High-Level Production of Astaxanthin by Xanthophyllomyces dendrorhous Mutant JH1 Using Statistical Experimental Designs

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Medium composition was optimized for high-level production of astaxanthin by Xanthophyllomyces dendrorhous mutant JH1 using statistical experimental designs. Glucose and yeast extract were the most important factors affecting astaxanthin production. Glucose 3.89%, yeast extract 0.29%, KH2PO4 0.30%, MgSO4 0.05%, MnSO4 0.02%, and CaCl2 0.01% were optimum for high-level production of astaxanthin. Under optimized conditions, the maximum concentration of astaxanthin obtained after 7 d of cultivation was 36.06 mg/l. The concentration of astaxanthin predicted by a polynomial model was 36.16 mg/l.

Key words: astaxanthin; carotenoids; high-level production; statistical experimental designs; Xanthophyllomyces dendrorhous

As lipid-soluble pigments, carotenoids constitute a wide range of natural pigments, and they are employed in the agrochemical industry as a food and feed additive. Among them, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is the principal carotenoid in marine animals such as crustaceans and salmonoids. Astaxanthin gives attractive pigmentation to many farmed animals and contributes to consumer appeal in the marketplace. To obtain a natural red-pink color, the use of astaxanthin for pigmentation in aquaculture, especially as a feed supplement in farmed salmon and trout, is necessary, since animals lack the ability to synthesize it de novo. Astaxanthin also has important metabolic functions in animals, including conversion to vitamin A, enhancement of immune response, and protection against diseases such as cancer by scavenging oxygen radicals. The antioxidant activity of astaxanthin has been reported to be approximately 10 times stronger than that of other carotenoids tested, including zeaxanthin, lutein, canthaxanthin, and β-carotene, and 100 times greater than that of α-tocopherol. Hence, astaxanthin has attracted commercial interest not only as a pigmentation source but also as a potent antioxidative reagent. A recent FDA communication admitted the use of astaxanthin as a supplement and listed it for use in salmonid fish feed.

Various natural biological sources of astaxanthin, including the crustacea and crustacean extracts, the green microalga Haematococcus pluvialis, and the red yeast Xanthophyllomyces dendrorhous, have been reported. Among them, H. pluvialis and X. dendrorhous are currently considered to be sources of astaxanthin for industrial production, because crustacean meals have relatively low contents of astaxanthin and high levels of moisture, ash, and chitin. H. pluvialis has high concentrations of astaxanthin, but industrial application is limited by lengthy autotrophic cultivation in open freshwater ponds and the necessity of disrupting the cell wall to liberate the carotenoid. X. dendrorhous has desirable properties and potential commercial value as a dietary source of natural astaxanthin, including rapid heterotrophic metabolism and production of high cell densities in bioreactors.

The cost of astaxanthin production is one of the main factors determining the economics of a process. The goal of basic research for industrial applications is to reduce the cost of astaxanthin production by optimizing the cultivation medium and conditions. Generally, the traditional “one-factor-at-a-time” technique for optimization is used. This method is determined by varying one factor while keeping the other factors at a constant level. Although it is simple, it often requires a considerable amount of time and effort. Recently, statistical designs for optimization have been used successfully in astaxanthin production. These statistical methods have proved to be powerful tools.

The aim of this study is to achieve high-level production of astaxanthin by X. dendrorhous mutant JH1 using statistical experimental designs. The effect of variables (glucose, yeast extract, KH2PO4, MgSO4, MnSO4, and CaCl2) on the production of astaxanthin

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Abbreviation: HPLC, high-performance liquid chromatography
was investigated by combining different statistical experimental designs.

Materials and Methods

Microorganism. *X. dendrorhous* ATCC 96594 was provided by Korea Research Institute of Bioscience and Biotechnology. The astaxanthin-overproducing mutant JH1 was derived from *X. dendrorhous* ATCC 96594 by mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine.\(^{18}\) The strain was cultivated in YM agar at 22 °C for 7 d and stored at -70 °C in 30% glycerol.

**Inoculation and culture conditions.** The basal medium (YM) consisted of 1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone, and the pH was adjusted to 7.0. The individual test media were made according to experimental design (Table 1), and the pH of these media was adjusted to 7.0. A colony of yeast was inoculated into a test tube containing 5 ml of YM broth, and further incubated for 36 h. Seed culture of *X. dendrorhous* was inoculated into a 250 ml baffled flask containing 30 ml of YM broth, and further incubated for 36 h. Seed culture (3%, v/v) was used as inoculum in the main cultures. The main cultures were carried out in 250 ml baffled flasks containing 30 ml of the individual test media. Inoculated baffled flasks were incubated in a rotary shaking incubator at 22 °C and 140 rpm for 7 d.

**Carotenoids and astaxanthin analysis.** For routine analysis of carotenoids, the washed cell pellets were mixed with dimethyl sulfoxide preheated to 55 °C, and agitated for 1 min. The broken cells were thoroughly stirred in acetone and centrifuged, and the pigments in the supernatant were transferred to petroleum ether with the addition of 20% NaCl solution.\(^{18,19}\) Total carotenoid concentration was determined using a spectrophotometer at 474 nm, based on a previously reported extinction coefficient.\(^{20}\) Astaxanthin was extracted from the concentrate and quantitatively analyzed by HPLC on a LUNA C\(_{18}\) column (250 × 4.6 mm; 5 μm, Phenomenex, Tokyo) at 25 °C at a flow rate of 1.0 ml/min with two 510 pumps and a 996 photodiode array detector. The mobile phase consisted of 85% methanol, 5% dichloromethane, 5.5% acetonitrile, and 4.5% water. Samples for HPLC analysis were diluted in the mobile phase, and peaks were measured at 480 nm. Astaxanthin was identified according to its retention time and spectrum by photodiode array detection.

**Cell mass.** Dry cell mass was measured gravimetrically. The cells were harvested and washed twice with distilled water. The washed cells were dried in a drying oven at 80 °C for 48 h.

**Glucose analysis.** The amount of glucose was measured using a glucose assay kit (Young-dong Co., Ltd., Seoul, Korea).

**Experimental design.** A 2\(^{6-2}\) fractional factorial design leading to 16 sets of experiments, performed in duplicate, was used to determine the most significant factor affecting astaxanthin production. The variables were coded according to eq. 1:

\[ x_i = (X_i - X_0) / \Delta X_i \] (1)

where \(x_i\) is the coded value of an independent variable, \(X_i\) is the real value of an independent variable, \(X_0\) is the real value of an independent variable at the center point, and \(\Delta X_i\) is the step change value. The range and the levels of the variables investigated in this study are given in Table 1. Astaxanthin production was taken as the dependent variable or response, \(Y_i\). Empirical fitting of the experimental data was by polynomial regression, based on analysis of variance (ANOVA).

In order to fit an empirical second-order polynomial model, a central composite design with five coded levels was performed (Table 4). The quadratic model for predicting the optimal point was expressed according to eq. 2:

\[ y = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j \] (2)

where \(y\) is the response variable, \(b\) is the regression coefficient of the model, and \(\chi\) is the coded level of the independent variable. An SAS package was used for regression analysis of the experimental data obtained.

The statistical significance of the second-order model equation was determined by \(F\)-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, \(R^2\).

**Results**

The six variables playing the most important role in *X. dendrorhous* cultivation were chosen (Table 1), and the effect of each variable on astaxanthin production was investigated. ANOVA was employed for the determination of significant variables. The experimental design and the results of the 2\(^{6-2}\) fractional factorial design are shown in Table 2. Astaxanthin concentration varied markedly with the conditions tested, in the range of 4.46–31.46 mg/l. The lowest value of astaxanthin concentration was obtained when the maximal level of

<table>
<thead>
<tr>
<th>Independent variables (X_i) (%)</th>
<th>Levels</th>
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</thead>
<tbody>
<tr>
<td>Glucose (%)</td>
<td>-1</td>
</tr>
<tr>
<td>Yeast extract (%)</td>
<td>0.05</td>
</tr>
<tr>
<td>KH(_2)PO(_4) (%)</td>
<td>0.05</td>
</tr>
<tr>
<td>MgSO(_4) (%)</td>
<td>0.01</td>
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<tr>
<td>MnSO(_4) (%)</td>
<td>0</td>
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<tr>
<td>CaCl(_2) (%)</td>
<td>0</td>
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Table 1. Range of Variables at Different Levels for the Fractional Factorial Design

\[ A_i = (X_i - X_0) / \Delta X_i \]

\[ [X]_{\text{max}} = \frac{[X]_0}{\text{density}} = \frac{[X]_0}{0.044} = \frac{2.5}{0.044} = 56.81 \text{ mg/ml} \]

\[ \text{Density} = 0.044 \text{ g/ml} \]

\[ \text{Concentration} = \text{Density} \times \text{Weight} \]

\[ \text{Weight} = \frac{\text{Concentration}}{\text{Density}} \]

\[ \text{Volume} = \frac{\text{Weight}}{\text{Density}} \]

\[ \text{Molarity} = \frac{\text{Concentration}}{\text{Volume}} \]

\[ \text{Moles} = \frac{\text{Molarity} \times \text{Volume}}{\text{Molecular Weight}} \]

\[ \text{Percentage} = \frac{\text{Moles}}{\text{Total Moles}} \times 100 \]

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\[ \text{Percentage} = \frac{\text{Moles}}{\text{Total Moles}} \times 100 \]
glucose and the minimal level of yeast extract were used (run 12). The highest value of astaxanthin concentration was obtained under conditions of maximal level of glucose and maximal level of yeast extract (run 15).

On the basis of these experimental values (Table 2), statistical testing was performed using Fisher’s statistical test for ANOVA. The $F$-value is the ratio of mean square due to regression to the mean square due to error and indicates the influence of each controlled factor on tested models. Also, the $P$-value corresponding to the $F$-value indicates the probability that differences between calculated and tabulated statistics are due only to random experimental error. When ANOVA analysis reflects the significance of a model with a confidence level greater than 99% ($P < 0.01$) in astaxanthin production, the $F$-value and $P$-value were 378.69 and $<0.0001$ respectively (Table 3). Thus the estimated models fit the experimental data adequately. The $F$ test applied on each factor, $\chi_1$, $\chi_2$, $\chi_3$, and $\chi_5$, is statistically significant at the 1% level of significance. Among these factors, the $F$-values of $\chi_1$ and $\chi_2$ were 1423.40 and 956.35 respectively, greater than other significant factors. The interaction of $\chi_1$ and $\chi_2$ was also greater than the others. Analysis of the ANOVA results showed that glucose ($X_1$) and yeast extract ($X_2$) were the two most important variables for the production of astaxanthin.

Response surface methodology was introduced to determine the optimal condition of the $X_1$ and $X_2$ variables. To obtain the optimal condition of these variables, variables $X_4$ and $X_6$ were set at 0.05% MgSO$_4$ and 0.01% CaCl$_2$ (the zero level in Table 1). Variables $X_3$ and $X_5$ were set at 0.25% KH$_2$PO$_4$ and 0.02% MnSO$_4$ (the +1 level in Table 1), because the highest value of astaxanthin concentration was obtained under conditions of maximal level of KH$_2$PO$_4$ and maximal level of MnSO$_4$ (Tables 2 and 3). The experiment was performed with two independent variables, glucose ($X_1$) and yeast extract ($X_2$), using a 2$^4$ full factorial design experiment with four star points ($\alpha = \pm 1.414$) and four replicates at the center point (Table 4).

Regression analysis was carried out to fit the response function with the experimental data. The $F$-value and $P$-value were 28.72 and 0.0004 respectively (Table 5). The tested model is statistically significant at the 1%
level of significance. The statistical significance of the second-order model equation was checked and the coefficient of determination ($R^2$) of the model was calculated to be 0.96, indicating that 96% of the variability in the response can be explained by the model. This indicates that the response equation provided a suitable model for the response surface of the experiment of astaxanthin production. The highest concentration of astaxanthin obtained is as follows: $Y = 34.1452 - 1.6205X_1 + 7.0915X_2 - 8.5405X_1^2 + 6.4016X_2^2 - 0.6123X_1X_2$, where $X_1 = \text{coded value of glucose}$, $X_2 = \text{coded value of yeast extract}$.

Figure 1 shows a three-dimensional diagram and a contour plot of calculated response surface. The optimum values of glucose ($X_1$) and yeast extract ($X_2$) obtained for astaxanthin production were $-0.0531$ and $-0.3892$ respectively. To calculate real values, coded values $X_1$ and $X_2$ were defined as: $X_1 = (X_1 - 4)/2$ and $X_2 = (X_2 - 0.35)/0.15$. According to these results, 3.89% glucose and 0.29% yeast extract were optimum for astaxanthin production. The maximum value of the concentration of astaxanthin predicted from the model was $36.16 \text{ mg/l}$. Consequently, glucose 3.89%, yeast extract 0.29%, KH$_2$PO$_4$ 0.25%, MgSO$_4$ 0.05%, MnSO$_4$ 0.02%, and CaCl$_2$ 0.01% were optimum for high-level production of astaxanthin.

To verify the optimal conditions, experimental re-checking was carried out using conditions representing these optimal factors. Figure 2 shows the time course profile of the production of carotenoids and astaxanthin by *X. dendrorhous* mutant JH1. The intense correlation between experimental and statistical results proves the validity of the response model and the existence of an optimal point. Cell mass greatly increased, and glucose concentration decreased dramatically in 4 d of cultivation. The highest concentration of astaxanthin obtained after 7 d of cultivation was 36.06 mg/l.

### Discussion

The red yeast *Xanthophyllomyces dendrorhous* appears to be the best candidate for producing a natural carotenoid astaxanthin of all the strains reported so far, due to its rapid heterotrophic metabolism and production of high cell densities in bioreactors. It can also potentially provide a biological source of astaxanthin for pigmentation and flavor in the aquaculture industry and supply nutrients required for the growth of the animals. Hence, considerable efforts have been made to improve astaxanthin production by strain development and high-cell density culture. Only a limited number of cases of production of astaxanthin, however, have been reported.

In the present study, statistical optimization methods were used for high-level production of astaxanthin by *X. dendrorhous* mutant JH1. For media formulation, glucose was used as the carbon source, because of its low price and high biomass yield. But, Johnson and Lewis reported that the growth of *X. dendrorhous* was inhibited when the glucose concentration was above 1.5% (w/v), and that the lag time and astaxanthin production were also influenced by high glucose concentrations. On the other hand, a high glucose concentration of 3.89% (w/v) increased, the astaxanthin production and cell growth of *X. dendrorhous* mutant.
In conclusion, _X. dendrorhous_ mutant JH1 is a potential microorganism for the production of astaxanthin, and the optimization using statistical methods appears to be a promising route for high-level production of astaxanthin.

**Acknowledgment**

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