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Optimization of essential oil and fucoxanthin extraction from *Sargassum binderi* by Supercritical Carbon Dioxide (SC-CO_2) extraction with ethanol as co-solvent Using Response Surface Methodology (RSM)

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Abstract

Supercritical carbon dioxide (SC-CO_2) extraction of fucoxanthin is more advantageous over conventional solvent extraction as it is less toxic, less hazardous to the environment and preserves the bioactivity of fucoxanthin. A face-centered central composite design (FCCCD) based on response surface methodology (RSM) was employed for SC-CO_2 extraction of oils and fucoxanthin from the brown seaweed *Sargassum binderi*, with ethanol as a co- solvent. Three independent parameters namely, extraction temperature (A: 40, 50, 60^{o}C), pressure (B: 2900, 3625, 4350 psig and particle size (C: 90, 500 and 1000 µm) were investigated to optimize extraction oil yields (EOY) and fucoxanthin yields (FY). A regression model was developed, tested for quality of fit (R^2) and expressed in the form of 3D response surface curve and 2D contour. The optimum extraction conditions were obtained at extraction temperature (A) 50^{o}C, pressure (B) 3625 psig and particle size (C) 500 µm. Under these conditions, optimal EOY and FY were 10.04 mg/g and 3188.99 µg/g, respectively. The difference between the lowest and the highest response in EOY and FY were 5.44 – 10.04 mg/g and 2109.10 - 3188.90 µg/g, respectively. The lowest yields were identified at 60^{o}C, 2900 psig and 1000 µm. The regression models generated showing interactions between the variables and EOY and FY response were significant as tested by ANOVA ($p < 0.0005$ and $p < 0.0007$, respectively) with high R^2 values (0.9848 and 0.9829, respectively). Interactions between the parameters had a strong synergistic effect on EOY and FY values, as indicated by the 3D response surface curve and 2D contour. The experimental results matched the predicted results closely. This indicated the suitability of the models developed and the success of FCCCD under RSM in optimizing the *S. binderi* extraction conditions.

Introduction

In an earlier study, reversed phase-HPLC (RP-HPLC) equipment was successfully used to quantify fucoxanthin content from three Malaysian brown seaweeds, *Sargassum binderi, S. duplicatum* and *Padina australis* (Noviendri *et al.*, 2011; Jaswir *et al.*, 2011). The fatty acid contents were analyzed by gas chromatography in the form of fatty acid methyl esters (FAMEs). *S. binderi* is a good candidate for fatty acid sources from seaweeds because the n6/n3 ratio is less than 1 (0.87) (Noviendri *et al.*, 2011), Contributed by high n-3 and low n-6. The n6/n3 ratio is beneficial for health due to several sources

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of information suggesting that human beings evolved a diet in which the ratio of n-6 to n-3 essential fatty acids (EFAs) was about 1 (Simopoulus, 2002). Fucoxanthin is a major marine carotenoid, found in edible seaweeds (Roh *et al.*, 2008). This compound is one of the most abundant carotenoids in nature (Matsuno, 2011; Miyashita, 2008), especially in the marine environment (Dembitsky and Maoka, 2007).

Currently, the most common method for fucoxanthin extraction is by liquid solvent extraction such as acetone, ethanol and dimethyl sulfoxide (DMSO). Fucoxanthin has been extracted from the microalgae *Phaeodactylum tricornutum* using ethanol (Kim *et al*., 2012), the brown seaweed *Laminaria japonica* using DMSO (Wang *et al.*, 2005) and ethanol (Zhang *et al*., 2008), *Undaria pinnatifida* using acetone (Sugawara et al., 2006) and chloroform/ methanol (Maeda *et al*., 2008), and *Hijikia fusiformis* (Yan *et al*., 1999), *Eisenia bicyclis,* respectively using acetone (Kim *et al*., 2011). However, conventional techniques can damage the functional properties of the extracts and are also potentially hazardous to the environment (Foster *et al*., 1993). Supercritical fluid extraction (SFE) eliminates the disadvantages of conventional technique which leads to degradation of thermally labile compounds (Roh *et al*., 2006), and leaves traces of toxic solvents in the solute (Döker *et al*., 2010).

SFE technology has been used in large-scale extraction of some essential oils (Abbas *et al*., 2008) and fucoxanthin from *Undaria pinnatifida* (Roh *et al*., 2008). The most commonly used SFE is supercritical carbon dioxide (SC-CO_2) extraction (Santoyo *et al*., 2006; Machmudah *et al*., 2007), because it has a favorable critical pressure (Pc = 1071 psig) and critical temperature (Tc = 31.1^{o}C) (Lang *et al*., 2001; Machmudah *et al.,* 2007; Romo- Hualde *et al*., 2012). SC-CO_2 extraction exhibits good density, high diffusivity, low surface tension and low viscosity, which play key roles in enabling the solvent to readily penetrate the solid biomass matrix as well as in extracting the solutes (Muthukumaran *et al*., 1999; Baek *et al*., 2004; Roh *et al*., 2008). SC-CO_2 extraction is also chemically inert under many conditions, non-flammable, non-toxic, and inexpensive (Lang *et al.*, 2001; Turner and Mathiasson, 2001; Machmudah *et al*., 2007; Roh *et al*., 2008; Abbas *et al*., 2008; Norulaini *et al*., 2009; Romo- Hualde *et al.*, 2012). These properties of SC-CO_2 make the products more advantageous in the field of food, pharmaceutical (Abbas *et al.*, 2008) or medicinal extract (Doker *et al*., 2010), and cosmetics (Machmudah *et al*., 2007).

Therefore, the purpose of this study was to optimize essential oil and fucoxanthin extraction from selected Malaysian brown seaweed *(S. binderi)* by SC-CO_2 extraction with ethanol as co-solvent under various: temperatures, pressures and particle sizes of the sample using response surface methodology (RSM).

Materials and methods

Materials

Plant material (brown seaweed) used in this study was *S. binderi*. It was freshly collected from Port Dickson, Negeri Sembilan, Malaysia in August 2011. Food grade carbon dioxide (99.9% pure) was used and ethanol as co-solvent as well as other reagents were of analytical grade.

Sample preparation

Fresh brown seaweed *(S. binderi)* was washed thoroughly with fresh water to remove salt and sand attached to the surface (Heo and Jeon, 2009). The cleaned sample was frozen at -80^{o}C and then dried in freeze-drier for 3 days (Roh *et al.*, 2008). The dried *S. binderi* was ground in a mill before treatment through a mesh sieve with sizes 90, 500 and 1000 µm.

Experimental design

RSM was used to optimize the conditions that enhanced oil and fucoxanthin extraction by SC-CO_2 extraction with ethanol as co-solvent. A face-centered central composite design (FCCCD) under RSM developed by the Design Expert software (Version 6.0.8, Stat-Ease Inc., Minneapolis, USA) (Muntari *et al*., 2012) was used to optimize the three significant extraction conditions: pressure, temperature and sample particle size, to yield of oil and fucoxanthin extract.

A set of 15 experimental runs with one center point (Run 13) was generated. Subsequently, three different levels, low (-1), medium (0) and high (+1) were used to study the independent variables. The extracted oil and fucoxanthin yield were considered as the response (Y_1) and (Y_2), respectively. The following second-order polynomial equation explains the relationship between dependent and independent variables (Muntari *et al*., 2012):

$$Y_1 \text{ or } Y_2 = \beta 0 + \beta 1A + \beta 2B + \beta 3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC \ldots\ldots\ldots (1)$$

Where Y_1 is the dependent variable (extracted oil yield, EOY), and Y_2 is the dependent variable (fucoxanthin yield, FY); A, B and C are independent variables (temperature, pressure and particle size of sample, respectively); β_0 is an intercept term; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are the interaction coefficients; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients.

The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), p- and F-values (Muntari *et al*., 2012). Then, the quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2 (Bari *et al*., 2009; Muntari *et al*., 2012). Furthermore, to explain the relationship between the experimental levels of each of variables under study and the responses, the fitted polynomial equation was expressed in the form of 3D response surface and contour (Muntari *et al.,* 2012).

Determination of extracted oil and fucoxanthin yield

The estimated yield of oil obtained from *S. binderi* by SC-CO_2 with ethanol as co-solvent was calculated directly as a ratio between the weight of oil obtained from extraction process and the weight of dried sample used in this optimization, in percentage. The determination of carotenoid (fucoxanthin) yield was adopted from the method of Gu *et al.* (2008) and Chen *et al*. (2006) with slight modifications. Briefly, the extract of S binderi was diluted with acetone and the solution was transferred to CELLSTAR® 96 well flat bottom plates (Greiner Bio-one) (Mori *et al.*, 2004) and measured by microplate reader (Tekan/ infinite M200, NanoQuan) at 450 nm (maximum wavelength for detecting fucoxanthin) (Yan *et al.*, 1999; Simopoulus, 2002; Mori *et al.*, 2004; Maeda *et al*., 2006; Nakazawa *et al*., 2009; Noviendri *et al.*, 2011a, 2011b; Jaswir *et al.*, 2011; Jaswir *et al*., 2013). The fucoxanthin yield (FY) (µg/g dried weight sample) was calculated according to the following formula (Chen *et al*., 2006):

FY (µg/g dried weight sample) = 1000ADV/0.16W ………………… (2)

Where A is the absorbance value of diluted extraction at 450 nm, D is the dilution ratio, V is the volume of the acetone. 0.16 is extinction coefficient of carotenoids (fucoxanthin), and W (g) is the weight of dried *S. binderi*.

Results and discussion

Optimization of SC-CO_2 extraction condition by RSM

The use of statistical experimental design is a vital tool in optimizing conditions (Muntari *et al*., 2012) that cause an increment of several folds in oil and carotenoid (fucoxanthin) yields. Wu *et al*. (2007) reported that the main advantage of RSM is to reduce the number of experimental trials needed to evaluate multiple variables and interactions. In addition, RSM is less laborious and time-consuming compared to other approaches (Hossain *et al*., 2011; Salihu *et al*., 2011).

In this study, an FCCCD under RSM was used to determine the optimal conditions of the three significant factors (temperature, pressure and particle

Table 1. An FCCCD of three independent variables with their actual and coded values and one center point showing the experimental and predicted response.

Run	T	P	PS	EOY (mg/g dw)		FY (µg/g dw)	
	(°C)	(psig)	(µm)	Ex.	Pr.*	Ex.	Pr.**
1	60 (+1)	2900 (-1)	1000 (+1)	5.44	5.46	2109.10	2109.30
2	50 (0)	3625 (0)	1000 (+1)	7.78	7.77	2600.30	2637.68
3	40 (-1)	4350 (+1)	90 (-1)	7.23	7.19	2589.40	2577.28
4	60 (+1)	3625 (0)	500 (+1)	8.87	9.12	3000.10	3025.69
5	50 (0)	2900 (-1)	500 (0)	8.51	8.72	3011.10	2942.32
6	60 (+1)	4350 (+1)	90 (-1)	7.91	7.93	2885.70	2860.28
7	40 (-1)	2900 (-1)	1000 (+1)	5.67	5.60	2300.00	2312.81
8	40 (-1)	2900 (-1)	90 (-1)	7.22	7.26	2579.10	2596.19
9	60 (+1)	4350 (+)	1000 (+1)	7.89	7.81	2645.90	2623.85
10	50 (0)	4350 (+1)	500 (0)	9.79	9.76	3029.30	3167.23
11	50 (0)	3625 (0)	90 (-1)	8.47	8.66	2988.90	2897.58
12	40 (-1)	4350 (+1)	1000 (+1)	7.39	7.53	2785.50	2757.16
13	50 (0)	3625 (0)	500 (0)	10.04	9.69	3188.90	3118.60
14	40 (-1)	3625 (0)	500 (0)	8.87	8.80	2954.80	2965.36
15	60 (+1)	2900 (-1)	500 (0)	7.78	7.58	2786.30	2808.98

T: temperature; P: pressure; PS: particle size; EOY: extracted oil yield; FY: fucoxanthin yield; Ex.: experiment; Pr.: predicted. *Second order polynomial (Eqn 3) was used to estimate the predicted response (extracted oil). ** Second order polynomial (Eqn. 4) was used to estimate the predicted response (carotenoid yield).

Table 2. ANOVA of a quadratic model for EOY.

Source	Sum of square	F-value	*p*-value
Model	22.61	36.01	0.0005
Temperature, *A*	0.22	3.17	0.1349
Pressure, *B*	3.24	46.46	0.0010
Particle size, *C*	1.98	29.39	0.0031
A^2	1.34	19.24	00071
B^2	0.52	7.24	0.0416
C^2	5.21	74.67	0.0003
AB	0.087	1.25	0.3140
AC	0.11	1.52	0.2723
BC	2.02	29.98	0.0030

$R^2 = 0.9848$, Adjusted $R^2 = 0.9575$, Adequate precision = 19.934, $p < 0.05$ was considered to be significant.

Table 3. ANOVA of a quadratic model for FY.

Source	Sum of square	F-value	*p*-value
Model	1.189E+006	32.01	0.0007
Temperature, *A*	3828.07	0.93	0.3798
Pressure, *B*	1.582E+005	38.35	0.0016
Particle size, *C*	1.740E+005	42.18	0.0013
A^2	38948.84	9.44	0.0277
B^2	10474.14	2.54	0.1720
C^2	2.932E+005	71.07	0.0004
AB	2462.02	0.60	04746
AC	86824.97	21.04	0.0059
BC	1.075E+005	26.06	0.0038

$R^2 = 0.9829$, Adjusted $R^2 = 0.9522$, Adequate precision = 20.171, $p < 0.05$ was considered to be significant.

size of the sample) to yield oil and fucoxanthin extract. For each run, the experimental (observed) results along with the predicted EOY and FY obtained from the regression equations for the 15 combinations are shown in Table 1.

In this study, a co-solvent ethanol was used in SC-CO_2 to extract the semi-polar carotenoid fucoxanthin. In the SC-CO_2 extraction of carotenoid from a natural source, the use of co-solvents such as ethanol (Nobre *et al*., 2006) has been used to improve the extraction efficiency (Naranjo- Mdad *et al*., 2000; Vassapollo *et al*., 2004). The presence of a polar co-solvent can increase the solubility of polar compounds and the selectivity of the process (Abbas *et al*., 2008) is due to the polar character of carotenoids. The formation of hydrogen bonds with ethanol present in the CO_2 stream and swelling of the biomass pore facilitates the release of the pigments from the samples (Nobre *et al*., 2006).

From this study, the results demonstrated that optimal extracted oil yield (EOY) and fucoxanthin yield (FY) by SC-CO_2 extraction with ethanol as co-solvent are 10.04 mg/g and 3188.99 µg/g, respectively (run 13, Table 1) achieved at 50°C, 3625 psig and 500 µm. Furthermore, the lowest amounts were observed in run 1 (5.44 mg/g and 2109.10 µg/g), where the factors such as temperature and particle size of the sample were at highest conditions (60°C and 1000 µm, respectively), whereas the pressure was at lowest condition (2900 psig). This study shows that the design matrix of FCCCD further improved the EOY and FY, such that the difference between the lowest and the highest response (5.44 – 10.04 mg/g; 2109.10 - 3188.90 µg/g), respectively.

Furthermore, a second order regression equation showed the dependence of EOY and FY on SC-CO_2 extraction components. The parameters of the equation were obtained by multiple regression analysis of the experimental data (Salihu *et al.*, 2011). An empirical relationship between the screened variables and response were expressed in terms of the second-order polynomial equation:

$$Y_1(\text{EOY, mg/g dried weight}) = +9.66 + 0.15A + 0.57B - 045C - 0.72A^2 - 0.45B^2 - 1.44C^2 + 0.10AB - 0.12AC + 0.50BC \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Where the EOY is the response (Y_1) and A, B and C are temperature, pressure, and particle size of the sample, respectively.

$$Y_2 (\text{FY, µg/g dried weight}) = +3106.93 + 19.57A + 123.94B - 131.95C - 123.07A2 - 61.64B^2 - 341.46C^2 + 17.25AB - 104.08AC + 113.8^2BC \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

Where the FY is the response (Y_2) and A, B and C are the temperature, pressure, and particle size of the sample, respectively.

The adequacy of the model for EOY and FY were checked using ANOVA which was tested using statistical analysis of Fisher and the results are shown in Table 2 and Table 3. For EOY (Table 2), the model F value of 36.01 and p-value of < 0.0005 imply that the model is significant, suggesting that there is only 0.05% chance that the model F value could occur due to noise. Model terms with Probability > F (less than 0.05) are considered significant, while those greater than 0.10 are insignificant (Saliu *et al*., 2011).

Furthermore, for FY (Table 3), the F value of

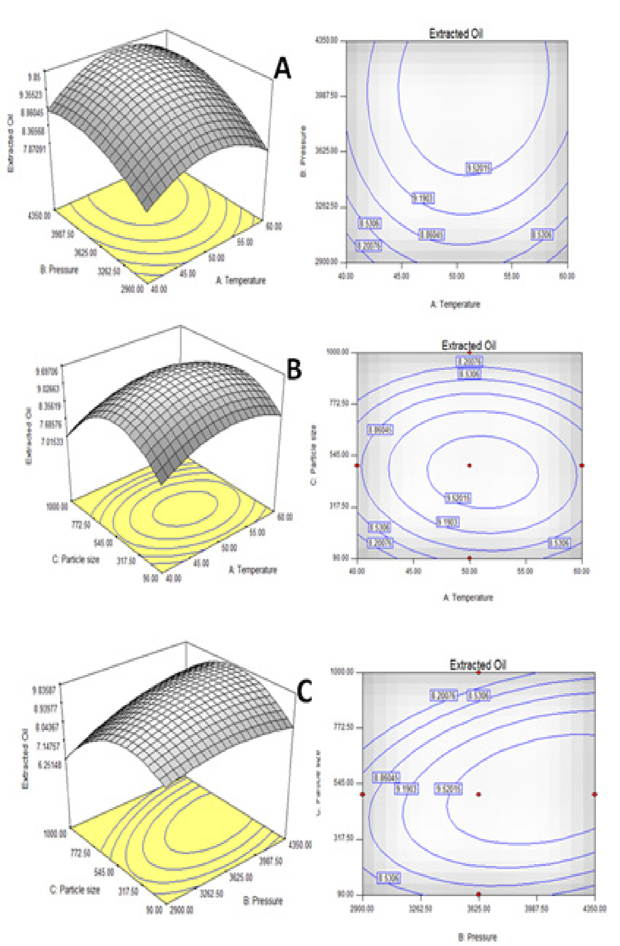

Figure 1. 3D response surface curves and 2D contour of the combined effects of temperature, pressure, the particle size of the sample on EOY by SC-CO_2 extraction with ethanol as cosolvent. Temperature and pressure at fixed level of particle size of sample (A), temperature and particle size at fixed level of pressure (B), pressure and particle size of the sample at fixed level of temperature (C).

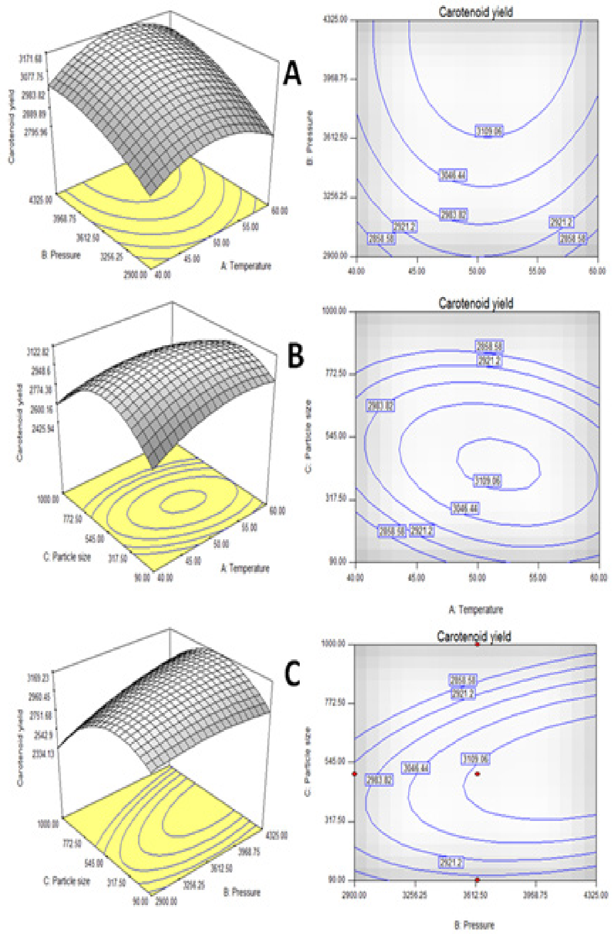

Figure 2. 3D response surface curves and 2D contour of the combined effects of temperature, pressure, the particle size of the sample on FY by SC-CO_2 extraction with ethanol as cosolvent. Temperature and pressure at fixed level of particle size of sample (A), temperature and particle size at fixed level of pressure (B), pressure and particle size of the sample at fixed level of temperature (C).

32.01 and p-value of < 0.0007 imply that the model is significant, suggesting that there is only 0.07% chance that the model F value could occur due to noise. Bari *et al.* (2009) have reported that a greater F-value indicates that the factors adequately explain the variation in data about its mean, and estimated factor effects are real. From this study, the model terms with Probability > F (less than 0.05) are considered significant.

The R^2 value closer to 1 denotes a better correlation between the experiment (observed) and predicted values (Salihu *et al.*, 2011). For EOY (Table 2), the higher values of R^2 (0.9848) and adjusted R^2 (0.9829) also indicated the efficacy of the model and 98.48% or 98.29% of variations could be accounted for by model equation. Thus, for a good statistical model, the R^2 value should be in the range of 0 – 1.0, and the closer the value is to 1.0, the more fit the model is deemed to be.

Moreover, adequate precision measures signal to noise ratio and a value of >4 is considered a prerequisite for desirable models. The adequate precision value of 19.937 for EOY indicates that the model can be used to navigate the design space. Furthermore, for FY (Table 3), the higher values of R^2 (0.9829) and adjusted R^2 (0.9522) also indicated the efficacy of the model and 98.29% or 95.22% of variations could be accounted for by model equation. The adequate precision value of 20.171 for FY also indicates that the model can be used to navigate the design space due to the adequate precision measures signal to noise ratio and a value >4. Thus, this is considered a prerequisite for desirable models.

The coefficient values of regression equation are listed below in Table 2 and 3. The p-value is used as a tool to check the significance of each coefficient, which also indicates the interaction strength between each independent variable (Li *et al.*, 2012). The smaller the p-value, the larger the significance of the corresponding coefficient (Li *et al.*, 2012).

For EOY (Table 2), the responses revealed that (B – pressure) and (C – particle size of sample), one interaction terms BC (pressure and particle size of sample), and all the quadratic coefficients (A^2, B^2 and C^2) were significant ($p < 0.05$) and had remarkable effects on the overall extracted oil. Similarly, for FY (Table 3), the responses revealed that (B – pressure) and (C – particle size of the sample) were significant ($p < 0.05$). However, for FY, there are two interaction terms AC (temperature and particle size of the sample) and BC (pressure and particle size of the sample), and the two quadratic coefficients (A^2 and C^2) were significant ($p < 0.05$) that had remarkable effects on the overall FY.

Tanyldizi *et al.* (2005) reported that the 3D response surface and 2D contour plots are the graphical representation of the regression equation used to determine the optimum values of the variables within the ranges considered. The 3D response surface and 2D contour plots of the combined effects of temperature, pressure and particle size of the sample for EOY and FY by SC-CO_2 extraction with ethanol as co-solvent are shown in Figures 1 and 2, respectively. The 3D plots are based on the function of the condition of two variables with the other variable being at its optimum level. The significance of the interaction between the corresponding variable is indicated by the elliptical or saddle nature of the contour plots (Muraldihar *et al.*, 2001; Salihu *et al.*, 2011).

Figure 1A represents the interaction between temperature and pressure conditions. Lower and higher levels of both temperature and pressure did not result in higher EOY. Figure 1B shows the 3D plot corresponding to temperature and particle size of the sample, where a moderate interaction between these tested variables occurred but was not significant. In the case of pressure and sample particle size (Figure 1C), the response plot was an elliptical indicating interaction between both with optimum EOY in SC-CO_2 extraction with ethanol as co-solvent.

Furthermore, Figure 2B and 2C show the 3D plots corresponding to temperature and particle size of the sample; and pressure and particle size of the sample, respectively. Lower and higher levels of pressure and sample particle size result in higher FY. Whereas, Figure 2A represents the interaction between temperature and pressure, lower and higher levels of temperature and pressure did not result in higher FY. The shape of the response surface curve showed a moderate interaction between the tested variables. Thus, it can be seen that the optimized combination of selected condition components (temperature, pressure and particle size of the sample) showed the strong synergistic effect on EOY and FY values.

Conclusion

In this study, SC-CO_2 extraction with ethanol as co-solvent was successful in extracting essential oil and carotenoid (fucoxanthin) from *S. binderi.* An FCCCD under RSM was used to determine the optimal conditions of the three significant factors (temperature, pressure and sample particle size) to yield oil and fucoxanthin extracts. The results demonstrated that optimal EOY and FY by SC-CO2 extraction with ethanol as co-solvent are 10.04 mg/g and 3188.99 µg/g achieved at 50°C, 3625 psig and 500 µm, respectively which represent the center point (run 13).

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