.76
23	0	0	0	−2	0	0	9.6	378.6	3.63
24	0	0	0	2	0	0	9.2	437.9	4.03
25	0	0	0	0	−2	0	0.3	504.4	0.15
26	0	0	0	0	2	0	9.1	432.9	3.94
27	0	0	0	0	0	−2	5.2	220.9	1.15
28	0	0	0	0	0	2	0.1	0.0	0.00
29	0	0	0	0	0	0	9.1	399.1	3.63
30	0	0	0	0	0	0	9.0	401.8	3.62
31	0	0	0	0	0	0	9.3	381.7	3.55
32	0	0	0	0	0	0	8.8	384.3	3.38
33	0	0	0	0	0	0	8.9	386.0	3.44

vent, A is the absorbance, and $E^{1\%}_{1\,cm}$ is the absorbance of a 1% w/v solution in a 1 cm path cuvette at a given wavelength. A value of 500 is usually used for $E^{1\%}_{1\,cm}$.

5. Response Surface Methodology

Carotenoid production in *R. aurantiaca* K-505 was optimized by means of RSM. A central composite design was used to evaluate the effects of the following six factors: concentration of glucose (x_1), concentration of yeast extract (x_2), concentration of peptone extract (x_3), concentration of ammonium sulfate (x_4), pH (x_5), and temperature (x_6) on cell mass yield (y_1), carotenoid content (y_2), and carotenoid production (y_3). Table 1 shows the experimental ranges and levels of the six factors tested in the central composite design (−1 and −2 for the lower levels, 1 and 2 for the upper levels, and 0 for the center point level). The design matrix, shown in Table 2, consisted of 28 experimental runs and five additional runs at the center point level to check reproducibility. The runs were conducted in randomized order to guard against systematic bias. In the experimental design, the six factors are coded according to the equation

$$x_i = \frac{(X_i - X_0)}{\Delta X}$$

where x_i is the coded value of the ith factor, X_i is the natural value of the ith factor, X_0 is the factor's natural value at the center point level, and ΔX is the step change value.

The three response variables-cell mass yield, carotenoid content, and carotenoid production-are also listed in Table 2. The carotenoid production was calculated from the experimentally measured cell mass yield and carotenoid content. Each response was used to develop an empirical model of the response surface in which each dependent variable was obtained as the sum of the contributions of the six investigated factors through first-order, second-order, and interaction terms, according to the following quadratic polynomial:

$$y = b_0 + \sum_i b_i x_i + \sum_i b_{ii} x_i^2 + \sum_i \sum_j b_{ij} x_i x_j \quad (1)$$

where y is the predicted response and b_0, b_i, b_{ii}, and b_{ij} (i<j) are the coefficients obtained by multiple regression of the experimental data. D.o.E. FUSION PRO software (S-Matrix Corp., Eureka, CA, USA) was used for statistical and regression analyses of the data obtained from the central composite design.

RESULTS AND DISCUSSION

1. Identification of Carotenoids

Five carotenoid pigments were separated by thin layer chromatography; their chromatographic properties are shown in Table 3 and Fig. 1. Three of the five carotenoids were identified by comparing their absorption spectra and polarity deduced from their R_f values with published data [16]. The absorption spectra of pigments 1, 2, and 3 shown in Fig. 1 matched those of plectaniaxanthin, torulene, and 2-hydroxyplectaniaxanthin, respectively. A previous study also reported the presence of pigments 1 and 3 in carotenoids produced by a strain of *R. aurantiaca* [17]. However, two of the five pigments could not be identified in this way. A small quantity of β-carotene (0.33% of total carotenoids) was detected through HPLC analysis (data not shown).

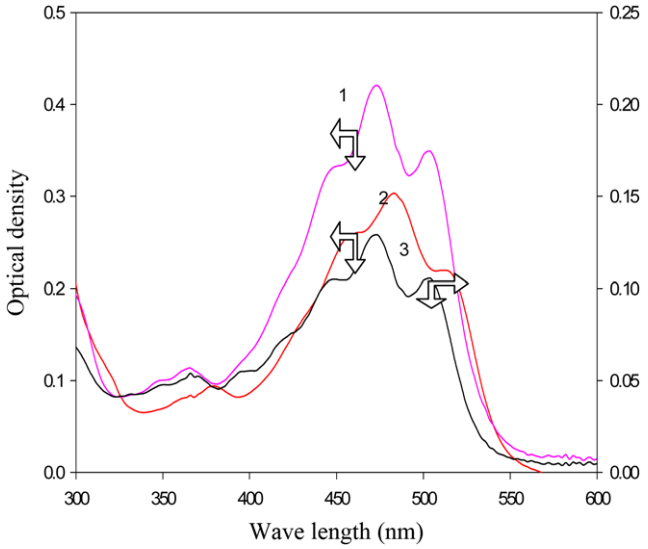

Fig. 1. Absorption spectra of three carotenoid pigments (see Table 3) separated by thin layer chromatography.

2. Optimization of Carotenoid Production

A central composite design was employed to evaluate the influence of six fermentation factors on carotenoid production in shake flasks. A typical central composite design is composed of a two-level fractional factorial design combined with a set of 2k axial or star points, where k is the number of independent variables studied. In this work a design comprising a 2^{6-2} fractional factorial design with 12 star points and 5 replicates at the central points was employed to generate experimental data, resulting in a total of 33 experiments. The design matrix and experimental responses are shown in Table 2. As can be seen, significant changes of cell mass and carotenoid yields exist within the 33 runs and that the highest values of

Table 3. Identification of carotenoid pigments

Pigment	R_f	Color	Maximum absorbance wavelengths (nm)	
			In acetone	In ethanol
Plectaniaxanthin (1)*	0.78-0.81	Orange-red	452;474;504	450;476;504
Torulene (2)*	0.91-1.00	Red	464;484;510	462;483;511
Hydroxyplectaniaxanthin (3)*	0.60-0.62	Orange	448;476;504	448;476;504
Unknown	0.33	Yellow	ND	ND
Unknown	0.27	Yellow	ND	ND

*Numbers in braces refer to spectra 1, 2, and 3 shown in Fig. 1
ND=Not detected

cell mass yield, carotenoid content, and carotenoid production were obtained in different runs: a maximum cell mass yield of 13.5 g/L was observed in run 18, carotenoid content reached the highest value of 504.4 μg/g in run 25, and run 12 produced the highest carotenoid production of 4.84 mg/L.

The results presented in Table 2 for cell mass yield, carotenoid content, and carotenoid production were subjected to regression and the analysis of variance (ANOVA). By using multiple regression analysis, the three experimental responses listed in Table 2 were correlated with the six factors according to Eq. (1).

$$\text{cell mass yield} = 8.25 + 1.56x_1 + 1.36x_2 + 0.23x_3 + 0.02x_4 + 0.65x_5 - 1.15x_6 + 0.37x_1^2 - 0.37x_2^2 + 0.42x_3^2 + 0.52x_4^2 - 0.65x_5^2 - 1.16x_6^2 + 0.50x_1x_2 + 0.21x_1x_3 + 0.14x_1x_4 - 0.08x_1x_5 - 0.56x_1x_6 + 0.15x_2x_4 - 0.22x_2x_6$$

$$\text{carotenoid content} = 390.5 - 6.40x_1 - 2.84x_2 + 2.43x_3 + 10.29x_4 - 16.43x_5 - 47.68x_6 - 7.65x_1^2 - 9.65x_2^2 + 1.39x_3^2 + 4.46x_4^2 + 19.56x_5^2 - 69.99x_6^2 - 4.05x_1x_2 + 5.39x_1x_3 - 4.61x_1x_4 + 8.90x_1x_5 + 6.87x_1x_6 - 5.63x_2x_4 - 12.36x_2x_6$$

$$\text{carotenoid production} = 3.33 + 0.50x_1 + 0.45x_2 + 0.10x_3 + 0.08x_4 + 0.21x_5 - 0.57x_6 - 0.001x_1^2 - 0.24x_2^2 + 0.11x_3^2 + 0.19_4^2 - 0.26x_5^2 - 0.63x^2{}_6 + 0.15x_1x_2 + 0.10x_1x_3 + 0.03x_1x_4 + 0.01x_1x_5 - 0.19x_1x_6 + 0.003x_2x_4 - 0.21x_2x_6$$

Table 4 shows the ANOVA for the three model equations at the 10% level of significance. The computed F values are higher than the theoretical F value of 2.02, indicating that the three model equations are adequate for approximating the true response surfaces of cell mass yield, carotenoid content, and carotenoid production. Fig. 2 shows the observed mass yield, carotenoid content, and carotenoid production in comparison with those from the corresponding empirical models. Although Fig. 2 shows some scatter, for all cases the agreement achieved is quite satisfactory.

The above three model equations were used for finding the optimum culture conditions that maximize cell mass yield, carotenoid content, and carotenoid production. According to Table 2, there is little or no cell growth when X_5=−2 or pH 1 (run 25) and x_6=2 or 35 °C (run 28). Consequently, optimization was carried out by restricting the six factors to the −1 and 1 range. The maximum cell mass yield, carotenoid content, and carotenoid production obtained from the three model equations are listed in Table 5, as are the combinations of the six factors in natural units giving these maximum values. The results in Table 5 reveal that the optimum culture conditions for maximum cell mass yield differ from the optimum culture conditions that maximize carotenoid content. As a result, differ-

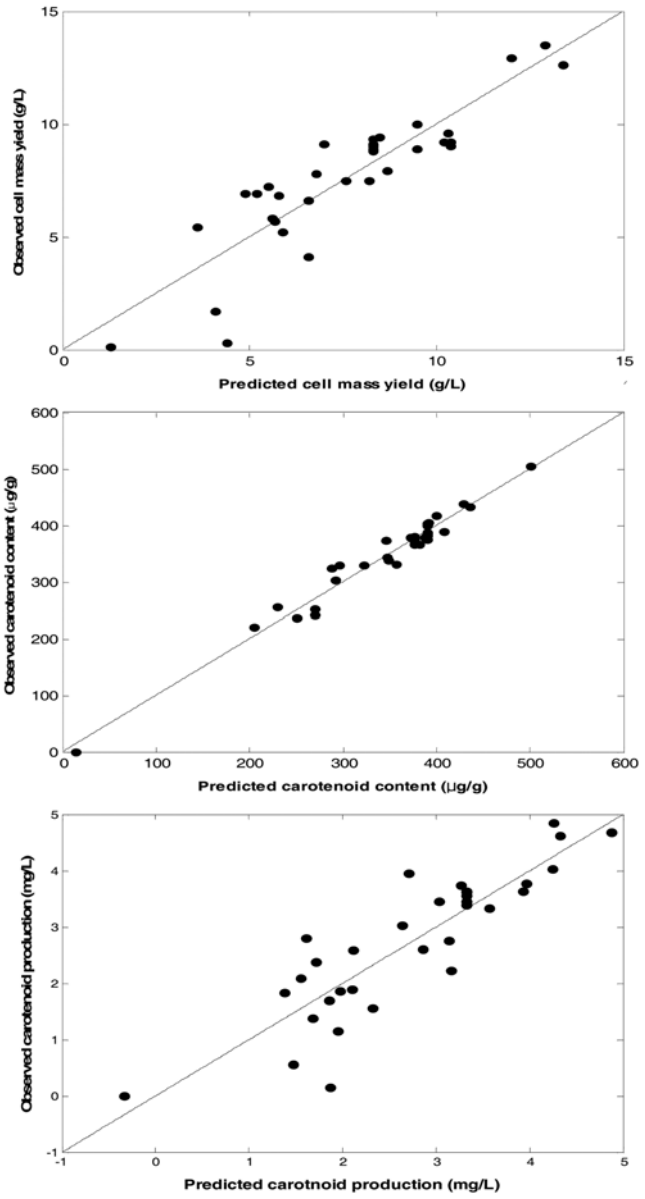

Fig. 2. Response variables calculated from the quadratic models vs. the corresponding experimentally measured values.

Table 4. Analysis of variance for the three model equations

Response	Source	Sum of square	Degree of freedom	Mean of square	*F*-Test
Cell mass yield	Regression	236.26	19	12.43	2.57
	Residual	62.83	13	4.83	
	Total	299.09	32		
Carotenoid content	Regression	239900	19	12627	21.63
	Residual	7588	13	584	
	Total	247500	32		
Carotenoid production	Regression	38.93	19	2.05	2.24
	Residual	11.89	13	0.91	
	Total	50.83	32		

Table 5. Maximum cell mass yield, carotenoid content, and carotenoid production identified by the three quadratic polynomial models and the optimum factors that result in the maximum values

Predicted maximum response	Optimum factors					
	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	X_4 (g/L)	X_5	X_6 (°C)
Cell mass yield (g/L)=14.2	20	6	6	1.5	7	20
Carotenoid content (μg/g)=468	10	4	2	1.5	4	23
Carotenoid production (mg/L)=4.9	20	6	6	1.5	5	22

ent optimum culture conditions exist for maximizing carotenoid production, which is calculated by multiplying the values of cell mass yield and carotenoid content. From a practical standpoint, maximizing the carotenoid production is of interest. The predicted maximum carotenoid production is 4.9 mg/L, as shown in Table 5. Experimental validation of the optimum culture conditions gave a carotenoid production of 5.3 mg/L. The measured result is in good agreement with the predicted value, and therefore corroborates the effectiveness of the response surface approach as a useful tool for fermentation modeling and optimization.

CONCLUSIONS

This study has demonstrated the feasibility of using response surface methodology to optimize the fermentation conditions for the production of carotenoids by *R. aurantiaca* K-505 in shake flasks. Polynomial models were constructed to model the relationships between six culture variables and three response variables. The models were successfully used to locate the optimum settings of the culture variables for maximizing cell mass yield, carotenoid content, and carotenoid production. A maximum carotenoid production of 4.9 mg/L can be obtained under the optimum culture conditions identified by this response surface approach. The predicted maximum value agreed well with the experimentally measured value of 5.3 mg/L.

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