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Original Article

Astaxanthin Production by Newly Isolated *Rhodosporidium toruloides*: Optimization of Medium Compositions by Response Surface Methodology

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Abstract

Astaxanthin is a valuable carotenoid pigment, which has been extensively used in various industries. In this study, *Rhodosporidium toruloides* was first used as a new microbial source for producing natural astaxanthin. Various carbon, nitrogen, and mineral sources were evaluated for their effect on astaxanthin production of *R. toruloides*. Response surface methodology (RSM) was then used to optimize the medium compositions for maximizing the astaxanthin concentration. Among the examined nutrients, glucose, peptone, and KH_2PO_4 were the most efficient carbon, nitrogen, and mineral source for astaxanthin production, respectively. Through RSM, a maximum astaxanthin concentration of 927.11 μg l^{-1} was obtained by using Hansen broth containing 83.74 g l^{-1} glucose, 20.01 g l^{-1} peptone, and 6.19 g l^{-1} KH_2PO_4. This study suggested that *R. toruloides* is a promising candidate to produce natural astaxanthin.

Keywords: astaxanthin production, *Rhodosporidium toruloides*, yeast, medium composition, response surface methodology

Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione), a carotenoid pigment, has been widely used as a feed additive in the poultry and aquaculture industry (Sarada *et al*., 2002; Panis and Carreon, 2016; Batghare *et al*., 2018). This pigment contributes to the attractive coloration of aquatic animals such as trout, salmon, and shrimps and improves their growth and survival (Ip *et al*., 2004; Panis and Carreon, 2016; Batghare *et al*., 2018). In addition, astaxanthin has much higher antioxidant activity than vitamin E and other carotenoids (Chen *et al*., 2017; Pan-utai *et al*., 2017; Sajjad *et al*., 2017). Studies have demonstrated that astaxanthin exhibits health-promoting effects against various diseases such as cancer, skin-related illness, and cardiovascular disease (Panis and Carreon, 2016; Pan-utai *et al*., 2017). Because of such health benefits, astaxanthin has great potential applications in cosmetic, nutraceutical, and pharmaceutical industries (Pan-utai *et al*., 2017; Batghare *et al*., 2018). Therefore, developing an efficient approach for astaxanthin production is an urgent need to meet the increasing market demand.

Astaxanthin is mainly produced by chemical synthesis (Panis and Carreon, 2016; Batghare *et al*., 2018). However, the chemical synthetic processes of astaxanthin negatively affect the environment and the use of synthetic astaxanthin raises the concern of food safety (Panis and Carreon, 2016). To address these problems, production of natural astaxanthin from microorganisms has attracted considerable attention since microorganisms can produce astaxanthin through their biosynthesis pathway (Ide *et al*., 2012; Zhou *et al*., 2017; Ma *et al*., 2018). Astaxanthin has been found in several microorganisms such as bacteria (e.g., *E. coli*, *Mycobacterium lacticola*, and *Brevibacterium* sp.) (Guyomarc'h *et al*., 2000; Fang and Wang, 2002; Zhang *et al*., 2018) and green microalgae (e.g., *Haematococcus pluvialis* and *Chlamydomonas nivalis*) (Choi *et al*., 2002;

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Mao *et al*., 2018). Among these microbial sources, green microalga *H. pluvialis* is recognized as a promising source of natural astaxanthin because of its high astaxanthin content (Kiperstok *et al*., 2017; Pan-utai *et al*., 2017; Christian *et al*., 2018). However, the use of *H. pluvialis* for biological production of astaxanthin has some limitations due to its slow growth, low cell concentration, and the high cost of cultivation technique (Li *et al*., 2011; Panis and Carreon, 2016; Chen *et al*., 2017). Yeast has been currently suggested as an alternative for astaxanthin production because of its rapid growth rate, high biomass productivity, and health benefits (Ukibe *et al*., 2009; Freitas *et al*., 2014 b; Lin *et al*., 2017 b). *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) is a red-pigmented heterobasidiomycetous yeast, which has been extensively studied for astaxanthin production (Domínguez-Bocanegra *et al*., 2007; Dermiki *et al*., 2010; Stoklosa *et al*., 2018). However, low astaxanthin productivity in wild strain *P. rhodozyma* limits its industrial application (Fang and Cheng, 1993; Domínguez-Bocanegra *et al*., 2007; Liu *et al*., 2008; Montanti *et al*., 2011). Therefore, studies have been attempted to find more candidates as new astaxanthin producers.

Rhodosporidium toruloides, oleaginous yeast, is extensively used as an oil producer (Dias *et al*., 2016; González-García *et al*., 2017; Díaz *et al*., 2018). In recent years, *R. toruloides* has been reported as a potential source of carotenoids (Freitas *et al*., 2014 a, b; Dias *et al*., 2016). In addition, the yeast can grow to very high density (100 g l^{-1} dry cell mass) (Yaegashi *et al*., 2017) and use various low-cost carbon sources (Freitas *et al*., 2014 b; González-García *et al*., 2017; Marques *et al*., 2018). Therefore, *R. toruloides* can be a potential source of astaxanthin and the use of *R. toruloides* can facilitate a low-cost process for astaxanthin production. However, most studies have focused on the production of carotenoid (Freitas *et al*., 2014 a, b; Dias *et al*., 2016; Lin *et al*., 2017 a) while the use of *R. toruloides* for producing astaxanthin is limited.

During the microorganism cultivation, the nutritional factors greatly influence cell growth and metabolism (Parreira *et al*., 2015; Singh *et al*., 2016; Nahidian *et al*., 2018). Studies have reported that carbon, nitrogen, and other nutrients in fermentation medium strongly affect the production of astaxanthin (Ramirez *et al*., 2001; Ip *et al*., 2004; Nahidian *et al*., 2018). Therefore, the optimal medium compositions are important for the industrial production of astaxanthin. In the current study, newly isolated yeast, *R. toruloides*, was studied for astaxanthin production. The effect of carbon, nitrogen, and mineral sources on the production of biomass and astaxanthin by *R. toruloides* was investigated. Response surface methodology (RSM) was also used to optimize the medium compositions for maximizing astaxanthin concentration.

Materials and Methods

Microorganism and inoculum preparation

Newly isolated yeast, *R. toruloides*, was obtained from Department of Biochemistry (University of Science, Vietnam National University Ho Chi Minh City, Vietnam). The *R. toruloides* was identified by using the rDNA-ITS sequencing with a single pair of primers ITS1-ITS4-5.8S rDNA synthesized by Nam Khoa BioTek (Ho Chi Minh City, Vietnam). DNA sequences were then compared to sequences deposited in the National Center of Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) with Entrez and BLAST. The yeast strain was maintained in glycerol at -80 °C before being cultivated in the basal medium (Hansen broth). The basal medium comprised 50 g l^{-1} sucrose, 10 g l^{-1} peptone, 3 g l^{-1} KH_2PO_4, and 3 g l^{-1} $MgSO_4$. The medium was adjusted to pH 6 and sterilized at 121 °C for 20 min. The yeast seed culture was prepared by inoculating *R. toruloides* cells (5%, v/v) into 250 ml Erlenmeyer flasks containing 100 ml of the basal medium and incubated at 30 °C with shaking at 200 rpm for 96 h. The prepared cultures were then used for further experiments.

Effect of various carbon, nitrogen, and mineral sources on cell growth and astaxanthin production

A series of experiments using Hansen broth containing different carbon sources (mannitol, sorbitol, glucose, fructose, maltose, sucrose, or lactose) at a concentration of 50 g l^{-1}, nitrogen sources (peptone, tryptone, ammonium sulfate, meat extract, or yeast extract) at a concentration of 10 g l^{-1}, and minerals (potassium hydrogen phosphate, potassium dihydrogen phosphate, calcium sulfate, magnesium sulfate, sodium hydrogen phosphate, calcium chloride, or zinc sulfate) at a concentration of 4 g l^{-1} for cultivation of *R. toruloides* were performed to investigate the effect of single nutrient on the biomass and astaxanthin production. The seed culture of *R. toruloides* (5%, v/v) was inoculated into 250-ml Erlenmeyer flasks containing 100 ml of the prepared medium. The *R. toruloides* was then grown at 30 °C with shaking at 200 rpm for 96 h. The culture was then withdrawn from the flask to determine the biomass and astaxanthin concentration. All experiments were conducted in triplicate.

Optimization of medium compositions using RSM

A five-level and three-factorial central composite design was employed to investigate the effects of medium compositions on the astaxanthin concentration. The medium containing various glucose concentrations (4.9 g l^{-1}-91.5 g l^{-1}), peptone concentrations (2.4 g l^{-1}-17.6 g l^{-1}), and KH_2PO_4 concentrations (1 g l^{-1}-7 g l^{-1}) were used to grow *R. toruloides*. After being cultivated at 30 °C for 96 h with shaking, the culture was taken to determine the astaxanthin concentration. The relationship between the determined astaxanthin concentration and the nutritional factors was established using the quadratic equation:

$$Y=\beta_0+\beta_1 X_1+\beta_2 X_2+\beta_3 X_3+\beta_{11} X_1^2+\beta_{22} X_2^2+\beta_{33} X_3^2+\beta_{12} X_1 X_2+\beta_{13} X_1 X_3+\beta_{23} X_2 X_3 \quad (1)$$

where Y is the astaxanthin concentration (μg l^{-1}); X_1 is the glucose concentration (g l^{-1}); X_2 is the peptone concentration (g l^{-1}); X_3 is the KH_2PO_4 concentration (g l^{-1}); β_0 is the regression coefficient for the intercept term; β_1-β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are interaction coefficients; and β_{11}, β_{22}, and β_{33} are quadratic coefficients. The least-squares method was used to determine the model coefficients (Nguyen *et al*., 2018a). An analysis of variance

(ANOVA) of model was carried out using Minitab 16 (Minitab Inc., State College, PA, USA). The developed model was then used to plot response surface curves by using OriginPro 8 (OriginLab Corp., Northampton, MD, USA) and to determine the optimal medium compositions for obtaining the maximal astaxanthin concentration using a canonical method (Nguyen *et al.*, 2018a).

Analysis

Measurement of biomass

Yeast biomass was harvested by centrifugation at 6000 rpm for 10 min. The harvested pellets were then rinsed twice with double-distilled water, dried overnight at 60 °C, and weighed. Yeast biomass was presented as grams dry weight per litter of culture medium. The dried biomass was subsequently used for determination of astaxanthin content.

Determination of astaxanthin content

Astaxanthin content was determined according to the methods of An *et al.* (1991) and Fang and Cheng (1993) with modifications. Dried biomass (100 mg) was resuspended in 5 ml dimethyl sulfoxide (DMSO). The mixture was mixed with 10 ml acetone and centrifuged at 6000 rpm for 15 min to obtain the supernatant. The pellet containing residual pigments was ground with a glass homogenizer and extracted again with acetone. All acetone extract was subsequently mixed with 10 ml petroleum ether, 2 ml of 20% NaCl solution, and 1 ml of distilled water and centrifuged at 6000 rpm for 15 min. The petroleum ether extract was obtained by removing the acetone layer and measured the absorbance at 474 nm using a scanning spectrophotometer (Genesis 10S UV-Vis, Thermo Fisher Scientific, USA). Astaxanthin content (µg l^{-1}) were calculated using Kelly-Harmon equation (Kelley and Harmon, 1972):

$$\text{Astaxanthin content } (\mu g\ g^{-1}) = \frac{A_{468} \times V \times 10^4}{E_{1cm}\% \times G} \quad (2)$$

$$\text{Astaxanthin concentration } (\mu g\ l^{-1}) = \frac{\text{Astaxanthin content } (\mu g\ g^{-1}) \times G}{\text{Volume of culture (l)}} \quad (3)$$

where A_{468} is the absorbance of petroleum ether extract at λ_{468}; V is the total volume of petroleum ether extract (ml); G is the total dry weight of yeast biomass (g); E_{1cm}% is the absorbance of 1% astaxanthin solution in petroleum ether (cuvette 1cm, E = 2100).

Statistical analysis

Data illustrated in Table 1, 2, and 3 were determined in triplicate and expressed as the mean ± standard deviation (SD). Those data were analyzed by an analysis of variance (ANOVA) with the Duncan's multiple range test at p values ≤ 0.05 using SAS software ver 8.2 (SAS Institute, Cary, NC, USA).

Table 1. Biomass and astaxanthin production of *R. toruloides* cultivated with different carbon sources

Carbon sources	Biomass concentration (g l^{-1})	Astaxanthin content (µg g^{-1})	Astaxanthin concentration (µg l^{-1})
Mannitol	3.84 ± 0.12 a	150.30 ± 4.51 c	577.60 ± 17.32 a
Glucose	3.29 ± 0.12 b	152.90 ± 5.28 c	503.30 ± 11.56 b
Fructose	2.96 ± 0.12 c	153.30 ± 6.13 bc	453.50 ± 18.14 c
Sorbitol	1.86 ± 0.04 d	210.60 ± 4.21 c	391.30 ± 7.83 d
Maltose	1.60 ± 0.07 d	157.80 ± 7.10 bc	253.00 ± 11.38 e
Sucrose	3.05 ± 0.12 bc	68.30 ± 2.73 d	208.40 ± 8.34 f
Lactose	1.25 ± 0.05 e	164.70 ± 6.59 b	205.80 ± 8.23 f

All values are the mean ± SD (n = 3). Means within a column with different letters are significant difference by Duncan's test a p <0.05.

Table 2. Biomass and astaxanthin production of *R. toruloides* cultivated with different nitrogen sources

Nitrogen sources	Biomass concentration (g l^{-1})	Astaxanthin content (µg g^{-1})	Astaxanthin concentration (µg l^{-1})
Peptone	3.19 ± 0.09 c	160.76 ± 2.71 a	513.00 ± 8.14 a
Ammonium sulfate	2.28 ± 0.10 d	143.66 ± 3.59 b	327.69 ± 8.19 b
Meat extract	6.84 ± 0.24 a	19.62 ± 0.39 c	134.16 ± 2.68 c
Yeast extract	5.67 ± 0.27 b	21.80 ± 0.87 c	123.54 ± 4.94 d
Tryptone	5.88 ± 0.11 b	20.50 ± 0.61 c	120.44 ± 3.61 d

All values are the mean ± SD (n = 3). Means within a column with different letters are significant difference by Duncan's test a p <0.05.

Table 3. Biomass and astaxanthin production of *R. toruloides* cultivated with different mineral sources

Mineral sources	Biomass concentration (g l^{-1})	Astaxanthin content (µg g^{-1})	Astaxanthin concentration (µg l^{-1})
KH_2PO_4	4.06 ± 0.12 b	159.26 ± 6.13 b	645.99 ± 18.14 a
$CaSO_4$	4.32 ± 0.05 b	118.94 ± 6.59 d	552.18 ± 8.23 b
$MgSO_4$	3.34 ± 0.11 c	135.81 ± 5.28 c	453.93 ± 11.56 c
Na_2HPO_4	2.52 ± 0.07 d	177.16 ± 7.10 a	445.57 ± 11.38 c
$CaCl_2$	4.12 ± 0.04 b	91.45 ± 4.21 e	376.68 ± 7.83 d
K_2HPO_4	5.25 ± 0.12 a	49.04 ± 2.73 f	257.31 ± 8.34 e
$ZnSO_4$	2.25 ± 0.12 e	89.56 ± 4.51 e	201.06 ± 7.32 f

All values are the mean ± SD (n = 3). Means within a column with different letters are significant difference by Duncan's test a p <0.05.

Results and Discussion

Effect of different carbon, nitrogen, and mineral sources on biomass and astaxanthin production

R. toruloides was cultivated in the Hansen broth containing different carbon sources to investigate the effect of those carbon sources on the growth and astaxanthin production. As shown in Table 1, carbon sources significantly affected the biomass concentration, astaxanthin content, and astaxanthin concentration. Among the carbon sources tested, mannitol resulted in the highest biomass (3.84 g l^{-1}) and astaxanthin concentration (577.6 μg l^{-1}) followed by glucose (3.29 g l^{-1} biomass and 503.3 μg l^{-1} astaxanthin). Although *R. toruloides* cultivated in medium containing sorbitol, maltose, and lactose as carbon source produced high astaxanthin content (157.8-210.6 μg g^{1}), low biomass concentration (1.25-1.86 g l^{-1}) was observed in these cultures, thus leading to low astaxanthin concentration (205.8-391.3 μg l^{-1}). Because the biomass and astaxanthin concentration obtained from mannitol did not dramatically differ from glucose, glucose was chosen as a potential carbon source for the cultivation of *R. toruloides* to minimize the cultivation cost.

The effect of nitrogen sources on the biomass and astaxanthin production of *R. toruloides* is presented in Table 2. Among nitrogen sources examined, meat extracts showed the most efficient source for biomass production with the highest biomass concentration of 6.84 g l^{-1}, but this nitrogen source resulted in very low astaxanthin content (19.62 μg g^{-1}); therefore, it is not suitable for the cultivation of *R. toruloides* for astaxanthin production. Although peptone resulted in lower biomass concentration (3.19 g l^{-1}) than meat extract, highest astaxanthin content (160.76 μg g^{-1}) and astaxanthin concentration (513.0 μg l^{-1}) were obtained by using peptone as nitrogen source for the cultivation of *R. toruloides*. Therefore, peptone was selected as the efficient nitrogen source for the cultivation of *R. toruloides*.

Table 3 illustrates the effect of minerals on the biomass and astaxanthin production of *R. toruloides*. As shown in Table 3, the biomass concentration and astaxanthin content varied among minerals tested. K_2HPO_4-based culture resulted in the highest biomass concentration (5.25 g l^{-1}), but it produced lowest astaxanthin content (49.04 μg g^{-1}). Na_2HPO_4 showed the adverse effect. *R. toruloides* cultivation using Na_2HPO_4 as mineral source produced high astaxanthin content (177.16 μg g^{-1}), but low biomass concentration (2.52 g l^{-1}). Among the minerals tested, KH_2PO_4 showed the most efficient nutrient for the cultivation of *R. toruloides* since it produced high biomass concentration (4.06 g l^{-1}) and astaxanthin content (159.26 μg g^{-1}). As a result, the highest astaxanthin concentration (645.99 μg l^{-1}) was obtained by cultivation of *R. toruloides* in the medium containing KH_2PO_4 as a mineral source. Therefore, KH_2PO_4 was selected for further studies.

Optimization of medium compositions using RSM

The relationship between astaxanthin concentration (measured response) and three nutritional factors (input variables), the glucose concentration, peptone concentration, and KH_2PO_4 concentration, was evaluated using RSM model. Table 4 shows the coded and uncoded values of the input variables for the central composite design. To obtain the optimal medium compositions, the *R. toruloides* was cultivated in the Hansen's medium containing the compositions according to the experimental design (Table 5). Based on the experimental results (Table 5), the relationship between measured response and input variables were modeled using a quadratic polynomial equation as follows:

$$Y = 874.38 + 69.83X_1 + 8.31X_2 + 90.81X_3 - 82.72X_1^2 - 76.63X_2^2 - \\ - 66.97X_3^2 + 2.21X_1X_2 - 67.97X_1X_3 - 11.03X_2X_3 \quad (4)$$

where X_1, X_2, X_3, and X_1X_2 have positive effects on the measured response, whereas the other parameters have adverse effects.

As shown in Table 5, the central runs (15–20) demonstrated reproducibility of the experiments. Table 6 shows the statistical significance of the model, which was evaluated using the F test for ANOVA. P value of the model was 0.0001 in the F test, indicating statistically significant of the model at the 95% confidence level. In addition, a high coefficient of determination (R^2) value was obtained (0.98), indicating high reliability of the model in predicting the astaxanthin concentration. As can be seen in Fig. 1, the predicted and actual values of the astaxanthin concentration were in agreement; therefore, the established model could provide accurate results.

Furthermore, the overall effects of the nutritional factors on the astaxanthin concentration were evaluated using t tests. As shown in Table 7, linear terms X_1 and X_3, all quadratic terms, and an interaction term X_1X_3, were significant factors in the medium compositions. The developed model was then used for plotting response surface curves to predict the optimal medium compositions for obtaining maximum astaxanthin concentration.

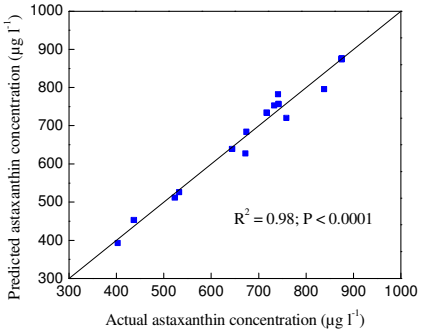

Fig. 1. Correlation between actual and predicted astaxanthin concentration

Table 4. Coded values of the variables for the central composite design

Variables	Symbols	Variable levels				
		-1.68	-1	0	1	1.68
Glucose concentration (g l^{-1})	X_1	4.9	23.2	50.0	76.8	91.5
Peptone concentration (g l^{-1})	X_2	2.4	5.5	10.0	14.5	17.6
KH_2PO_4 concentration (g l^{-1})	X_3	1.0	2.2	4.0	5.8	7.0

Table 5. Central composite design matrix for the effect of the three variables on the astaxanthin content in coded values and experimental results

Run	Variables			Response, *Y*
	X_1	X_2	X_3	
1	-1	1	-1	453.46
2	0	1.68	0	628.32
3	-1.68	0	0	512.42
4	1	1	1	782.82
5	0	-1.68	0	639.91
6	-1	-1	-1	392.99
7	-1	1	1	754.21
8	0	0	-1.68	526.36
9	1	-1	1	757.66
10	1	-1	-1	684.65
11	1.68	0	0	721.36
12	1	1	-1	734.81
13	0	0	1.68	796.50
14	-1	-1	1	757.01
15	0	0	0	875.61
16	0	0	0	874.86
17	0	0	0	877.06
18	0	0	0	876.60
19	0	0	0	874.56
20	0	0	0	875.66

Table 6. Analysis of variance for the empirical model

Source	DF [b]	SS [b]	MS [b]	F value	Probability (P) > F
Model[a]	9	425411	47268	54.29	<0.0001
Residual (error)	10	8707	871		
Total	19	434118			

[a] Coefficient of determination (R^2) = 0.98; adjusted R^2 = 0.96.
[b] DF, degree of freedom; SS, sum of squares; MS, mean square.

Table 7. Significance of the coefficients in the empirical model

Model term	Parameter estimate	Standard error	*t* value[a]	*P* value
β_0	874.38	12.03	72.66	0.000 [b]
β_1	69.83	7.99	8.75	0.000 [b]
β_2	8.31	7.99	1.04	0.322
β_3	90.81	7.99	11.37	0.000 [b]
β_{11}	-82.72	7.77	-10.64	0.000 [b]
β_{22}	-76.63	7.77	-9.86	0.000 [b]
β_{33}	-66.97	7.77	-8.62	0.000 [b]
β_{12}	2.21	10.43	0.21	0.837
β_{13}	-67.97	10.43	-6.52	0.000 [b]
B_{23}	-11.03	10.43	-1.06	0.315

[a] $t_{\alpha/2,n-p} = t_{0.025,10} = 2.23$.
[b] P <0.05 indicates that the model terms are significant.

Effect of medium compositions on the astaxanthin concentration

Fig. 2 illustrates the combined effects of the glucose concentration and peptone concentration on the astaxanthin production. At a given peptone concentration, increasing the concentration of glucose resulted in an increase in astaxanthin concentration. This is because glucose promotes cell growth and metabolism in microorganisms (Ip *et al.*, 2004; Nguyen *et al.*, 2018b). Studies have reported that a sufficient glucose concentration enhanced astaxanthin production in yeast through the metabolic pathway (Fang and Wang, 2002; Ip *et al.*, 2004). Yeast cells produce intermediates such as acetic acid and ethanol through the fermentation by using glucose as a substrate. The produced ethanol is then converted to acetyl CoA (a precursor for carotenoids synthesis) by alcohol dehydrogenase, thus facilitating the biosynthesis of carotenoids (Fang and Wang, 2002). However, a further increase in glucose concentration adversely affected astaxanthin production. This could be explained that the excess glucose inhibits the cell growth and high sugar osmotic pressure favored the synthesis of energetic

substance such as protein instead of astaxanthin to resist the environmental stress (Ip *et al.*, 2004; Nguyen *et al.*, 2018b), thus lowering the astaxanthin concentration. This phenomenon is similar to the astaxanthin production in microalgae *C. zofingiensis* (Ip *et al.*, 2004) and yeast *P. rhodozyma* (Fang and Wang, 2002).

Fig. 3 shows the combined effects of the peptone concentration and KH_2PO_4 concentration on the astaxanthin production while maintaining glucose concentration as a constant. At any KH_2PO_4 level, the astaxanthin concentration increased when the peptone concentration was increased. This is because peptone supported the astaxanthin production in yeast (Fang and Cheng, 1993). However, the astaxanthin concentration decreased with a further increase in the peptone concentration. This could be because a decrease in carbon to nitrogen ratio suppresses the cell growth and the formation of secondary pigments (Ip *et al.*, 2004; Saenge *et al.*, 2011). Studies have reported that initial carbon to nitrogen ratio in the medium significantly affects the formation of astaxanthin in microorganisms including yeast (Saenge *et al.*, 2011). A high carbon to nitrogen ratio suppresses the protein synthesis, thus reducing NADPH consumption for primary metabolism; therefore, more NADPH is available for the biosynthesis of astaxanthin (Johnson, 1996; Ip and Chen, 2005; Saenge *et al.*, 2011). Therefore, high carbon to nitrogen ratio is required for the biosynthesis of astaxanthin in yeast (*P. rhodozyma*) (Yamane *et al.*, 1997; Ramirez *et al.*, 2001; Stoklosa *et al.*, 2018) and microalgae (*C. zofingiensis*) (Ip and Chen, 2005). In this study, high carbon to nitrogen ratio is also favorable for the synthesis of astaxanthin by *R. toruloides*.

Fig. 4 presents a response surface curve obtained by plotting the astaxanthin concentration against the glucose and KH_2PO_4 concentrations. At any level of glucose, increasing KH_2PO_4 concentration caused a marked increase in astaxanthin concentration. However, a further increase in KH_2PO_4 caused a decrease in astaxanthin concentration. This result is in agreement with astaxanthin production of *P. rhodozyma*. Batghare *et al.* (2018) reported that KH_2PO_4 is an important component for the growth of yeast and astaxanthin production. Potassium ion is required for the activation of enzymes catalyzing the phosphoryl transfer or elimination reactions, which are involved in carotenogenesis (Domınguez-Bocanegra *et al.*, 2004; Batghare *et al.*, 2018). However, an excess KH_2PO_4 causes a suppression of cell growth and carotenoids biosynthesis, thus lowering the astaxanthin concentration (Domınguez-Bocanegra *et al.*, 2004; Batghare *et al.*, 2018).

Obtaining optimal medium compositions

The model [Eq. (4)] represents the maximization of the response function; therefore the optimal medium compositions can be obtained by solving the Eq. (4). The maximal astaxanthin concentration was predicted to be 907.32 μg l^{-1} at 83.74 g l^{-1} glucose, 20.09 g l^{-1} peptone, and 6.19 g l^{-1} KH_2PO_4. An experiment was conducted under the optimal medium compositions to verify the prediction. An astaxanthin concentration of 927.11 μg l^{-1} ± 4.21 μg l^{-1} was obtained, indicating agreement with the empirical model prediction. Therefore, the developed RSM model can be used for representing the relationship between the variables and the response in the cultivation of *R. toruloides* for astaxanthin production. *R. toruloides* revealed a comparable astaxanthin concentration to the wild strain *P. rhodozyma* (<570 μg l^{-1}) (Fang and Cheng, 1993; Xie *et al.*, 2014).

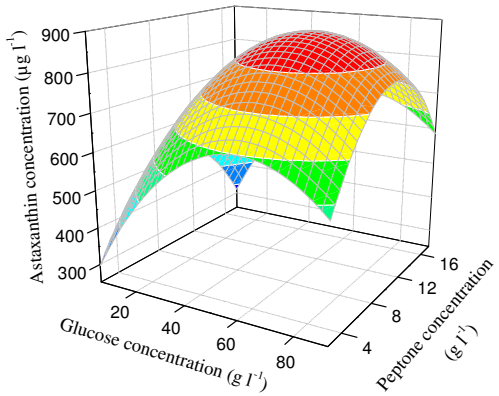

Fig. 2. Response surface plot of the combined effects of the glucose concentration and peptone concentration on the astaxanthin concentration

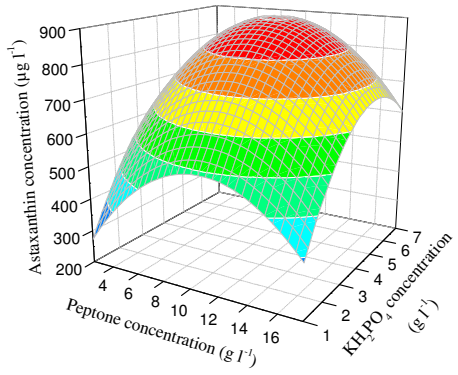

Fig. 3. Response surface plot of the combined effects of the peptone concentration and KH_2PO_4 concentration on the astaxanthin concentration

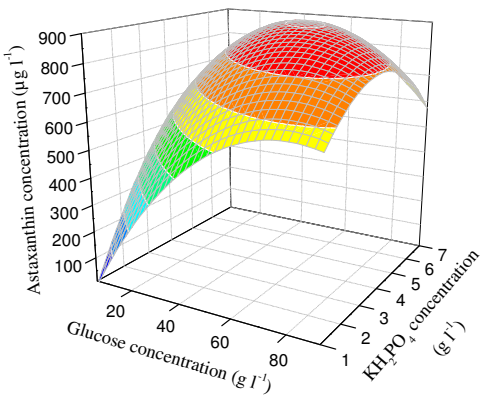

Fig. 4. Response surface plot of the combined effects of the glucose concentration and KH_2PO_4 concentration on the astaxanthin concentration

Although the astaxanthin concentration produced by *R. toruloides* was lower than microalgae *H. pluvialis* (up to 31.81 mg l^{-1}), *R. toruloides* has more advantages than microalgae due to its higher growth rate and shorter cultivation time (Kiperstok *et al.*, 2017; Christian *et al.*, 2018; Nahidian *et al.*, 2018). Those results suggested that *R. toruloides* can be an alternative for the astaxanthin production. Further studies are also required to improve the astaxanthin production of *R. toruloides* through optimization of cultivation conditions or generation of mutant strains.

Conclusions

In this paper, we report the astaxanthin production by the cultivation of a new yeast strain, *R. toruloides*, in Hansen's medium containing different carbon, nitrogen, and mineral sources. Among the nutrients tested, glucose, peptone, and KH_2PO_4 exhibited the most efficient carbon, nitrogen, and mineral source, respectively, for astaxanthin production by *R. toruloides*. The medium compositions were subsequently optimized for maximizing the astaxanthin concentration using RSM. A maximal astaxanthin concentration of 927.11 μg l^{-1} was obtained and verified. The results suggested that the *R. toruloides* can be a promising microbial source for producing natural astaxanthin.

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