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Original article

Optimization of arachidonic acid production from *Mortierella alpina* PRAO7-10 by response surface methodology

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ABSTRACT

Arachidonic acid (ARA) is an essential fatty acid in animal nutrition. The filamentous fungus *Mortierella alpina* has been widely used for production of ARA. The strain *M. alpina* PRAO7-10 was isolated from a soil sample in northern Thailand. Seven parameters—temperature, pH, percentage of medium volume per flask volume (% v/v) and glucose, KNO_3, K_2HPO_4 and soy isolate concentrations—affected the biomass and ARA production of this fungus and were determined using the Plackett-Burman statistical design technique. The results revealed that temperature and % v/v played a significant role in the biomass and ARA production while glucose and soy isolate only affected the ARA production. Therefore, they were chosen for optimization using a central composite design and response surface methodology to maximize the dried cell weight (DCW) and ARA production. The optimal values for the temperature, % v/v, glucose concentration and soy isolate concentration were 25.06 °C, 14.16%, 6.67% and 0.48%, respectively. Under these optimal culture conditions, the maximum DCW and ARA production were 52.64 g/L and 6.76 g/L, respectively. Validation of the optimal conditions showed that deviations in DCW and ARA of the experimental data from the predicted values were 1.72% and 2.42%, respectively, suggesting the suitability of the model employed and that the experimental designs were effective for the optimization of the DCW and ARA production.

Introduction

There has been increasing interest in the production of lipids containing polyunsaturated fatty acids (PUFAs) from microbial sources in the past decade (Ratledge, 2004, 1993). Arachidonic acid (ARA, C20:4 n-6) is an omega-6 polyunsaturated fatty acid (ω-6 PUFA), which is increasingly in demand for its biological activity and clinical effects (Sakuradani and Shimizu, 2009). It is important as a natural constituent of biological membranes and a precursor of numerous eicosanoids, such as prostaglandins, thromboxanes and leukotrienes (Goodnight et al., 1982; Marx, 1982; Das et al., 1987). ARA is necessary for the neurological and neurophysiological development of both term and pre-term infants (Brick et al., 2000; Bougle et al., 1999). In addition, ARA has wide applications in the medicinal, pharmaceutical, cosmetic, infant nutrition and food industries, and recently in agriculture (Eroshin et al., 2000; Dyal and Narine, 2005; Ward and Singh, 2005).

Although ARA is found in animal viscera and adrenal glands, the only conventional commercial sources of ARA are marine fish oils (Yamada et al., 1987; Singh and Ward, 1997). However, over fishing is becoming a global problem resulting in a diminishing supply of the oil (Armstrong et al., 1994). In addition, it has an undesirable taste and odor, contains high cholesterol and is frequently contaminated with heavy metals; hence, non-conventional sources from microorganisms in particular, are more desirable to be used as food additives and supplements (Cheng et al., 1999). The filamentous fungus *Mortierella alpina*, belonging to the Phycomycota, has been identified as a promising producer of ARA due to its high intracellular content of the fatty acid (Bajpai et al., 1991; Li et al., 1995; Higashiyama et al., 1998, 2002; Eroshin et al., 2000). There are no reliable toxicology research reports showing toxic responses to this species or its products (Nisha et al., 2009). Many species of the genus *Mortierella* are known to be rich in ARA when grown under suitable culture conditions (Totani and Oba, 1988; Shinmen et al., 1989; Sakuradani and Shimizu, 2009).

Biomass productivity and fatty acid accumulation of the microorganism can be increased by manipulating nutritional requirements and culture conditions. Chodok et al. (2010) reviewed a

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methodology based on the Plackett-Burman design that provides an efficient way of screening a large number of variables and identifying those that are more important. They noted that such a method facilitates the prediction of responses to values not yet tested, while the conventional approach of one factor at a time does not reveal the interaction these factors. The factors chosen can be either nutritional components or environmental conditions. After finding the critical factors, the next step optimizes the levels of these components in the cultivation process.

In the current study, a response surface methodology (RSM) using a central composite design was used. RSM has by now been established as a convenient method for developing the optimum process with precise conditions and has also minimized the cost of production of many processes with efficient screening of process parameters (Francis et al., 2003). The models developed also indicate the interactions among the selected variables. Recently, statistical optimization designs for biomass and ARA production by *M. alpina* have been reported (Nisha et al., 2011).

The objective of the current study was to maximize the biomass and ARA production of *M. alpina* PRAO7-10 by screening the significant growth parameters using the Plackett-Burman design and further optimize the levels of the selected variables by employing the central composite design (CCD) with RSM.

Materials and methods

Microorganisms

M. alpina PRAO7-10 used in this study was isolated from soil samples collected from Prao district, Chiang Mai province, northern Thailand. The stock culture was maintained on potato dextrose agar containing 200 g/L potato, 20 g/L dextrose and 15 g/L agar.

Cultivation conditions

The starter culture was prepared in 100 mL of medium containing 50 g/L glucose, 8.4 g/L soy isolate, 3 g/LKNO_3, 4 g/L$K_2HPO_4 \cdot 3H_20$, 0.05 g/L$CaCl_2 \cdot 2H_2O$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 5.5. The culture was grown at 25 °C for 7 d at 120 revolutions per minute (rpm) in an incubator shaker (Bio-Shaker BR-300 LF; TAITEC; Koshigaya, Japan). The basal medium in this study contained 50 g/L glucose, 4.5 g/L soy isolate, 3 g/L KNO_3, 4 g/L $K_2HPO_4 \cdot 3H_20$, 0.05 g/L $CaCl_2 \cdot 2H_2O$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$ (Zhu et al., 2004). The pH of the medium was adjusted to 5.5 before autoclaving. Then, the experimental medium in each flask was inoculated with 1% volume per volume of the starter culture. All the experiments were carried out for 10 d in three replications. Samples were taken daily for analyses of the studied parameters.

Cell dry weight (biomass) determination

The biomass was determined using the gravimetric method. Cell suspension was filtered through GFC filter paper (Whatman No. 1822-047). The cell mat was washed twice with distilled water and dried at 80 °C to constant weight.

Fatty acid analysis

Lipid extraction was carried out based on the procedure of Blight and Dyer (1959). Transmethylation of lipid was accomplished by reaction with 6% sulfuric acid in methanol at 80 °C for 15 h (Holub and Skeaff, 1987). Fatty acid methyl esters were then analyzed using a gas chromatographer (GC–14B; Shimadzu; Kyoto, Japan) equipped with a flame ionization detector and a split injector, using a capillary column of 30 m length, 0.25 mm internal diameter and 0.25 μm of film thickness (J&W, DB–225; Agilent Technologies; Santa Clara, CA, USA). Identification of fatty acids profile was obtained by comparison of the retention time with authentic fatty acid standards (Sigma; St. Louis, Mo, USA). An internal standard (pentadecanoic acid) was used to assist in quantitation of the fatty acids. The amounts of ARA were calculated from peak areas compared with authentic fatty acid standards (Sigma; St. Louis, MO, USA).

Experimentals and data analysis

Plackett-Burman design

The Plackett-Burman design was used to screen the factors that played significant roles on biomass and the production of ARA of *M. alpina* PRAO7-10. In total, seven variables—temperature (X_1), pH (X_2), percentage of the medium volume per flask volume (X_3), glucose (X_4), KNO_3 (X_5), $K_2HPO_4 \cdot 3H_2O$ (X_6) and soy isolate (X_7)—were evaluated and are listed in Table 1. Each variable was tested at two levels—high (+) and low (−). Eight experiments were generated with seven variables and one dummy variable (X_8) according to Karim et al. (2011) and Rao et al. (2002). The Plackett-Burman design matrix and response values are listed in Table 2. Statistical analyses were employed to identify the variables that had significant effects on the responses.

The effect of each variable on the response was determined using Equation (1):

$$E_{(xi)} = \left(\sum M_{i+} - M_i \right) / N \quad (1)$$

where $E_{(xi)}$ is the effect of the tested variable. M_{i+} and M_{i-} are the production of biomass and ARA production where the variables were at high and low levels, respectively. N is the total number of experiments (N = 8).

Table 1
Experimental codes, ranges and levels of factors in the Plackett–Burman design.

Variable	Unit	Code	Level	
			Low (−)	High (+)
Temperature	°C	X_1	15	25
pH		X_2	5	6.50
Percentage of medium volume per flask volume	%v/v	X_3	15	20
Glucose concentration	%	X_4	2	6
KNO_3 concentration	%	X_5	0.30	0.60
$K_2HPO_4 \cdot 3H_2O$ concentration	%	X_6	0.30	0.60
Soy isolate concentration	%	X_7	0.45	0.90

Basal medium contained 50 g/L glucose, 4.5 g/L soy isolate, 3 g/L KNO_3, 4 g/L $K_2HPO_4 \cdot 3H_20$, 0.05 g/L $CaCl_2 \cdot 2H_2O$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$ (Zhu et al., 2004). The pH of the medium was adjusted to 5.5 before autoclaving.

Table 2
Plackett–Burman design matrix for screening of critical factors for dried cell weight (DCW) and arachidonic acid (ARA)production by *M. alpina* PRAO7-10.

Run	X_1[a]	X_2	X_3	X_4	X_5	X_6	X_7	DCW (g/L)	ARA (g/L)
1	25 (+1)	6.5 (+1)	20 (+1)	2 (−1)	0.6 (+1)	0.3 (−1)	0.45 (−1)	8.34	0.46
2	15 (−1)	6.5 (+1)	20 (+1)	6 (+1)	0.3 (−1)	0.6 (+1)	0.45 (−1)	25.59	0.77
3	15 (−1)	5.0 (−1)	20 (+1)	6 (+1)	0.6 (+1)	0.3 (−1)	0.90 (+1)	13.90	0.68
4	25 (+1)	5.0 (−1)	15 (−1)	6 (+1)	0.6 (+1)	0.6 (+1)	0.45 (−1)	40.76	1.39
5	15 (−1)	6.5 (+1)	15 (−1)	2 (−1)	0.6 (+1)	0.6 (+1)	0.90 (+1)	12.10	0.21
6	25 (+1)	5.0 (−1)	20 (+1)	2 (−1)	0.3 (−1)	0.6 (+1)	0.90 (+1)	7.77	0.36
7	25 (+1)	6.5 (+1)	15 (−1)	6 (+1)	0.3 (−1)	0.3 (−1)	0.90 (+1)	32.76	1.23
8	15 (−1)	5.0 (−1)	15 (−1)	2 (−1)	0.3 (−1)	0.3 (−1)	0.45 (−1)	13.36	0.27

[a] X_1 = temperature (°C); X_2 = pH; X_3 = percentage of medium volume per flask volume (% volume per volume); X_4 = glucose concentration (%); X_5 = KNO_3 concentration (%); X_6 = $K_2HPO_4 \cdot 3H_2O$ concentration (%); X_7 = soy isolate concentration (%).

Table 3
The effects for dried cell weight (DCW) and arachidonic acid (ARA) production of variables in Plackett–Burman design experiment.

Term	DCW			ARA		
	Effect	t value	*p* value	Effect	t value	*p* value
X_1[a]	0.110	3.532	0.003	0.490	6.846	0.000
X_2	−0.022	−0.713	0.486	−0.095	−1.323	0.205
X_3	−0.086	−2.766	0.014	−0.281	−3.928	0.001
X_4	0.035	1.118	0.280	0.748	10.439	0.000
X_5	0.002	0.062	0.951	0.042	0.581	0.569
X_6	−0.030	−0.973	0.345	−0.010	−0.139	0.891
X_7	−0.026	−0.818	0.425	−0.169	−2.357	0.032

[a] X_1 = temperature (°C); X_2 = pH; X_3 = percentage of medium volume per flask volume (% volume per volume); X_4 = glucose concentration (%); X_5 = KNO_3 concentration (%); X_6 = $K_2HPO_4 \cdot 3H_2O$ concentration (%); X_7 = soy isolate concentration (%).

The effect of the dummy variable was used to calculate the SE determined using Equation (2):

$$SE = \sqrt{\frac{\sum (E_d)^2}{n}} \quad (2)$$

where E_d is the effect of each dummy variable and n is the number of dummy variables ($n = 1$).

The significance of each variable was determined using Equation (3):

$$\text{t value} = \frac{E_{(xi)}}{SE} \quad (3)$$

The experimental design and statistical analysis of the data used SPSS for Windows version 12.0 (SPSS Inc.; Bangkok, Thailand). The factors significant at the 95% level ($p < 0.05$) were considered reliable.

Central composite design

Optimization of the biomass and ARA production was carried out using a central composite design (CCD) based on significant variables identified by the Plackett-Burman design. In total, 27 experiments were generated from four selected variables. The significant variables were assessed at five coded levels (−2, −1, 0, +1 and +2), as shown in Table 4. The experimental design and the results of CCD are shown in Table 5.

Table 5
Central composite design of the significant factors for dried cell weight (DCW) and arachidonic acid (ARA) production.

Run	X_1[a]	X_2	X_3	X_4	DCW (g/L)		ARA (g/L)	
					Experimental	Predicted	Experimental	Predicted
1	−1	−1	−1	−1	16.49	15.79	2.26	2.43
2	1	−1	−1	−1	20.06	19.00	1.65	1.69
3	−1	1	−1	−1	12.28	13.96	2.42	2.39
4	1	1	−1	−1	18.25	18.96	1.52	1.74
5	−1	−1	1	−1	15.31	14.92	2.88	3.04
6	1	−1	1	−1	30.10	29.73	3.80	3.90
7	−1	1	1	−1	12.04	14.65	1.70	1.85
8	1	1	1	−1	33.23	31.24	2.80	2.79
9	−1	−1	−1	1	17.25	19.38	2.26	2.42
10	1	−1	−1	1	27.58	25.51	1.72	1.83
11	−1	1	−1	1	16.55	17.46	1.66	1.83
12	1	1	−1	1	24.85	25.39	1.34	1.33
13	−1	−1	1	1	20.74	20.56	4.03	4.08
14	1	−1	1	1	39.83	38.29	4.91	5.09
15	−1	1	1	1	19.01	20.21	2.23	2.34
16	1	1	1	1	38.48	39.72	3.33	3.43
17	−2	0	0	0	20.17	16.89	1.60	1.34
18	2	0	0	0	37.01	39.62	1.84	1.69
19	0	−2	0	0	23.61	26.04	4.39	4.12
20	0	2	0	0	28.76	25.65	2.58	2.44
21	0	0	−2	0	10.55	9.82	1.66	1.45
22	0	0	2	0	23.23	23.28	4.38	4.17
23	0	0	0	−2	14.41	14.51	2.81	2.62
24	0	0	0	2	27.35	26.58	3.47	3.25
25	0	0	0	0	50.46	49.13	6.50	6.37
26	0	0	0	0	47.70	49.13	6.31	6.37
27	0	0	0	0	49.23	49.13	6.29	6.37

[a] X_1 = temperature (°C); X_2 = pH; X_3 = percentage of medium volume per flask volume (% volume per volume); X_4 = glucose concentration (%); X_5 = KNO_3 concentration (%); X_6 = $K_2HPO_4 \cdot 3H_2O$ concentration (%); X_7 = soy isolate concentration (%).

Table 4
Experimental codes, ranges and levels of the independent variables in the central composite design.

Independent Variables	Unit	Code[a]	−2	−1	0	1	2
Temperature	°C	X_1	15	20	25	30	35
Percentage of medium volume per flask volume	%v/v[b]	X_2	9	12	15	18	21
Glucose	%	X_3	2	4	6	8	10
Soy isolate	%	X_4	0.15	0.30	0.45	0.60	0.75

[a] X_1 = temperature (°C); X_2 = pH; X_3 = percentage of medium volume per flask volume (% volume per volume); X_4 = glucose concentration.
[b] Percent volume per volume.

The relationship of the independent variables and the responses (biomass and ARA production) was calculated using a second-order polynomial (Equation (4)):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2{}_i + \sum \beta_{ij} X_i X_j \quad (4)$$

where Y is the predicted response, β_0 is the model constant, β_I is the linear coefficient, $\beta_{ii\ z}$ is the quadratic coefficient and β_{ij} is the interaction coefficient. ANOVA for the experimental data and the model coefficients were calculated using the software Design-Expert 7.0 (Stat-Ease Inc.; Minneapolis, MN, USA).

Results and discussion

Screening of significant variables using Plackett-Burman design

The results of the Plackett-Burman design for the seven variables revealed that run 4 in the design matrix produced the highest production of both biomass and ARA production at 40.76 g/L and 1.39 g/L, respectively, as shown in Table 2. The coefficient values from the regression analysis for the Plackett-Burman design are shown in Table 3. Variables with *p*-values less than 0.05 were considered to have a significant effect on the response. The data revealed that X_1 (temperature) and X_3 (percentage of medium volume per flask volume) played significant roles in the biomass and ARA production while X_4 (glucose concentration) and X_7 (soy isolate concentration) only affected ARA production. The positive effects of four factors (X_1, X_4, X_5 and X_6) on the biomass and ARA production were greater at high concentration, while X_2, X_3 and X_7 had a negative effect and their influence was greater at the lower levels. However, the *p* values of less than 0.05 were considered to have a significant effect on the response (Table 3). Therefore, the variables X_1, X_3, X_4 and X_7 were selected for further optimization using CCD.

Temperature is one of the most important environment parameters affecting cell growth and ARA production. Peng et al. (2010) reported that temperature was a critical factor for high level production of ARA by *M. alpina* in batch cultivation. ARA was an intracellular metabolite and the concentration was associated with the cell growth. Park et al. (1999) indicated that to improve ARA productivity, it is necessary to increase both the mycelial concentration and ARA yield, based on the dried cell weight. The degree of unsaturation in the fatty acid composition is known to be influenced by temperature; when the growth temperature is lowered, the proportion of unsaturated fatty acids to saturated fatty acids tends to increase as a result of increased membrane fluidity as an adaptation to the cold environment (Suutari and Laasko, 1994). Yuan et al. (2002) reported that a lower temperature was suitable for the accumulation of ARA. The percentage of medium volume per flask volume implied the rate of aeration during cultivation, that is, lowering the percentage increased the oxygen solubility. Ratledge (1992) mentioned that ARA production requires adequate oxygen because ARA is formed through enzymatic desaturation which comprises oxygenation. Thus, an adequate oxygen supply is a key factor in obtaining a higher ARA content in the cells. The basic physiology of lipid overproduction is that the organism is cultivated on medium consisting of an excess carbon source and a limited quantity of other important nutrients, especially nitrogen. Yamada et al. (1987) indicated that among carbon sources, glucose was found to be the most effective and the ARA production increased in parallel with the cell growth in the medium containing a glucose concentration as high as 10%. Wassef (1977) mentioned that glucose was used as the sole carbon and energy source and it is the most commonly used sugar for fungal fat production and is efficiently converted into lipids by many fungi. The rapid and efficient conversion of glucose into lipid is explained by an assumption that glucose is metabolized exclusively via the glycolysis pathway to pyruvate (Karim et al., 2011). Wynn et al. (1999) suggested that the concentration of the nitrogen source is essential for the production of ARA because it plays an important role in maintaining the high activity of the malic enzyme which in turn plays an important role in the provision of NADPH for lipid biosynthesis and thus regulates the extent of lipid accumulation in *M. alpina*. Park et al. (1999) also indicated that the nitrogen source affected the mycelial morphology and ARA production of *M. alpina* CBS 754.68. Feather-like morphology with soybean meal as the nitrogen source was suitable for ARA production. High carbon and low nitrogen levels are known to support good lipid accumulation (Ratledge, 2002).

Optimization of significant variables using response surface methodology

Based on the above screening test using the Plackett-Burman design, the RSM using CCD was used to determine the optimum levels of the four significant factors. These factors were tested at five levels (−2, −1, 0, 1, 2), as shown in Table 4. The design matrix and the corresponding results of the RSM experiments to determine the effects of the four independent variables are shown in Table 5.

The experimental results and predicted values of dried cell weight (DCW) and ARA production are shown in Table 5. Multiple regression analysis was used and the data were fitted to a second-order polynomial equation. The response biomass production by *M. alpina* PRAO7-10 can be expressed in terms of regression Equation (5):

$$\begin{aligned} Y_{DCW} = {} & +49.13 + 5.68X_1 - 0.099X_2 + 3.37X_3 + 3.02X_4 \\ & + 0.45X_1X_2 + 2.90X_1X_3 + 0.73X_1X_4 + 0.39X_2X_3 \\ & - 0.022X_2X_4 + 0.51X_3X_4 - 5.22{X_1}^2 - 5.82{X_2}^2 \\ & - 8.14{X_3}^2 - 7.15{X_4}^2 \end{aligned} \quad (5)$$

where Y_{DCW} is the predicted dried cell weight, X_1 is the temperature, X_2 is the percentage of medium volume per flask volume, X_3 is the glucose concentration and X_4 is the soy isolate concentration.

Table 6
ANOVA for dried cell weight (DCW) and arachidonic acid (ARA) production for response surface methodology factors.

Source[a]	DCW			ARA		
	Estimate	*f* value	*p* value	Estimate	*f* value	*p* value
X_1	5.68	133.95	<0.0001	0.09	3.55	0.0841
X_2	−0.099	0.04	0.844	−0.42	81.77	<0.0001
X_3	3.37	47.00	<0.0001	0.68	211.45	<0.0001
X_4	3.02	37.75	<0.0001	0.16	11.33	0.0056
X_1X_2	0.45	0.55	0.4717	0.02	0.13	0.7245
X_1X_3	2.90	23.22	0.0004	0.40	48.50	<0.0001
X_1X_4	0.73	1.47	0.2485	0.04	0.44	0.5175
X_2X_3	0.39	0.42	0.5295	−0.29	25.40	0.0003
X_2X_4	−0.022	1.32E-03	0.9716	−0.14	5.84	0.0325
X_3X_4	0.51	0.73	0.4091	0.26	20.98	0.0006
X_1^2	−5.22	100.45	<0.0001	−1.17	559.17	<0.0001
X_2^2	−5.82	124.92	<0.0001	−0.73	217.10	<0.0001
X_3^2	−8.14	244.56	<0.0001	−0.85	291.81	<0.0001
X_4^2	−7.15	188.33	<0.0001	−0.82	271.47	<0.0001
Model		42.39	<0.0001		80.35	<0.0001

[a] X_1 = temperature (°C); X_2 = pH; X_3 = percentage of medium volume per flask volume (% volume per volume); X_4 = glucose concentration (%); X_5 = KNO_3 concentration (%); X_6 = $K_2HPO_4 \cdot 3H_2O$ concentration (%); X_7 = soy isolate concentration (%).

For Equation (5), the values of the coefficients, f values and p values are listed in Table 6. The f value of the model was 42.39 and the p value level was 0.0001, suggesting that the model was significant. The multiple correlation coefficient (R^2) was 0.9802 for the biomass production, indicating good agreement between the experimental and the predicted values. The value of the adjusted R^2 (0.957) for Equation (5) suggested that the model was capable of explaining 95.7% of the variation response. In this model, X_1, X_3, X_4, X_1X_3, X_1^2, X_2^2, X_3^2 and X_4^2 were significant variables for biomass production. The interactions between temperature and the percentage of medium volume per flask volume were significant for biomass production.

The optimal value of each variable was clearly represented in the three dimensional surface plots and contour plots as shown in Figs. 1 and 2 for the biomass and ARA production, respectively. Each response surface plot represented the effect of two independent variables, while maintaining other variables at the central point level. Interactions between the temperature and glucose concentrations were significant for the biomass production, as shown by the low p values (less than 0.05) for the interactive terms. As shown in Fig. 1B, an increase in the glucose concentration resulted in an increase in the biomass production.

Fig. 1 (representing the model using Equation (5) to predict the biomass production) shows the relative effect of variable interactions. Fig. 1A shows the effect of temperature and the percentage of medium volume per flask volume on biomass production. Fig. 1B shows the effect of the temperature and glucose concentration. Fig. 1C shows the effect of the temperature and the soy isolate concentration. Fig. 1D shows the effect of the percentage of medium volume per flask volume and the glucose concentration. Fig. 1E shows the effect of the percentage of medium volume per flask volume and the soy isolate concentration. Fig. 1F shows the effect of the glucose and soy isolate concentrations. A similar profile was observed in Fig. 2 for the production of ARA.

Similarly, multiple regression analysis was used to analyze the experimental data and a second-order polynomial equation for ARA production can be expressed in terms of Equation (6):

$$\begin{aligned} Y_{ARA} = {} & +6.37 + 0.088X_1 - 0.42X_2 + 0.68X_3 + 0.16X_4 \\ & + 0.021X_1X_2 + 0.4X_1X_3 + 0.038X_1X_4 - 0.29X_2X_3 \\ & - 0.14X_2X_4 + 0.26X_3X_4 - 1.21{X_1}^2 - 0.77{X_2}^2 \\ & - 0.89{X_3}^2 - 0.86{X_4}^2 \end{aligned} \quad (6)$$

where Y_{ARA} is the predicted ARA production.

For Equation (6), the f value and p value of the model were calculated as 80.35 and 0.0001, respectively, which suggested that the obtained experimental data were a good fit for the model. Each coefficient was determined to be significant. The ANOVA results of the second-order response surface model are given in Table 6. The regression equation obtained from the ANOVA showed that the multiple correlation coefficient (R^2) for ARA production was 0.9898 (a value above 0.75 indicates a good fit of the model according to Karim et al., 2011). The value of the adjusted R^2 was 0.9780 for Equation (6) indicating approximately 98% of the variation could be explained by the model. In this model, X_2, X_3, X_4, X_1X_3, X_2X_3, X_2X_4, X_1^2, X_2^2, X_3^2 and X_4^2 were significant variables for the ARA production. The interactions between temperature and the glucose concentration, and the percentage of medium volume per flask volume and the glucose concentration, and percentage of medium volume per flask volume and the soy isolate concentration, and the glucose and soy isolate concentrations were significant for the ARA production.

In order to determine the optimal levels of each variable for the maximum biomass and ARA production, contour plots were constructed by plotting the response against each of the two independent variables, while maintaining the other variables at their fixed (zero) levels. The optimal values for the temperature, percentage of medium volume per flask volume and the glucose and percent soy isolate percentages were 25.06 °C, 14.16%, 6.67% and 0.48%, respectively. Under these optimal culture conditions, the maximum DCW and ARA production were 52.64 g/L and 6.76 g/L, respectively.

Table 7 shows the fatty acid profiles of lipids produced from *M. alpina* PRAO7-10 grown in the basal medium and the optimized medium for 7 d. Lipids of *M. alpina* PRAO7-10 contained saturated and unsaturated fatty acids with the carbon chain length from C_{14} to C_{20} similar to the earlier reports (Eroshin et al., 2002; Fakas et al., 2009). The degree of desaturation of fatty acids was clearly increased as the combined percentage of oleic acid (18:1) and linoleic acid (18:2) reduced from 30.8% in the basal medium to 21.5% in the optimized medium while the content of ARA increased from 40.3% to 48.7%, respectively. The results coincided with those reported by Fakas et al. (2009) who grew the fungus in a solid substrate medium.

The ARA production of *M. alpina* PRAO7-10 in this study was 6.76 g/L which was much higher than that reported by Chen et al. (1997). They investigated the effect of culture conditions on ARA production by *M. alpina* Wuji-H4 using RSM, with the optimal concentration as soluble starch being 99.7 g/L, yeast extract being 12.6 g/L and KH_2PO_4 being 3.0 g/L. The ARA production increased to 3.85 g/L. Karim et al. (2011) improved the ARA production by *M. alpina* CBS754.68 using the Plackett-Burman design and RSM. Five significant variables—the glucose concentration, yeast extract concentration, temperature, agitation rate and fermentation time—were used for optimization. The results indicated that carrying out the fermentation under conditions of glucose 50 g/L, yeast extract 14 g/L, temperature 22 °C, agitation rate 180 rpm and fermentation time 8 d would increase the ARA production to 6.22 g/L. Bajpai et al. (1991) reported that the fungus *M. alpina* had high cell growth at 15–25 °C, and it decreased at a temperature higher than 28 °C or lower than 8 °C. The effect of temperature agreed with the study of Singh and Ward (1997) who reported that *M. alpina* ATCC 32222 had a high biomass and ARA content at 25 °C. A similar report by Nisha et al. (2011) improved the ARA production by *M. alpina* using RSM, with four independent variables selected (glucose, corn solids, KH_2PO_4 and KNO_3) and the optimal conditions resulted in a maximum production of ARA of 1.39 g/L and biomass of 12.49 g/L. Samadlouiel et al. (2012) improved the ARA production by *M. alpina* CBS754.68 using RSM, with their the results indicating that glucose and soybean were the major impact factors with optimal concentrations at 50.35 g/L and 18.30 g/L, respectively. The ARA production increased to 5.64 g/L.

Validation of the optimal conditions

Validation of the statistical model and regression equation were conducted using the optimal levels of the four factors (6.67% glucose, 0.48% soy isolate, 14.16% volume per flask volume and temperature at 25.06 °C). The experimental data were compared with the predicted values. Under these optimized conditions, the predicted response for the ARA and biomass production were 6.60 g/L and 51.75 g/L, respectively, while the observed experimental values were 6.76 g/L and 52.64 g/L, respectively. The corresponding results of the deviations of the experimental and predicted values for the production of DCW and ARA were 1.72% and 2.42%, respectively. These results confirmed the validity of the

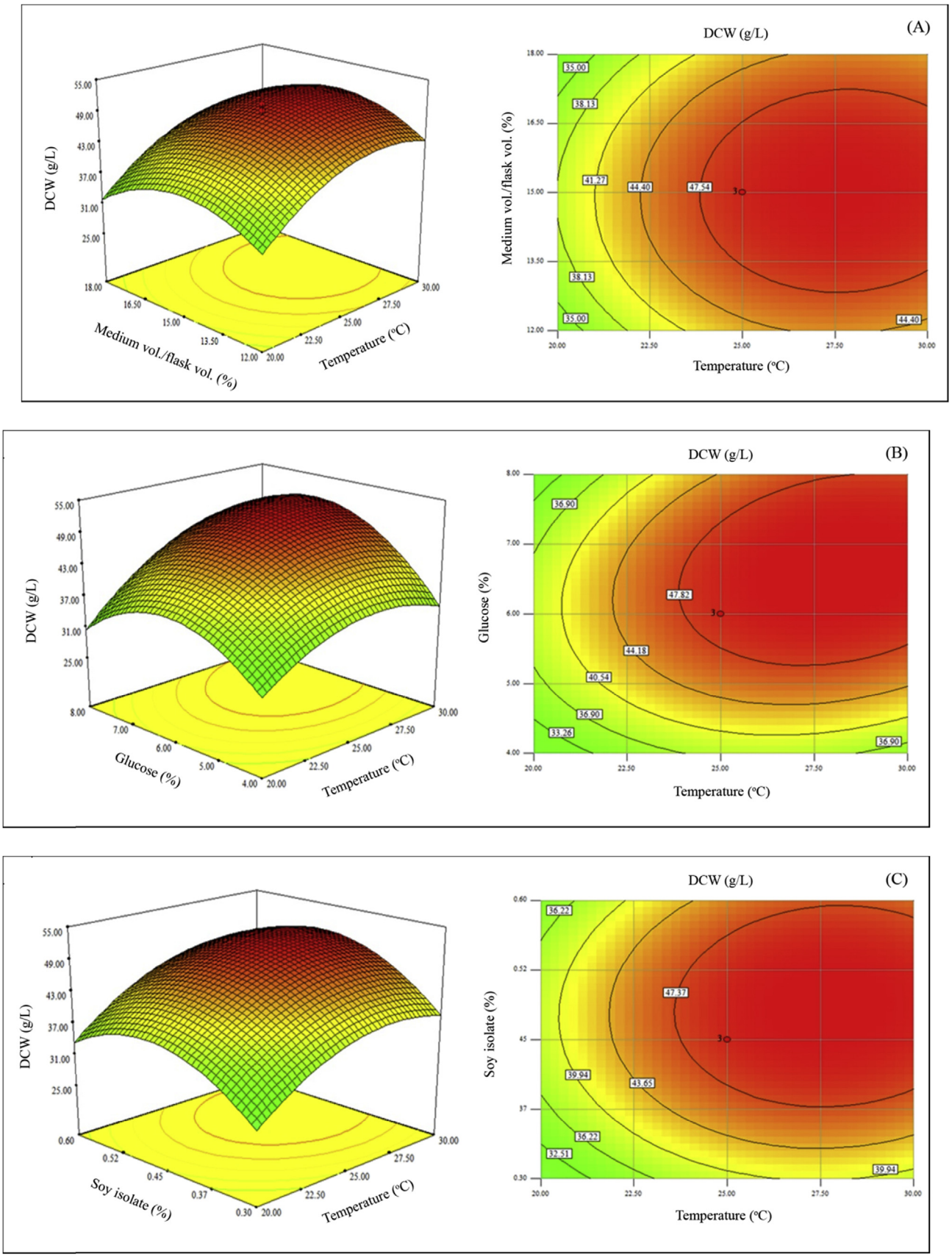
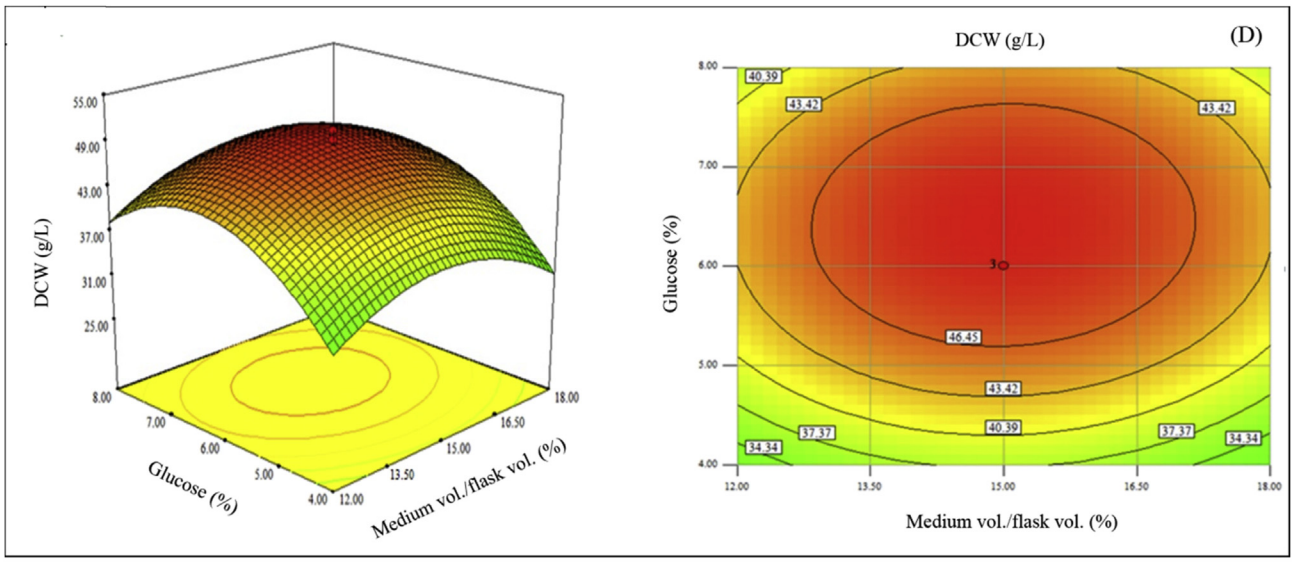
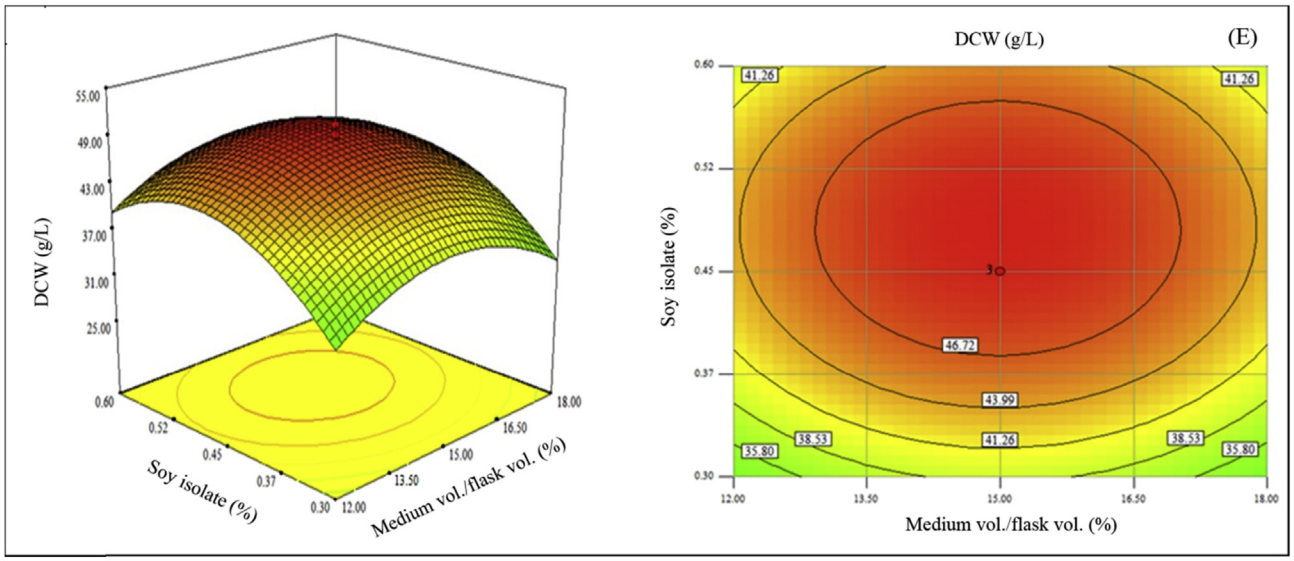


Fig. 1. Three-dimensional response surface plots and two-dimension contour plots for dried cell weight (DCW) production by *M. alpina* PRAO7-10 showing interaction of two variables while the remaining factors were held constant: (A) temperature and percentage of medium volume (vol.) per flask vol.; (B) temperature and glucose concentration; (C) temperature and soy isolate concentration. Three-dimensional response surface plots and two-dimension contour plots for dried cell weight (DCW) production by *M. alpina* PRAO7-10 showing interaction of two variables while the remaining factors were held constant: (D) glucose concentration and percentage of medium volume (vol.) per flask vol.; (E) soy isolate concentration and percentage of medium volume per flask volume; (F) glucose concentration and soy isolate concentration.





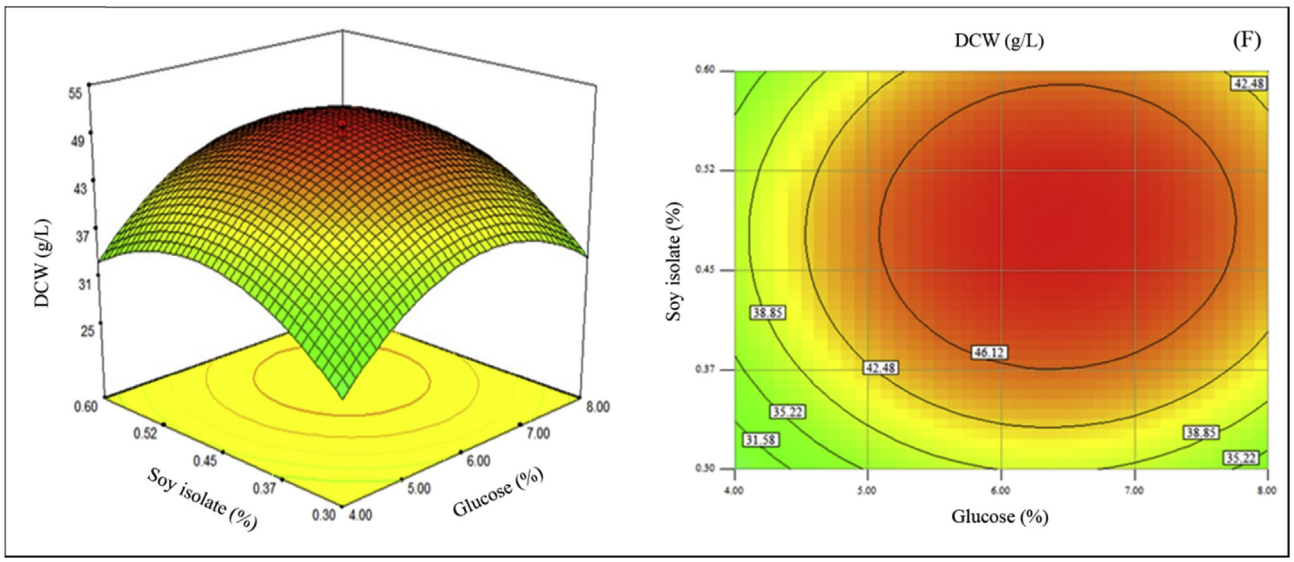


Fig. 1. (*continued*).

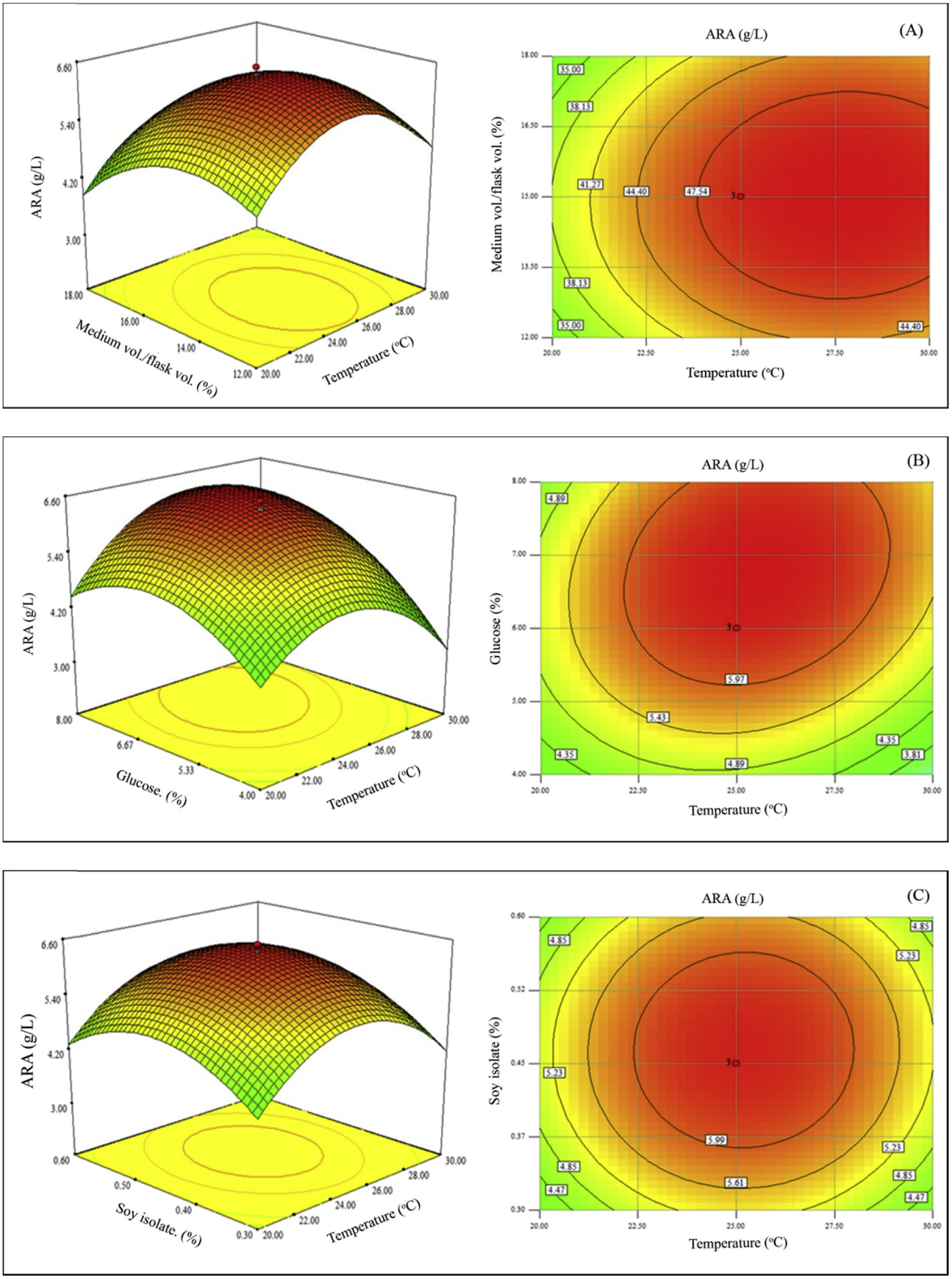
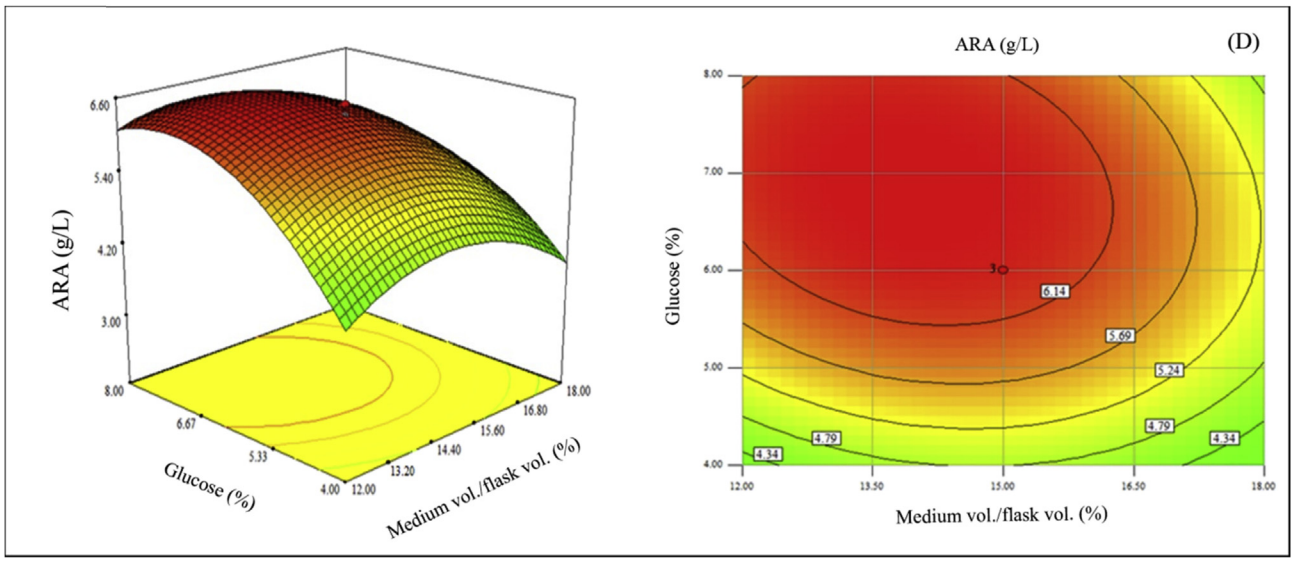
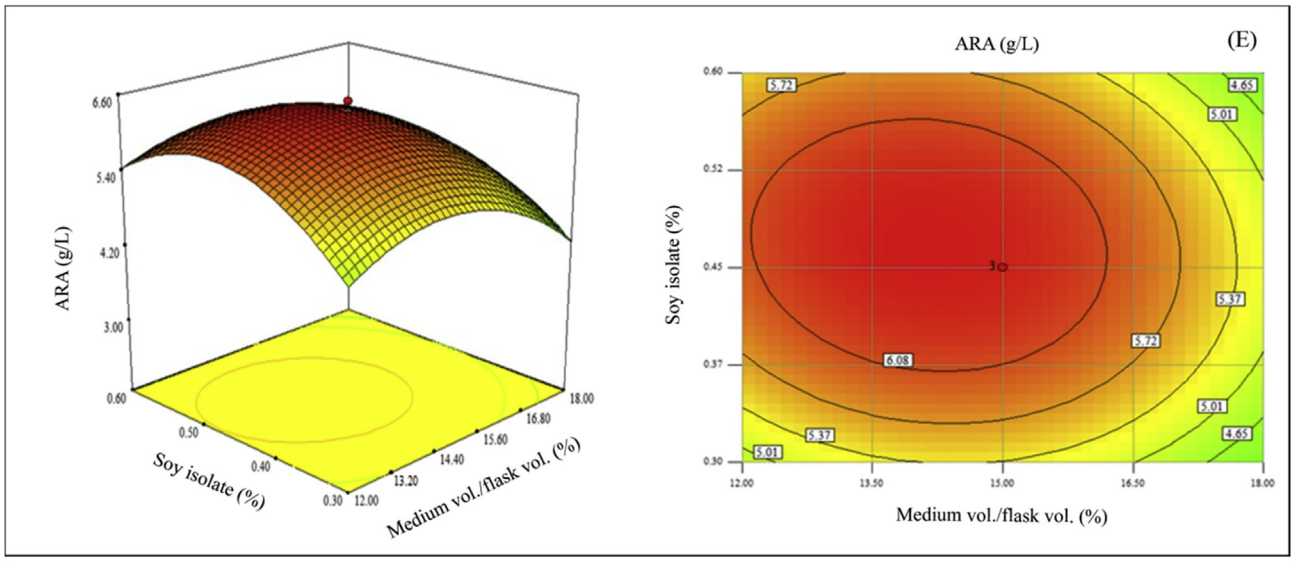


Fig. 2. Three-dimensional response surface plots and two-dimension contour plots for arachidonic acid (ARA) production by *M. alpina* PRAO7-10 showing interaction of two variables while the remaining factors were held constant: (A) temperature and percentage of medium volume (vol.) per flask vol.; (B) temperature and glucose concentration; (C) temperature and soy isolate concentration. Three-dimensional response surface plots and two-dimension contour plots for arachidonic acid (ARA) production by *M. alpina* PRAO7-10 showing interaction of two variables while the remaining factors were held constant: (D) glucose concentration and percentage of medium volume (vol.) per flask vol.; (E) soy isolate concentration and percent of medium volume per flask volume; (F) glucose concentration and soy isolate concentration.





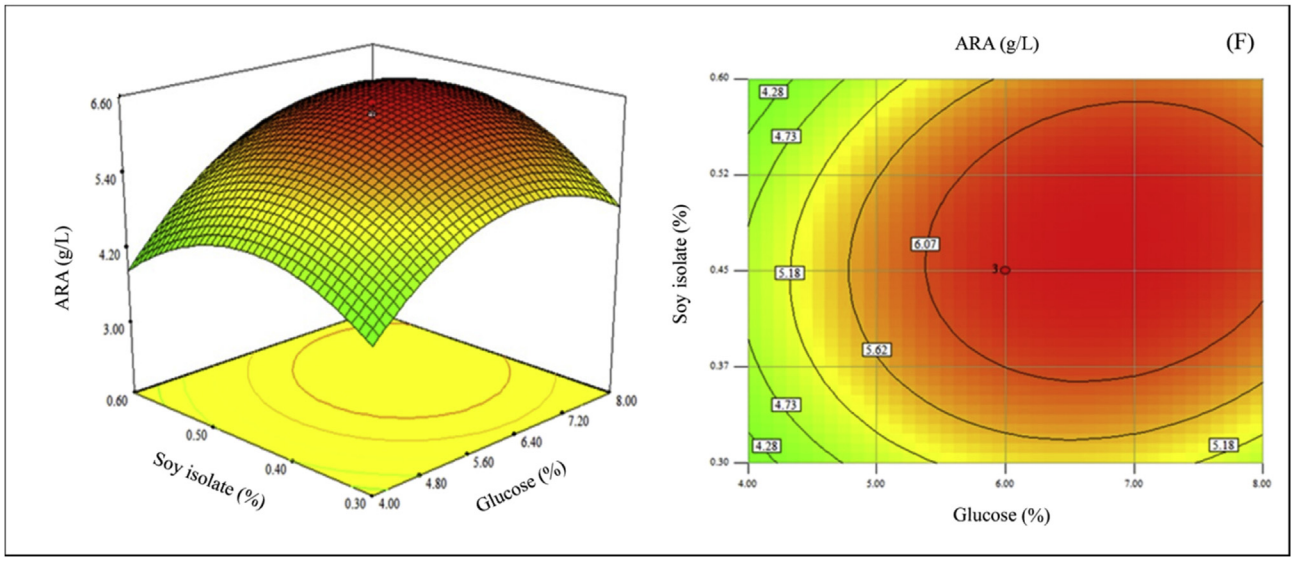


Fig. 2. (*continued*).

Table 7
Fatty acid profiles of lipids produced from *M. alpina* PRAO7-10.

M. alpina PRAO7-10	Percentage of total fatty acids[a]							
	14:0	16:0	18:0	18:1	18:2	18:3α	20:4	Others
Basal medium	0.6	14.1	8.7	19.5	11.3	4.2	40.3	1.3
Optimized medium	1.0	15.2	9.2	15.4	6.1	4.1	48.7	0.3

[a] 14:0 (myristic acid), 16:0 (palmitic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 18:3α (α-linolenic acid), 20:4 (arachidonic acid).

model and that the experimental designs used in this work were appropriate for predicting the optimized culture conditions.

Comparison of the DCW and ARA production in the basal medium and in the optimized medium showed that the maximum DCW and ARA production in the optimized medium were 52.64 g/L and 6.76 g/L, respectively, while in the basal medium they were 40.76 g/L and 5.89 g/L, respectively. It was clear that the temperature, percentage of medium volume per flask volume, percentage glucose and percentage soy isolate were suitable for cultivation of *M. alpina* PRAO7-10 and resulted in improving the DCW and ARA production. The period for the growth and ARA production in the basal medium and the optimized medium are shown in Fig. 3. *M. alpina* had two distinct growth phases. The first phase consisted of rapid cell proliferation that continued until probably one of the essential nutrients became a limiting growth factor. At this point of nutritional depletion, the cell growth ceased. The second stage of growth then commenced as lipid synthesis dominated, resulting in the accumulation of storage lipids (Eroshin et al., 2002).

In this study, statistical analyses were used to optimize the culture variables for *M. alpina* PRAO7-10 in order to estimate the biomass and ARA production. The most significant factors were identified using a Plackett-Burman design. Among the seven variables tested, the temperature and the percentage of medium volume per flask volume played a significant role in the biomass and ARA production; glucose and soy isolate only affected ARA production. The optimum culture conditions for the biomass and ARA production by *M. alpina* PRAO7-10 cultures were further derived using RSM. The optimal levels of culture conditions to obtain the maximum biomass and ARA production were 6.67% glucose, 0.48% soy isolate, 14.16% medium volume per flask volume and a temperature of 25.06 °C. Under these optimal culture conditions, the maximum DCW and ARA production were 52.64 g/L and 6.76 g/L, respectively. The second order polynomial equations for DCW and ARA production were validated and provided deviations for DCW and ARA of 1–3%. Therefore, the results suggested that the statistically optimum strategy was an effective tool for the optimization of the process parameters on the biomass and ARA production. The information from this study is currently being used for the development of a larger scale cultivation process for *M. alpina* PRAO7-10.

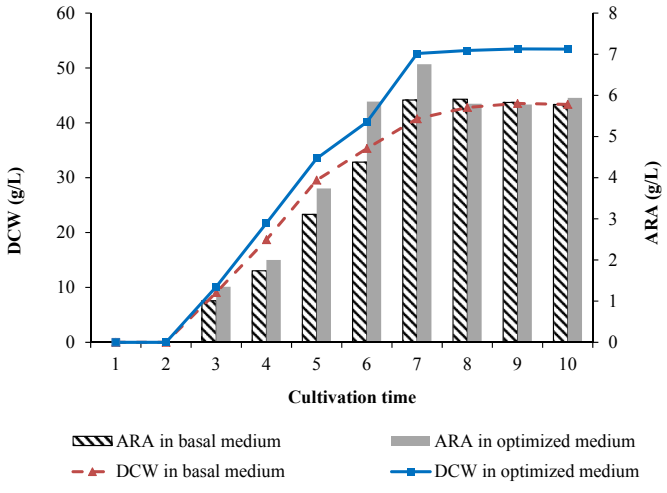


Fig. 3. Time course of dried cell weight (DCW) and arachidonic acid (ARA) production in basal medium and optimized medium.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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