OPTIMIZATION OF LIPID PRODUCTION BY OLEAGINOUS YEAST USING RESPONSE SURFACE METHODOLOGY

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Abstract

The effects of growth parameters of *Rhodotorula graminis* TISTR 5124 in batch fermentation were studied and optimized for lipid production by using response surface methodology via a Box-Behnken Design. Values of the fermentation parameters affecting the lipid production were varied as follows: carbon sources (glucose, glucose and glycerol and glycerol), temperatures (28, 30, and 32°C) and shaking speeds (150, 200, 250 rpm). Fermentation was carried out in 100 mL Erlenmeyer flasks with a 24 h cultivation time. After eliminating insignificant terms, we found that a good fit (R^2 = 0.7555) for lipid production was given by the quadratic regression relationship:Lipid = (4.00* Temperature)- (4.75*Carbon) + (0.70*Shaking speed) – (0.02*Temperature*Shaking speed) -116.0. The results showed that lipid production was significantly influenced by carbon source ($p < 0.0001$). For the range of conditions studied, we found that the highest yield of lipid was 17.40 g/L, which was obtained using glycerol as sole carbon source at 10 g/L, a temperature of 28°C and a shaking speed of 239 rpm. We found that a good fit (R^2 = 0.9107) for the biomass production results was given by the quadratic regression relationship: Biomass = 64.29 -(41.13*Carbon) – (0.19* Temperature) – (0.48*Shaking speed) + (1.38*Carbon* Temperature) – (0.03* Carbon*Shake speed) + (1.28E - 003* Shake speed 2). The highest biomass concentration produced was 27.99 g/L, which was obtained using glycerol as sole carbon source, a temperature of 28°C and a shaking speed of 239 rpm. The mathematical relationship of lipid production can be scaled up to apply to fermentation in a laboratory scale bioreactor.

Keywords: Rhodotorulagraminis, response surface methodology, lipid, oleaginous yeast

Introduction

The second generation biodiesel is currently received significant attention especially the oils from the microbial cells through transesterification to replace the traditional technologies that are based on oil crops and plants. However, these agricultural materials

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are inadequate as their production capacity and rate of production are limited. This alternative fuels can be obtained from oleaginous microorganisms that produce lipids up to the 60% of the total cell dry weight under cultivation conditions (Li *et al*., 2008). Approximately 1500 species of yeasts belonging to over 100 genera have been described so far (Satyanarayana and Kunze, 2010). Yeast species are very efficient in the accumulation of intracellular oil as many reports of the anamorphic genus *Rhodotorula* have often been used as biological catalyze to convert renewable resources into microbial oils (Ageitos *et al*., 2011; Beopoulos *et al*., 2011; Kosa and Ragauskas, 2011) in form of neutral lipids as triacylglycerols (TAGs). The derivatives of long-chain fatty acid methyl estersand alkanesin TAGs have potentially explored as raw materials for biofuels (Li *et al*., 2010; Madsen *et al*., 2011). However, microbial oils are different in fatty acid compositions since the oleaginous yeasts are species dependence. The diverse composition of the medium affected not only the lipid/ biomass yield, but also the TAGs composition, in terms of ration of saturated, monounsaturated, and polyunsaturated fatty acids (Easterling *et al*., 2009). Although glucose is a very good carbon source for lipid production with oleaginous yeasts, other alternative sources of wastes and by-products of argo-industrial residues have a great potential to exploite as substrate. Nowadays the lipid production is focused on selection and development of yeasts as converters of crude glycerol into lipid for biodiesel production, since it is the major side-product of the biodiesel production process. In order to optimize the production of lipidby *Rhodotorula graminis* TISTR 5124, the effects of growth parameters of this strain in batch fermentation were studied and optimized through a multivariate approach for lipid production by using response surface methodology via a Box-Behnken design.

Materials and Methods

Oleaginous Yeast Inoculums Preparation and Morphological Study

R. graminis TISTR 5124 were obtained from Culture Collection of Thailand Institute of Scientific and Technology Research (Bangkok, Thailand). Yeast cultures were maintainedon yeast extract-malt extract (YM) agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose and 20 g/L agar) and stored at 4°C. Cells were cultured on yeast malt broth (YMB) medium to the exponential growth phase and used as seed culture to a designed medium. Single colony of yeast culture incubated at 30°C for 48 h was observed and photographed by using the light microscopy with amplification at 1000x.

Substrates, Fermentation Conditions and Determination of Biomass and Lipid Content

Crude glycerol was obtained as by-product of biodiesel production process from KOH-catalyzed transesterification of commercial palm oil, Pathum Vegetable Oil Co., Ltd, Thailand. A preliminary chemical analysis of the crude glycerol showed 85.90% glycerol, 4.8% NaCl, 9.9% moisture and 0.04% of fatty acid and esters, pH 6.4 with the density of 1.2564 g/cm^3. Seed preparation was transferred from the stock solution to YM agar slants and incubated for 2 days at 30°C. Following this period, colonies were transferred into the YMB broth and incubated for 48 h before transferring the 10% of inoculums in to three levels of carbon sources, which contained thedifferent compositions of carbon sources in 250 mL Erlenmeyer flasks containing 100 mL of media as the following in g/L: Medium I (glucose 10, yeast extract 3, malt extract 3 and peptone 5); Medium II (glucose 5 g with 0.197 ml of crude glycerol, yeast extract 3, malt extract 3 and peptone 5) and Medium III (0.401 mL of crude glycerol, yeast extract 3, malt extract 3 and peptone 5). The final pH of all there medium were adjusted to 7. These three Mediums contributed towith the three levels of coded values of -1, 0 and 1, respectively. Cultures were then incubated at three temperature levels: 28, 30, and 32°C in an orbital shaking incubator with three levels of shake speeds: 150, 200, and 250 rpm for

48 h. Each sample was analyzed in triplicate. Biomass was determined by measuring the dry weight after drying at 75°C for 5 h to constant weight. To determine total fatty acid, lipids were extracted from cells according to Folch *et al*. (1957) using chloroform/methanol system. Cellular lipid content was reported as total fatty acid content.

Response Surface Methodology

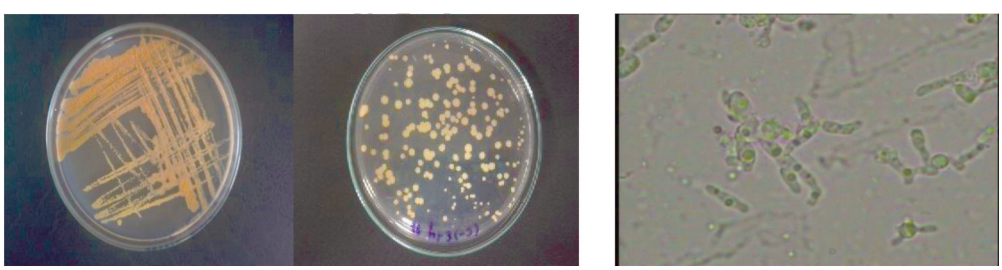
Response surface methodology was adopted for the production of lipid to derive a statistical model for the individual and interactive effects of carbon source, temperature and shaking speed. Levels of these factors were optimized for maximum lipid production (the response) using the Box-Behnken statistical design (Box and Behnken 1960). Table 1 represents a 17-trial experimental design, where each variable was tested in three different coded levels: low (–1), middle (0) and high (+1). When lipid content in biomass cells were measured, a second-order polynomial model was fitted to the response data obtained from the design. The polynomial equation is in the following form:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_i X_i X_j + \Sigma\beta_{ii} X_i^2 \quad (1)$$

where X_1, X_2 and X_3, represent the coded levels of the independent variables as described in Table 1 and β_0, β_i, and β_j (i, j = 1, 2, 3, 4) are the coefficient estimates, where, β_0 is the interception term, β_i is the linear term, β_{ii} is the quadric term, and β_{ii} is the interaction term. For the predicted responses, y_1 stands for lipid content whereas y_2 stands for biomass concentration. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination (R^2). The experimental data was analyzed using the statistical software, Design-Expert software version 8.0.6 (STAT-EASE Inc., Minneapolis, MN, USA), for regression analysis to fit the equations developed and also for the evaluation of the statistical significance of the equations.

Results

R. graminis TISTR 5124 (UPCC 2004) belongs to the species of the ballistoconidia-forming genera. This strain was isolated from a decaying fruit. It grewon YM agar after 24 h of 30°C incubation and showed orange, smooth, glistening, convex and the entried edge (Figure. 1(a)). This oleaginous red yeast accumulatee both lipid and carotenoid as observed in Figure 1(a, b). Cells were observed and showed single or in pair ovoidal to globose (Figure 1(b)). As shown in Figure 1(b), some cells were elongate, fusiform, curved, and some showed the reproduction stage by polar budding, single or in pairs and contain oil droplets.



(a) (b)

Figure 1. Microscopic morphology of *R. graminis* TISTR 5124 (a) growth on YM agar, (b) cells observed under a light microscope (1000x)

In order to investigate the ability of consumption of carbon sources by *R. graminis* as Oleaginous yeast by batch cultures in shake flasks were performed on two different carbon sources with the different levels of shaking speeds and temperatures. The data reported in Table 1 were converted into a second order polynomial equation with three independent variables forthe lipid production model. The models were then submitted to statistical analysis (ANOVA) to neglect all terms that were statistically non-significant ($P > 0.01$). As shown in Table 2, where the model coefficients are listed together and associated probabilities, in case of biomass and lipid production we can explore 91.00 and 75.55 of the variation, respectively.

This study shows that the three independent variable (carbon source, temperature and shaking speed), that were chosen, only two fermentation parameters, carbon source and shaking speed had a substantial influence on biomass production. Moreover, the interactive effects of carbon source and temperature and carbon source and shaking speed had the positive effect to biomass production. In case of biomass production had only two fermentation parameters affected to the yields which are carbon source and shaking speed with the p-values < 0.0001 and 0.0082, respectively, while no main effect of the temperature was significantly detected (Table 2). The second-order polynomial model for biomass and lipid production as a function of carbon source, temperature and shaking speed generated a regression

Table 1. Experimental design for lipid production

Run	Factor 1:X_1	Factor 2:X_2	Factor 3:X_3	Res.1:Y_1	Res 2:Y_2
	Carbon (g/L)	Temperature (^oC)	Shake speed (rpm)	Biomass (g/L)	Lipid (g/L)
1	Glucose+glycerol	32	250	18 ± 0	6 ± 0
2	glycerol	32	200	10 ± 4.24	4 ± 1.41
3	Glucose+glycerol	30	200	15 ± 5.66	9 ± 2.83
4	Glucose	32	200	16 ± 2.83	10 ± 4.24
5	Glucose+glycerol	30	200	13 ± 1.41	8 ± 0
6	glycerol	30	150	12 ± 1.41	6 ± 1.41
7	glycerol	28	200	7 ± 0	3 ± 0
8	Glucose	28	200	24 ± 5.66	16 ± 1.41
9	Glucose+glycerol	30	200	14 ± 1.41	7 ± 1.41
10	Glucose	30	250	28 ± 2.83	18 ± 1.41
11	Glucose+glycerol	28	150	14 ± 2.83	5 ± 2.83
12	Glucose+glycerol	32	150	17 ± 4.24	8 ± 2.83
13	Glucose+glycerol	30	200	16 ± 2.83	10 ± 4.24
14	Glucose	30	150	21 ± 0	14 ± 2.83
15	glycerol	30	250	13 ± 1.41	7 ± 4.24
16	Glucose+glycerol	28	250	19 ± 1.41	12 ± 4.24
17	Glucose+glycerol	30	200	16 ± 1.41	9 ± 2.83

relationship as given in Equations 2 and 3.

For biomass production; Biomass (y_1) = *64.29-41.13*Carbon-(0.19*Temp)-(0.48* Shaking speed) + (1.38 *Carbon*Temp) - (0.03 *Carbon*Shake speed) + (1.28E- 003* Shake speed²)*

$$(R^2 = 0.9107) \quad (2)$$

Normally lipid production requires a medium with an excess sugar or similar components, in this case once compared between glucose and glycerol once carbon to nitrogen (C/N) ratio of the culture in these experiments kept in the same mole ratio. Result found that glycerol as a carbon source was more preferable than that of glucose for the lipid production. Figure 2 gives the contour plots for biomass production at varying temperature/carbon values (a), carbon sources/shaking speed values and shaking speed/temperature (c), respectively.

For lipid production; Lipid (y_2) = *-116.06-4.75*Carbon+ (4.00*Temp) + (0.70* Shaking speed) - (0.02 Temp * Shaking speed)*

$$(R^2 = 0.7555) \quad (3)$$

In order to give a visual overview of the multivariate influence of cultivating conditions on biomass and lipid production, selected response surface diagrams were plotted (Figure 2(a-f)). From a geometrical point of view, the response of biomass exhibited a convex-shaped upward pattern suggesting a synergistic effect between the increasing glycerol composition in media from the glucose as carbon source and the decreasing of temperature (Figure 2(a)). For lipid production, the yield was significantly influenced by the carbon source with the p-value of < 0.0001. The maximum production of lipid content at 17.405 g/L can be seen to occur at 27.99 g/L of biomass by using glucose as sole source under a temperature of 28°C and a shaking speed of 239 rpm. The relationship of the effect of influencial parameters under optimization condition (Figure 3(a,b)), temperature and shaking speed with the positive effect on both biomass and lipid production on this oleaginous yeast.

Table 2. Estimated coefficients of multiple determinations (R2) for biomass and lipid production using coded values

Term **(A) Biomass [S = / R^2 = 0.91 / $R^2_{(ad)}$ = 0.9743]**	**Coefficient**	**SE coefficient**	**p value**
Carbon source	- 41.13	9.16	< 0.0001
Temp.	- 0.19	9.16	0.4977
Shake speed	- 0.48	9.16	0.0082
Carbon source × Temp.	1.38	10.24	0.0045
Carbon source × Shake speed	- 0.030	10.24	0.0746
(B) Lipid [S= /R^2 = 0.7555 / $R^2_{(ad)}$ = 0.9858]			
Carbon source	0.1	0.029	< 0.0001
Temp.	0.97	0.029	0.1898
Shake speed	- 0.081	0.029	0.1079
Temp. x Shake speed	0.07	0.033	0.0472

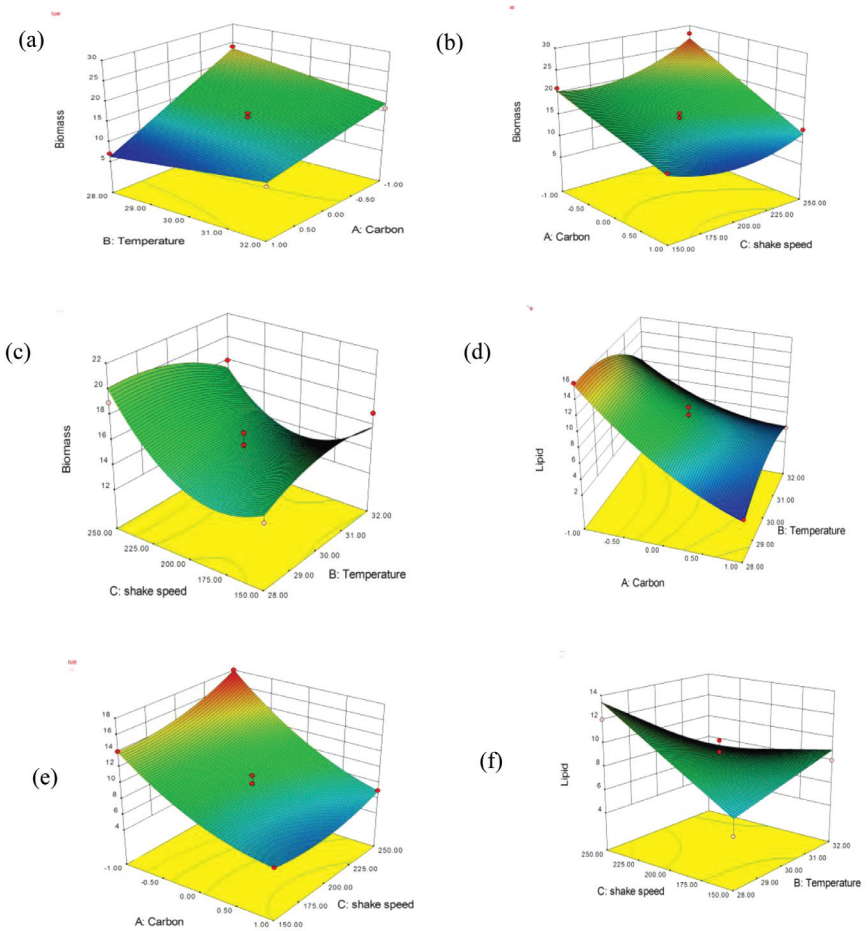


Figure 2. The contour plots for biomass production at varying (a) carbon source and temperature, (b) carbon source and shaking speed, (c) shaking speed and temperature and for lipid (fat) (d) carbon source and temperature, (e) carbon source and shaking speed, (f) shaking speed and temperature. Under this model, the optimum biomass of 30 g/L was obtained once the fermentation broth contained the glycerol as carbon source and the experiment was run under 28°C with 250 rpm shaking speed. This condition would yield 60% of lipid (18.19 g/L)

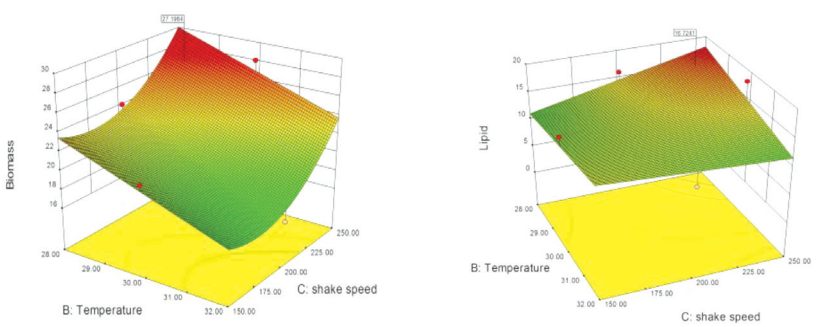


Figure 3. Response surface plots for the effect of temperature and shaking speed on biomass (a) and lipid (b)

Discussion

Response surface methodology application to use glucose and glycerol for improving the growth and production of oleaginous yeast under the reduction of process variability have been studied by several researchers (Li *et al*., 2007; Emaily *et al*., 2009; Saenge *et al*., 2011). The *R. graminis* strains showed a wide range of carbon sources utilization and growing on hexoses and pentosessuch as galactose, xylose, mannose and cellobiose as well as from glycerol (Galafassi *et al*., 2012). Therefore, it is a suitable candidate oleaginous microorganism as abiological catalyst for application touse in fermentation processes by using renewable sources industrially-derived crude glycerol. The utilization of glycerol has been studied and explained the mechanism that it passes into the microbial cell by facilitated diffusion and is assimilated via either the phosphorylation or the oxidative pathway (Papanikolaou and Aggelis, 2011; Saenge *et al*., 2011). This yeast strain produced the fatty acids profiles which were similar to butanoic acid (C4:0)) and capric acid (C10:0), with palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) as the minor component (data from fatty acid analysis, GCMS is not shown). In general, de novo synthesized yeast lipid is composed of C16 and C18 fatty acids. Plamitic acid (C16:0) constitutes the 15-25% w/w, oftotal lipids, whilst palmitoleic ($^{\Delta 9}$C16:1) is, in general, presented inpercentages inferior than 5% w/w. Likewise, stearic acid (C18:0) is generally a minor component of the yeast lipid (5-8% w/w). Oleic acid ($^{\Delta 9}$C18:1) is the principal fatty acid accumulated inside the yeast cells (amounts sometimes higher than 70% w/w), while linoleic ($^{\Delta 9,12}$C18:2) is found in the second position (15-25% w/w). More unsaturated fatty acids (e.g. α-linolenic acid -$^{\Delta 9,12,15}$C18:3) are not frequently synthesized into the yeast lipid reserves (Davies, 1988; Ratledge, 1994; Aggelis *et al*., 1996; Papanikolaou and Aggelis, 2009, 2011) This the significantly influence of temperature and shaking speed finding about on the biomass production resulting in higher lipid productivities production is in agreement with other reports (Li *et al*., 2008; Ageitos *et al*., 2011). This indicates that optimum temperature and oxygen availability is an important parameter affecting lipid synthesis. The concern of optimization to use the renewable substrate especially raw glycerol from the biodiesel production is still in interest of many researchers because the cost of feed stock or carbon source required for the production of microbial lipids accounts for 60 to 75% of the total costs of the biodiesel. Thus, the cost of lipid production was influenced strongly by the cost of medium nutrients (50%) needed for cultivation of cells and the cost of solvent (25%) for the extraction of lipids from biomass (Subramaniam *et al*., 2010). Economic analyses have indicated the need to minimize costs of medium components and for further research dealing with microbial systems capable of producing lipids at relatively high productivities in minimal media

Conclusions

The model describing the relationship between carbon sources both glucose and crude glycerol, temperature and shaking speed to lipid production by *R. graminis* is in good agreement with experimental data and the high R^2 values for the equations expressing the biomass and lipid production well support our model, and make it a valuable tool in optimizing the production of microbial oil by acting on selected cultural conditions.

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