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Research paper

Optimization of Supercritical CO_2 Natural Dye Extraction from Brown Seaweed (*Sargassum Sp.*) via Response Surface Methodology

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Abstract

This paper discusses the effects of two main parameters (pressure and temperature) in supercritical carbon dioxide extraction of *Sargassum sp.* through response surface methodology (RSM). Perturbation plot shown significant effects of all process parameters on the yield of extract. An experimental design software was designated to achieve optimization on the process situations pertaining maximum extraction yield. The optimal conditions perceived were at pressure of 4500 psi and temperature of 65°C. The highest yield of extract achieved was 2.7 mg-extract/g-dried sample under the optimum conditions. The yield of extract was then further analysed via Gas Chromatography Mass Spectrometry (GCMS) and it was found that *Sargassum sp.* contains sterols, pentadecanoic acid, 14-methyl ester, 9-Hexadecenoic acid, methyl ester and phytol which are the constituents of bioactive compounds and antimicrobial properties.

Keywords: *Sargassum sp.; brown seaweed; SC-CO_2; RSM; GCMS; natural dyes.*

1. Introduction

Since ancient history, numerous colouring matters have been exploited from various parts of plants including flowers, seeds, stems, roots, barks, leaves and fruits [1-4]. Later on, the innovation of economical synthetic dyes took place and natural dyes with its limitations eventually struggled to compete against mass production of synthetic dyes. However, in recent decades, the demand for eco-friendly natural dyes has been increasing due to the hazardous and carcinogenic implications of synthetic dyes in food and textile application. In present study, seaweed, also known as macroalgae, was selected as the source of natural dyes as they comes in many colours and highly abundance, thus become a subject of interest for various research projects mainly for food and textile application with the potential source of dye pigments particularly chlorophylls and carotenoids [6, 8]. Yet, there were limited reports on the utilization of *Sargassum sp*. and its application as natural colorant on textile fabrics.

SC-CO_2 extraction is an alternative advanced green technology, which continuously increases its application field with beneficial factors including highly pure yield of extracts with no waste produced, shorter extraction time, automation and lower solvent consumption [10-18]. In addition, the use of CO_2 as the extraction solvent provides nontoxic, non-polluting, high efficiency and operational flexibility with modest quality [2, 15, 17, 19, 20].

This study therefore, deals with the optimization of SC-CO_2 extraction through Response Surface Methodology (RSM) in order to determine the best optimized pressure and temperature during extraction. The objective is to achieve the highest yield of extract for textile coloration purposes. In addition, the contents of *Sargassum sp*. extract were screened via Gas Chromatography Mass Spectrometry (GCMS) to verify the presence of bioactive compounds and antimicrobial properties which will be beneficial in textile application.

2. Material and Method

2.1. Materials and Sample Preparation

Sargassum sp. were collected from Mabul Island, Sabah, Malaysia. Then, the samples were cleaned thoroughly with tap water to remove debris, sand and unwanted particles. All parts of *Sargassum sp.* were oven-dried at 50°C for 48 h to eradicate moisture before storing them in at ambient temperature in sealed container. The samples were ground in a mill-grinder and further sieved to 1 mm using a sieve shaker (AS200 Retch, Germany). A high purity CO_2 (99.995%) purchased from Pure Dimension Sdn. Bhd. (Malaysia) and absolute ethanol 99.99% of analytical grade purchased from RCI Labscan Limited, Thailand were used in the SC-CO_2 extraction.

2.2. SC-CO_2 Extraction

The SC-CO_2 extraction was performed using (OV-SCF-10000 Model from Taiwan Supercritical Technology Co. Ltd.) which comprises a 60 mL extraction canister, a pressure regulator (Techmaster, UK), a high pressure booster pump (Zook, Canada) and a recirculating cooler model RC10 UNISS. Samples of ground *Sargassum sp.* (15 g) of 1 mm diameter size were placed into the extraction canister. The temperature was allowed to rise until the exact required temperature was reached followed by the reach of required pressure. Then, the liquefied CO_2 was transmitted into the extraction canister with the help of compressed air boosted by

the high pressure pump. After the desired pressure and temperature has been reached, the extract will be released from the unit and collected into an extraction flask.

2.3. Yield of Extract

The yield of extract in the form of mg-extract/g-dried sample was weighed after the extraction using an analytical balance (±0.0001g) (Sartorius, Germany) and calculated according to equation (1):

$$Y = m_c / m_e \times 1000 \quad (1)$$

Where m_c is the mass extract of the crude yield(g) and m_e is the mass of sample used (g).

2.4. Optimization of SC-CO_2 Extraction via Experimental Design Approach

The experimental design approach was adapted by response surface methodology (RSM) via Design-Expert Software version 6.0.4 in order to optimize the process variables to achieve the highest amount of extraction yield from *Sargassum sp.* Two main variables namely pressure and temperature, were manipulated in this study. The central composite design (CCD) from the above software was preferred as it provides at its best efficiency limited to two variables rather than Box-Behnken design which is more proficient for 3-21 factors as supported by Design Expert [21]. From the CCD, 13 sets of experiments were conducted with pressure between 1500 to 5000 psi and at temperature between 40 to 70°C. Each of the variables was set at five coded levels of -α, -1, 0, +1 and +α. The general equation of second-order polynomial is given in Equation (2):

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A^2 + \beta_{22} B^2 + \varepsilon \quad (2)$$

Y is the dependent variables (response), β_o is the constant coefficient, $\beta_1 A$ and $\beta_2 B$ are the linear coefficients, $\beta_{11} A^2$ and $\beta_{22} B^2$ are the quadratic coefficients, $\beta_{12} AB$ is the interaction effect, A and B are the factors (independent variables) and ε is the error [22].

Table 1: CCD designated for the SC-CO2 extraction of *Sargassum sp*.

	Temperature (°C)	Pressure (psi)
α	40	3250
1	44	2000
0	55	5000
1	65	4500
α	70	3250

The optimum level of independent variables was predicted via numerical optimization that leads to the anticipated main response. The adequacy of the model was measured using the lack of fit test, model analysis, adjusted-R^2 and coefficient of determination (R^2). The significant terms which is based on p-value ($p < 0.05$) in designed model were instigated by the variance analysis (ANOVA).

2.5. GCMS Screening of Compound from *Sargassum Sp.*

Samples were analysed using Agilent Technologies 6890N Network GC coupled with an Agilent Technologies 5973 Inert Mass Selective Detector and Agilent 7683 Series Injector [23]. A HP-5MS column was used for the GCMS analysis. The preliminary column temperature was set at 80 °C while the temperature of the injector and detector were set at 350°C. A volume of 2 µL sample was inserted into the column and ran using split less mode. The temperature of the oven was upraised to 150°C at a ramp rate of 10°C/min after 2 min followed by 250°C at a ramp rate of 5 °C/min and finally to 280°C at a ramp rate of 20°C/min and the condition was maintained for 40 min. Helium was used at a flow rate of 1.0 mL/min in splitless mode as the carrier gas. Identification of the compounds was done by comparing their relative retention indices and mass spectral fragmentation pattern with NIST data inbuilt library provided by the instrument software [23, 24].

3. Result and Discussion

3.1. Effect of the Reaction Factors against Yield of Extract

The influence of varied pressures and temperatures on the extracted yield was measured. The individual influence of independent variables; temperature (A) and pressure (B) was established by a perturbation plot. The plot did not illustrate the effect of an interaction. The perturbation plot assists to relate the interaction of all independent parameters at a certain point in the space of design [12]. The response was plotted by varying one factor and at the same time the other factor held constant. A curvature in a factor confirmed that the response was subtle to that particular factor [12]. Hence, these prove that the yield of extract is sensitive to both pressure and temperature. The perturbation plot for the extracted yield is presented in Figure 1.

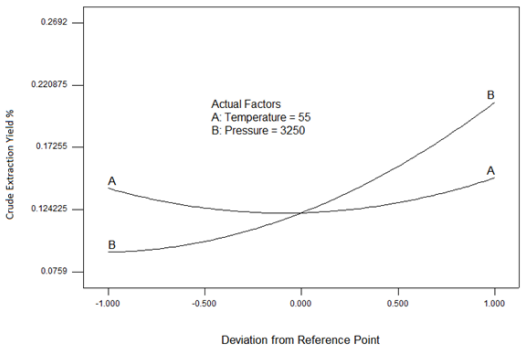

Fig. 1: Perturbation graph demonstrates the effect of process variables on crude extract yield

3.2 SC-CO_2 Extraction Optimized Conditions and Model Verification

The results based on experimental design are shown in Table 2. The yield of extract were in the range of 0.8 to 2.7 mg-extract/g-dried sample. A multiple linear regression analysis was applied to purify the second-order polynomial model for expecting the yield from the data of experiment. The predictable statistical significance of linear, quadratic and interaction of process variables and regression equation coefficients along with the corresponding R^2, adjusted-R^2 and lack of fit test for the final reduced model were displayed in Table 3. Probability values (*p*-value) showed that all linear, quadratic and interaction terms had significant ($p < 0.05$) result on the extraction yield. The final reduced model indicate the *p*-value of less than 0.05 which ultimately confirmed the significance of the model fitness (Table 3). Likewise, the lack of fit shows a non-significant value ($p > 0.05$) which indicates the model could adequately fit the experiment. The established final reduced model of extraction yield was attained according to equation (3) as follows:

$$Y = 0.12 + 0.005228A + 0.058B + 0.022A^2 + 0.028B^2 + 0.013 \quad (3)$$

Where A and B are the coded variables for pressure and temperature, respectively.

The Model F-value of 13.60 suggested the model was significant. There was only a 0.17% chance that a "Model F-Value" this large might occur due to noise. Values of "Prob> F" less than 0.0500 specify model terms were significant. In this case B, A^2, B^2 were substantial model terms. Values greater than 0.1000 indicate the model terms were not significant. The "Lack of Fit F-Value" of 4.07 proposed the Lack of Fit was not significant comparative to the pure error. 10.45% chance of the "Lack of Fit F-Value" might be due to noise. Non-significant lack of fit was good because the model is necessary to be fit. The Adequate Precision measured the ratio of signal to noise that desire a ratio greater than 4. Since the ratio of 10.531 was obtained, it specifies an adequate signal.

Hence, the model was suitable to navigate the design space. Figure 2 specifies that the predicted values were adjacent to the actual values. Certainly, the high value of coefficient of determination, R^2 (0.9105) and adjusted-R^2 (0.8466) confirmed enough accuracy of the polynomial reduced model. The three-dimensional (3D) response surface plot was produced to visualize the graphical description on the effect of independent variables interaction toward the response [12]. A 3D graph in Figure 3 demonstrates the effect of pressure and temperature on the extraction yield at a fixed extraction time of 60 min. There were significant interactions between variable factors and the response surface graphs as variation of the process conditions. It is certain that there is an optimal value for pressure and temperature to acquire the maximum yield of extract.

Table 2: Experimental design and results of response variable

Process variables				Yield of Extract (%)			
Run	Temperature (°C)	Pressure (psi)		Actual Value	Predicted Value		Yield of Extract (mg-extract/g-dried sample)
1	55	1500		0.076	0.095		0.8
2	44	2000		0.14	0.12		1.4
3	65	2000		0.13	0.11		1.3
4	40	3250		0.14	0.16		1.4
5	55	3250		0.11	0.12		1.1
6	55	3250		0.12	0.12		1.2
7	55	3250		0.12	0.12		1.1
8	55	3250		0.15	0.12		1.5
9	55	3250		0.11	0.12		1.2
10	70	3250		0.15	0.17		1.5
11	44	4500		0.23	0.21		2.3
12	65	4500		0.27	0.25		2.7
13	55	5000		0.24	0.26		2.4

Table 2: Analysis of variance (ANOVA) and coefficients of the final reduced regression equation

Source	df		Yield of Extract in percentage (%)		
		Coefficient estimate	Sum of squares	F Value	*p*-value
Model	5		0.036	13.6	0.0017
A	1	5.23E-03	0.0002187	0.41	0.5416
B	1	0.058	0.027	51.67	0.0002
A^2	1	0.022	0.003241	6.1	0.0429
B^2	1	0.028	0.00547	10.29	0.0149
AB	1	0.013	0.0006554	1.23	0.3034
Residual	7		0.003719		
Lack of Fit	3		0.002801	4.07	0.1045
Pure Error	4		0.0009186		
Corrected Total	12		0.04		
Total					
	S.D.	0.023		R^2	0.9105
	Mean	0.15		Adjusted R^2	0.8466
	C.V.	15.1		Adequate Precision	10.5313

The regression equation achieved was applied to maximize the yield of extract from *Sargassum sp.* as the preferred optimal circumstance of the extraction method. The optimum conditions for the anticipated area were predicted as 4500 psi and 65°C and the acquired value were compared with the predicted result. The mean value of 2.7mg-extract/g-dried sample acquired from the actual experiment was very adjacent to the predicted value (2.5mg-extract/g-dried sample) thus, the outcome specifies the adequacy and validity of the reduced model to imitate the anticipated optimization.

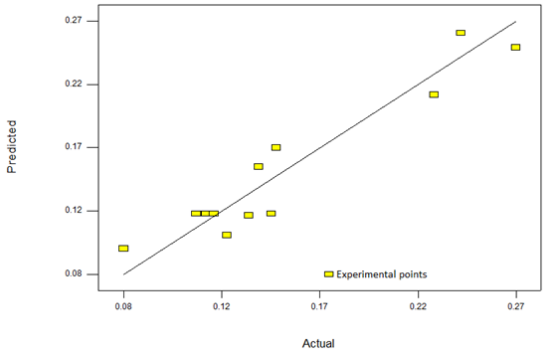

Fig. 2: Plot of predicted crude extraction yield related with experimental values

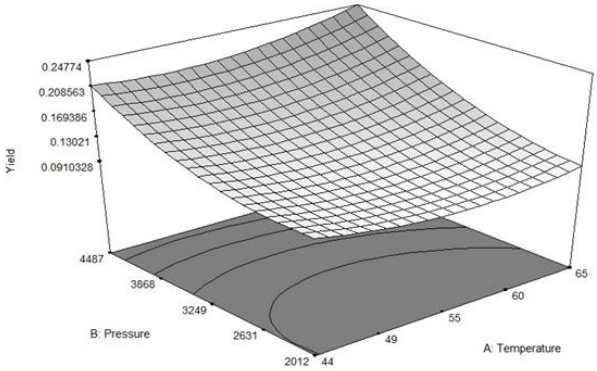

Fig. 3: Response surface plots for extraction yield (%) as a function of pressure (psi) and temperature (°C)

3.3. GCMS Analysis

The GCMS data of the identified chemical components present in *Sargassum sp.* is summarized in Table 4 according to their order of elution from a HP-5MS column. Fourteen compounds were identified in *Sargassum sp.* extract obtained from SC-CO_2 extraction based on NIST library. Major compounds found were fucosterol, cholesterol, pentadecanoic acid 14-methyl ester, ergost-22-en-3-one (ergosterol) and 9-Octadecenoic acid, methyl esther (methyl 9-octadecenoate) while the minor compounds presented were 27-norergosta-5, 22-dien-3-ol (occelasterol), phytol, 5,8,11,14-eicosatetraenoic acid, ethyl esther (arachidonic acid methyl ester) and 9-Hexadecenoic acid, methyl ester (methyl palmitoleate). The findings were supported by Thirunavukkarasu [24] and Conde et al. [15] who studied the chemical compositions of *Sargassum wightii* and *Sargassum muticum* (similar genus but different species from *Sargassum sp.*) and reported that the main constituents in the brown seaweeds resulted from GCMS were sterols, palmitic acid (hexadecanoic acid) and arachidonic acid which contains bioactive compounds and microbial activities [15, 24].

Table 4: List of fragmented compounds for the *Sargassum sp.* by GCMS

Peak Name	Molecular Weight	Formula	Retention Time	Peak Area
9-Hexadecenoic acid, methyl ester	268.4348	$C_{17}H_{32}O_2$	18.657	27255313
Pentadecanoic acid, 14-methyl ester	270.4507	$C_{17}H_{34}O_2$	19.015	17706 3023
Arachidonic acid	304.4669	$C_{20}H_{32}O_2$	21.036	16450615
10,13-Octadecadienoic acid, methyl ester	294.479	C19H 34O_2	22.06	28753500
9-Octadecenoic acid, methyl ester	296.4879	$C_{19}H_{36}O_2$	22.15	10313 8930

Phytol	296.54	$C_{20}H_{40}O$	22.375	74350138
Octadecanoic acid, methyl ester	294.4721	$C_{19}H_{34}O_2$	22.606	25197337
5,8,11,14-Eicosatetraenoic acid, ethyl ester	332.52	$C_{22}H_{36}O_2$	24.891	47997976
17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta(a)phenanthren-3-ol	414.718	$C_{29}H_{50}O$	33.284	379487876
Ergost-22-en-3-one	398.664	$C_{28}H_{46}O$	34.159	115643966
27-Norergosta-5, 22-dien-3-ol	384.648	$C_{27}H_{44}O$	35.577	76446264
Cholesterol	386.664	$C_{27}H_{46}O$	36.209	372928434
Fucosterol	412.702	$C_{29}H_{48}O$	37.751	526061876

4. Conclusion

The SC-CO_2 extraction of *Sargassum sp.* had been accomplished under several operating conditions. The approach of RSM via CCD was effectively functional for the optimization of the SC-CO_2 extraction process. The effects of pressure and temperature on the yield of extract were studied which showed positive significant effect on the response. The empirical polynomial equation was proposed to predict the optimum operating conditions of a particular process. The highest yield of extract of 2.7 mg-extract/g-dried sample was achieved when the experiment was executed at 4500 psi and 65°C in 60 min of extraction time. Under these optimized conditions, the experimental yield of extract agreed closely with the predicted yield. Apparently, the green SC-CO_2 extraction method is a promising technology in extracting colorant from natural dyes particularly from *Sargassum sp.* Therefore, *Sargassum sp*. can be suggested as a potential source of natural dyes with additional advantage of its beneficial bioactive compounds and antimicrobial properties for food and textile coloration.

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