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Optimisation of Ammonium Tartrate and Glucose Concentration for Gamma Linolenic Acid Production by *Cunninghamella* sp. 2A1

(Pengoptimuman Kepekatan Ammonium Tartarat dan Glukosa Terhadap Penghasilan Asid Gamma Linolenik oleh *Cunninghamella* sp. 2A1)

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ABSTRACT

The effects of ammonium tartrate and glucose concentration on biomass, lipid and GLA accumulation in Cunninghamella *sp. 2A1 were investigated using Response Surface Methodology (RSM). Cultivation was carried out in 250 mL shake flask containing 100 mL of nitrogen limiting medium (with various combinations of concentration of ammonium tartrate (1-3 g/L) and glucose (30-60 g/L) at 30°C and 250 rpm agitation for 120 h. The concentration of both compounds significantly affected the biomass, lipid and GLA yield ($p<0.05$), with the production of each of them being represented by quadratic models. Higher concentration of ammonium tartrate and glucose (2.99 and 59.33 g/L, respectively) was required for enhanced biomass production whereas low nitrogen content with excess glucose was otherwise favoured for lipid and GLA production. Ammonium tartrate and glucose concentration at 1 and 43 g/L, respectively were estimated by the model and proven to give the highest lipid production and GLA yield of 31.06 % (g/g biomass) and 4.15 $\times 10^{-2}$ (g/g lipid less biomass), respectively.*

Keywords: C/N ratio; Cunninghamella *sp.; gamma linolenic acid; lipid; Response Surface Methodology (RSM)*

ABSTRAK

Kesan kepekatan ammonium tartarat dan glukosa terhadap penghasilan biojisim, lipid dan GLA oleh Cunninghamella *sp. 2A1 dikaji menggunakan Kaedah Gerak balas Permukaan (RSM). Pengkulturan dilakukan dalam kelalang goncangan 250 mL yang mengandungi 100 mL medium terhad nitrogen (dengan pelbagai kombinasi kepekatan ammonium tartarat (1-3 g/L) dan glukosa (30-60 g/L) pada 30°C dengan kadar goncangan 250 rpm selama 120 j. Kepekatan kedua-dua sebatian ini memberi kesan signifikan terhadap penghasilan biojisim, lipid dan GLA ($p<0.05$) yang masing-masing diwakili oleh model kuadratik. Kepekatan ammonium tartarat dan glukosa yang lebih tinggi iaitu masing-masing pada 2.99 dan 59.33 g/L, didapati perlu untuk merangsang penghasilan biojisim sedangkan kandungan ammonium tartarat yang rendah dengan kepekatan glukosa yang tinggi pula diperlukan untuk membantu meningkatkan penghasilan lipid dan GLA. Kepekatan ammonium tartarat dan glukosa masing-masing pada 1 dan 43 g/L telah dianggarkan oleh model tersebut dan terbukti memberikan penghasilan lipid dan GLA tertinggi iaitu masing-masing sebanyak 31.06 % (g/g biojisim) dan 4.15 $\times 10^{-2}$ (g/g biojisim tanpa lipid).*

Kata kunci: Asid gamma linolenik; Cunninghamella *sp.; Kaedah Gerak Balas Permukaan (RSM); lipid; nisbah C/N*

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) such as γ-linolenic acid (GLA), are essential fatty acids in human nutrition. This is due to human's lack of desaturase involved in the formation of linoleic acid from oleic acid (Anderson & Wynn 2001). Unsaturated fatty acids in organisms are elongated or further unsaturated to physiologically active substances such as prostaglandins or leukotrienes which play various vital physiological role in human (Certik & Shimizu 1999). Based on the potential need for PUFAs in human nutrition and medicine, searches for new sources of these compounds have been conducted. The potential of microbes as alternative sources for PUFAs production has led studies related to optimisation of process parameters being intensively carried out. Of particular importance are studies dealing with media optimisation for enhanced lipid and GLA production.

Macronutrients required by microbes include carbon and nitrogen which are vital for enzyme, other proteins and nucleic acids synthesis which finally affect growth (Stanbury et al. 1984). In the development of any biotechnological processes, one of the most crucial aspects to be addressed is the generation as much biomass as possible in the shortest period of time, which would directly affect the production of desired products. Hence, the concentrations of these elements are crucial in any media formulation applied in biotechnological processes.

In the case of microbial lipid production, the key to lipid accumulation lies in allowing the amount of nitrogen supplied to the medium culture to become exhausted

(Ratledge 1997). The excess carbon which is available to the culture after nitrogen exhaustion continues to be assimilated by the cells and converted into lipids. Therefore, generally, the strategy applied in the production of microbial lipid is to produce as much biomass as possible in the first phase followed by allowing a nitrogen deficient condition to occur to promote lipid biosynthesis. As most processes developed involve a single-stage batch cultivation system, the introduction of these two phases is achieved by using a nitrogen-limiting media. This allows microbes to grow in the presence of nitrogen followed by lipid accumulation after exhaustion of nitrogen occurs, in the presence of excess carbon source. Therefore, in order to achieve enhanced production of microbial lipid, determination of concentrations of nitrogen and carbon in media formulation is of paramount importance.

Many reports have been concerned with determining the optimal carbon and nitrogen ratios for various fungi in a single stage batch cultivation system for enhanced PUFA production (Chen & Chang 2008; Koike et al. 2001). However, in this paper, we investigated the effects of carbon and nitrogen concentration, using RSM, on three responses; biomass concentration, lipid and GLA production. This enables the optimal nitrogen and carbon concentrations for enhanced production of each response to be determined and could be applied for further enhancement of the process.

Materials and Methods

Microorganism and inoculum preparation

Cunninghamella sp. 2A1 was maintained on potato dextrose agar (PDA) at 4°C. The standard inoculum used, was in order of 10^5 spores/mL medium, harvested from 7-day-old plates. Seed culture was prepared by transferring the spore suspension into 500 mL conical flask containing 200 mL of modified nitrogen-limited medium (Kendrick & Ratledge 1992) at 30°C, 250 rpm for 48 h cultivation.

Culture medium and conditions

Culture medium contained the following constituents (g/L): $NH_4C_4H_4O_6$ 1.0; KH_2PO_4 7.0; Na_2HPO_4 2; $MgSO_4.7H_2O$ 1.5; yeast extracts 1.5; $CaCl_2$ 0.1; $Co(NO_3)_2.6H2O$ 0.0001; $FeCl_3.6H_2O$ 0.008; $ZnSO_4.7H_2O$ 0.0001; $CuSO_4.5H_2O$ 0.0001; $MnSO_4.5H_2O$ 0.0001; and Glucose 30g/L which was sterilised and added separately. A 10% (v/v) of seed culture was added into thirteen 250 mL conical flask containing 100 mL of the same composition of nitrogen limited medium with various concentrations of glucose and ammonium tartrate according to response surface experimental design. All cultures were incubated at 30°C and 250 rpm and harvested after 120 h.

Experimental design

Response surface methodology (RSM) in *Design Expert software (version 6.0 Stat-Ease, Inc)* was used in the present study involving 2 factors: glucose and ammonium tartrate concentration. The range of concentrations used was based on common concentrations of both factors reported in the studies of lipid production by oleaginous fungi, as well as from our own preliminary data related to biomass production of *Cunninghamella* sp. 2A1 (unpublished data). The chosen ranges are: glucose (30 – 60 g/L) and ammonium tartrate (1 – 3 g/L). Coded and actual value of each factors were shown in Table 1 and 13 experiments were designed based on central composite design in RSM (Table 2). Experiments were randomised in order to minimise the effects of unexplained variability in the observed responses due to extraneous factors. The center point in the design was repeated five times to calculate the repeatability of the method (Montgomery 2001). The following second order polynomial response surface model (Eq. 1) was fitted to each of the response variable (Yk) with the independent variables (X)

$$Y_k = b_{k0} + \sum_{i=1}^{2} b_{ki}X_i + \sum_{i=1}^{2} b_{ii}X_i^2 + \sum_{i \neq j=1}^{2} b_{kij}X_iX_j, \quad (1)$$

where b_{k0}, b_{ki}, b_{kii}, and b_{kij} are the constant, linear, quadratic and cross-product regression coefficients, respectively and X_i's are the coded independent variables of X_1 and X_2.

Regression analysis and analysis of variance (ANOVA) were conducted for fitting the models represented by (1) and to examine the statistical significance of the model terms. The adequacy of the models were determined using model analysis, lack-of fit test and R^2 (coefficient of determination) analysis as outline by (Lee et al. 2000; Weng et al. 2001). The lack of fit is a measure of the failure of a model to represent data in the experimental domain at which points were not included in the regression or variations in the models cannot be accounted for by random error (Montgomery 2001). If there is a significant lack of fit, as indicated by a low probability value, the response

TABLE 1. level and code for each factors for CCD

Factor (g/L)	Codded value				
	-α	-1	0	1	α
	Actual value				
Ammonium tartrate concentration, X_1	0.59	1.00	2.00	3.00	3.41
Glucose concentration, X_2	23.79	30.00	45.00	60.00	66.21

TABLE 2. Treatment combinations for biomass, lipid and GLA production by *Cunninghamella* sp. 2A1 with 2 variables RSM design

run	Concentration (g/L)		Responsers					
	X_1	X_2	biomass concentration		lipid yield (%)		GLA yield ($\times 10^{-12}$)	
	g/L	g/L	g/L		(g/g biomass)		(g/g lipid less biomass)	
			actual	predicted	actual	predicted	actual	predicted
1	3.41	45.00	16.08	15.62	20.87	22.06	2.98	3.19
2	2.00	45.00	14.38	14.87	28.27	29.83	3.75	4.05
3	3.00	60.00	18.58	18.77	29.37	29.30	4.15	4.07
4	1.00	60.00	11.02	10.45	26.72	28.87	3.19	3.60
5	2.00	45.00	14.77	14.87	29.13	29.83	3.98	4.05
6	1.00	30.00	10.72	10.36	27.98	28.82	3.49	3.82
7	3.00	30.00	10.67	11.07	15.92	14.54	2.31	2.15
8	2.00	45.00	14.73	14.87	31.07	29.83	4.36	4.05
9	2.00	45.00	15.35	14.87	31.07	29.83	4.27	4.05
10	0.59	45.00	8.60	9.23	33.81	31.86	4.50	4.03
11	2.00	66.21	15.43	15.66	30.36	29.04	3.99	3.81
12	2.00	23.79	10.21	10.14	18.03	18.58	2.69	2.62
13	2.00	45.00	15.12	14.87	29.60	29.83	3.86	4.05

predictor is discarded. The R^2 is defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit (Haber & Runyon 1977) Coefficient of variation (CV) indicates the relative dispersion of the experimental points from the prediction of the model. Response surfaces and contour plots were generated with the help of commercial statistical package, Design Expert software (version 6.0 Stat-Ease, Inc).

The numerical and optimisations were also performed by the same software. Numerical optimisation technique of the Design-Expert software was used for simultaneous optimisation of the multiple responses. The desired goals for each variables and response were chosen. All the independent variables were kept within range while the responses were either maximised or minimised. The numerical optimisation finds a point that maximises the desirability function.

ANALYTICAL METHODS

The fungal mycelia were harvested by filtration through filter paper (Whatman no. 1) and washed with 200 mL of distilled water. The filtered mycelia were then stored for 24 at -4°C and freeze-dried for 24 for determination of dry weight. Dried mycelia were crushed using a pestle and mortar, followed by lipid extraction using chloroform/methanol 2:1 (v/v) (Folch et al. 1957). Lipid extracted was transesterified with 5% sodium methoxide methanol solution. The resultant colorless or pale-yellow transparent methyl esters were analysed by gas chromatography. A Shimadzu GC-14A gas chromatograph equipped with a 3.1 m glass column of 3.2 mm bore packed with Shimadzu Shinchrom E71 5%/Shimalite 80-100 was used. The instrument was fitted with a flame ionisation detector. Identification of peaks was based on standards from (Orbiting Scientific Technology Sdn Bhd).

RESULTS AND DISCUSSIONS

Response surface analysis was applied to the experimental data (Table 2) and the second order polynomial response surface model (Eq. 1) was fitted to each of the response variable (Yk). Regression analysis and ANOVA were conducted for fitting the model and to examine the statistical significance of the model terms. The lack of fit test measures the fitness of the model, did not result in a significant F-value in case of biomass concentration, lipid and GLA yield indicating that these models are sufficiently accurate for predicting those responses. The coefficient of determination (R^2) values for all responses are quite high (>0.84) indicating a high proportion of variability was explained by the data and the RSM models were adequate. As a general rule, the coefficient of variation (CV) should not be greater than 10%. In this study, the coefficients of variation were less than 10% for all the responses.

EFFECTS OF AMMONIUM TARTRATE AND GLUCOSE CONCENTRATION ON BIOMASS CONCENTRATION OF *CUNNINGHAMELLA* SP. 2A1.

The biomass production by *Cunninghamella* sp. 2A1 with different combinations of glucose and ammonium tartrate concentration varied from 8.60 to 18.58 g/L within the combination of variables studied (Table 2). Values predicted by the software were also close to the actual values obtained in the experiment. When RSM analysis was applied, quadratic model was shown to be the most significant ($p<0.05$) model describing the interaction of ammonium tartrate and glucose for biomass production, according to the sequential model of sum of squares table (Table 3).

From Table 4, it can be observed from ANOVA that both ammonium tartarate and glucose are significant in affecting the biomass concentration ($p<0.001$) at linear

TABLE 3. Sequential model of sum of squares

Responses	Model	Sum of Squres	DF	$(Mean)^2$	F Value
Biomass concentration (g/L)	Mean	2373.06	1	2373.06	
	Linear	71.22	2	35.61	11.26
	2F1	14.51	1	14.51	7.63
	Quadratic	15.23	2	7.62	28.41
	Cubic	1.26	2	0.63	5.08
Lipid yield, % (g/g biomass)	Mean	9542.36	1	9542.36	
	Linear	205.59	2	102.80	7.07
	2F1	54.11	1	54.11	5.34
	Quadratic	70.64	2	35.32	12.02
	Cubic	13.31	2	6.66	4.58
GLA yield (g/g lipidless biomas)	Mean	172.24	1	172.24	
	Linear	1.96	2	0.98	3.01
	2F1	1.13	1	1.13	4.80
	Quadratic	1.42	2	0.71	7.12
	Cubic	0.34	2	0.17	2.40

TABLE 4. Analysis of varians (ANOVA) for response surface quadratic model for biomass concentration (g/L) after 120 h fermentation

Sources	Sum of squares	DF	$(Mean)^2$	F Value	Prob > F
Model	100.97	5	20.19	75.32	< 0.0001
X_1	40.86	1	40.86	152.39	< 0.0001
X_2	30.37	1	30.37	113.28	< 0.0001
X_1^2	10.41	1	10.41	38.83	0.0004
X_2^2	6.75	1	6.75	25.18	0.0015
X_1X_2	14.51	1	14.51	54.13	0.0002
Lack of Fit	1.31	3	0.44	3.10	0.1512

$R^2 = 0.98$; Adjusted $R^2 = 0.97$; significant at $p<0.05$

terms (X_1 and X_2), interaction order (X_1X_2) and quadratic terms (X_1^2) except for glucose (X_2^2) which was significant at $p<0.01$ in quadratic term. This agrees to the fact that carbon and nitrogen source are crucial macronutrients necessary for growth as any deficiency in any of these nutrients could cause in cessation of growth. The value of R^2 for biomass concentration achieved (Table 4) was high (0.98) indicating that 98% proportion of variability was explained by the data and the RSM quadratic models were adequate.

The regression coefficients, variance inflation factor (VIF) and standard error for the quadratic model of biomass concentration, lipid and GLA yield are presented in Table 5. The value of VIF equals to one, indicating the factors were independent and the variance of the model was not inflated by the lack of orthogonality in the design. The estimated equation for the model according to regression analysis (Table 5) which describe the effect of the process variables on biomass production in terms of coded level of the variables are given as:

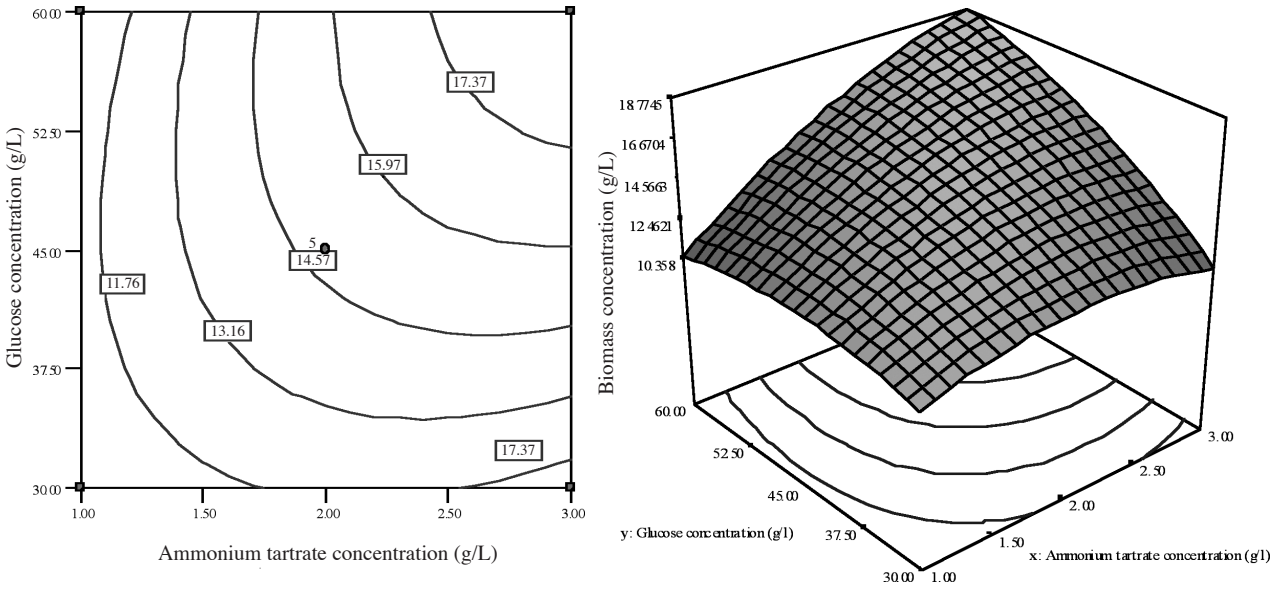
$$\text{Biomass concentration (g/L)} = 14.87 + 2.26X_1 + 1.95X - 1.22\,X_1^2 - 0.99\,X_2^2 + 1.90\,X_1X_2 \quad (2)$$

Biomass production was shown to have significant positive effects from ammonium tartrate and glucose concentration at linear terms (X_1 and X_2) indicating that the addition of these variables respectively increase the biomass production by *Cunninghamella* sp. 2A1. Addition of both ammonium tartrate and glucose concentration simultaneously in interaction terms (X_1X_2) also increases the biomass production. However, the negative coefficients of ammonium tartrate and glucose at quadratic terms indicated that biomass concentration decreases with the extra increase of these variables.

Figure 1 shows the biomass concentration of *Cunninghamella* sp. 2A1 as a function of ammonium tartrate and glucose concentration. Maximum biomass concentration of 17.37 g/L (Figure 1a) achieved when 2.4-3.0 g/L ammonium tartrate and 50-60 g/L glucose was used. To obtain the maximum optimum activity, the factor levels were set at the values given by the numerical optimisation process in the Design expert software under global solution of desirability that equal to one. The desired goals for each variable and response were chosen. One solution for optimum conditions of independent variables with the predicted values of responses was given by the software (Table 6). That is, ammonium tartrate were set

TABLE 5. Regression result of quadratic model for biomass, lipid and GLA production in coded unit

Responses	Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Biomass concentration	Intercept	14.87	1	0.23	14.32	15.42	
	X_1	2.26	1	0.18	1.83	2.69	1.00
	X_2	1.95	1	0.18	1.52	2.38	1.00
	X_1^2	-1.22	1	0.20	-1.69	-0.76	1.02
	X_2^2	-0.99	1	0.20	-1.45	-0.52	1.02
	X_1X_2	1.90	1	0.26	1.29	2.52	1.00
Lipid yield	Intercept	29.83	1	0.77	28.01	31.64	
	X_1	-3.46	1	0.61	-4.90	-2.03	1.00
	X_2	3.70	1	0.61	2.27	5.13	1.00
	X_1^2	-1.43	1	0.65	-2.97	0.10	1.02
	X_2^2	-3.01	1	0.65	-4.55	-1.47	1.02
	X_1X_2	3.68	1	0.86	1.65	5.70	1.00
GLA yield	Intercept	4.05	1	0.14	3.71	4.38	
	X_1	-0.26	1	0.11	-0.52	0.00	1.00
	X_2	0.42	1	0.11	0.16	0.69	1.00
	X_1^2	-0.26	1	0.12	-0.54	0.03	1.02
	X_2^2	-0.40	1	0.12	-0.69	-0.12	1.02
	X_1X_2	0.53	1	0.16	0.16	0.91	1.00



(a) (b)

FIGURE 1. Contour plots (a) and 3D response surface (b) for the effect of ammonium tartrate and glucose concentration on biomass concentration (g/L) of *Cunninghamella* sp. 2A1

at 2.99 g/L and glucose at 59.33 g/L incorporated in the culture medium. This global solution would result in 18.67 g/L biomass as a predicted value of response given by the software. Repeated experiments were performed for the production of biomass of *Cunninghamella* sp. 2A1 cultivated in the suggested optimised medium. The experimental results from the three replicates gave biomass concentration of 18.79 g/L, which is close to the predicted value. Therefore, according to the result, the optimal ammonium tartrate and glucose concentration was 2.99 g/L and 59.33 g/L, respectively.

TABLE 6. Optimum conditions of independent variables with the predicted values and actual values obtained after numerical optimisation process of responses given by Design Expert software (version 6.0 Stat-Ease, Inc)

	Concentration		biomass production (g/L)
	ammonium tartrate (g/L)	glucose (g/L)	
Goal	in range	in range	maximum
Expected value by *Design Experts*	2.99	59.33	18.67
Actual value	2.99	59.33	18.79

EFFECTS OF AMMONIUM TARTRATE AND GLUCOSE CONCENTRATION ON LIPID YIELD

Lipid yield (% g/g biomass) produced by *Cunninghamella* sp. 2A1 at different combinations of ammonium tartrate and glucose concentration are presented in Table 2. Minimum lipid yield (15.9%) was found to be when the cultures were grown in medium which contained ammonium tartrate at 3.0 g/L and 30.0 g/L glucose while maximum lipid yield (33.81%) was recorded when concentration of ammonium tartrate was 0.59 and glucose was 45.0 g/L. When RSM analysis was applied, quadratic model was shown to be the most significant ($p<0.05$) describing the interaction of ammonium tartrate and glucose for lipid yield, according to sequential model of sum of squares (Table 3).

It can be observed from ANOVA (Table 7) that ammonium tartrate and glucose are significant in affecting the lipid yield ($p<0.001$) at linear order (X_1 and X_2), while, quadratic terms of glucose (X_2^2) and contribution (X_1X_2) at interaction term are significant at $p<0.01$. However, ammonium tartrate gave no significant effect on lipid yield at quadratic terms (X_1^2). The quadratic model were adequate (94% proportion of variability) to explain the data according to the value of R^2 (0.94) for lipid yield (Table 7).

The estimated equation for the model according to regression result (Table 5) which describe the effect of the process variables on lipid production in terms of coded level of the variables are given as:

$$\text{Lipid yield (\% g/g biomass)} = 29.83 - 3.46X_1 + 3.70X_2 - 1.43\,X_1^2 - 3.01\,X_2^2 + 3.68X_1X_2 \quad (3)$$

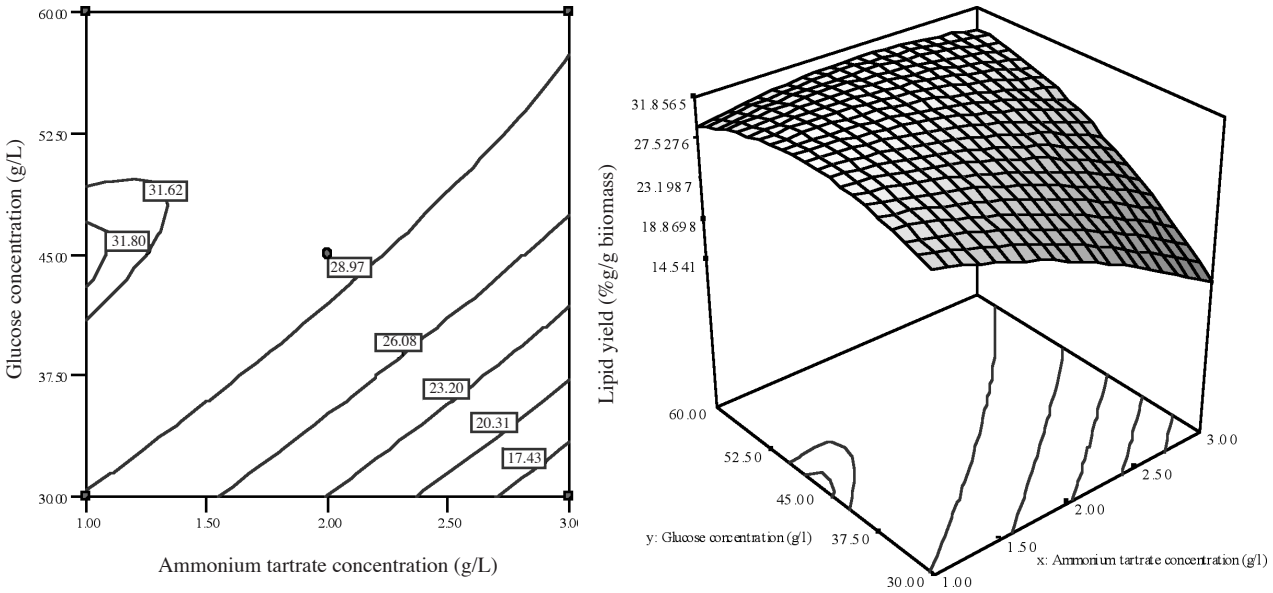
Lipid yield was shown to have significant positive effects from glucose concentration at linear terms (X_2) indicating that the addition of glucose concentration would increase lipid production by *Cunninghamella* sp. 2A1. Addition of both ammonium tartrate and glucose concentration simultaneously in interaction terms (X_1X_2) also increases the lipid production. However, the negative coefficients for ammonium tartrate at linear order as well as glucose and ammonium tartrate at quadratic terms indicated that lipid yield decreases with the extra increase of these variables.

Figure 2 shows the best concentration of ammonium tartrate for lipid production was 1 g/L at 45 g/L glucose concentration. The increase in ammonium tartrate concentration resulted in the decrease of the lipid yield of *Cunninghamella* sp. 2A1. Maximum optimum activity was obtained by setting the factor levels at the values given by the numerical optimisation process in the Design expert software under global solution of desirability that equal to one. The desired goals for each variable and response were chosen. One solution for optimum conditions of independent variables with the predicted values of responses was given by the software (Table 8). That is, ammonium tartrate were set at 1.00 g/L and glucose at 45.00 g/L incorporated in the culture medium. This global solution would result in 31.86% lipid (g/gbiomass) as a predicted value of response given by the software. Repeated experiments were performed for the lipid production by *Cunninghamella* sp. 2A1 cultivated in the suggested optimised medium. The experimental results from the three replicates gave lipid yield of 31.72% (g/g biomass) which gave no significant different with the value predicted by the software.

TABLE 7. Analysis of varians (ANOVA) for response surface quadratic model for lipid yield (% g/g biomass) after 120 h fermentation

Sources	Sum of squares	DF	(Mean)^2	F Value	Prob > F
Model	330.34	5	66.0676	22.4809	0.0004
X_1	96.00	1	96.0039	32.6674	0.0007
X_2	109.59	1	109.589	37.2899	0.0005
X_1^2	14.32	1	14.3202	4.87275	0.0630
X_2^2	62.95	1	62.9501	21.4201	0.0024
X_1X_2	54.11	1	54.1055	18.4105	0.0036
Lack of Fit	14.51	3	4.83624	3.19059	0.1459

R^2=0.94; Adjusted R^2= 0.90; significant at $p<0.05$



(a) (b)

FIGURE 2. Contour plots (a) and 3D response surface (b) for the effect of ammonium tartrate and glucose concentration on lipid yield (%g/g biomass) of *Cunninghamella* sp. 2A1

TABLE 8. Optimum conditions of independent variables with the predicted values and actual values obtained after numerical optimisation process of responses given by Design Expert software (version 6.0 Stat-Ease, Inc)

	Concentration		Lipid yield
	ammonium tartrate (g/L)	glucose (g/L)	(g/L)
Goal	minimum	in range	maximum
Expected value by *Design Experts*	1.00	45.00	31.86
Actual value	1.00	45.00	31.72

Therefore, compared to optimal values of both factors for biomass production, enhanced lipid yield occurred in cultures which had lower ammonium concentration (1 g/L). Lower nitrogen composition in the medium would limit growth and therefore more glucose was being converted into lipids. However, although excess carbon is crucial for enhanced lipid yield, optimum glucose concentrations was not achieved when glucose concentration was higher than 45 g/L where no significant differences in lipid yield was observed when glucose concentration was increased beyond 45 g/L (Figure 2b). This is also similar to what was reported in *Mucor circinellodes* where cessation of lipid accumulation occurred in a culture although glucose was still present in the medium. In *Mucor circinelloides*, this was shown to be caused by deactivation of malic enzyme, which is one of the key enzymes implicated in lipogenesis (Wynn et al.1999).

EFFECTS OF AMMONIUM TARTRATE AND GLUCOSE CONCENTRATION ON GLA YIELD

Sequential model of sum of squares (Table 3) shows that quadratic model was the most significant ($p<0.05$) model for describing the interaction of ammonium tartrate and glucose for GLA yield. Glucose concentration significantly positively affects GLA yield ($p<0.05$) at linear order (X_2), followed by interaction terms with ammonium tartrate (X_1X_2) and quadratic terms (X_2^2), while ammonium tartrate gave no significant effect at linear (X_1) and quadratic term (X_1^2). The value of R^2 for GLA yield achieved (Table 9) was 0.86 indicating that 86% proportion of variability was explained by the data and the RSM quadratic models were adequate.

The estimated equation for the model according to regression result (Table 5) which describe the effect of the process variables on GLA yield in terms of coded level of the variables are given as:

$$\text{GLA yield (\% g/g lipidless biomass)} = 4.05 - 0.30X_1 + 0.42X_2 - 0.22\,X_1^2 - 0.42X_2^2 + 0.53X_1X_2 \quad (4)$$

Similar to lipid yield data, GLA yield was also shown to have significant positive effects from glucose concentration at linear terms (X_2) indicating that the addition of glucose concentration increase the GLA production by *Cunninghamella* sp. 2A1. Addition of both ammonium

TABLE 9. Analysis of varians (ANOVA) for response surface quadratic model for GLA yield (g/g lipid less biomass) after 120 h fermentation

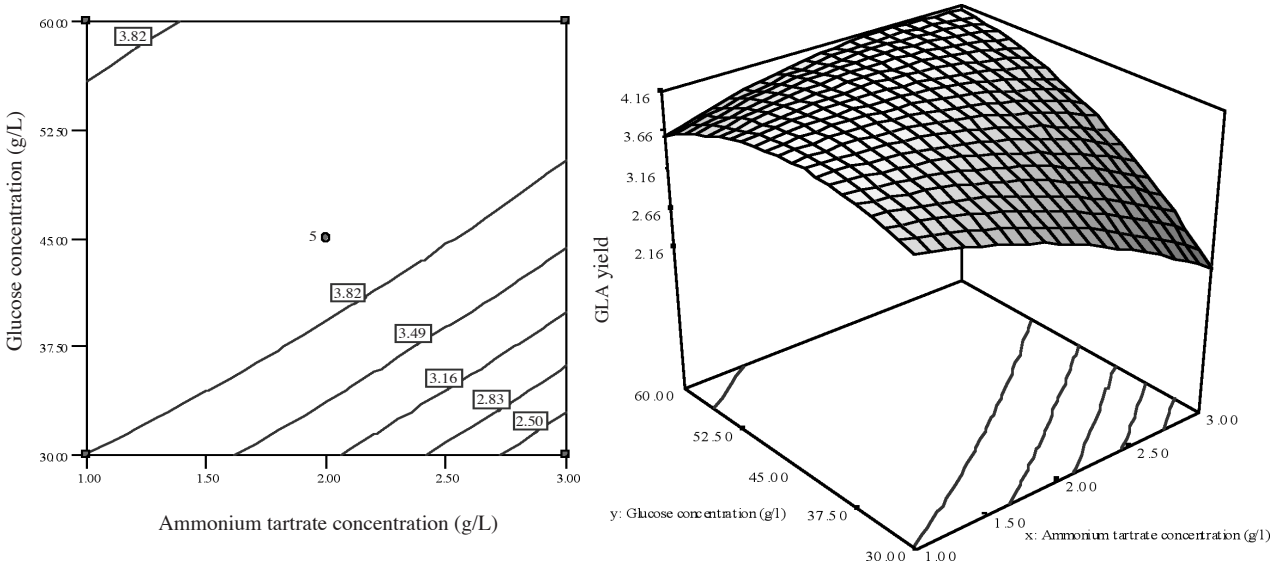
Sources	Sum of squares	DF	$(Mean)^2$	F Value	Prob > F
Model	4.51	5	0.90	9.04	0.0058
X_1	0.54	1	0.54	5.39	0.0532
X_2	1.42	1	1.42	14.23	0.0070
X_1^2	0.45	1	0.45	4.54	0.0705
X_2^2	1.13	1	1.13	11.33	0.0120
X_1X_2	1.13	1	1.13	11.33	0.0120
Lack of Fit	0.42	3	2.06	2.06	0.2488

R^2= 0.86; Adjusted R^2=0.77; significant at $p<0.05$

tartrate and glucose concentration simultaneously in interaction terms (X_1X_2) also increases the GLA production. Whereas, a negative linear terms (X_1) indicates that an increase in ammonium tartrate concentration would result in a decreased GLA yield. The best concentration of ammonium tartrate for GLA production as shown in Figure 3 was 1 g/L with the glucose concentration of 43 g/L and the increase in ammonium tartrate concentration resulted in the decrease of the GLA yield achieved.

Numerical optimisation was carried out for the process parameters for obtaining the optimal GLA production by *Cunninghamella* sp. 2A1. To perform this operation, Design Expert software (version 6.0 Stat-Ease, Inc) software was utilised for simultaneous optimisation of the multiple responses. The desired goals for each variable and response were chosen. One solution for optimum conditions of independent variables with the predicted values of responses was given by the software (Table 10). The solution has the maximum desirability value which was selected as the optimum conditions for GLA production by *Cunninghamella* sp. 2A1. A set of experiment was performed using the optimum condition suggested by the numerical optimisation process and the result obtained was presented in Table 10. There was no significant different between the results obtained compared to the responses predicted by the software showing the reproducibility of the data and the software was really useful in optimising ammonium tartrate and glucose concentration by this isolate.

Therefore, results obtained showed that condition that promotes GLA and lipid production was almost similar. As minimum GLA yield (0.023 g/g lipid less biomass) was produced by cultures which had ammonium tartrate concentration at 3.0 g/L and glucose concentration of 30.0 g/l this shows that increasing nitrogen content which promotes growth affected GLA production negatively, as it affects lipid yield.



(a) (b)

FIGURE 3. Contour plots (a) and 3D response surface (b) for the effect of ammonium tartrate and glucose concentration on GLA yield (× 10^{-2} g/g lipidless biomass) of *Cunninghamella* sp. 2A1

TABLE 10. Optimal conditions of independent variables with the predicted values and actual values obtained after numerical optimisation process of responses given by Design Expert software (version 6.0 Stat-Ease, Inc)

	Concentration		GLA yield
	ammonium tartrate (g/L)	glucose (g/L)	$\times 10^{-2}$ (g/g lipidless biomass)
Goal	minimum	in range	maximum
Expected value by *Design Experts*	1	43	4.06
Actual value	1	43	4.15*

*biomass concentration = 11.43 g/L; lipid content = 31.06% (g/g biomass)

CONCLUSIONS

Central composite design was found suitable for the process optimisation of ammonium tartrate and glucose concentration for biomass, lipid and GLA production by *Cunninghamella* sp. 2A1. The quadratic models for biomass, lipid and GLA production obtained using Design Expert software (version 6.0 Stat-Ease, Inc*)* were found to be statistically significant. After optimisation of the process conditions by numerical optimisation methods, optimal glucose and ammonium tartrate concentration medium for biomass production differ to the medium composition for lipid and GLA production by *Cunninghamella* sp. 2A1. Ammonium tartrate at a concentration of 2.99 g/L and 59.33 g/L glucose are needed to obtain optimal biomass production (18.79 g/L) while 1 g/L ammonium tartrate and 43 g/L glucose are needed in obtaining optimal lipid (31.06% g/g biomass) and GLA (0.0415 g/g lipidless biomass) production. It can be concluded that more nitrogen source are needed for biomass production compared to lipid and GLA production and that limitation of nitrogen source and excess carbon source, up to a certain level, are important to the lipid and GLA biosynthesis. In addition, these results showed that enhancement of lipid and GLAproduction can possibly be achieved in a two-stage batch system, with medium designed for biomass production in the first stage, followed by lipid accumulating stage.

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