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Medium Optimization for the Production of Lipidless Biomass By *Cunninghamella* sp. 2A1 Using Response Surface Methodology

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ABSTRACT

A statistical design approach has been used to optimize the production of biomass by *Cunninghamella* sp. 2A1, evaluated based on lipidless biomass. A 2^3 full factorial central composite design (CCD) was chosen to study the combined effects of three factors; ammonium tartrate, peptone and glucose concentrations. The p-value for each factor was <0.05 suggesting that these factors have significant effects on the production of lipidless biomass. The production is represented by a linear model with p-value <0.0001. The optimized medium consisting of 3.86g/L ammonium tartrate, 55.84g/L glucose and 7.73g/L peptone predicted a lipidless biomass of 16.83g/L. Results from four replications based on the optimized medium produced an average of 18.48g/L lipidless biomass, which is in close agreement with the predicted value. The coefficient for glucose was the highest indicating it to be the most significant factor affecting lipidless biomass production.

Keywords: Medium optimization, *Cunninghamella* sp. 2A1, Lipidless biomass, Response surface.

INTRODUCTION

Polyunsaturated fatty acids (PUFA) play an important role as precursors for a variety of metabolites (such as prostaglandins and leukotrienes) that regulate critical biological functions. The first commercial-scale microbial lipid production was developed in 1985 in the United Kingdom using *Mucor circinelloides*, an oleaginous fungus producing lipid containing 15-18% γ-linolenic acid (GLA) (of total fatty acid) (Ratledge 1992). Production of lipid is very much dependent on medium composition and for a new isolate, this aspect needs intensive investigation especially in relation to biomass concentration and lipid content. Our preliminary data involving the investigation of four medium components (ammonium tartrate, peptone, yeast extract and glucose) indicated that ammonium tartrate, peptone and glucose affect the biomass production of *Cunninghamella* sp. 2A1 which is an important aspect in the optimization of lipid production (Siti Aminah *et al*., 2004). Data on lipidless biomass, usually not reported in literature, gave new insight into effect of medium composition on lipid production. Therefore, these three factors were chosen for further optimization for lipidless biomass production using response surface methodology (RSM).

Optimizations of media are normally carried out by varying one parameter at a time whilst keeping the others constant. RSM is a technique for studying the effect of several factors acting together and affecting the responses by varying them in a number of experiments (Maddon and Richard 1977). RSM had been successfully applied in the optimization of medium composition for the production of glucosyltransferase by *Aspergillus niger* (Lee and Chen 1997), optimization of growth medium for the production of CGTase from *Bacillus* sp. (Rahman *et al*., 2004; *Ibrahim et al.,* 2005) and optimization of culture medium for production of lovastatin by *Monascus ruber* (Chang *et al.,* 2002).

This study reports the application of RSM to optimize lipidless biomass production using oleaginous GLA-producing local fungal isolate, *Cunninghamella* sp. 2A1. The assessment of the actual biomass concentration was carried out based on lipidless biomass as lipid content contributed up to 30% (w/w) of biomass. The relationship between the selected factors (concentrations of ammonium tartrate, glucose and peptone), and their interactions and influences on the measured responses were established.

MATERIALS AND METHODS

Microorganism and culture condition

Cunninghamella sp. 2A1 was obtained from the School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia. The cultures were maintained at 4°C and were subcultured at regular intervals. Inoculum was prepared by transferring 1ml of spore suspension into 100ml of nitrogen-limited medium (Kendrick and Ratledge 1992) and incubating at 30°C, 250 rpm for 48 h. The composition (g/L) of medium comprises: ammonium tartrate, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001; $CuSO_4.5H_2O$, 0.0001; $Co(NO_3)_2.6H_2O$, 0.0001; $MnSO_4.5H_2O$, 0.0001. Glucose,

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30g/L was sterilized (121°C for 15min) and added separately.

10% (v/v) of the seed culture was used as inoculum for batch fermentation in a 500 ml flask containing 100 ml of medium. Medium composition was varied based on the experimental design using Design Expert Version 6.0.10 (Section 2.3.). Cultivation was then carried out at 250 rpm and 30°C for 120 h. Cultures were harvested after 120 h of fermentation and the biomass concentration and lipid content were determined.

Analytical methods

Determination of cell dry weight

Biomass was harvested by filtering 100ml of the culture through a filter paper (Whatman No.1), washing extensively with distilled water and freeze-drying for 24 h. The dry weight of cell was determined using a balance (AND GR-200). Lipidless biomass was calculated by subtracting the amount of lipid per liter culture from the biomass produced per liter culture.

Determination of ammonium and glucose concentration

Ammonium concentration was measured using indophenol method (Chaney and Marbach 1962). The glucose concentration was determined using a glucose oxidase Perid-test kit (Boehringer Mannheim). The optical density (OD) for ammonium and glucose determination was carried out at 625 nm and 500 nm (JASCO UV-VIS Spectrophotometer), respectively.

Extraction of lipid

Lipid was extracted using chloroform and methanol in a ratio of 2:1 (v/v) (Folch *et al.,* 1957) overnight before filtering. The filtrate was washed with 150 ml of NaCl (1%) followed by 150 ml of distilled water. The chloroform layer was obtained and evaporated using rotary evaporator (BUCHI Rotavapor R-124). Lipid residue was dissolved in a minimal amount of diethyl ether and transferred to a tared vial.

Experimental design

Experimental design was determined using Design Expert Software Version 6.0.10 (State-Ease Inc., Minneapolis, USA). A 2^3 full factorial CCD was used for three independent factors with six replication of the central points and six axial points, leading to a total of 20 sets of experiments. Low and high factor settings were coded as –1 and +1 respectively, the centre point was coded as 0 and the design was extended up to $+\alpha$ and $-\alpha$ (α=1.682) (Table 1). The value of alpha represents the distance from the centre of the design space to an axial point. The optimal concentrations of factors were obtained by a numerical optimization procedure and analysing the response surface plots (Myers and Montgomery 1995).

Table 1: Factor settings in the form of coded values

Factors	Level of factors				
	$-\alpha$	-1	0	+1	$+\alpha$
Ammonium tartrate (X_1, g/L)	1.32	2	3	4	4.68
Glucose (X_2, g/L)	23.18	30	40	50	56.82
Peptone (X_3, g/L)	2.64	4	6	8	9.32

From the experimental results, an approximate polynomial relationship for dependent factors of lipidless biomass production was obtained. The result of this design was used to fit a first-order model,

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \quad .....(1)$$

Y is the predicted response; β_0, β_I, are the constant coefficients, and X_i is the coded independent factor.

RESULTS AND DISCUSSION

Table 2 shows the predicted, actual and residual values of twenty runs. The results showed that the predicted values closely matched the actual values.

Model selection

Table 3 shows the sequential model sum of squares for the lipidless biomass and how terms of increasing complexity contribute to the total model. From that, the linear coefficient showed a significant result of p-value was <0.0001. P-values for two-factor interaction (2FI), quadratic and cubic models for lipidless biomass production were >0.05, meaning that the interactions among factors were not significant. This indicates that the linear model was accurate in describing or predicting the effect of significant factors on the production of lipidless biomass from *Cunninghamella* sp. 2A1.

Model fitting

ANOVA was used to evaluate the adequacy of the fitted model (Table 4). The fisher F-test with a very low probability value (<0.0001) for response (lipidless biomass) demonstrated a high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2) (Haalland 1989). The R-squared value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of one represents the ideal case at which 100% of the variation in the observed value can be explained by the model (Khuri and Cornell 1987). In this case, the value of R^2 for lipidless biomass was 0.9511 indicating that only 4.89% of the total variations were not explained by the model.

Table 2: Experimental design of 20 runs of 2^3 full factorial CCD with predicted, actual and residual values of each run

Run	Factors			Response		
	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	Lipidless biomass (g/L)		
				Actual	Predicted	Residual
1	-1	-1	-1	9.89	9.11	0.78
2	+1	-1	-1	9.68	10.12	-0.44
3	-1	+1	-1	12.85	13.37	-0.52
4	+1	+1	-1	14.72	14.37	0.35
5	-1	-1	+1	10.91	10.50	0.41
6	+1	-1	+1	11.10	11.50	-0.40
7	-1	+1	+1	14.69	14.76	-0.07
8	+1	+1	+1	15.37	15.76	-0.39
9	-α	0	0	11.51	11.59	-0.08
10	+α	0	0	14.07	13.28	0.79
11	0	-α	0	8.67	8.86	-0.19
12	0	+α	0	16.40	16.01	0.39
13	0	0	-α	11.28	11.27	0.01
14	0	0	+α	13.98	13.60	0.38
15	0	0	0	12.11	12.44	-0.33
16	0	0	0	10.25	12.44	-2.19
17	0	0	0	13.34	12.44	0.90
18	0	0	0	12.43	12.44	-0.01
19	0	0	0	13.06	12.44	0.62
20	0	0	0	12.40	12.44	-0.04

Table 3: Sequential model sum of squares for lipidless biomass (g/L)

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P-value	
Mean	2992.80	1	2992.80			
Linear	71.78	3	23.93	97.18	<0.0001	Suggested
2FI	0.90	3	0.30	1.30	0.3207	
Quadratic	0.18	3	0.059	0.20	0.8920	
Cubic	1.37	4	0.34	1.38	0.3611	Aliased
Residual	1.24	5	0.25			
Total	3068.28	19	161.49			

* 2FI - 2-factor interaction

Table 4: ANOVA for response surface linear model for lipidless biomass (g/L) after 120h fermentation

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
Model	71.78	3	23.93	97.18	< 0.0001
Ammonium tartrate	3.42	1	3.42	13.89	0.0020
Glucose	61.79	1	61.79	250.97	< 0.0001
Peptone	6.57	1	6.57	26.67	0.0001
Residual	3.69	15	0.25		
Lack of Fit	2.65	11	0.24	0.92	0.5897
Pure Error	1.05	4	0.26		
Correlation Total	75.48	18			

R^2=0.9511, R=0.9752, Adjusted R^2=0.9413 * significance (%) =p<0.05

The value of the adjusted R^2 is also high, which indicates a high significance of the model. A higher value of the correlation coefficient (R=0.9752) signifies an excellent correlation between the independent factors. An insignificant lack of fit indicated that the model fits the data. The lack of fit tests compares the residual error to the pure error from replicated design points. The lack of fit F-value of 0.92 for lipidless biomass implies it is not significant relative to the pure error.

The key to lipid accumulation lies in allowing the amount of nitrogen supplied to the culture to become exhausted which means that cell proliferation stopped. The excess available carbon continues to be assimilated by the cells and are converted directly into lipid (Ratledge 1997). Our results (Siti Aminah *et al.,* 2004) indicated that high lipidless biomass concentration does not necessarily correspond to high lipid content is also high. Although, lipid synthesis are not growth-associated, the lipid accumulation and yields however, could be significantly affected by the growth media in different species (Dyal *et al.,* 2005). Our results are in agreement with these reports and that there exist a ranged direct correlation between lipidless biomass and lipid content. An increase in lipidless biomass beyond the critical value does not result in a corresponding increase in lipid content.

Based on Table 4, the concentration of ammonium tartrate, glucose and peptone are significant factors ($p<0.05$) affecting lipidless biomass. Generally, nitrogen and carbon sources which are supplied from these medium components are required for biomass production (Stanbury *et al.,* 1984). The carbon source (glucose) was used as an energy supply for both biosynthesis and viable cell maintenance as well as for cell biosynthesis (Adour *et al.,* 2006). Nitrogen is known to be an essential component of nearly all complex macromolecules in fungal cells, such as protein, nucleic acids and cell wall components (Burkovski 2003). Our previous study showed that increasing the concentration of ammonium tartrate in medium led to an increase in biomass concentration (Siti Aminah *et al.,* 2004). Supplementation with a nitrogen source in a peptide form (peptone) was more positive for yeast metabolism, inducing higher biomass and metabolite production (da Cruz *et al.,* 2002). Other *Mortierella* sp. and *Mucor* sp. showed similar observation as determined in this study where glucose concentration of 30g/L up to 50g/L yielded good growth and lipid production (Wynn *et al.,* 2001).

From the regression equation, it is predicted that increasing the concentrations of ammonium tartrate (X_1), glucose (X_2) and peptone (X_3) should enhance lipidless biomass production. The regression equation of the model for lipidless biomass in terms of coded values showed significant positive linear effects for all three factors. The factor with the largest effect was glucose concentration (X_2) followed by peptone concentration (X_3) and ammonium tartrate concentration (X_1).

The regression equation:

$$\text{Lipidless biomass (g/L)} = 12.55 + 0.50\,X_1 + 2.13\,X_2 + 0.69\,X_3$$

The one factor plot for the lipidless biomass shows the linear effect of changing the level of a single factor with the other two being at their zero level (Figure 1-3). As can be seen, an increase in ammonium tartrate, glucose and peptone led to an increase in lipidless biomass production.

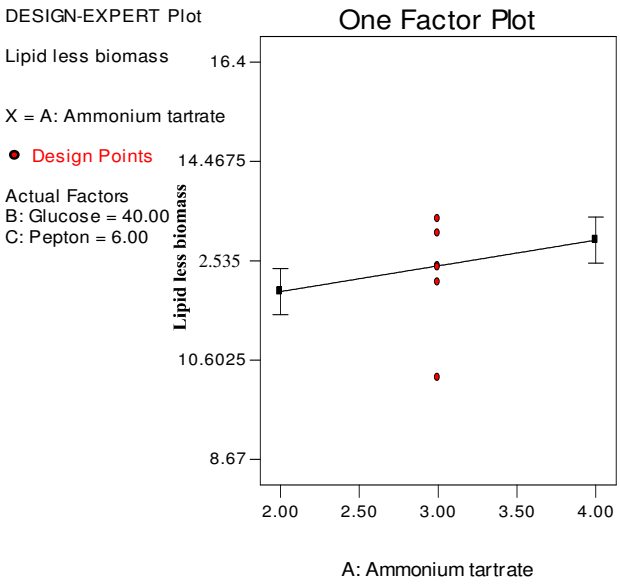

Figure 1: Effect of ammonium tartrate concentration on the lipidless biomass by *Cunninghamella* sp.2A1

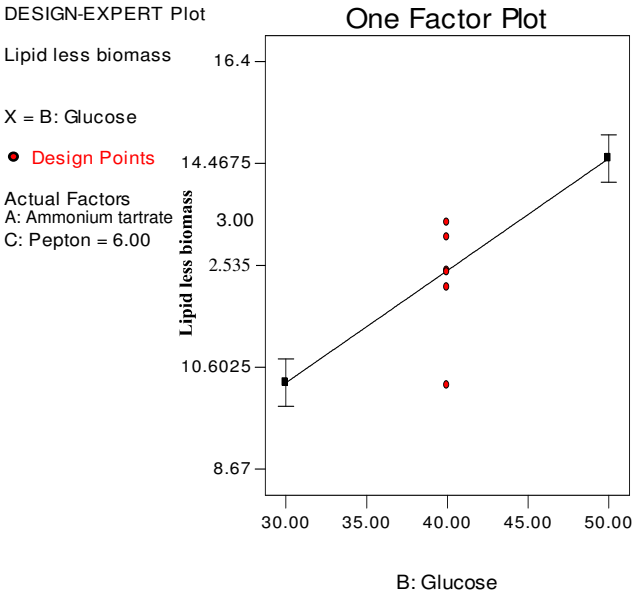

Figure 2: Effect of glucose concentration on the lipidless biomass by *Cunninghamella* sp.2A1

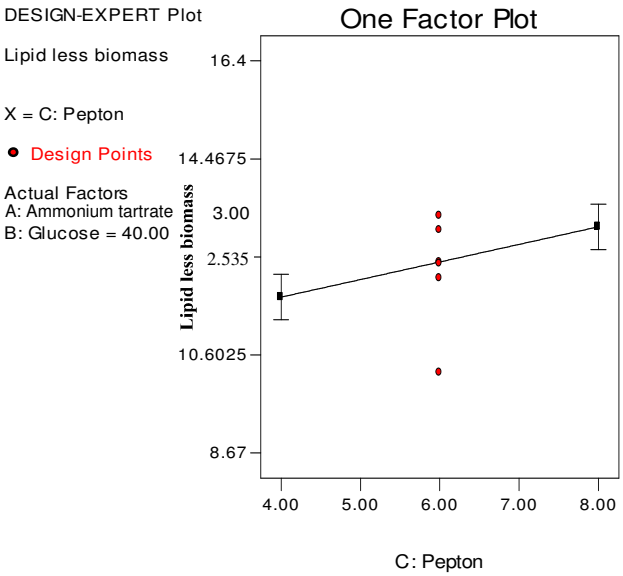

Figure 3: Effect of peptone concentration on the lipidless biomass by *Cunninghamella* sp.2A1

Numerical optimization of factors

Based on Table 2 (run no.12), the highest concentration of lipidless biomass (16.4g/L) and gave lipidless biomass yield (0.35g/g glucose utilized) from *Cunninghamella* sp. 2A1 was obtained when the concentration of ammonium tartrate, glucose and peptone were 3.0, 56.82 and 6.0g/L, respectively. To obtain the maximum optimum activity, the factor levels and response were set at the desired goal using Design Expert's Numerical Optimization with desirability level equal to one. Optimal concentrations of ammonium tartrate, glucose and peptone were established as 3.86g/L, 55.84g/L and 7.73g/L, respectively. This solution gives the predicted response for lipidless biomass as 16.83g/l. From four replications of the experiment under suggested optimal concentrations, an average lipidless biomass of 18.48g/L and lipidless biomass yield of 0.41g/g glucose utilized was achieved. This indicates an increase of 17% in efficiency of glucose utilization under optimized medium condition. The result correlates well with the predicted value and the model was proven to be adequate.

CONCLUSION

CCD and response surface methodology were useful to determine the optimum concentration levels of medium components that significantly influence the yield of lipidless biomass from *Cunninghamella* sp. 2A1. The final composition of the defined medium to produce 18.48g/L of lipidless biomass and lipidless biomass yield of 0.41g/g glucose utilized after the optimization procedure was as follows: 3.86g/L ammonium tartrate; glucose 55.84g/L and peptone 7.73g/L.

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