Scientific Bulletin. Series F. Biotechnologies, Vol. XX, 2016
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EFFECT OF EXTRACTION VARIABLES ON THE OMEGA-3 EICOSAPENTAENOIC ACID (EPA) CONTENT OF (*Nannochloropsis oculata*) MICROALGA OIL

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Abstract

Microalgae are a recognized source of fatty acids and fatty acid-based lipids of potential interest in preparation of functional health products. Unlike terrestrial crops, these photoautotrophic microorganisms can directly produce polyunsaturated fatty acids (PUFA) and, although microalgae are not suitable for direct human consumption, their nutritional value can also be exploited if added to animal feeds. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) belongs to the omega-3 fatty acids group. In recent years, omega-3 fatty acids rich oil has attracted much attention because of its recognized beneficial effect on human health. In this study, response surface methodology was used to investigate the effect of ultrasound-assisted extraction variables including extraction temperature (25-6°C), extraction time (30-90 min.) and solvent: microalga ratio (10:1-30:1 ml:mg) on the omega-3 EPA content of N. oculata microalga. The experimental results showed that the extraction temperature and time were the significant parameters for the EPA rich oil extraction, while the solvent:microalga ratio was insignificant. The optimum oil extraction parameters for the maximum omega-3 EPA content were as follows: extraction temperature, 27.6°C; extraction time, 34.00 min. and solvent:microalga ratio, 21:3 ml:mg. Under the above predicted optimum conditions, the experimental oil yield and omega-3 EPA content were 62.8 % and 16.25%, respectively. The drying of algal biomass by freeze dryer improved the green color intensity and omega-3 EPA content.

Key words*: Omega-3 EPA, oil extraction, optimization, microalga oil, N. oculata.*

INTRODUCTION

The important health benefits are associated with omega-3 polyunsaturated fatty acids (PUFA) particularly with eicosapentaenoic (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*:3) (Gogus & Smith, 2010). Traditionally, main commercial omega-3 fatty acids source is fish. Concerns about the potential danger of contaminants such as mercury, however, often discourage people from eating fish. Another more recently recognized and serious issue is the global decline in wild-harvest fish stocks. Thus, new sources of omega-3 fatty acids must be found in order to reply this growing omega-3 rich oil demand. Microalgae, krill and genetically modified crops are considered as alternative omega-3 fatty acids rich oil sources (Ryckebosch, Bruneel, Termote-Verhalle, Goiris, Muylaert, & Foubert, 2014). Marine microalga *Nannochloropsis oculata* possesses valuable nutrients particularly omega-3 EPA for human health (Lee, Chuang, Su, & Wu, 2013). *N. oculata* contains high amount omega-3 EPA with as much as 5% of its dry biomass (Khozin-Goldberg & Boussiba, 2011). Besides, due to its high omega-3 PUFA levels and its ability to grow easily and rapidly, *N. oculata* is commonly utilized as feed to rear rotifers that are then fed to marine finfish larvae for growth transformation (Chaturvedi, Uppalapati, Alamsjah, & Fujita, 2004). Extraction is one of the fundamental processing steps used for recovering oil from microbial cell for the microalga oil industrialized production. Several methods such as acid heating, autolyze, Soxhlet's, organic solvent, supercritical fluid extraction, mechanical presses, and enzymatic oil extraction have been used to extract the algal oil from the microbial cell and plant materials (Zhou, Zhu, & Ren, 2013). Among these, ultrasound-assisted extraction (UAE) is an alternative to conventional extraction techniques with its inherent advantages (reduction of extraction time, solvent volume,

energy and better extraction efficiency). The enhancement of extraction obtained by using ultrasound is mainly attributed to the effects of acoustic cavitations produced in the solvent by the passage of an ultrasonic wave (Topuz, Gokoglu, Yerlikaya, Ucak, & Gumus, 2015).
In this study, response surface methodology was used to investigate the effect of ultrasound-assisted extraction variables ncluding extraction temperature (25-65°C), extraction time (30-90 min) and solvent: microalga ratio (10:1-30:1 ml:mg) on the omega-3 EPA content of *N. oculata* microalga.

MATERIALS AND METHODS

Materials
The microalga (*N. oculata*, CCMP525) was obtained from NCMA Bigelow lab (East Boothbay, Maine, USA). It was cultivated in 50 lt polyetilen bubble column photobioreactor at a temperature 24±1°C. The culture was enriched with f/2 medium (Guillard, 1975) that is widely used for marine algae, and composed of (mg/l): $NaNO_3$, 75; NaH_2-$PO_4.H_2O$, 5; $Na2SiO_3.9H_2O$, 30; Na_2EDTA, 4.36; $CoCl_2.6H_2O$, 0.01; $CuSO_4.5H_2O$, 0.01; $FeCl_3.6H_2O$, 3.15; $MnCl_2.4H_2O$, 0.18; $Na_2MoO_4.2H_2O$, 0.006; $ZnSO_4.7H_2O$, 0.022; Thiamine HCl, 0.1; Biotin, 0.0005; B_{12}, 0.0005. The ultra-filtrated water with salinity of 25 g/l was used for cultivation. Air was aerated with a flow rate of 250 ml/min for about 20 days continuously. The growth was monitored by counting the cells with the help of Neubauer haemocytometer. The cultured microalgal biomass was concentrated by centrifugation (4000×g, 5 min) and then freeze dried to final water activity of aw: 0.6.

Ultrasound-assisted extraction of omega-3 EPA rich microalgal oil
Hexane was selected as oil extracting solvent according to preliminary trial and study of dos Santos, Moreira, Kunigami, Aranda, and Teixeira (2015). Ultrasound-assisted extraction was performed in an ultrasonic bath. Frequency of ultrasonic bath was fixed at 250 W, 40 kHz and microalga sample was placed into a volumetric flask, made up to volume with the extracting solvent and sonicated for different times at the required temperature. After the extraction, the flask was transferred and cooled to room temperature. The algal oil extract were filtered through Whatman no:1 filter paper. Solvent was evaporated with rotary evaporator at 45ºC and algal oil rich extract was obtained.

Experimental design
In order to obtain the optimal conditions for the extraction of omega-3 EPA rich oil from *N. oculata* biomass and examine the effect of solvent:microalgae ratio, extraction temperature and time on the yield and omega-3 EPA content, a three-variable, three-level Box & Behnken Design (Box & Behnken, 1960) was applied in a response surface methodology (RSM) study by generating second-order polynomial equations (Eq. 1):

$$Y = \beta_0 + \Sigma\beta_i X_i + \beta_{ii} X^2 i + \Sigma\beta_{ij} X_i X_j$$

Where Y represents the experimental response, β_0, β_i, β_{ii} and β_{ij} are constants and regression coefficients of the model, and X_i and X_j are uncoded values of independent variables. The experimental design variables and responses are represented in Table 1. The responses obtained from the experimental design were subjected to multiple nonlinear regressions using the software Design-Expert 9.0 (State-Ease, MN, USA) to obtain the coefficients of the second polynominal model.

Table 1. Box & Behnken's experimental design and responses

	Parameters*			Responses	
Test	X_1 (°C)	X_2 (Min.)	X_3 (ml:mg)	Oil yield (%)	Omega-3-EPA (%)
A_1	25	60	10	63.92	16.18
A_2	25	30	20	65.36	16.25
A_3	25	60	30	65.85	16.09
A_4	25	90	20	66.14	15.94
A_5	45	90	30	71.54	15.08
A_6	45	30	30	68.65	15.77
A_7	45	60	20	69.42	15.63
A_8	45	60	20	69.61	15.58
A_9	45	30	10	66.43	15.86
A_{10}	45	60	20	71.16	15.47
A_{11}	45	90	10	71.45	15.32
A_{12}	65	30	20	73.18	15.02
A_{13}	65	90	20	75.60	14.11
A_{14}	65	60	30	74.15	14.45
A_{15}	65	60	10	73.67	14.58

*X_1:Extraction temperature, X_2: Extraction time, X_3: Solvent::microalgae ratio

Analyses

Oil content and oil yield analyses
Total lipid content analysis was performed according to method of Bligh and Dyer (1959). Lipid yield was calculated as below:

LY (%): (L_1 x 100)/L_0)

Where LY represents the lipid yield, L_0 is total lipid content of algal and L_1 is lipid content of algae extracts.

Fatty acid composition analysis
Methyl esters were prepared by transmethylation using 2 M KOH in methanol and n-hexane, according to the method of (Özogul and Özogul, 2007). The fatty acid composition was analyzed by a gas chromatography device (Clarus 500 Perkin-Elmer, USA) equipped with a flame ionization detector and a fused silica capillary SGE column (30 m x 0.32 mm ID x 0.25 µm BP20 0.25 UM, USA). The fatty acid composition analyses were performed in triplicate and the results were given in chromatography area % as mean values.

Optical microscopy
Optical microscopy images were taken from surface of algae extract residue using stereo microscope.

Statistical analysis
Data were subjected to multiple nonlinear regression using the software Design-Expert 9.0 (State-Ease, Inc., Minneapolis, USA), to obtain the coefficients of the second polynomial model.

RESULTS AND DISCUSSIONS

The effect of variables on the extraction of lipids from N. oculata biomass
Total lipid content of *N. oculata* dried biomass was 31.07 g/100 g. Table 1 shows the experimental conditions and the results of lipid extractions according to design. The extracted total lipid yield of alga biomass ranged between 63.9 and 75.6%. Maximum lipid yield (75.6%) was determined under the experimental parameters of extraction temperature of 65 °C, extraction time of 90 min and solvent:alga ratio of 20:1 (ml:mg). The statistical analysis revealed that extraction temperature and extraction time significantly affected total lipid yield (P<0.05), while solvent:alga ratio had no effect on the lipid yield.

The effect of variables on the omega-3 EPA content of N. oculata.
The omega-3 EPA content of lipids extracted from *N. oculata* biomass ranged between 14.1 and 16.3% of algal lipid. Maximum omega-3 EPA content was found under the experimental parameters of extraction temperature of 25°C, extraction time of 30 min. and solvent:alga ratio of 20:1 (ml:mg) (Table 1). The extraction temperature and extraction time significantly affected omega-3 EPA content of algal biomass (P<0.05), while solvent:alga ratio had no effect on the EPA content of algal lipid. Fig. 1 shows the effect of extraction temperature and extraction time on the omega-3 EPA content of extracted algal lipid. Decreasing extraction temperature and time at the moderate solvent:alga ratio (20:1, ml:mg) significantly increased the omega-3 EPA content of extracted algal oil.

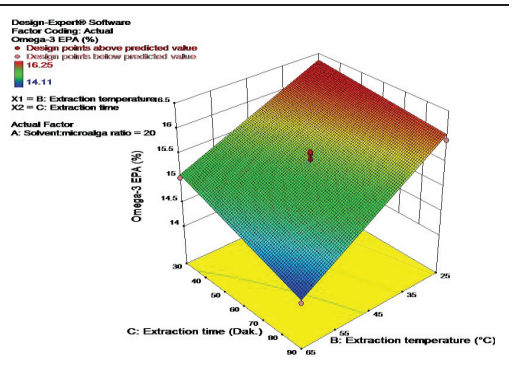

Figure 1. Effect of extraction temperature and extraction time on the omega-3 EPA content.

Fig. 2 shows the effect of extraction temperature and solvent:microalga ratio on the omega-3 EPA content of extracted algal lipid. Decreasing of extraction temperature at moderate extraction time (60 min) and solvent ratio (20:1, ml:mg) significantly increased omega-3 EPA content, whereas increasing of solvent:alga ratio did not increased during extraction (Fig. 2).

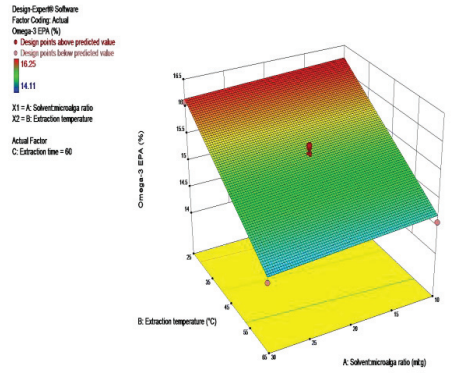

Figure 2. Effect of extraction temperature and solvent:alga ratio on the omega-3 EPA content.

Fig. 3 shows the effect of extraction time and solvent:microalga ratio on the omega-3 EPA content of extracted algal lipid. Both increasing of extraction time and solvent:alga ratio did not change the omega-3 EPA content of extracted lipid of *N. oculata* (Fig.3).

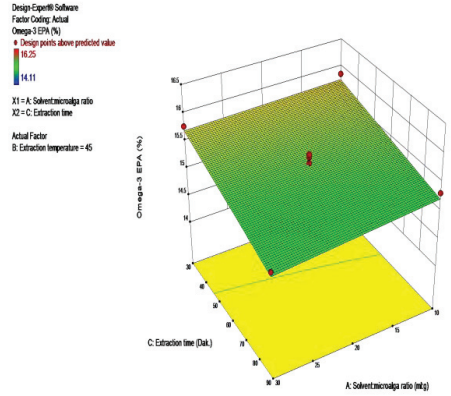

Figure 3. Effect of extraction time and solvent:alga ratio on the omega-3 EPA content.

Optical microscopy images

As clearly seen in optical microscopy images given in Fig. 4, freeze dried extraction residue kept its green color after the *extraction* (Fig. 4a). Drying of concentrated *N. oculata* biomass with heat treatment turned its green color to yellowish (Fig. 4b) and dark brown color (Fig. 4c). Drying of algal biomass in conventional oven made dried algal cell more solid and fragile with irregular shape.

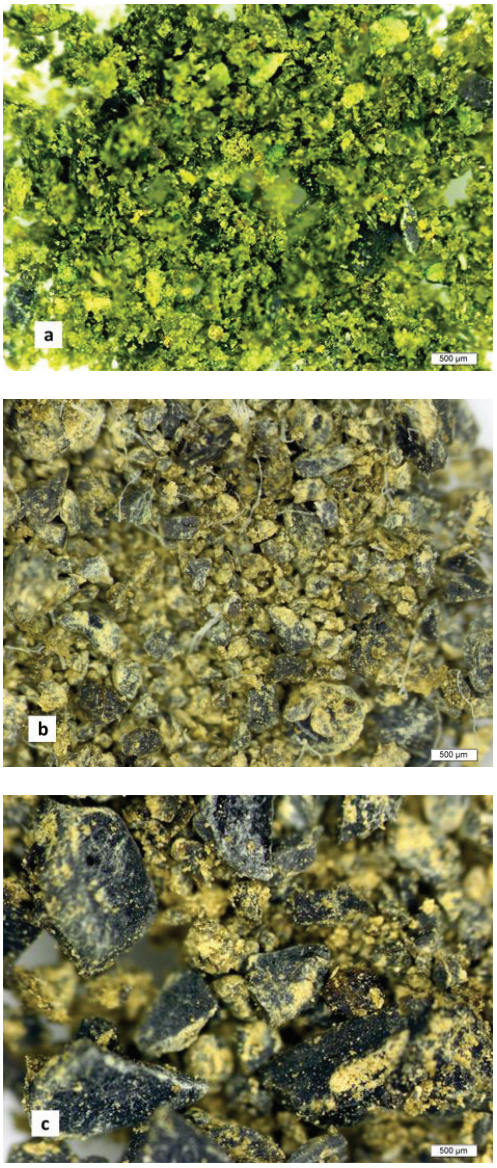

Figure 4. Optical microscopy images of dried and lipid extracted algal biomass

Optimum lipid extraction conditions

The optimum extraction conditions were determined and used for calculating the predicted values of response variables using the prediction equations derived by response surface methodology. Verification experiments performed at the predicted conditions derived

from ridge analysis of RSM demonstrated that experimental values were reasonably close to the predicted values confirming the validity and adequacy of the predicted models. The optimum conditions obtained using the model for maximum omega-3 EPA content of *N. oculata* lipid was follows: extraction temperature, 27.67°C; extraction time, 34 min and solvent alga ratio, 21.3:1 (Table 2). Under these conditions, the model predicted maximum extraction yield and omega-3 EPA content were 62.85 of 16.25%, respectively. To verify the predicted result with the practical value and compare to biomass drying methods including, conventional oven drying (ı), fluidized bed drying (ıı) and freeze drying (ııı), new extractions at optimal conditions were performed. Results of experimental extractions carried out in optimum conditions were given in Table 2. Highest omega-3 EPA content and lowest extraction yield were determined in freeze dried samples (Table 2). Both extraction yield and omega-3 EPA content of samples were significantly (P<0.05) affected biomass drying methods. Highest extraction yield of BD sample could be stemmed from fluidized bed drying methods which allows heat treatment in a short time. However, omega-3 EPA content of BD samples was at a moderate level in comparison with FD and OD samples. If both freeze and fluidized bed drying methods are important in alga biomass drying, a combination of fluidized bed drying and freeze drying treatments might be more effective than conventional oven treatment.

Table 2. Optimum conditions for omega-3 EPA rich algal lipid extractions from *N. oculata* microalga

Optimum conditions*			Responses**					
X_1 (°C)	X_2 (Min.)	X_3 (ml:mg)	Extraction yield (%)			Omega-3 EPA content (%)		
			FD	BD	OD	FD	BD	OD
27.67	34.00	21.34	63.17±0.35^{C}	68.49±0.55^{A}	65.82±0.87^{B}	17.28±0.21^{A}	16.75±0.29^{B}	16.19±0.32^{C}

*X_1:Extraction temperature, X_2: Extraction time, X_3: Solvent::microalgae ratio
**FD: Freeze dried biomass, BD: Fluidized bed dried biomass, OD: Oven dried biomass. Means±standard deviation (n:3). Means with different capital (A, B, C) in rows are significantly different (P<0.05).

CONCLUSIONS

The ultrasound-assisted extraction of omega-3 EPA rich lipid from dried *N. oculata* biomass was performed with a three-variable, three levels Box-Behnken design based on the RSM. The experimental results showed that the extraction temperature and extraction time were the major contributing factor to extraction of algal lipids from *N. oculata*.

The drying of algal biomass by freeze dryer improved the green color intensity and omega-3 EPA content.

Although the drying of biomass by fluidized bed dryer maximized the extraction yield, its omega-3 EPA content was lower than its freeze dried. Considering industrial demand for 'minimal processed' products, freeze drying and fluidized bed drying methods could be served as an effective biomass drying methods.

ACKNOWLEDGEMENTS

The authors would like to express their sincere thanks to Scientific Research Project Coordination Unit of Akdeniz University and Scientific and Technological Research Council of Turkey (TUBİTAK project code: 214O721) for the support.

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