Astaxanthin production by *Phaffia rhodozyma* fermentation of cassava residues substrate

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Abstract: Cassava residues as main materials were fermented with Phaffia rhodozyma to produce astaxanthin. Using the Box-Behnken design, the effects of sugar content, initial pH and nitrogen content were studied with the yield of astaxanthin as response value, which was evaluated to optimize the fermentation conditions of astaxanthin production. The optimal fermentation conditions have been reached by the study: sugar content was 40 g/L, the initial pH was at 4 and nitrogen content was 8 g/L. By validation test, the astaxanthin yield under the optimal condition, which was basically corresponded to the model prediction, was 96.83%.

Keywords: astaxanthin, cassava recidues, *Phaffia rhodozyma*

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1 Introduction

Astaxanthin (3,3'-dihydroxy-b,b-carotene-4,4'-dione) was a carotenoid pigment, which was found throughout the animal kingdom, especially in marine species. It existed in lobsters, crabs, shrimp, trout and salmon[1-4]. There has been a growing interest in the use of astaxanthin as a pigment for the aquaculture industry, and as a functional food and pharmaceutical supplement because of its proven and potent antioxidant activity[5,6]. In aquaculture, it was employed as a source of natural pigmentation and dietary supplement for trout and salmon[7]. However, the costs in natural or synthetic processes for astaxanthin production were high (U.S. $2,500–3,000 per kg)[8]. As a consequence, researchers had been studying the use of alternative carbon sources for the production of astaxanthin. *Phaffia rhodozyma* was an excellent astaxanthin-producing yeast and had been regarded as a potential source of dietary astaxanthin[9] production. The yeast was able to ferment glucose and other sugars and thus produces carotenoids, such as astaxanthin during its growth[10]. It could be used in industrial scale productions, to cut costs by exercising less expensive substrates. There had been reports on cane molasses[11], sugar cane juice[12], corn wet-milling products[13], alfalfa residual juice[14], grape juice[15] and hydrolyzed peat[16,17] etc., which were frequently used as additional carbon sources to enrich the substrates.

Cassava is one of the major economic crops in tropical areas, and is mainly used to produce starch. Cassava residues, by-products of cassava processing, contain a little bit of starch, protein, cellulose and other nutrients. A means of utilizing the cassava residues to produce astaxanthin with crude saccharidase preparation and *Phaffia rhodozyma* was proposed and studied.

2 Materials and methods

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2.1 Microorganism and culture conditions

Phaffia rhodozymaxy was obtained from Culture Collection, which is cultured in the laboratory of College of Food and Technology. The yeast was maintained on malt (YM) agar plates at 4℃ and transferred every month. The fixed growth conditions used during submerged fermentation were selected from the optimal values found in previous works[18]: a temperature of 22℃ and an agitation speed of 160 rpm.

2.2 Aspergillus preparation (crude saccharidase preparation)

20 g bran, 5 g flour, 20 mL water was taken and mixed into 500 ml Erlenmeyer flask, sterilizing at 121℃ for 20 min. Then the solution would be cooled to 30℃ and inoculated *Aspergillus nige* AS. 3.278 and *Aspergillus flavus* AS. 3.800 respectively under aseptic conditions. Mix it again and culture it at 30℃ for 12 h. Close the flask till mycelium grow over shake flask and form into shape of pie in 24 h, continue culturing till substrate were fully grown in spore. The overall procedure would take 72 h or so.

2.3 Saccharification of Cassava Residues

Put 100 g cassava residues into 1000 ml triangular flask, add 700 mL water and sterilize at 121℃ for 20 min. After sterilization, adding cultured aspergillus preparation (3 g AS. 3.800 and 7g AS.3.2783) and saccharify at 58℃ for 5 h.

2.4 Nitrogen sources medium

Filter saccharified cassava residues for solution. Adjust sugar degree to 30 g/L through glucose, add $KHRRRR_2PO_4$ of 1.5 g/L, $MgSO_4$ of 0.5 g/L, and add 5g/L of peptone, yeast extract, beef extract, KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3, respectively. Then, put it into 100 mL/ 500 mL triangular flask respectively, and sterilize at 121℃ for 20 min.

2.5 Growth medium

Filter saccharified cassava residues for solution. Adjust sugar degree to 30 g/L through glucose, add yeast extract of 3 g/L, $(NH_4)_2SO_4$ of 2 g/L, KH_2PO_4 of 1.5 g/L, and $MgSO_4$ of 0.5 g/L and put it into 100 mL/500 mL triangular flask respectively, and sterilize at 121℃ for 20 min.

3 Analytical methods

3.1 Pigment extraction[18]

The extraction and analysis of Astaxanthin content in the yeast cells followed the methods as described by Du S. J., et al. Yeast biomass was separated from the liquid medium by centrifuging and rinsed twice with double distilled water, the yeast was disrupted with dimethyl sulphoxide (DMSO) and then extracted with Acetone. Astaxanthin was calculated from the absorbance measured at 480 nm multiplying an extinction coefficient of 2150.

3.2 Dry weight determination

Yeast biomass was separated from the liquid medium by centrifuging and rinsed twice with double distilled water, and then dried at 105℃ overnight to constant weight, yielding the dry weight.

3.3 Experimental design

The optimal operational conditions for the fermentation of *P. rhodozyma* using Cassava Residue as substrate were determined by means of RSM. The dependent variables selected for this study were astaxanthin yield. Table 1 lists the independent variables, their units, dimensionless and normalized independent variables (coded variables) at levels −1, 0, and 1 that were defined for the purposes of these calculations.

Table 1 Independent variables and their levels in experimental design

Variable	Coded	Factor levels		
		−1	0	1
Sugar content	X_1	30	35	40
Nitrogen content	X_2	4	6	8
Initial pH	X_3	4	5	6

3.4 Statistical analysis

All data presented are mean values of three determinations. Linear regressions and experimental data plotting were done by using Minitab15 and Microsoft Excel 7.0.

4 Results and discussion

4.1 Effect of temperature shift fermentation on astaxanthin synthesis

The optimal temperature range for growth of yeast is 28-30℃, while synthesis temperature of astaxanthin was 22℃. Temperature shift was adopted during fermentation with higher temperature to promote growth of yeast in the prophase and lower temperature to improve synthetise of astaxanthin in the anaphase. The experiment subject were fermented at original temperature of 30℃, and the fermenting temperature were shifted to 22℃ in 12, 24, 36, 48, 60, and 72 h respectively to cultivate synthetise of astaxanthin. It was shown in figure 1 that fermenting after 36 hours growth leads to the highest astaxanthin yield of 2.8056 mg/L, a 14.0% increase compared with those fermentation (2.4603 mg/L) without temperature shift.

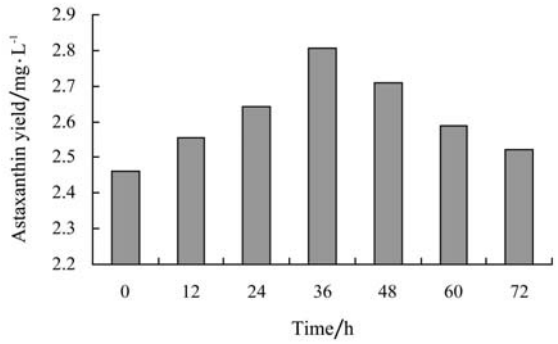

Figure 1 Effect of temperature shift fermentation on astaxanthin yield

5 Effect of nitrogen sources on astaxanthin synthesis

5.1 Effect of different nitrogen sources on astaxanthin synthesis

It was necessary to add nitrogen source into saccharified fluid of cassava residues for the growth of yeast. Experiments were made to test the effects of different nitrogen sources on astaxanthin yield through fermentation, of which added respectively, are peptone, yeast extract, beef extract, KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3. The astaxanthin yield is 2.65 mg/L by adding yeast extract as its nitrogen source, higher than those of adding beef extract and peptone. $(NH_4)_2SO_4$ was a good inorganic nitrogen source which can foster astaxanthin yield at 2.5 mg/L. Thus, yeast extract and $(NH_4)_2SO_4$ were chosen as nitrogen source for the growth of *Phaffia rhodozyma.*

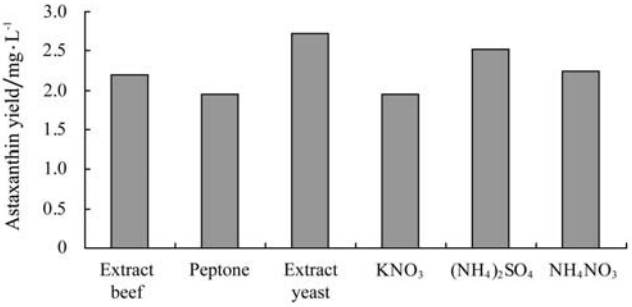

Figure 2 Effect of different nitrogen source addition on astaxanthin yield

5.2 Density ratio effect of yeast extract and $(NH_4)_2SO_4$ on astaxanthin synthesis

Experiments were made with yeast extract and $(NH_4)_2SO_4$ as mixed nitrogen source to finalize ratio of organic and inorganic nitrogen source as was shown on Figure 3. The density ratio of the yeast extract and $(NH_4)_2SO_4$ were 3:1, 3:2, 2:1, 1:1, 1:2, 2:3, 1:3 (mass ratio). With the increasing ratio of $(NH_4)_2SO_4$, astaxanthin yield showed the tendency of increasing at first and a decrease after a certain value, demonstrating a decrease of yeast extract density made contributions to the improvement of astaxanthin yield. The yeast extract and $(NH_4)_2SO_4$ at the ratio of 2 and 3 (mass ratio) was found to be the best nitrogen source for yeast growth. The astaxanthin yield was the highest, up to 2.70 mg/L.

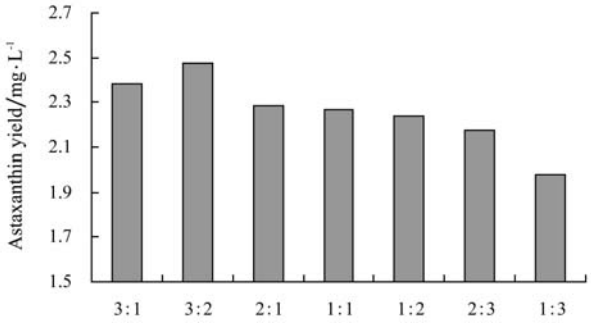

Figure 3 Effect of yeast extract and $(NH_4)_2SO_4$ density ratio on astaxanthin synthesis

6 Design matrix and experimental results of Plackett-Burman design

Regression analysis was performed to appropriate the response function with the experimental data. The response function was related to the coded variables (x_i, $i=1, 2, 3$) by a second-degree polynomial using the method of least squares. By applying multiple regression analysis methods and the data shown in Table 2 analysis by Minitab15, the predicted response Y for polysaccharide yield was obtained as

$$Y=-7.181+0.475X_1-0.241X_2+1.18X_3-0.005X_1^2-0.051X_2^2-0.204X_3^2-0.003X_1X_2-0.015X_1X_3-0.219X_2X_3.$$

Table 2 Central composite design with observed responses and predicted values for yield of astaxanthin

Experiment numbers	X_1 (sugar content)	X_2 (nitrogen content)	X_3 (initial pH)	Astaxanthin yield/mg·L^{-1}
1	30	4	5	2.31
2	40	4	5	2.26
3	30	8	5	2.26
4	40	8	5	2.89
5	30	6	4	2.58
6	40	6	4	2.72
7	30	6	6	2.27
8	40	6	6	2.70
9	35	4	4	2.35
10	35	8	4	2.78
11	35	4	6	2.05
12	35	8	6	2.36
13	35	6	5	2.53
14	35	6	5	2.55
15	35	6	5	2.60

The values are listed along with the parameter estimates in Table 3. The significance of each coefficient was determined by *T* test and *P* values. The P values were used as a tool to check the significance of each of the coefficients in order to understand the pattern of mutual interactions between the test variables. In Table 3 it can be seen that all the three effects (X_1, sugar contention, X_2, nitrogen content, X_3, initial pH) were highly significant. This suggested that the sugar contention and nitrogen content were related to the astaxanthin yield.

Table 3 Regression coefficients and their significance of the squadratic model

Term	Coef	SECoef	*T*	*P*
Constant	2.56	0.04068	62.928	0
X_1	0.14375	0.02491	5.77	0.002
X_2	0.165	0.02491	6.623	0.001
X_3	-0.13125	0.02491	-5.268	0.003
X_1^2	0.02625	0.03667	0.716	0.506
X_2^2	-0.15625	0.03667	-4.268	0.008
X_3^2	-0.01875	0.03667	-0.511	0.631
X_1X_2	0.17	0.03523	4.825	0.005
X_1X_3	0.0725	0.03523	2.058	0.095
X_2X_3	-0.03	0.03523	-0.852	0.433

In order to screen out significant model terms, the statistical technique of analysis of variance (ANOVA) with the F test was applied to the simulation, giving the data shown in Table 4. The ANOVA results confirmed that this model was appropriate. The model *F* value of 16.94 indicates that the model was significant. The determination coefficient, R^2= 96.83%, indicated that the second-order model was well adjusted to the experimental data.

Table 4 Analysis of variance for the quadratic model

Source	*DF*	*SS*	*MS*	*F*	*P*
Regression	9	0.757068	0.084119	16.94	0.003
Linear	3	0.520925	0.173642	34.97	0.001
Square	3	0.095918	0.031973	6.44	0.036
Interaction	3	0.140225	0.046742	9.41	0.017
Lack-of-Fit	3	0.022225	0.007408	5.70	0.153
Pure Error	2	0.002600	0.001300		
Total	14	0.781893	0.084119		

Response surface plots are graphical representations of a regression equation that illustrate the main and interactive effects of independent variables on a dependent variable.

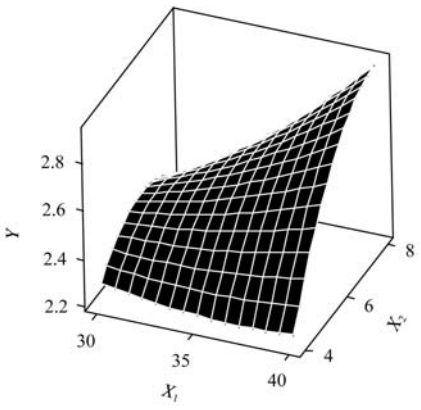

Figure 4 Response surface plot of function $Y=(X_1, X_2)$

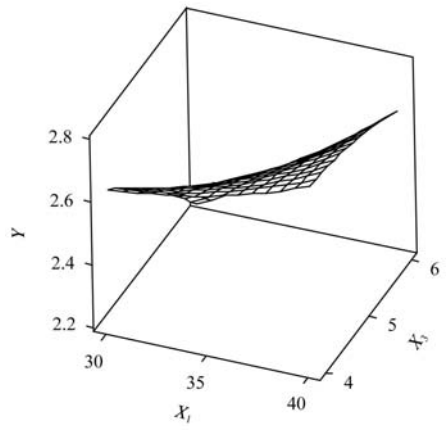

Figure 5 Response surface plot of function $Y=(X_1, X_3)$

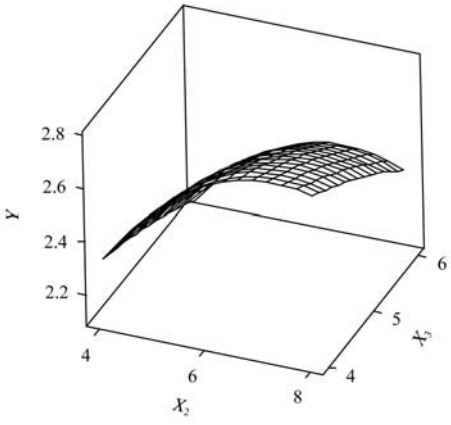

Figure 6 Response surface plot of function $Y=(X_2, X_3)$

Figure 4 shows the response surface for effects of sugar content (X_1) and nitrogen content (X_2) on astaxanthin yield with initial pH (X_3) are fixed at its central level. It can be seen that the interactive effect of sugar content (X_1) and nitrogen content (X_2) was significant and the astaxanthin yield increased with increasing nitrogen content.

Figure 5 shows the interactive effect of sugar content (X_1) and initial pH (X_3) was not significant.

Figure 6 shows the interactive effect of nitrogen content (X_2) and initial pH (X_3) on astaxanthin yield was not significant.

The optimal values of the selected variables were obtained by solving the regression equation. The procedure involved equating the derivatives to zero and then solving the resulting equation system. The values thus obtained were X_1=40, X_2=8, X_3=4, with the corresponding Y=2.99. Using RSM, the optimal fermentation conditions of astaxanthin were sugar content 40 g/L, nitrogen content 8 g/L, initial pH 4. To confirm these results, a test was performed under optimized conditions, the astaxanthin yield was 2.98 g/L, which showed that the model fitted the experimental data.

6 Growth curve of *Phaffia rhodozyma*

Figure7 shows the growth of parameters of *P. rhodozyma*, grown in cassava residues media, at various fermentation times. There is no noticeable increase in axtaxanthin before 36 h, but thereafter there is a noticeable increase in biomass and axtaxanthin concentration. At 72 h, a maximum cell biomass of 8.6 g/L, the maximum astaxanthin yield of 2.98 mg/L is obtained at 96 h.

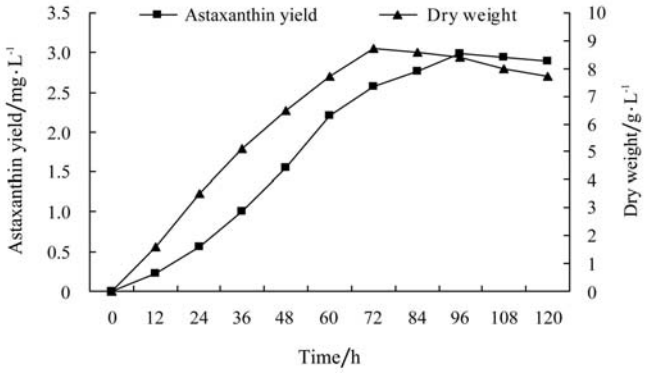

Figure 7 Growth curve of *Phaffia rhodozyma*

7 Conclusions

This study shows that cassava residue, an inexpensive raw material for fermentation, is a promising substrate for producing *Phafia rhodozyma* with increased astaxanthin. The yeast extract and $(NH_4)_2SO_4$ at the ratio of 3 and 2 was found to be the best nitrogen source for yeast growth. The optimum growth conditions determined in this work were sugar content 40 g/L, nitrogen content 8 g/L, initial pH 4 the time which temperature was changing is at the 36th hour. The yield of astaxanthin could be up to 2.98 mg/L.

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References

Bjerkeng, B., T. Storebakken, J. S. Liaaen. 1990. Response to carotenoids by rainbow trout in the sea: Resorption and metabolism of dietary astaxanthin and canthaxanthin. Aquaculture 91: 153–162.

Johnson, E. A., T. Villa, M. Lewis. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture 20: 123–134.

Sigurgisladottir, S., C. C. Parrish, R. G. Ackman, S. P. Lall. 1994a. Tocopherol deposition in the muscle of atlantic salmon (Salmo salar). J Food Sci, 59: 256–259.

Sigurgisladottir, S., C. C. Parrish, S. P. Lall, R. G. Ackman. 1994b. Effects of natural tocopherols and astaxanthin on atlantic salmon (Salmosalar) fillet quality. Food Res. Int. 27: 23–32.

Johnson, E. A., and W. A. Schroeder. 1995. Microbial carotenoids production. Adv Biochem Eng 53, 119–178.

Guerin, M., M. E. Huntley and M. Olaizola. 2003. Haematococcus astaxanthin: applications for human health and nutrition. Trends Biotechnol 21, 210–216.

An, G. H, J. Bielich, R. Auerbach, E. A. Johnson. 1991. Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. Biotechnology 9:70–73

Olaizola, M. 2000. Commercial production of astaxanthin from Haematococcus pluvialis using 25,000-liter outdoor photobioreactors. J Appl Phycol 12:499–506

Andrewes, A. G., H. J. Phaff and M. P. Starr. 1976. Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry 15, 1003–1007.

Johnson, E. A., G. H. An. 1991. Astaxanthin from microbial sources. Crit.Rev. Biotechnol. 11: 297–326.

Haard, N. F. 1988. Astaxanthin formation bythe yeast *Phaffia rhodozyma* on molasses. Biotechnol Lett 10: 609–614.

Fontana, J. D., M. F. Guimaraes, N. T. Martins, C. A. Fontana, M. Baron. 1996. Culture of the astaxanthin ogenic yeast *Phaffia rhodozyma* in low-cost media. Appl Biochem Biotechnol 57–58:413–422.

Hayman, T. G., B. N. Mannarelli, T. D. Leathers. 1995. Production of carotenoids by *Phaffia rhodozyma* grown on media composed of corn wet-milling co-products. J Ind Microbiol 115:173–183.

Okagbue, R. N., M. W. Lewis. 1984. Autolysis of the red yeast *Phaffia rhodozyma:* a potential tool to facilitate extraction of astaxanthin. Biotechnol Lett 6: 247.

Meyer, P. S., J. C. du Preez. 1994. Astaxanthin production by Phaffia rhodozyma mutant on grape juice. World J MicrobiolBiotechnol, 10: 178–183.

Acheampong, E., A. Martin. 1995. Kinetic studies on the yeast *Phaffia rhodozyma.* J Basic Microbiol, 35: 147–155.

Martin, A. M., E. Acheampong, T. R. Patel. 1993. Production of astaxanthin by *Phaffia rhodozyma* using peat hydrolysates assubstrate. J Chem Tech Biotechnol, 58: 223–230.

Du, S. J., Y. Z. Mei, Y. H. Hu, et al. 2008. Optimization of Culture Conditions for Producing Astaxanthin by *Phaffia rhodozyma*. Food Science, 29(8): 441–444. (China)