

Optimization of Ultrasound-assisted Extraction Procedure to Determine Astaxanthin in *Xanthophyllomyces dendrorhous* by Box-Behnken Design

Wei Wu and Xin Yu

School of Medicine, Hubei Polytechnic University, Huangshi CN-435003, P.R. China

Abstract: An ultrasonic-assisted extraction method has been developed for the effective extraction of astaxanthin from *Xanthophyllomyces dendrorhous*. Single-factor experiment design was employed to optimize the disruption temperature, disruption time, ethanol concentration, extraction time. The ethanol concentration was 60% ethanol in ethyl lactate and temperature for disrupting (40-60°C), time for disrupting extraction time (15-35 min), extraction time (20-40 min) were used for further optimization of extraction conditions. The optimal conditions for the ultrasound-assisted extraction of astaxanthin were determined using Response Surface Methodology (RSM) by Box-Behnken design. The optimal extraction conditions were that the yeast was disrupted at 49.08°C for 26.09 min under ultrasound irradiation of 200 W and then extracted for 32.43 min. Under optimal conditions, the astaxanthin content was 1472.85±43.64 µg/g DW. This study introduces a simple, green and highly efficient method for extraction of astaxanthin from *X. dendrorhous*.

Keywords: Astaxanthin, response surface methodology, ultrasound-assisted extraction, *xanthophyllomyces dendrorhous*

INTRODUCTION

Astaxanthin (3, 3'-dihydroxy- β,β -carotene-4,4'-dione) is one of the best-selling products in the carotenoid market with 100-130 tons per year and it is a powerful biological antioxidant, which is common in crustacean shells, salmon, fish eggs and asteroideans (Inoue *et al.*, 2012). It is reported that astaxanthin has various beneficial bioactive properties for human and animal health, including the prevention of cardiovascular disease, the promotion of immune responses and anti-oxidative actions (Higuera-Ciapara *et al.*, 2006; Hussein *et al.*, 2006), so it is also used in food, cosmetic and medical applications. Previous studies report that *Xanthophyllomyces dendrorhous* is one of the most promising microorganisms for biotechnological production of dietary astaxanthin (Cruz and Parajó, 1998).

But the efficiency of astaxanthin extraction from *X. dendrorhous* is not very high for its rigid cell wall. It is reported that several methods such as enzymatic method (Gentles and Haard, 1991; Storebakken *et al.*, 2004) and high-pressure homogeneity method (Gentles and Haard, 1991) have been proved not sufficiently definitive. Recently, much wider attention has been given to applications of Ultrasound-Assisted Extraction (UAE), which can more easily be scaled up for commercial production (Ma *et al.*, 2012). And the UAE

is one of the promising extraction techniques that can offer high reproducibility in a shorter time, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Jiao and Zuo, 2009; Zuo *et al.*, 2004). Ultrasound enhancement of extraction is attributed to the disruption of cell walls, particle size reduction and the enhancement on the mass transfer of the cell content to the solvent caused by the collapse of the bubbles produced by cavitations (Paniwnyk *et al.*, 2001; Rodrigues and Pinto, 2007). It was reported that UAE is a very useful method for extracting astaxanthin from marine life (Zou *et al.*, 2013). However, it was unknown whether the UAE will help to improve the extraction efficiency of astaxanthin from *X. dendrorhous*.

Response Surface Methodology (RSM) is a useful statistical technique that uses sequential experimental techniques to survey a domain of interest, focusing on the most important variables and their effects, to build an empirical model. It has been proved that RSM can be used to optimize the total flavonoid compound from many medicinal plants (Liu *et al.*, 2010). In this study, ultrasonic device was applied to help disrupt the yeast cell wall and astaxanthin was qualified by HPLC. The effects of several experimental parameters, such as temperature for disrupting, time for disrupting and extraction time, on the extraction efficiency of astaxanthin from *X. dendrorhous* were optimized by RSM.

MATERIALS AND METHODS

Microorganisms and culture condition: *X. dendrorhous* AS 2.1557 (obtained from China General Microbiological Culture Collection Center, Beijing, China) was used in this study, which was maintained on slants of Yeast Malt (YM) agar at 4°C and transferred monthly. YM agar medium, which contained the following components (per liter): 3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10.0 g glucose and 20.0 g agar, was used to maintain the yeast strains. The medium for liquid culture of the yeast was made of 40.0 g glucose, 3.0 g KH₂PO₄, 0.5 g MgSO₄, 1.0 g Na₂HPO₄ and 4.0 g yeast extract (per liter). The medium was adjusted to pH 5.0 and sterilized by autoclaving at 121°C for 20 min. *X. dendrorhous* AS 2.1557 was grown in 250-mL Erlenmeyer flask containing 30 mL growth medium for 4 d at 22°C in shaking incubators on a shaker operating at 200 rpm.

Astaxanthin extraction: In this study, ultrasonic device was applied to help disrupt the yeast. For the routine extraction of astaxanthin, the culture media suspensions were centrifuged and the resulting pellets were lyophilized. The resultant dried yeast cells were ground into fine powder using a mortar and pestle and passed through 60-mesh screen. The lyophilized cells powder (0.2 g) was accurately weighed and placed in a 20 mL capped tube and then mixed with 10 mL of 5 mol/L lactic acid. The tube was mixed vigorously and immersed into water in the temperature-controlled ultrasonic device (SY360, Ninson Ultrasonic Instrument Company, Shanghai, China). After cell disruption, the water phase was removed by centrifugation and the fraction of disrupted cells was extracted by 15 mL of mixture of ethanol and ethyl lactate at room temperature.

The conventional extraction of astaxanthin from *X. dendrorhous* was that yeast was disrupted by lactic acid or hydrochloric acid and then extracted by ethyl lactate or acetone (Wu *et al.*, 2011).

Experimental design: In this study, the extraction parameters of were optimized using Response Surface Methodology (RSM) (Bezerra *et al.*, 2008). A Box-Behnken design was used to evaluate the main and interaction effects of the factors: temperature for disrupting (X_1), time for disrupting (X_2) and extraction time (X_3) on astaxanthin yield. Seventeen experiments were performed with five experiments as the repeatability of the measurements at the center of the experimental domain. All factors and levels tested were reported in Table 1. Astaxanthin yield was selected as the responses for the combination of the independent variables given in Table 2. Experimental runs were randomized, to minimize the effects of unexpected variability in the observed responses. A second order

Table 1: Factors and levels tested for the designed experiment

Independent variables	Coded levels		
	-1	0	1
Temperature for disrupting (X_1 , °C)	40	50	60
Time for disrupting (X_2 , min)	15	25	35
Extraction time (X_3 , min)	20	30	40

Table 2: Experimental designs using Box-Behnken and results

Run	X_1	X_2	X_3	Astaxanthin content (µg/g DCW)
1	-1	0	1	1140.59
2	1	-1	0	833.17
3	1	1	0	1004.65
4	-1	0	-1	1039.49
5	0	0	0	1485.45
6	1	0	-1	836.40
7	-1	-1	0	1030.83
8	0	-1	-1	1052.11
9	-1	1	0	1051.06
10	0	0	0	1464.52
11	0	0	0	1494.74
12	0	0	0	1477.18
13	0	0	0	1458.39
14	0	-1	1	1211.89
15	0	1	1	1264.66
16	0	1	-1	1154.91
17	1	0	1	1025.82

polynomial equation was then fitted to the data using the Design-Expert software. To verify the validity of the model, additional confirmation experiments were subsequently conducted.

Analytical procedures: Astaxanthin was determined by High-Performance Liquid Chromatography (HPLC) with a Waters 2695 instrument equipped with a 5-µm Dikma Diamonsil TM-C18 reversed-phase column (250×4.6 mm; Dikma Technologies Inc., Beijing, China), using UV detection at 480 nm. The eluting solvent was methanol/methyl cyanide (9:1, v/v) and the flow rate was 1 mL/min (Wu *et al.*, 2010). All samplings and assays were carried out in triplicate and the results were determined as the mean values±standard deviation.

RESULTS AND DISCUSSION

Effect of temperature for disrupting on the astaxanthin yield: Extracting astaxanthin from *X. dendrorhous* usually include two steps:

- Disrupting cell wall
- Extracting by organic solvent and it is necessary to disrupt the yeast for extracting astaxanthin considering the yeast accumulating carotenoids in cell. Temperature is a key factor in the extraction of heat sensitive compounds. Along with the increase of temperature, some thermal labile components can decompose (Dong *et al.*, 2010).

In this study, the yeast cell was treated with lactic acid, not only did it support a high-disrupting efficiency

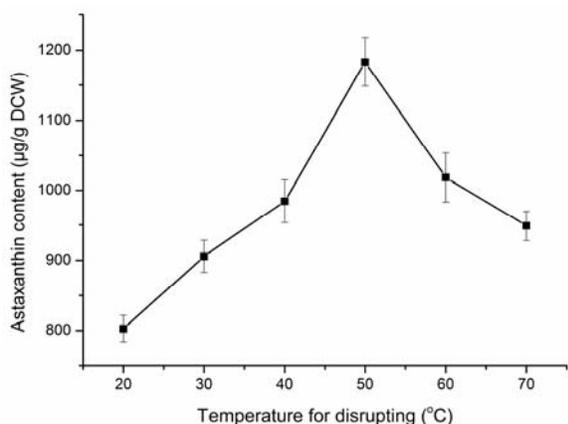


Fig. 1: Effect of temperature for disrupting on the astaxanthin yield, other conditions were fixed: Time for disrupting was 15 min, percentage of ethanol was 40% and extraction time was 20 min

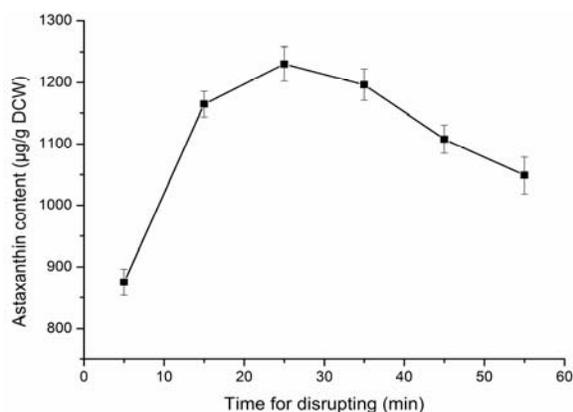


Fig. 2: Effect of time for disrupting on the astaxanthin yield, other conditions were fixed: Temperature for disrupting was 50°C, percentage of ethanol was 40% and extraction time was 20 min

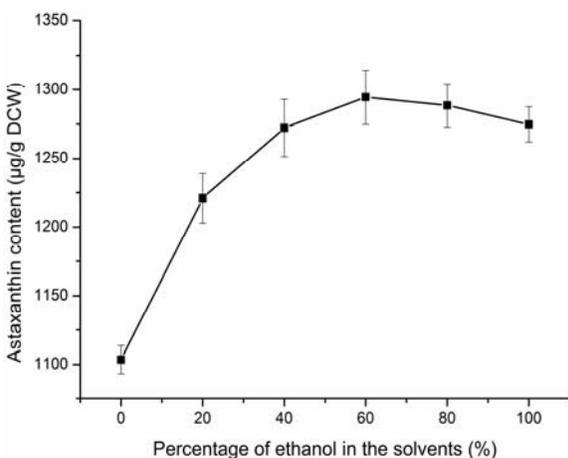


Fig. 3: Effect of percentage of ethanol on the astaxanthin yield, other conditions were fixed: temperature for disrupting was 50°C, time for disrupting was 25 min, and extraction time was 20 min

but also it is safe for people (Wu *et al.*, 2011). The effect of temperature on the astaxanthin yield was investigated (Fig. 1), while the yeast was treated by 5 mol/L lactate acid. The amounts of astaxanthin were enhanced with increasing temperature to the maximum at 50°C, after which the astaxanthin content decreased. Temperature at 50°C supported the highest astaxanthin content of (1283.15±34.16) µg/g DCW. Therefore, 40-60°C was chosen for the further optimization of extraction conditions.

Effect of time for disrupting on the astaxanthin yield:

The effect of time on astaxanthin yield was examined, while the yeast was disrupted by 5 mol/L lactate acid. As shown in Fig. 2, astaxanthin content increased from 5 min to 25 min and then gradually decreased with prolonged incubation, since a long time for disrupting may lead to oxidative degradation of astaxanthin. So disrupting time of 25 min resulted in the maximal astaxanthin yields at (1180.24±28.01) µg/g DCW. Therefore, the optimal time for disrupting the yeast cell wall by lactate acid was 15-35 min.

Effect of percentage of ethanol on the astaxanthin yield:

Extracting by organic solvent was also a crucial step towards parameter optimization, which has a strong impact on the yield of extraction. Different solvents will yield different amount and composition of extract. Therefore, suitable extracting solvent should be selected for the extraction. In this test, a mixture of ethanol and ethyl lactate was employed as extraction solvents (Wu *et al.*, 2011). As depicted in Fig. 3, the effect of various ratios of ethanol and ethyl lactate as solvents on the amounts of astaxanthin were prominent. When the percentage of ethanol increased from 0 to 60%, the yield of astaxanthin was improved rapidly and then the increase in the amount of astaxanthin seemed to reach a plateau slowly after 60% of ethanol. The maximum yield obtained was (1194.47±19.72) µg/g DCW at 60%. Therefore, 60% of ethanol was used in the subsequent experiments.

Effect of extraction time on the astaxanthin yield:

Data on the amounts of astaxanthin extracted by 60% ethanol in ethyl lactate for periods up to 60 min of incubation was depicted in Fig. 4. The extraction time of 30 min resulted in the maximal astaxanthin yields at (1164.52±28.23) µg/g DW and then rapidly decreased with prolonged incubation due to the degradation of astaxanthin. Therefore, the optimal extraction time of astaxanthin from *X. dendrorhous* by 60% ethanol was 20-40 min.

Optimization of cell disruption and astaxanthin extraction:

Temperature for disrupting, time for disrupting and extraction time were chosen as independent variables for further optimization of the astaxanthin extraction procedure. A three-level three-factor factorial design was adopted to optimize the

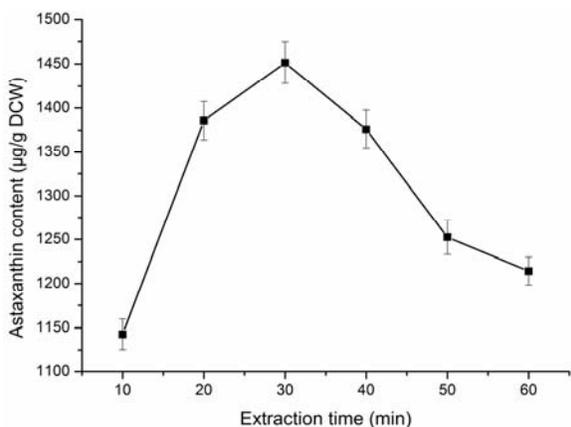


Fig. 4: Effect of extraction time on the astaxanthin yield, other conditions were fixed: Temperature for disrupting was 50°C, time for disrupting was 25 min and percentage of ethanol was 60%

extraction conditions, as shown in Table 1. The number of experiments required to investigate the above three parameters at three levels would be 27 (3³). However, it was reduced to 17 by Box-Behnken design, among which 12 were factorial experiments and 5 were zero-point tests performed to estimate the errors.

All experimental data obtained was shown in Table 2. The experimental data were fitted into the second-order polynomial equations and the regression coefficients were calculated. The astaxanthin yield ranged from 833.17 to 1494.74 µg/g DW. The maximum astaxanthin content was achieved under the experimental conditions of X₁ = 49.08°C, X₂ = 26.09 min and X₃ = 32.43 min. By applying multiple regression analysis to the experimental data, the response variable and the test variables are related by the following second-order polynomial equation:

$$Y = 1476.06 - 70.24X_1 + 43.41X_2 + 70.01X_3 + 37.81X_1X_2 + 22.08X_1X_3 - 12.51X_2X_3 - 328.22X_1^2 - 167.91X_2^2 - 137.26X_3^2$$

Table 3 shows the Analysis of Variance (ANOVA) for the regression equation. R² value for the model was 0.9978 which indicated that 99.78% of variation observed in the yield of astaxanthin could be attributed to temperature for disrupting, time for disrupting, extraction time and the interactions among these variables. A signal to noise ratio of 52.909 as estimated by adequate precision measure indicated an adequate signal for the model. The predicted R² value of 0.9807 was in reasonable agreement with the adjusted R² value of 0.9950, which indicated a good agreement between the experimental and predicted values of astaxanthin. The F value of the model was 355.79, which indicated that the model was significant. Values of ‘probability> F’ less than 0.05 indicated X₁, X₂, X₃, X₁², X₂², X₃², X₁X₂ and X₁X₃ were the model terms which significantly influenced astaxanthin yields. The lack of fit F-value of 1.36 showed that lack of fit was insignificant. A low value of coefficient of the variance (C.V.%) (1.35) clearly indicated a high degree of precision and reliability of the experimental values.

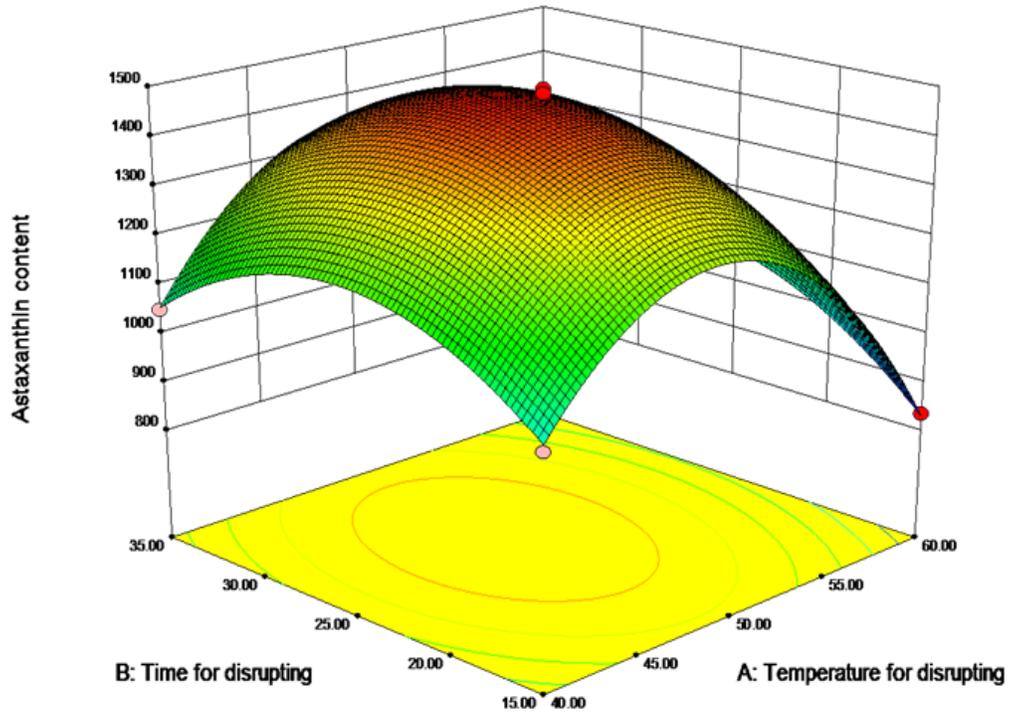
The three dimensional response surface curves given in Fig. 5 show the relative effect of any two variables, when the concentration of the third variable is maintained at its middle level. An increase of temperature for disrupting (X₁), time for disrupting (X₂) and extraction time (X₃) result in an initial increase of astaxanthin yield, which then decrease with the rising of X₁, X₂ and X₃. The optimal values of the selected three variables for maximizing astaxanthin yield were determined by solving the model equation using the numerical optimization function in Design-Expert software. The model predicted a maximal astaxanthin yield of 1490.18 µg/g DW, while the optimal extraction conditions of astaxanthin were disrupted at 49.08°C for 26.09 min and then extracted for 32.43 min. To confirm these results, tests were performed in triplicate under the above optimized conditions and the astaxanthin yield was 1472.85±43.64 µg/g DW. This implied that there was a high fit degree between the value observed

Table 3: Analysis of variance (ANOVA) for the regression equation

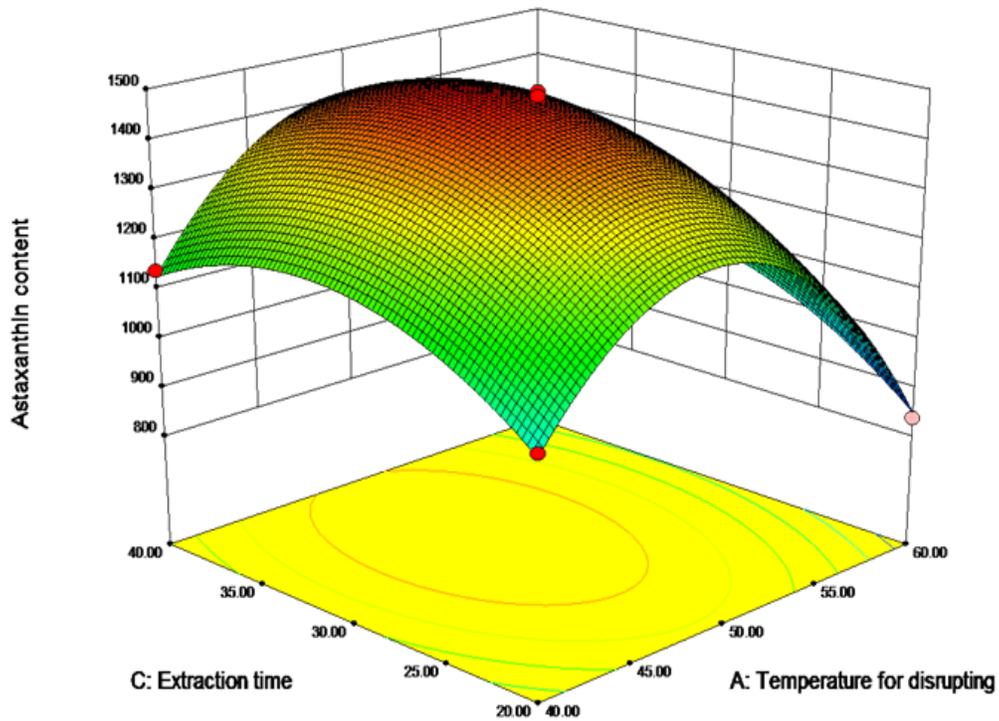
Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
Model	8.155E+005	9	90610.27	355.79	<0.0001
X ₁	39470.67	1	39470.67	154.98	<0.0001
X ₂	15075.42	1	15075.42	59.19	0.0001
X ₃	39207.00	1	39207.00	153.95	<0.0001
X ₁ X ₂	5719.14	1	5719.14	22.46	0.0021
X ₁ X ₃	1950.11	1	1950.11	7.66	0.0278
X ₂ X ₃	625.75	1	625.75	2.46	0.1610
X ₁ ²	4.536E+005	1	4.536E+005	1781.10	<0.0001
X ₂ ²	1.187E+005	1	1.187E+005	466.10	<0.0001
X ₃ ²	79325.30	1	79325.30	311.48	<0.0001
Residual	1782.72	7	254.67		
Lack of fit	898.96	3	299.65	1.36	0.3756
SD	15.96	R ²	0.9978		
Mean	1177.99	Adjusted R ²	0.9950		
C. V. %	1.35	Predicted R ²	0.9807		
PRESS	15764.18	Adequate precision	52.909		

in experiment and the value predicted from the regression model. Therefore, the response surface modeling could be applied effectively to predict the extraction condition.

Comparison with other conventional methods: The yeast was extracted by Ultrasound-Assisted Extraction (UAE) and conventional extraction, respectively. Method A, B and C share the same disrupting time,



(a)



(b)

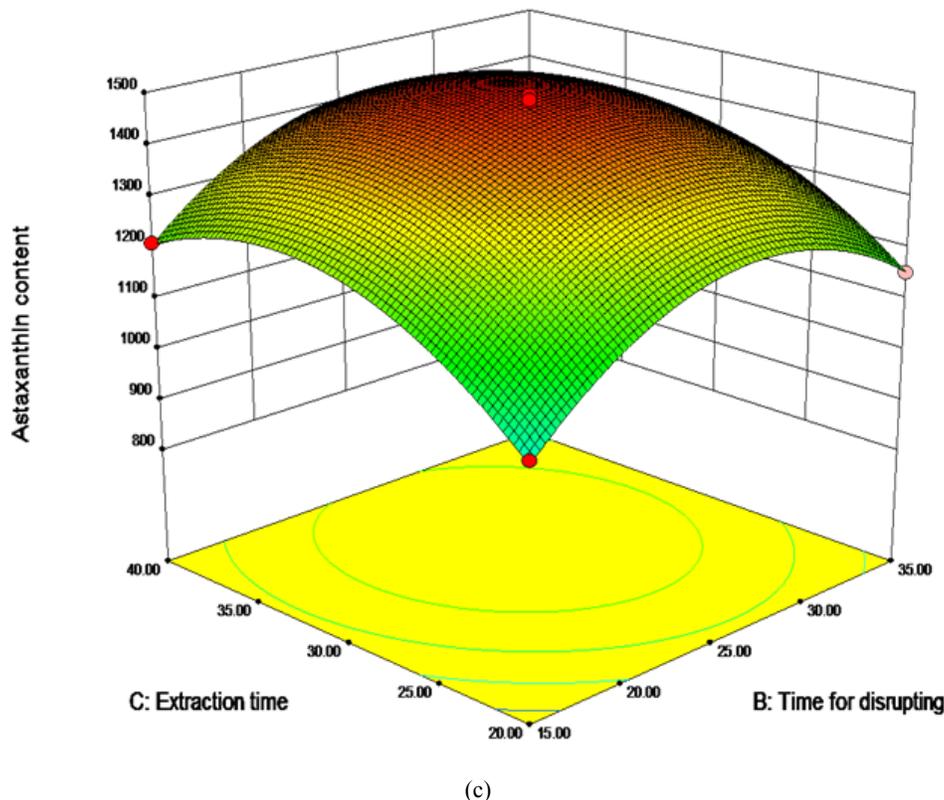


Fig. 5: Response surface graphs for the effects of temperature for disrupting, time for disrupting, and extraction time on the astaxanthin yield: (a) temperature for disrupting (X_1) and time for disrupting (X_2); (b) temperature for disrupting (X_1) and extraction time (X_3); (c) time for disrupting (X_2) and extraction time (X_3)

Table 4: Astaxanthin yields for different conventional extraction approaches

Extraction approaches	Astaxanthin content ($\mu\text{g/g}$, $n = 3$)
A: DMSO+acetone	1464.28 \pm 37.31
B: hydrochloric acid+acetone	1137.56 \pm 52.18
C: lactate acid+ethyl lactate	1384.38 \pm 43.49
D: Ultrasound-assisted extraction	1472.85 \pm 43.64

disrupting temperature and extracting time, which are 1 h, 65°C and 30 min, respectively. While for UAE, they are 25 min, 50°C and 30 min. Table 4 shows that UAE gave a higher astaxanthin content than other three method and needed a shorter disrupting temperature and a lower disrupting temperature. Therefore, UAE is a more efficient extraction method for astaxanthin from *X. dendrorhous*.

CONCLUSION

The experimental design approach allowed the determination of the significant effects and polynomial functions that describe the effects of Temperature for disrupting, time for disrupting and extraction time on the astaxanthin extraction from *X. dendrorhous*. Ultrasonic device is a powerful tool, which can efficiently improve the efficiency of astaxanthin extraction. A polynomial regression model was proposed to reasonably describe the experimental

results and based on the proposed model, the optimal condition for astaxanthin yields that was within the experimental range was found to be disrupted at 49.08°C for 26.09 min and then extracted for 32.43 min. At this condition, the predicted astaxanthin yield was 1490.18 $\mu\text{g/g}$ DW.

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