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High cell density cultivation for docosahexanoic acid production of *Schizochytrium* sp. BCC 25505

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Abstract

Docosahexanoic acid (22:6 *n*-3; DHA) is a polyunsaturated fatty acid that is essential for functional development and maintenance of human brain and is also involved in the prevention of cardiovascular diseases, improving neural and retinal development in infants. *Schizochytrium* sp. BCC 25505, a marine protist, can produce DHA at high yield.In this study, biomass and DHA production of this marine protist were carried out to avoid glucose repression and nitrogen limitation using high cell density cultivation technique. The use of high glucose concentration resulted in a slow growth rate and low biomass concentration. By employing central composite design and by pulsing 40 $g{\cdot}L^{-1}$ glucose at a time yielded maximum biomass (77.3 $g.L^{-1}$) and DHA (18.5 $g{\cdot}L^{-1}$) productions.Theoptimal conditions for maximizing DHA production of *Schizochytrium* sp. BCC 25505 calculated using Design expert software were validated in a 5L bioreactor cultivation, yielding at 97 h a maximum concentration of biomass and docosahexanoic acid of 70.8 and 14.1 $g{\cdot}L^{-1}$ respectively. This was further scaled up in 300 L bioreactor and 115.9 $g{\cdot}L^{-1}$ biomass, 15.0 $g{\cdot}L^{-1}$ DHA at 119 h were obtained. This optimal fermentation process can provide a low cost production of DHA, which can be used in industrial scale.

Keywords: *docosahexanoic acid, Schizochytrium sp, Plackett-Burman design, Central composite design, Single cell oil.*

1. Introduction

Docosahexanoic acid (22:6 *n*-3; DHA) is a polyunsaturated fatty acid that is essential for functional development, maintenance of human brain and retina (Connor et al. 1992). Furthermore, DHA is also involved in the prevention of cardiovascular diseases and in improving neural and retinal development in infants (Das and Fams 2003; Horrocks and Yeo 1999; Nordoy et al. 2001). DHA has been added to infant formulae (Arterburn et al. 2000), food and beverage for human consumption (Ward and Singh 2005) and animal and aquaculture feed (Miller et al. 2007; Simopoulos et al. 1999a, 1999b).

A large source of this fatty acid is marine fish. Other organisms, which can synthesize DHA at high yield, include

thraustochytrid (Bajpai et al. 1991a, 1991b; Kyle 1997; Bowles et al. 1999; Singh and Ward 1996, 1997), *Crypthecodiniumcohnii* (Kyle 1994; deSwaaf et al. 1999, 2003), and *Schizochytrium* (Yokochi et al. 1998; Unagul et al. 2007; Fan et al. 2001; Bailey et al. 2003). *Schizochytrium* sp. is a marinethraustochytrid that synthesize polyunsaturated fatty acids of which DHA represents 30-40% of the total fatty acids (Yokochi et al. 1998).Although this organism is reported to produce DHA at low cost and in high production (Ward and Singh 2005), there is still a need to develop an optimal fermentation process that can provide increased biomass and DHA yield.

The present study describes the screening of significant variables using Plackett-Burman design and identification of optimal values for these factors using a response surface methodology for DHA production by *Schizochytrium* sp. BCC 25505. Individual and interactive effects as well as the optimal level of glucose, yeast extract, ammonium dihydrogenphosphate concentration in culture medium, and harvesting time were investigated. Glucose was pulsed in order to avoid glucose inhibition as well as to increase the range of nitrogen concentration used in order to have enough nitrogen for cell production. Finally, the optimized media components obtained from experimental design were confirmed in a bioreactor cultivation to validate the model and to improve process productivity.

2. Materials and methods

2.1 Microorganism strain and cultivation

Schizochytrium sp. BCC 25505, a marine microorganism, was collected from mangrove leaf in the gulf of Thailand and identified by Dr. PanidaUnagul, NationalCenter for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand. The microbe was deposited in BIOTEC Culture Collection, which is publicly accessible. *Schizochytrium*sp. BCC 25505 was maintained at 22°C by monthly sub-cultivation on GPY medium (glucose 10 $g \cdot L^{-1}$; peptone 1 $g \cdot L^{-1}$; yeast extract 1 $g \cdot L^{-1}$) supplemented with 15 $g \cdot L^{-1}$ agar (Difco, Franklin Lakes, USA) and 15 $g \cdot L^{-1}$ artificial sea salts (Sigma, St. Louis, USA).

2.2 Fermentation condition

About 10 percent (v/v) of seed culture were transferred into 50 mL of liquid medium (30 $g \cdot L^{-1}$ glucose, 5 $g \cdot L^{-1}$ yeast extract, 50 mM $MgSO_4 \cdot 7H_2O$ and 1 $mL \cdot L^{-1}$ trace element solution) in a 250 mL Erlenmeyer flask and incubated at 25 ^oC on a rotary shaker at 200 rpm for 2 days and then used as inoculum. Trace element solution consisted of 4.5 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.3 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, 0.3 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 3.0 $mg \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$, 1.0 $mg \cdot L^{-1}$ H_3BO_4, 0.1 $mg \cdot L^{-1}$ KI, 1.0 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$,0.4 $mg \cdot L^{-1}$ $NaMoO_4 \cdot 2H_2O$, and 4.5 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$. The medium used in the shake flask (for Plackett-Burman design) consisted of 100-150 $g \cdot L^{-1}$ glucose, 7.5-12.5 $g \cdot L^{-1}$ yeast extract, 0-5 $g \cdot L^{-1}$ $(NH_4)_2H_2PO_4$, 0-5 $mL \cdot L^{-1}$ vitamin mixture, 1-5 $mL \cdot L^{-1}$ Ami AmiG, 10-50 mM $MgSO_4$, 1-5 $mL \cdot L^{-1}$ trace element solution and 20-25 ^oC production temperature. The initial pH of the culture medium was 7.0. The medium used in the shake flask (for central composite design) consisted of 100-250 $g \cdot L^{-1}$ glucose (by pulsing at 40 $g \cdot L^{-1}$), 17.5 $g \cdot L^{-1}$ yeast extract, 2.5 $g \cdot L^{-1}$ $(NH_4)_2H_2PO_4$, 5 $mL \cdot L^{-1}$ vitamin mixture, 1 $mL \cdot L^{-1}$Ami AmiG (glutamic acid waste),

10 mM $MgSO_4$ and 1 mL·L^{-1} trace element solution. The initial pH of the culture medium was 7.0. The medium used in 5 Lbioreactor (B. Braun Biotech International GmbH, Melsungen, Germany) was a working volume of 4 L containing 280 g·L^{-1} glucose (by pulsing at 40 g·L^{-1}), 17.5 g·L^{-1} yeast extract, 2.5 g·L^{-1} $(NH_4)_2H_2PO_4$, 5 mL·L^{-1} vitamin mixture, 1 mL·L^{-1} Ami AmiG, 10 mM $MgSO_4$ and 1 mL·L^{-1} trace element solution. The initial pH of the culture medium was 7.0, agitation speed of 500 rpm, and aeration rate of 1 vvm. The medium used in a 300 L bioreactor was 180 L with a starting volume of 100 L. The initial pH of the culture medium was 7.0, agitation speed of 120 rpm, and aeration rate of 1 vvm.

2.3 Experimental design

A Plackett-Burman design was applied on 9 selected factors influencing biomass and DHA production to obtain primary optimal range, namely, glucose, yeast extract, temperature, vitamin solution, Ami Ami G (glutamic acid waste), $MgSO_4$, trace element solution, artificial sea salt, and ammonium dihydrogen phosphate. Each factor was tested at two levels, high (+1) and low (-1) (Table 1). An experiment was conducted in 30 runs including fold-over and 6 center points were added in order to increase the power of the test. Response from a design of 30 runs was measured in terms of biomass and DHA production (Table 2). A central composite design was applied on the 4 selected factors influencing biomass and DHA production (levels of each factor are shown in Table 3). Response from a design of 30 runs including 6 center points was measured also in terms of biomass and DHA production (Table 4). Factors influencing biomass and DHA production were analyzed based on ANOVA statistical analysis. Design Expert software (Version 7.0.b1.1, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis, and linear model building. Optimal fermentation conditions for enhanced yield of biomass and DHA were obtained by solving regression equation and also by analyzing interactions.

2.4 Biomass determination

Cells were sedimented by centrifugation at 12000 g for 10 min at 4 ºC. Sugar content in supernatantwas analyzed and cell pellet was lyophilized for assays of dry weight and fatty acid content.

2.5 Fatty acid analysis

Two mL aliquot of 4% sulfuric acid in methanol, butylatedhydroxytoluene (0.1 g·L^{-1}) (as anti-oxidant) and C17:0 (as internal standard) were added to freeze dried cell pellet (10 and 20 mg) in vial with tightly fitting Teflon-lined cap. The vial was heated at 90 ºC for 1 h. Following rapid cooling, fatty acid methyl esters produced were extracted twice with 2 mL of hexane: water (1:1). Hexane layer was collected and a few grains of sodium sulfate added to absorb any remaining water. Then the sample was injected using an auto-injector with a 1:100 split into Shimadzu GC-17A instrument (Osaka, Japan) equipped with Supelco Omegawax-250 (Supelco, Bellefonte, USA) fused silica capillary column (30 m in length, 0.25 mm internal diameter). Injector and detector temperature was 250 and 260 °C respectively. Carrier gas was helium with a linear flow of 30 cm s^{-1}. Column temperature was kept at 200 °C for 10 min and then increased to 230 °C at a rate of 10 °C min^{-1}. After 17 min at 230 °C, the column was cooled to 200 °C and a new cycle started. Calibration was performed using a mixture of fatty acid methyl ester (FAME-) standards (Sigma no

189–19) as well as DHA-FAME (Sigma). Identity of fatty acids was confirmed by GC/MS. In addition, the procedure was checked by methylation of C17:0 and DHA (Sigma) and recovery was determined against FAME (Fatty acid methyl ester) standards. Repeated methylation of residue and/or repeated hexane extraction did not increase fatty acid yield.

2.6 Sugar determination

Supernatant was centrifuged at 10,000 *g* for 10 min and filtered through a 0.22 mm filter paper. Filtrate was subjected to HPLC analysis using Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) at 60 ^{o}C, using 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL·min^{-1}, and sugars were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA, USA).

3. Results

3.1 Biomass and DHA production in *Schizochytrium* sp. BCC 25505 using Plackett-Burman design

Nine variables (glucose concentration, yeast extract concentration, temperature, vitamin solution, Ami Ami G, $MgSO_4$, trace element solution, artificial sea salt and ammonium dihydrogen phosphate) were selected in order to study their effects on biomass and DHA production by *Schizochytrium*sp. BCC 25505 using a Plackett-Burmandesign. Levels of each factors used in the design, which were assigned as high (+1), low (-1), and center points level (0), are shown in Table 1. The highest biomass production (37.38 g·L^{-1}), DHA production (9.08 g·L^{-1}) and percent DHA/TFA (37.38 %) at a cultivation temperature of 20 °C were obtained with 100 g·L^{-1} glucose, 12.5 g·L^{-1} yeast extract, 5 mL·L^{-1} vitamin, 1 mL·L^{-1} Ami AmiG, 10 mM $MgSO_4$, 1 mL·L^{-1} trace element solution, and 8 g·L^{-1} sea salt (Table 2). Highest percent DHA/biomass (24.99%) and percent TFA/biomass (70.46%) were obtained with 100 g·L^{-1} glucose, 7.5 g·L^{-1}

Table 1. Factors and levels used in Plackett-Burman design.

Variable	Variable code	High level (+1)	Low level (-1)
Glucose (g·L^{-1})	A	150	100
Yeast Extract (g·L^{-1})	B	12.5	7.5
Temperature (°C)	C	25	20
Vitamin (mL·L^{-1})	D	5	0
Ami Ami G (mL·L^{-1})	E	5	1
$MgSO_4$ (mM)	F	50	10
Trace (mL·L^{-1})	G	5	1
Artificial sea salt (g·L^{-1})	H	8	0
Ammonium dihydrogen phosphate (g·L^{-1})	J	5	0

yeast extract, 5 mL·L^{-1} vitamin, 5 mL·L^{-1} Ami AmiG, 50 mM $MgSO_4$, 1 mL·L^{-1} trace element solution, and 8 g·L^{-1} sea salt. By applying multiple regression analysis on theexperimental data, the following equation (Eq. (1)) describes biomass production:

Biomass(g·L^{-1})=24.554-0.88A +1.05B+ 0.57C+0.15D-0.06E-0.20F-0.04G -0.40H-9.80J.....(1)

where*A* = glucose concentration, *B* = yeast extract concentration, *C* = temperature, *D* = vitamin, *E* = Ami G, *F* = $MgSO_4$, *G* = trace element, *H* = artificial sea salt, *J* = ammonium dihydrogen phosphate.

The estimated coefficient of factors *A, B, C, D, E, F, G, H* and *J* were -0.88, 1.05, 0.57, 0.15, -0.06, -0.20, -0.04, -0.40 and -9.80, respectively. This equation indicated that yeast extract concentration (12.5 g·L^{-1}), temperature (25 °C) and vitamin (5 mL·L^{-1}) at high level had a positive effect on biomass production, while glucose concentration (150 g·L^{-1}), Ami G (5 mL·L^{-1}), $MgSO_4$ (50 mM), trace element (5 mL·L^{-1}), artificial sea salt (8 g·L^{-1}), and ammonium dihydrogen phosphate (5 g·L^{-1}) at high level had negative effect on biomass production. Multiple regression analysis of theexperimental data resulted in the following equation (Eq. (2)) describing DHA production:

DHA (g·L^{-1})= 3.72-0.31A+0.15B +0.10C+ 0.22D-0.25E-0.10F-0.44G-0.03H-3.20J.....(2)

where*A* = glucose concentration, *B* = yeast extract concentration, *C* = temperature, *D* = vitamin, *E* = Ami G, *F* = $MgSO_4$, *G* = trace element, *H* = artificial sea salt, *J* = ammonium dihydrogen phosphate.

The estimated coefficient of factors *A, B, C, D, E, F, G, H* and *J* was -0.31, 0.15, 0.10, 0.22, -0.25, -0.10, -0.44, -0.33, and -0.320, respectively. This equation indicated that yeast extract concentration (12.5 g·L^{-1}), temperature (25°C) and vitamin (5 mL·L^{-1}) at high level had a positive effect on DHA production, while glucose concentration (150 g·L^{-1}, Ami G (5 mL·L^{-1}), $MgSO_4$ (50 mM), trace element (5 mL·L^{-1}), artificial sea salt (8 g·L^{-1}), and ammonium dihydrogen phosphate (5 g·L^{-1}) at high level had a negative effect on biomass production.

3.2 Optimization of biomass production by *Schizochytrium* sp. BCC 25505 using central composite design.

Using a central composite design, 4 factors (glucose concentration, yeast extract concentration, ammonium dihydrogenate phosphate concentration and harvest time) were selected based on the results obtained from Plackett-Burman design for their effects on biomass and DHA production by *Schizochytrium* sp. BCC 25505 (except for harvest time, which was selected to ensure that glucose was completely consumed). Table 3 shows the levels of factors studied. Highest biomass (77.31 g·L^{-1}) and highest DHA production (18.45 g·L^{-1}) were obtained on 228 g·L^{-1} glucose, 17.56 g·L^{-1} yeast extract, 2.56 g·L^{-1}ammonium dihydrogen phosphate and harvest time at 142.68 h (standard condition 14 in Table 4), whereas the highest amount of DHA/TFA (w/w) (48.33 g·L^{-1}) was obtained with 121 g·L^{-1} glucose, 44.44 g·L^{-1} yeast extract, 2.56 g·L^{-1} ammonium dihydrogen phosphate and 142.68 h harvest time.

Analysis of variance (ANOVA) of the central composite design of biomass production by *Schizochytrium* sp. BCC 25505 is summarized in Table 5, in which models are significant with low values of "Prob>F" (0.0251). A value of "Prob>F" less than 0.0500 indicates model terms are significant. Interactions between yeast extract and ammonium dihydrogen phosphate (*BC*) concentrations and harvested time (D^2) are significant model terms with the value of "Prob> F" 0.0237 and 0.0124 respectively. "Adeq precision" measures the signal to noise ratio, with a ratio greater than 4 being desirable for model prediction. From Table 5, the ratio of 6.74 indicated an adequate signal, and therefore this model can be used to navigate the design space even though the lack of fit is significant.By applying multiple regression analysis on theexperimental data, the following equation (Eq. (3)) was found to describe biomass production:

$$\text{Biomass } (g\cdot L^{-1}) = 61.72 + 2.55A - 3.23B + 0.55C + 2.90D - 3.32AB + 2.40AC + 1.26AD - 4.64BC + 0.08BD + 1.85CD - 2.71A^2 - 3.73B^2 - 0.36C^2 - 6.85D^2 \quad (3)$$

where *A* = glucose concentration, *B* = yeast extract concentration, *C* = ammonium dihydrogen phosphate concentration, and *D* = harvest time

Table 2. Plackett-Burman design for biomass and DHA production by *Schizochytrium* sp. BCC 25505 (*TFA=Total Fatty Acid).

STD	Run	Variable									Response				
		A	B	C	D	E	F	G	H	J	Biomass ($g\cdot L^{-1}$)	DHA ($g\cdot L^{-1}$)	DHA (% w/w)	TFA* (% w/w)	% DHA/ TFA
1	8	1	1	-1	1	1	1	-1	-1	-1	32.72	6.48	19.79	56.36	35.12
2	3	-1	1	1	-1	1	1	1	-1	-1	35.65	6.25	17.54	53.94	32.52
3	15	1	-1	1	1	-1	1	1	1	-1	34.78	6.66	19.15	64.13	29.87
4	6	-1	1	-1	1	1	-1	1	1	1	15.58	0.61	3.89	15.41	25.24
5	14	-1	-1	1	-1	1	1	-1	1	1	13.82	0.33	2.38	22.53	10.55
6	11	-1	-1	-1	1	-1	1	1	-1	1	14.37	0.96	6.65	24.46	27.18
7	1	1	-1	-1	-1	1	-1	1	1	-1	29.48	3.86	13.09	54.37	24.08
8	10	1	1	-1	-1	-1	1	-1	1	1	11.67	0.36	3.09	12.17	25.38
9	5	1	1	1	-1	-1	-1	1	-1	1	17.22	0.34	1.99	15.8	12.62
10	12	-1	1	1	1	-1	-1	-1	1	-1	37.38	9.08	24.29	63.77	38.09
11	7	1	-1	1	1	1	-1	-1	-1	1	14.37	0.64	4.45	21.26	20.93
12	13	-1	-1	-1	-1	-1	-1	-1	-1	-1	33.92	7.84	23.12	68.34	33.84
13	9	0	0	0	0	0	0	0	0	0	21.38	3.58	16.75	46.09	36.35
14	4	0	0	0	0	0	0	0	0	0	22.26	3.63	16.3	44.95	36.26
15	2	0	0	0	0	0	0	0	0	0	19.16	2.75	14.33	38.99	36.75
16	27	-1	-1	1	-1	-1	-1	1	1	1	13.35	0.33	2.46	20.53	11.97
17	30	1	-1	-1	1	-1	-1	-1	1	1	12.32	0.17	1.37	13.25	10.35
18	19	-1	1	-1	-1	1	-1	-1	-1	1	19.47	0.60	3.08	21.53	14.29
19	28	1	-1	1	-1	-1	1	-1	-1	-1	33.46	7.26	21.71	62.73	34.62
20	25	1	1	-1	1	-1	-1	1	-1	-1	34.23	6.70	22.96	65.42	35.1

Table 2. Plackett-Burman design for biomass and DHA production by *Schizochytrium* sp. BCC 25505 (*TFA=Total Fatty Acid) (continued).

STD	Run	Variable									Response				
		A	B	C	D	E	F	G	H	J	Biomass ($g \cdot L^{-1}$)	DHA ($g \cdot L^{-1}$)	DHA (% w/w)	TFA* (% w/w)	% DHA/ TFA
21	24	1	1	1	-1	1	-1	-1	1	-1	35.26	7.49	21.25	60.18	35.32
22	22	-1	1	1	1	-1	1	-1	-1	1	16.32	1.05	6.42	33.01	19.45
23	17	-1	-1	1	1	1	-1	1	-1	-1	34.51	5.85	16.89	69.47	24.31
24	21	-1	-1	-1	1	1	1	-1	1	-1	34.47	8.61	24.99	70.46	35.46
25	23	1	-1	-1	-1	1	1	1	-1	1	13.22	0.40	2.99	18.93	15.81
26	18	-1	1	-1	-1	-1	1	1	1	-1	36.34	6.94	19.1	60.2	31.74
27	26	1	1	1	1	1	1	1	1	1	15.39	0.54	3.52	16.1	21.84
28	29	0	0	0	0	0	0	0	0	0	23.26	2.87	12.35	36.6	33.75
29	20	0	0	0	0	0	0	0	0	0	20.16	2.93	14.51	41.04	35.35
30	16	0	0	0	0	0	0	0	0	0	21.73	3.71	17.09	47.32	36.13

Table 3. Factors and levels used in central composite design.

Variable	-α	-1	0	+1	+α
Glucose concentration ($g \cdot L^{-1}$)	100.00	121.97	175.00	228.03	250.00
Yeast extract concentration ($g \cdot L^{-1}$)	12	17.56	31.00	44.44	50.00
Ammonium dihydrogen phosphate concentration ($g \cdot L^{-1}$)	0	0.44	1.50	2.56	3.00
Harvest time (h)	100.00	107.32	125.00	142.68	150.00

The estimated coefficient of variable *A, B, C, D, AB, AC, AD, BC, BD, CD,* A^2, B^2, C^2 and D^2 was 2.55, -3.23, 0.55, 2.90, -3.32, 2.40, 1.26, -4.64, 0.08, 1.85, -2.71, -3.73, 0.36, and -6.85, respectively. Three dimensional surface responses were plotted to illustrate the relationships among responses and variables. Statistical analysis indicated that the interaction between yeast extract and ammonium dihydrogen phosphate concentrations has a more significant effect on biomass production than the individual effects of glucose, yeast extract, ammonium dihydrogen phosphate concentrations and harvest time (Table 5). When response (biomass production) was plotted as functions of glucose and yeast extract concentrations, the effect of the interaction between yeast extract and ammonium dihydrogen phosphate was clearly shown (Figure 1). Increases inglucose and yeast extract concentrations in culture medium could not maximize biomass production in the presence of ammonium dihydrogen phosphate as a result of this interaction. At a high level of ammonium dihydrogen phosphate (2.56 $g \cdot L^{-1}$), maximum biomass production obtained was lower than that at a low level (0.44 $g \cdot L^{-1}$) (Figure 1). The maximum response value of biomass

(73.62 g·L^{-1}) was derived using Design expertsoftware. Optimum conditions for maximizing biomass production of *Schizochytrium* sp. BCC 25505 were calculated by setting the partial derivatives of Eq.3 to zero with respect to the corresponding variables, yielding 228.03 g·L^{-1} glucose, 17.57 g·L^{-1} yeast extract, 2.56 g·L^{-1} ammonium dihydrogen phosphate and harvest time of 132.51 h.

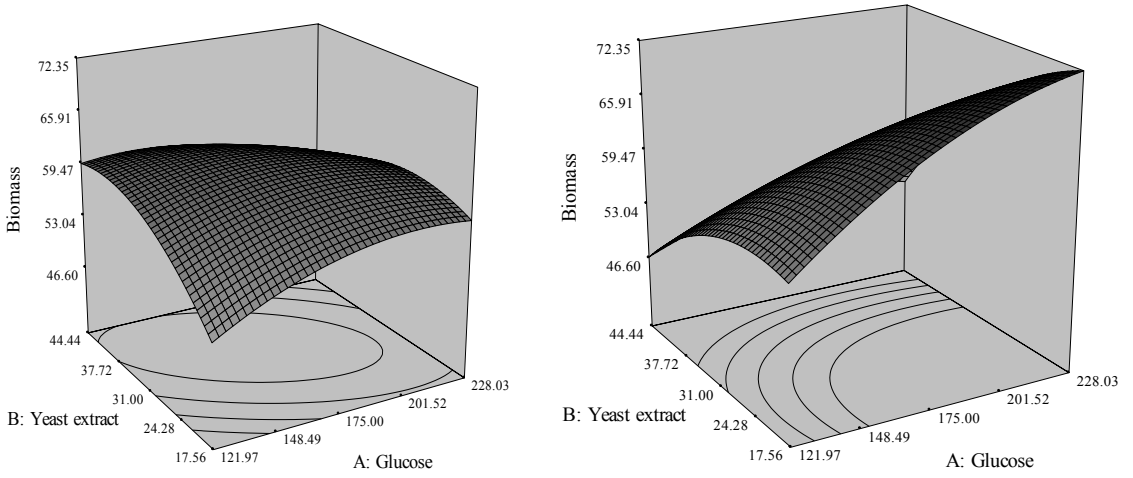


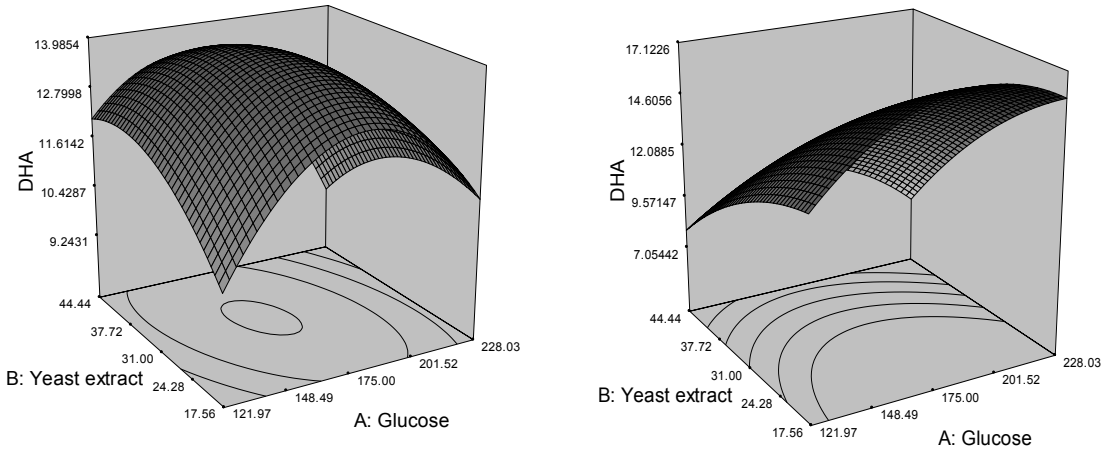
Figure 1. Three-dimensional and contour plots of biomass vs. glucose and yeast extract concentration at high (a) and low (b) level of ammonium dihydrogen phosphate.

3.3 Optimization of DHA production by *Schizochytrium* sp. BCC 25505 using central composite design.

Results of analysis of variance (ANOVA) of the central composite design of DHA production by *Schizochytrium*sp. BCC 25505 are summarized in Table 6, in which models are shown to be significant with low values of "Prob> F" (0.0025). The concentration of yeast extract (*B*) and interaction between yeast extract and ammonium dihydrogen phosphate (*BC*) are significant model terms for DHA production with value of "Prob> F" 0.0018, 0.0036 and 0.0027, respectively. The signal to noise ratio of 8.691 indicated that this model could be used to navigate the design space. By applying multiple regression analysis on theexperimental data, the following equation (Eq. (4)) was found to describe DHA production:

$$\text{DHA (g·L}^{-1}) = 14.12-0.01A-1.74B+0.18C+073D-0.91AB+0.50AC+0.33AD-1.77BC+0.23BD+0.83CD-2.41A^2-0.91B^2+0.02C^2-1.39D^2 \quad (4)$$

where*A* = glucose concentration, *B* = yeast extract concentration, *C* = ammonium dihydrogen phosphate concentration, and *D* = harvest time.



(a) (b)

Figure 2. Three-dimensional and contour plots of DHA vs. glucose and yeast extract concentration at high (a) and low (b) level of ammonium dihydrogen phosphate.

The estimated coefficient of variable *A, B, C, D, AB, AC, AD, BC, BD, CD, A2, B2, C2* and D^2 was 14.12, -0.01, -1.74, 0.18, 0.73, -0.91, 0.50, 0.33, -1.77, 0.23, 0.83, -2.41, 0.91, 0.02, and -1.39, respectively (Table 6). Similar to biomass production analysis, three dimensional surface responses were plotted to illustrate the relationships among responses and variables. Interaction between yeast extract and ammonium dihydrogen phosphate had a more significant effect on DHA production than the individual effects of glucose, ammonium dihydrogen phosphate and harvest time (Table 6). At high level of ammonium dihydrogen phosphate (2.56 $g \cdot L^{-1}$) (Figure 2a), maximum DHA production obtained was lower than that at low level (0.44 $g \cdot L^{-1}$) (Figure 2b). Increase of glucose and yeast extract concentrations in culture medium did not maximize DHA production in the presence of ammonium dihydrogen phosphate as a result of the interaction between yeast extract and ammonium dihydrogen phosphate. Derived maximum response value of DHA was 17.49 $g \cdot L^{-1}$ (Design expertsoftware). Optimum conditions for maximizing DHA production of *Schizochytrium* sp. BCC 25505 biomass (calculated in a similar manner as for biomass) were 192.19 $g \cdot L^{-1}$ glucose, 17.57 $g \cdot L^{-1}$ yeast extract, 2.56 $g \cdot L^{-1}$ ammonium dihydrogen phosphate and 134.12 h harvest time.

3.4 Biomass and DHA production in a 5-L bioreactor.

The optimal media conditions obtained from a central composite design were applied in the production of DHA (intracellular polyunsaturated fatty acid) by *Schizochytrium* sp. BCC 25505 in a 5-L bioreactor. Although batch cultivation is the most widely used strategy for DHA production in microalgae, however, there is a problem of substrate inhibition when using high initial substrate concentrations (Yan et al. 2013). Time pulsing of substrate (glucose) was adopted to overcome this problem. Time profiles of growth and DHA production of *Schizochytrium* sp. in the 5-L bioreactor showed a specific growth rate (μ) of *Schizochytrium* sp. BCC 25505 of

0.0597 h^{-1} (calculated from semi-log plot), biomass yield (Y_{XS}) 0.25 $g \cdot g_{glucose}^{-1}$, total fatty acid production rate (q_{TFA}) 3.42 $g \cdot L^{-1} \cdot day^{-1}$ and DHA production rate (q_P) 2.27 $g \cdot L^{-1} \cdot day^{-1}$ (Figure 3). Maximum concentration of biomass and DHA obtained at 97 h was 70.79 and 14.09 $g \cdot L^{-1}$ respectively, which was slightly lower than the values predicted from the central composite design.

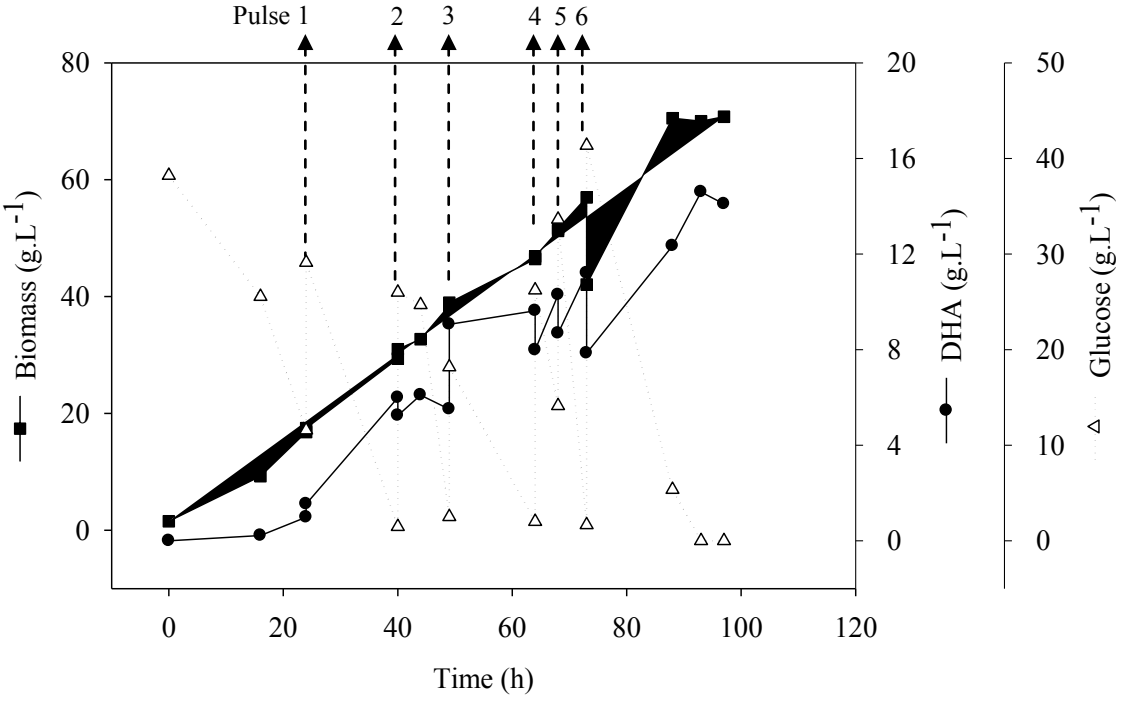


Figure 3. Time profile of biomass and DHA production by *Schizochytrium* sp. BCC25505 in a 5-L bioreactor.

Table 4. Central composite design for biomass and DHA production by *Schizochytrium* sp. BCC 25505.

Std	Run	A: Glucose	B: Yeast extract	C: Ammonium dihydrogen phosphate	D: Harvest time	Biomass ($g \cdot L^{-1}$)	DHA ($g \cdot L^{-1}$)	DHA/ TFA* (%w/w)
1	5	-1	-1	-1	-1	47.96	10.87	36.95
2	19	+1	-1	-1	-1	46.33	10.48	37.18
3	22	-1	+1	-1	-1	50.14	9.95	45.45
4	30	+1	+1	-1	-1	45.09	7.83	46.09
5	1	-1	-1	+1	-1	46.35	11.22	44.08
6	20	+1	-1	+1	-1	52.98	10.33	41.84
7	28	-1	+1	+1	-1	36.40	4.79	46.71

Table 4. Central composite design for biomass and DHA production by *Schizochytrium* sp. BCC 25505 (continued).

Std	Run	A: Glucose	B: Yeast extract	C: Ammonium dihydrogen phosphate	D: Harvest time	Biomass ($g \cdot L^{-1}$)	DHA ($g \cdot L^{-1}$)	DHA/ TFA* (%w/w)
8	26	+1	+1	+1	-1	32.82	4.39	47.03
9	21	-1	-1	-1	+1	50.33	8.62	31.63
10	16	+1	-1	-1	+1	47.00	8.29	30.83
11	11	-1	+1	-1	+1	53.85	11.41	47.26
12	2	+1	+1	-1	+1	51.14	9.15	44.27
13	10	-1	-1	+1	+1	45.92	10.67	43.61
14	7	+1	-1	+1	+1	77.31	18.45	39.05
15	13	-1	+1	+1	+1	48.24	8.43	48.33
16	18	+1	+1	+1	+1	39.47	4.78	44.35
17	8	-α	0	0	0	45.23	8.88	46.12
18	9	+α	0	0	0	72.08	10.30	43.78
19	25	0	-α	0	0	59.32	14.91	34.55
20	6	0	+α	0	0	53.92	10.25	46.87
21	4	0	0	-α	0	56.53	11.89	35.65
22	23	0	0	+α	0	73.07	17.00	44.49
23	17	0	0	0	-α	49.40	10.02	44.48
24	3	0	0	0	+α	51.33	13.24	45.52
25	14	0	0	0	0	61.63	15.01	40.67
26	12	0	0	0	0	58.46	13.48	45.36
27	29	0	0	0	0	59.87	14.00	43.89
28	15	0	0	0	0	61.66	14.97	43.99
29	27	0	0	0	0	64.17	15.58	44.30
30	24	0	0	0	0	55.14	10.52	42.35

***TFA=Total Fatty Acid**

Table 5. Analysis of variance (ANOVA) of central composite design for biomass production by *Schizochytrium* sp. BCC 25505.

Source	Coefficient estimate	Sum of squares	df	Mean square	F -Value	Probability (P) >F
Model		2197.95	14	1567	2.89	0.0251
Intercept	61.72					
A-Glucose	2.55	129.70	1	129.70	2.39	0.1432
B-Yeast extract	-3.23	209.14	1	209.14	3.85	0.0686
C-Ammonium dihydrogen phosphate	0.55	6.08	1	6.08	0.11	0.7427
D-Harvest time	2.90	167.71	1	167.71	3.09	0.0994
AB	-3.32	176.71	1	176.71	3.25	0.0915
AC	2.40	92.16	1	92.16	1.70	0.2125
AD	1.26	25.55	1	25.55	0.47	0.5034
BC	-4.64	344.29	1	344.29	6.34	0.0237
D	0.08	0.11	1	0.11	0.002	0.9652
CD	1.85	54.71	1	54.71	1.01	0.3316
A^2	-2.71	68.48	1	68.48	1.26	0.2793
B^2	-3.37	129.53	1	129.53	2.38	0.1435
C^2	0.36	1.22	1	1.22	0.02	0.8827
D^2	-6.85	438.20	1	438.20	8.063	0.0124
Lack of fit		766.56	10	76.66	7.88	0.0172
Std. Dev.	7.37	R-squared			0.73	
Mean	53.11	Adj R-squared			0.48	
C.V. %	13.88	Pred R-squared			-0.48	
PRESS	4454.37	Adeq precision			6.74	

3.5 Biomass and DHA production in a 300-L bioreactor.

Time profile of growth and DHA production of *Schizochytrium* sp. BCC 25505 in the 300 L bioreactor is shown in Figure 4. Maximum concentration of biomass and DHA obtained at 119 h was 115.9 and 15.0 $g \cdot L^{-1}$respectively.The value obtained from the 300-L bioreactor was slightly higher than that obtained from the cultivation of the microorganism in the 5-L bioreactor because of better controlled of physical parameters.

Table 6. Analysis of variance (ANOVA) of central composite design for DHA production by *Schizochytrium* sp. BCC 25505.

Source	Coefficient estimate	Sum of squares	df	Mean square	F -Value	Probability
Model		280.27	14	20.02	4.74	0.0025
Intercept	14.12					
A-Glucose	-0.01	0.003	1	0.003	0.001	0.9788
B-Yeast extract	-1.74	60.54	1	60.54	14.33	0.0018
C-Ammonium dihydrogen phosphate	0.18	0.67	1	0.67	0.16	0.6964
D-Harvest time	0.73	10.52	1	10.52	2.49	0.1354
AB	-0.91	13.36	1	13.36	3.16	0.0955
AC	0.50	3.94	1	3.94	0.93	0.3492
AD	0.33	1.78	1	1.78	0.42	0.5261
BC	-1.77	50.33	1	50.33	11.92	0.0036
BD	0.23	0.85	1	0.85	0.20	0.6607
CD	0.83	11.0	1	10.99	2.60	0.1276
A^2	-2.41	54.16	1	54.16	12.82	0.0027
B^2	-0.91	7.79	1	7.79	1.85	0.1945
C^2	0.01	0.003	1	0.003	0.001	0.9782
D^2	-1.39	18.02	1	18.02	4.27	0.0566
Lack of Fit		46.55	10	4.66	1.39	0.3780
Std. Dev.	2.06			R-squared		0.8156
Mean	10.99			Adj R-squared		0.6436
C.V. %	18.70			Pred R-squared		0.0574
PRESS	323.91			Adeq precision		8.6912

4. Discussion

As ammonium dihydrogen phosphate had the highest negative estimated coefficient value obtained from Plackett -Burmann design, this factor had the negative effect on both biomass and DHA production, resulting in lower amounts of biomass and DHA production at high level of ammonium dihydrogen phosphate used (Eq. 1 and 2). This might be due to the low pH (below 4.0) in culture medium (flask) when ammonium dihydrogen phosphate was used as the sole nitrogen source and pH decreased after growing in the log phase without pH controlled. The well controlled bioreactor must be studied to avoid this effect. Furthermore, adjusting the initial pH and using buffers are suggested. At neutral initial pH, *Schizochytrium* sp.S31

accumulates lipids of up to 40% of biomass with 13% (w/w) DHA of lipid content (Wu et al. 2005; Zhu et al. 2008; Unagul et al. 2005). Glucose had the second highest negative effect from ammonium dihydrogen phosphate on biomass and this due to high initial glucose was used in Plackett-Burman design and glucose pulsed-experiment was performed in central composite design to avoid glucose inhibition.Harvest time was selected as an additional parameter in central composite design to ensure glucose was completely consumed because glucose was pulsed to obtained high cell density and production of DHA by *Schizochytrium* sp. BCC 25505 in glucose non-repression conditon. The cultivation process of *Schizochytrium* as an industrial cell factory for DHA production includes two stage, cell growth or biomass production and lipid accumulation phases. The early stage of cell growth by nitrogen supplied and cell enlargement for lipid accumulation phase by carbon concentration controlled were necessary for DHA production (Kim et al. 2013, Qu et al. 2013). Pulsing experiment in central composite design clearly demonstrated growth and lipid accumulation by nitrogen and carbon concentration control.Yeast extract had the highest positive effect on both biomass and DHA production and it served as nitrogen sources for growth of *Schizochytrium* sp. BCC 25505 at the biomass accumulation stage.

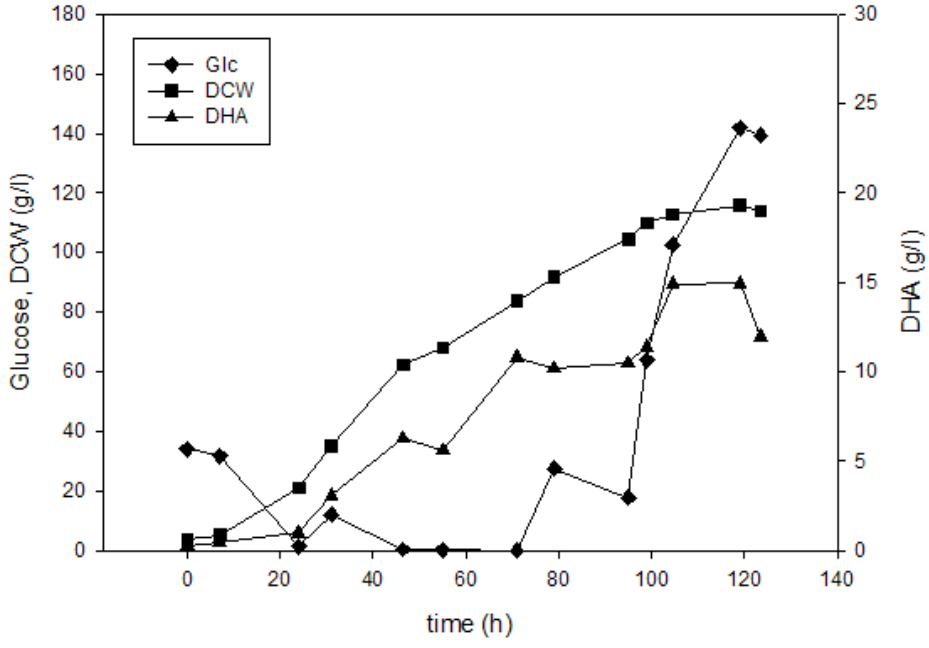


Figure 4. Time profile of biomass and DHA production by *Schizochytrium* sp. BCC25505 in a 300-L bioreactor.

The phenomenon of lipid accumulation in yeast is triggered by cell exhausting nitrogen from the culture medium, but glucose continues to be assimilated (Ratledge 2002). Moreover, the amount of carbon in relation to the nitrogen source is also important. A high C/N ratio is generally preferred for lipid accumulation

(Singh and Ward 1997). Similarly, this study indicated that increasing yeast extract concentration does not improve DHA yield (data obtained from central composite design). In order to achieve lipid accumulation in microorganisms, the culture medium needs to be formulated such that the supply of nitrogen becomes exhausted (at which cell proliferation ceases) but the carbon feed stock (usually glucose) continues to be present (Ratledge and Wynn 2002). Thus, the maximum nitrogen concentration producing the highest biomass production and lipid (DHA) accumulation in *Schizochytrium* sp. BCC 25505 was determined in this study.

High production of biomass and DHA by *Schizochytrium* sp. BCC 25505 provides an alternative source of DHA from Single Cell Oil (SCO). Cultivation was conducted at high sugar concentration and in the absence of NaCl, a major cause of fermentor corrosion (Zhang et al. 2011). Glucose inhibition and nitrogen limitation (Yan et al. 2013) in DHA production by *Schizochytrium*sp. BCC 25505 were overcome by pulsing glucose at low concentration and by increasing the range of nitrogen concentration (pulsing experiment in central composite design). Media conditions for optimizing biomass and DHA production obtained from a central composite design were successfully applied to production in bioreactors.

5. Conclusion

Pulsed-batch process was successfully developed for DHA production by *Schizochytrium*sp. BCC 25505 based on the optimization using Plackett-Burman and central composite designs. The production of DHA and biomass in 5 and 300 L bioreactor represented the industrial production of the oleaginous cell factory used in food and feed applications. This high-cell density may lead to industrial production of DHA by *Schizochytrium*sp. BCC 25505 in the near future.

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7. References

(1) Arterburn LM, Boswell KD, Henwood SM, Kyle DJ. A developmental safety study in rats using DHA- and ARA-rich single cell oils. Food ChemToxicol. 2000; 38:763-771.

(2) Bailey RB, DiMasi D, Hansen JMM, Mirrasoul PJ, Ruecker CM, Veeder III GT. Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermenters. United States Patent 6,607,900. 2003.

(3) Bajpai P, Bajpai P, Ward OP. Production of docosahexanoic acid (DHA) by *Thraustochytriumaureum*. ApplMicrobiolBiotechnol. 1991a; 35:706-10.

(4) Bajpai P, Bajpai P, Ward OP. Optimisation of production of docosahexanoic acid (DHA) by *Thraustochytriumaureum*ATCC 34304. J Am Oil Chem Soc. 1991b; 68:509-14.

(5) Bowles RD, Hunt AE, Bremer GB, Duchars MG, Eaton RA. Longchain n-3 polyunsaturated fatty acid production by members of the marine protistan group the *thraustochytrids*: screening of isolates and optimization of docosahexanoic acid production. J Biotechnol. 1999;70:193-202.

(6) Connor WE, Neuringer M, Reisbick S. Essential fatty acid: the importance of n-3 fatty acids in the retina and brain. Nutr Rev. 1992;50:21-29

(7) Das UN, Fams MD. Long-chain polyunsaturated fatty acids in the growth and development of the brain and memory. Nutr. 2003;19:62-65.

(8) deSwaaf ME, deRijk TC, Eggink G, Sijtsma L. Optimisation of docosahexanoic acid production in batch cultivations by *Crypthecodiniumcohnii*. J Biotechnol. 1999;70: 185-92.

(9) deSwaaf ME, Pronk JT, Sijtsma L. (2003) Fed-batch culture of the docosahexanoic-acid-producing marine alga *Crypthecodiniumcohnii* on ethanol. ApplMicrobiolBiotechnol. 2003;61:40–43.

(10) Fan KW, Chen F, Jones EBG, Vrijmoed LLP. Eicosapentaenoic and docosahexanoic acids production by and okara-utilizing potential of *thraustochytrids*. J IndMicrobiolBiotechnol. 2001;27: 199-202.

(11) Horrocks LA, Yeo YK. Health benefits of docosahexanoic acid (DHA). Pharmacol Res. 1999;40: 211-225.

(12) Kim K,Kim EJ, Ryu BG, Park S, Yang YC. A novel fed-batch process based on the biology of *Aurantiochytrium* sp.KRS101 for the production of biodiesel and docosahexaenoic acid. Bioresou Technol. 2013; 135: 269-274.

(13) Kyle DJ. Microbial oil mixtures and uses thereof. United States Patent 5,374,657. 1994.

(14) Kyle DJ. Arachidonic acid and methods for the production and use thereof. United States Patent 5,658,767. 1997.

(15) Miller MR, Nichols PD, Carter CG. Replacement of fish oil with thraustochytrid*Schizochytrium*sp. L oil in Atlantic salmon parr (*Salmosalar* L) diets. CompBiochem Physiol. 2007;148:382-392.

(16) Nordoy AR, Marchioli R, Amesen H, Videbaek J. n-3 polyunsaturated fatty acids and cardiovascular diseases. Lipids. 2001;36:127-129.

(17) Qu L, Ren LJ, Sun GN, Ji XJ, Nie ZK, Huang H. Batch, fed-batch and repeated fed-batch fermentation processes of the marine thraustochytrid*Schizochytrium* sp. for producing docosahexaenoic acid. Bioprocbiosysteng. 2013; 1-8.

(18) Ratledge C. Regulation of lipid accumulation in oleaginous micro-organisms. BiochemSoc Trans. 2002;30:1047-1050.

(19) Ratledge C, Wynn JP. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. AdvApplMicrobiol. 2002;51:1-5.

(20) Simopoulos AP, Leaf A, Salem JrN. Essentiality of and recommended-dietary intakes for omega-6 and omega-3 fatty acids. Ann NutrMetab. 1999a;43:127-130.

(21) Simopoulos AP, Leaf A, Salem JrN. Workshop on the essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. Food Aust. 1999b;5:332-333.

(22) Singh A, Ward OP. Production of high yields of docosahexanoic acid by *Thraustochytriumroseum*ATCC 28210. J IndMicrobiol. 1996;16: 370-373.

(23) Singh A, Ward OP. Microbial Production of Docosahexanoic Acid (DHA, C22:6). AdvApplMicrobiol. 1997;45:271-312.

(24) Unagul P, Assantachai C, Phadungruengluij S, Suphantharika M, Tanticharoen M, Verduyn C. Coconut water as a medium additive for the production of docosahexanoic acid (C22:6 n3) by *Schizochytrium mangrovei*Sk-02. Bioresour Tech. 2007;98:281-287.

(25) Ward OP, Singh A (2005) Omega -3/6 fatty acids: Alternative sources of production. Process Biochem. 2005;40:3627-3652.

(26) Wu ST, Yu ST, Lin EC. Effect of culture conditions on docosahexaenoic acid production by *Schizochytrium* sp. S31. Process Biochem. 2005;40:3103-3108.

(27) Yan J, Cheng R, Lin X, Li SYK, Rong H, Ma Y. Overexpression of acetyl-CoA synthetase increased the biomass and fatty acid proportion in microalga*Schizochytrium*. Applmicrobiolbiotechnol. 2013; 1-7.

(28) Yokochi T, Honda D, Higashihara T, Nakahara T. Optimization of docosahexanoic acid production by SchozochytriumlimacinumSR21. ApplMicrobiolBiotechnol. 1998;49:72-76.

(29) Zhang Y, Arends JBA, Van de Wiele T, Boon N. Bioreactor technology in marine microbiology: From design to future application. Biotechnol Adv. 2011; 29:312–321.

(30) Zhu L, Zhang X, Ren X, Zhu Q. Effect of culture conditions on growth and docosahexanoic acid production from *Schizochytriumlimacinum*. J Ocean Univ Chin. 2008; 7:83-88.