Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Studies on optimization of nitrogen sources for astaxanthin production by *Phaffia rhodozyma**

NI Hui[†1,2], CHEN Qi-he[1], RUAN Hui[1], YANG Yuan-fan[2], LI Li-jun[2], WU Guang-bin[2], HU Yang[2,3], HE Guo-qing[†‡1]

([1]*School of Biosystem Engineering and Food Science, Zhejiang University, Hangzhou 310029, China*)

([2]*School of Bioengineering, Jimei University, Xiamen 361021, China*)

([3]*School of Information and Engineering Technique, Sichuan Agricultural University, Ya'an 625014, China*)

[†]E-mail: nihui@jmu.edu.cn; gqhe@zju.edu.cn

Received Sept. 21, 2006; revision accepted Feb. 5, 2007

Abstract: Fermentation of *Phaffia rhodozyma* is a major method for producing astaxanthin, an important pigment with industrial and pharmaceutical application. To improve astaxanthin productivity, single factor and mixture design experiments were used to investigate the effects of nitrogen source on *Phaffia rhodozyma* cultivation and astaxanthin production. Results of single factor experiments showed nitrogen source could significantly affect *P. rhodozyma* cultivation with respect to carbon source utilization, yeast growth and astaxanthin accumulation. Further studies of mixture design experiments using $(NH_4)_2SO_4$, KNO_3 and beef extract as nitrogen sources indicated that the proportion of three nitrogen sources was very important to astaxanthin production. Validation experiments showed that the optimal nitrogen source was composed of 0.28 g/L $(NH_4)_2SO_4$, 0.49 g/L KNO_3 and 1.19 g/L beef extract. The kinetic characteristics of batch cultivation were investigated in a 5-L pH-stat fermentor. The maximum amount of biomass and highest astaxanthin yield in terms of volume and in terms of biomass were 7.71 mg/L and 1.00 mg/g, respectively.

Key words: *Phaffia rhodozyma*, Astaxanthin, Nitrogen source, Optimization
doi:10.1631/jzus.2007.B0365 **Document code:** A **CLC number:** Q939

INTRODUCTION

Astaxanthin (3,3-dihydroxy-β,β-carotene-4,4'-dione) is a novel carotenoid with high market value for the pharmaceutical and feed industries (Wang and Li, 1997; Guerin *et al*., 2003; Johnson, 1991). Astaxanthin is currently chemically synthesized and added into some animal feeds for pigmentation of animals, especially for marine fishes. However, synthetic astaxanthin is expensive. It was reported (Johnson, 1991) that synthetic astaxanthin accounts for approximately 10% of the total cost of fish feed. There is growing interest in the use of astaxanthin as a pigment for the aquaculture and poultry industries (Verdoes *et al*., 1999). In addition, other diverse biological functions of astaxanthin have attracted more and more interest because of its health benefits to human beings due to its roles in cancer prevention, enhancement of immune response, and as free radical quencher (Paul *et al*., 1997).

Studies showed that fermentation of *Phaffia rhodozyma* is one of the most promising methods for producing natural astaxanthin (Johnson, 1991; 2003). Therefore, the optimal fermenting conditions should be very important for the industrialization of astaxanthin production. Nitrogen source is one of the necessary components in the fermentation medium, and different components of the nitrogen source could greatly affect the yield of astaxanthin (Du *et al*., 2005).

‡ Corresponding author

* Project supported by the National Natural Science Foundation of China (No. 30571450) and the Foundation for Young Professors of Jimei University of Xiamen, China

In this study, different nitrogen sources were used in the fermentation of *P. rhodozyma* and the optimal nitrogen source for high astaxanthin yield was obtained by regressive analysis.

MATERIALS AND METHODS

Microorganism and inoculums development

Phaffia rhodozyme 7B12 originated from *P. rhodozyma* Past-1 that was generously provided by Professor Ulf Stahl, Berlin Industrial College, Germany. The yeast was maintained and activated on yeast/malt (YM) agar slants containing 10.0 g/L glucose, 5.0 g/L bacto-peptone, 3.0 g/L malt extract, 3.0 g/L yeast extract and 20.0 g/L agar, and inoculated in 250 ml shake flasks containing 25 ml sterilized YM medium and cultured on a rotary shaker at 22 °C, 200 r/min for 72 h (Johnson, 1991).

Reagents and chemicals

Astaxanthin and β-carotene were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and stored at −20 °C. Yeast extract, peptone and beef extract were purchased from Shanghai Biochemical Agent Co. (China). Methanol used for determination of astaxanthin was HPLC grade. Other chemicals used in the study were analytical grade.

Experimental design

Single factor design experiments were used to analyze the influence of several nitrogen sources on astaxanthin production by the yeast. Mixture design experiments (Montgomery, 1997) were adopted to optimize the complex nitrogen compositions for *P. rhodozyma* fermentation of astaxanthin production.

The basic medium for *P. rhodozyma* fermentation was composed of 20 g/L glucose, 1 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}H_2O$, 0.5 mg/L $MnSO_4{\cdot}H_2O$, 0.14 mg/L $ZnCl_2$, 0.27 mg/L $FeCl_3{\cdot}6H_2O$, 1.25 mg/L $CuSO_4{\cdot}5H_2O$ and pH was initially controlled at 6.0. To investigate effects of different nitrogen sources on astaxanthin synthesis, $(NH_4)_2SO_4$ was replaced by various nitrogen sources. The optimal experiments were carried out in 250 ml flasks containing 25 ml medium, inoculated with 1% inoculum and then incubated on a rotary shaker at 22 °C, 200 r/min for 96 h (Zhu and Liang, 2005).

Kinetics experiments on *P. rhodozyma* cultivation were carried out in a 5 L pH-stat fermentor (Bio. Braun Biotech International Co., Germany) containing 3.5 L fermentation medium and inoculated with 1% (v/v) inoculum and cultured at 22 °C, 200 r/min, 1.2 VVM, pH 5.0 (Hu *et al*., 2005).

Analytical methods

Yeast cells were harvested by centrifugation and biomass was calculated by dry weight (g/L). Residual sugar was determined by the 3,5-dinitrosalicylic acid (DNS) method (Zhang *et al*., 1997) and carotenoid was extracted by dimethylsulfoxide method (Sedmak *et al*., 1990; Ni *et al*., 2004).

Waters HPLC system (Waters Corporation, Milford, MA, USA) including the 1525 pump, Nova-Pak C_{18} reverse phase column (3.9 mm×150 mm, 4 μm) and 2478 UV detector were used to analyze carotenoids. Carotenoids were isolated in a Nova-Pak C_{18} reverse phase column under the conditions listed in Table 1 with flow rate at 1.2 ml/min, column pressure at 0~3000 psi and column temperature at 40 °C. Astaxanthin concentration was detected at the wavelength of 474 nm.

Table 1 HPLC condition for astaxanthin analysis

Time (min)	Methanol (%)	Water (%)
0.00	80.0	20.0
3.00	80.0	20.0
5.00	100.0	0.0
8.00	100.0	0.0
9.00	80.0	0.0

RESUTLS

Single nitrogen sources affected the astaxanthin yield

Different nitrogen sources listed in Table 2 were used to investigate their effects on *P. rhodozyma* growth and pigmentation. The total amount of nitrogen of these sources was equal to the amount of $(NH_4)_2SO_4$ in the basic medium. Biomass and the yield of astaxanthin were measured and are summarized in Table 2. As shown in Table 2, using beef extract can result in the highest biomass yield, but the astaxanthin yield in terms of broth volume or biomass

were quite lower compared to the yields when using KNO_3 or $(NH_4)_2SO_4$ as nitrogen sources. It is indicated that the yield of astaxanthin was not proportional to the biomass. Using $(NH_4)_2SO_4$ as the nitrogen source produced the highest astaxanthin yield in terms of biomass compared to use of other nitrogen sources. And using KNO_3 produced the highest astaxanthin yield in terms of broth volume. Thus, KNO_3 and $(NH_4)_2SO_4$ were selected to do the following mixture experiments. Beef extracts were also selected in the further study since the largest amount of biomass was obtained when beef extracts were used as the nitrogen source.

Different proportions of three selected nitrogen sources affected the astaxanthin yield

In order to optimize nitrogen source for astaxanthin production by *P. rhodozyma* cultivation, mixture experiment design was carried out by using the three selected nitrogen sources (i.e. beef extract, potassium nitrate and ammonium sulfate) at different levels. The results of mixture experiments showed that the yield of astaxanthin and biomass changed with the proportion of three nitrogen sources (Table 3). Table 4 shows the ANOVA results for the mixture experiment design. It is indicated that the proportion of these three nitrogen sources could significantly affect the amounts of the residual sugar, biomass and astaxanthin. Pro>F were 0.06%, 4.31% and 0.09%, respectively. The astaxanthin yield in terms of volume was selected as response variable for regressive analysis of the influence of the three nitrogen sources. Results in Table 5 demonstrated that X_1 ($(NH_4)_2SO_4$, $P>T$=0.29%), X_2 (KNO_3, Pro>T=3.11%), X_3 (beef extract, $P>T$=0.43%) and $X_2 \times X_3$ ($P>T$=4.38%) could significantly affect the response variables, compared to $X_1 \times X_2$ ($P>T$=62.81%) and $X_1 \times X_3$ ($P>T$=74.77%). Therefore, the effects of $X_1 \times X_2$ ($P>T$=62.81%) and $X_1 \times X_3$ can be neglected. According to the parameters listed in Table 5, Eq.(1) in a second-order polynomial prediction model was established. This model can be used to predict the influence of various proportions of these three nitrogen sources on astaxanthin yield.

Table 2 Nitrogen source significantly affects growth and pigmentation of *Phaffia rhodozyma*

Nitrogen sources	Commercial values ($/kg)	Average biomass of three repeats (g/L)	Average astaxanthin yield of three repeats (mg/L)	Average astaxanthin yield in terms to biomass of three repeats (mg/g yeast)
Carbamide	2.11	3.74^{c}	3.44^{d}	0.92^{b}
Ammonium acetic	2.83	2.88^{e}	1.93^{h}	0.67^{c}
NH_4Cl	1.84	4.95^{b}	5.19^{b}	1.05^{ab}
KNO_3	2.47	3.06^{d}	3.75^{d}	1.23^{a}
$(NH_4)_2SO_4$	1.96	4.96^{b}	5.51^{a}	1.11^{a}
Ammonium oxalic	2.86	3.13^{d}	2.92^{e}	0.93^{b}
Peptone	10.06	4.00^{c}	4.16^{c}	1.04^{ab}
Beef extract	13.23	5.62^{a}	2.29^{g}	0.41^{d}
Yeast extract	8.21	3.14^{d}	2.62^{f}	0.83^{bc}

The same letters behind the values in the same column mean $P>0.05$, different letters mean $P<0.05$

Table 3 Results of mixture experiment by using compounding nitrogen sources

Runs	X_1	X_2	X_3	Average residual sugar of three repeats (g/L)	Average biomass of three repeats (g/L)	Average astaxanthin yield of three repeats (mg/L)
1	1	0	0	0.12	3.94	4.43
2	0	1	0	1.79	3.43	2.61
3	0	0	1	0.46	6.84	4.53
4	0.333	0.667	0	0.17	3.90	2.78
5	0.333	0	0.667	0.40	4.86	4.18
6	0.333	0.333	0.333	0.30	3.88	4.71
7	0.667	0.333	0	0.18	3.82	5.51
8	0.667	0	0.333	0.23	5.38	4.77
9	0	0.333	0.667	0.35	6.66	5.67
10	0	0.667	0.333	0.20	6.01	5.68

X_1, X_2, X_3 are the proportions of the nitrogen content from ammonium sulfate, potassium nitrate and beef extract respectively in all nitrogen sources experimentation

Table 4 Proportion of nitrogen sources significantly affects the astaxanthin biosynthesis by ANOVA analysis

	Biomass	Residual sugar	Astaxanthin production velocity
F-value	67.22	6.73	54.34
Pro>*F* (%)	0.06	4.31	0.09
C.V.	16.14	91.12	17.86
R^2	0.990	0.910	0.988

Table 5 Regressive analysis of the mixture experiments

	Estimate	*T* for H_0	Pro>*T* (%)
X_1	4.88	6.47	0.29
X_2	2.46	3.26	3.11
X_3	4.41	5.84	0.43
$X_1 \times X_2$	1.75	0.52	62.81
$X_1 \times X_3$	−1.15	−0.34	74.77
$X_2 \times X_3$	9.70	2.91	4.38

Average astaxanthin yield of three repeats:

$$Y=4.88X_1+2.46X_2+5.48X_3+0.52X_1\times X_2-0.34X_1\times X_3+2.91X_2\times X_3 \quad (1)$$

Validation experiments for high astaxanthin yield using compounding nitrogen source

Eight compounding nitrogen sources were predicted maximum response values by Eq.(1), so they were selected to confirm if the predicted results were biased to the practical values. The results of validation experiment are shown in Table 6. The response value (astaxanthin yield in terms of volume) reached its maximum value when X_1=0.284, X_2=0.323 and X_3=0.393, and the maximum response values was 6.40 mg/L. Thus, for high astaxanthin yield by *P. rhodozyma* cultivation, the optimal nitrogen source was composed of 13.11% $(NH_4)_2SO_4$, 22.82% KNO_3 and 64.07% beef extract (containing 6% nitrogen). To make same amount of nitrogen equal to 0.1% $(NH_4)_2SO_4$, the optimal nitrogen sources were composed of 0.28 g/L $(NH_4)_2SO_4$, 0.49 g/L KNO_3 and 1.19 g/L beef extract. In addition, a quadratic model showed as Eq.(2) was proposed to predict the response values of the stationary area.

High astaxanthin yield was obtained using the optimized nitrogen source

The optimal compounding nitrogen source was used to culture *P. rhodozyma* as a batch in a 5 L pH-stat bioreactor. As shown in Table 7, the sugar consumption was synchronous with the growth of the yeast, but astaxanthin synthesis lagged sugar consumption and yeast growth. The exponential phase began at 12 h and ended at 42 h after inoculation. Sugar consumption and growth of yeast were so fast in the exponential phase that the biomass reached to the maximum point (8.36 g/L) at 42 h, and then no significant sugar consumption was detected. Astaxanthin was mainly synthesized during 18~54 h after inoculation. The astaxanthin yields in terms of broth volume and in terms of biomass were 7.71 g/L and 1 mg/g, respectively after 54 h cultivation.

DISCUSSION AND CONCLUSION

Some studies showed that *Phaffia rhodozyma* can utilize many kinds of nitrogen sources including ammonium, nitrate and organic nitrogen sources (Johnson, 1991; Du *et al*., 2005). In order to test the effects of nitrogen sources on the growth of *P. rhodozyma* and yield of astaxanthin, single factor experiments were carried out and three nitrogen sources—$(NH_4)_2SO_4$, KNO_3 and beef extract were selected for further studies of compounding nitrogen sources.

Table 6 Validation experiments on composed nitrogen sources for *P. rhodozyma* 7B12 cultivation

Runs	X_1	X_2	X_3	Astaxanthin yield (mg/L)	
				Predicted values	Average values of three repeats
1	0.333	0.333	0.333	5.06	5.79^{b}
2	0.284	0.323	0.393	5.17	6.40^{a}
3	0.236	0.326	0.440	5.29	5.48^{c}
4	0.183	0.334	0.482	5.41	6.11^{ab}
5	0.134	0.345	0.522	5.54	5.84
6	0.084	0.356	0.559	5.68	6.06^{ab}
7	0.035	0.368	0.596	5.83	5.90^{b}
8	0	0.333	0.667	5.12	5.54^{c}

The same letters behind the values in the same column mean $P>0.05$. Average astaxanthin yield of three repeats:

$$Y=-527.28+1313.16X_1+4732.54X_2-1167.49X_3-1034.77X_1^2-3363.25X_1\times X_2-6545.85X_2^2+1015.82X_3^2 \quad (2)$$

Table 7 High astaxanthin yield was obtained using optimized nitrogen source

Time (h)	pH	Residual sugar (g/L)	Biomass (g/L)	Astaxanthin in terms of batch volume (mg/L)	Astaxanthin in terms of biomass (mg/g)
6	6.02	19.06	2.20	0.11	0.05
12	5.06	18.97	2.36	0.16	0.07
18	3.13	11.87	5.26	0.36	0.07
24	2.50	6.55	6.87	2.73	0.40
30	2.42	4.74	7.08	5.28	0.75
36	2.41	3.05	7.58	6.61	0.87
42	2.42	1.04	8.36	6.91	0.83
48	2.47	0.48	8.06	7.45	0.92
54	2.48	0.44	7.71	7.71	1.00
60	2.51	0.43	7.64	7.42	0.97
66	2.53	0.44	7.59	7.29	0.96
72	2.54	0.38	7.21	6.78	0.94
78	2.56	0.53	7.62	7.33	0.96
84	2.57	0.50	7.66	7.49	0.98
90	2.58	0.56	7.51	7.40	0.99
96	2.58	0.55	7.48	7.31	0.98

Mixture experiment design is the most important method for optimal medium composition (Zhang *et al*., 1997). Using this method, compounding nitrogen sources composed of three nitrogen source components [i.e. $(NH_4)_2SO_4$, KNO_3 and beef extract] were used for cultivation of *P. rhodozyma*. Results of mixture experiments in Tables 3 and 4 indicated that different proportions of three components could affect *P. rhodozyma* cultivation and astaxanthin production significantly. After regressive analysis and validation experiments, the most effective nitrogen source was confirmed to be composed of 0.28 g/L ammonium sulfate, 0.49 g/L potassium nitrate and 1.19 g/L beef extract.

P. rhodozyma was batch cultivated in a 5 L pH-stat fermentor to investigate the kinetic characteristics. The results shown in Table 7 showed that highest astaxanthin yield in terms of broth volume and biomass were 7.71 mg/L and 1.00 mg/g, respectively. The maximum amount of biomass was 7.71 g/L. Compared with other fermentation experiments using the same strain, although the amount of biomass was less than that of original medium, astaxanthin yields in terms of volume and biomass were much higher than those of original nitrogen source (Ni *et al*., 2005a; 2005b; 2005c). Additionally, the kinetic characteristics of growth, carbon source consumption and astaxanthin synthesis were consistent with other researches reported (Ni *et al*., 2005a; 2005b; 2005c; Kusdiyantini *et al*., 1998; Jesus *et al*., 2001).

References

Du, X.L., Zhu, M.J., Wu, Z.Q., Liang, S.Z., 2005. Effects of nitrogen sources on astaxanthin production by *Phaffia rhodozyma*. *Sci. Technol. Food Ind.*, **26**(9):73-75, 77 (in Chinese).

Guerin, M., Huntley, M.E., Olaizola, M., 2003. *Haematococcus* astaxanthin: application for human health and nutrition. *Trends Biotechnol.*, **21**(5):210-216. [doi:10.1016/S0167-7799(03)00078-7]

Hu, Z.C., Zheng, Y.G., Wang, Z., Shen, Y.C., 2005. Effect of sugar-feeding strategies on astaxanthin production by *Xanthophyllomyces dendrorhous*. *World J. Microbiol. Biotechnol.*, **21**(5):771-775. [doi:10.1007/s11274-004-5566-x]

Jesus, R., Humberto, G., Anne, G., 2001. Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology. *J. Biothechnol.*, **88**:259-268.

Johnson, E.A., 1991. Astaxanthin from microbiol sources. *Crit. Rev. Biotechnol.*, **11**(4):297-326.

Johnson, E.A., 2003. *Phaffia rhodozyma*: colorful odyssey. *Int. Microbiol.*, **6**(3):169-174. [doi:10.1007/s10123-003-0130-3]

Kusdiyantini, E., Gaudin, P., Goma, G., Blanc, P.J., 1998. Growth kinetics and astaxanthin production of *Phaffia rhodozyma* on glycerol as a carbon source during batch fermentation. *Biotechnol. Lett.*, **20**(10):929-934. [doi:10.

1023/A:1005345224510]

Montgomery, D.C., 1997. Design and Analysis of Experiments, 4th Ed. John Wiley & Sons Inc., New York.

Ni, H., He, G.Q., Yang, Y.F., Wu, G.B., Chen, S.R., Cai, L.S., 2004. Optimization of condition for extracting astaxanthin from *Phaffia rhodozyma*. *Trans. Chin. Soc. Agric. Eng.*, **20**(2):204-208 (in Chinese).

Ni, H., He, G.Q., Ruan, H., Chen, Q.H., Chen, F., 2005a. Application of derivative ratio spectrophotometry for the determination of β-carotene and astaxanthin from the *Phaffia rhodozyma* extract. *J. Zhejiang Univ. Sci.*, **6B**(6):514-522. [doi:10.1631/jzus.2005.B0514]

Ni, H., Yang, Y.F., Yang, Q.M., Guo, C.H., Cai, H.N., 2005b. Studies on the determination of carotenoids of *Phaffia rhodozyma* by spectrophotometric method. *J. Jimei Univ. (Nat. Sci.)*, **10**(4):318-323 (in Chinese).

Ni, H., He, G.Q., Yang, Y.F., Cai, H.N., 2005c. Studies on effects of metal ions on astaxanthin production by *Phaffia rhodozyma*. *Food Fermentation Ind.*, **31**(12):4-7 (in Chinese).

Paul, D.F., Yutaka, M., Misawa, N., 1997. In vitro characterization of astaxanthin biosynthetic enzymes. *J. Bioeng. Chem.*, **272**(10):6128-6135.

Sedmak, J.J., Weerasinghe, D.K., Jolly, S.O., 1990. Extraction and quantification of astaxanthin from *Phaffia rhodozyma*. *Biotechnol. Tech.*, **4**(2):107-112. [doi:10.1007/BF00163282]

Verdoes, J.C., Misawa, N., van Ooyen, A.J.J., 1999. Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthphyllomyces dendrorhous*. *Biotechnol. Bioeng.*, **63**(6):750-755. [doi:10.1002/(SICI)1097-0290(19990620)63:6<750::AID-BIT13>3.0.CO;2-7]

Wang, Q.Y., Li, Q.S., 1997. Natural Carotenoids: Advancements of Studies, Production and Applications. China Press of Pharmaceutical Science and Technology, Beijing, China (in Chinese).

Zhang, L.X., Zhang, T.F., Li, L.Y., 1997. Methodology of Biochemical. Higher Education Press, Beijing, China (in Chinese).

Zhu, M.J., Liang, S.Z., 2005. Optimizations culture parameter for growth and astaxanthin accumulation by *Phaffia rhodozyma*. *J. Zhengzhou Univ. Light Ind.*, **20**(2):17-19 (in Chinese).