Optimization on Yeast Lipid Production of Psuedozyma sp. with Response Surface Methodology for Biodiesel Manufacturing

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***Abstract*—The microbial lipid was interested to be an alternative feedstock of biodiesel production. The lipid producing condition of oleaginous yeast *Psuedozyma* sp. was optimized. The response surface methodology (RSM) was applied with central composite design (CCD) in this study. The experiment results showed that a second-order polynomial regression equation was achieved with good coefficient of determination in analysis of variance. The validation tests were performed and the experimented results were not significant different to the predicted value, thus confirming the reliability of empirical model in describing lipid production. Moreover, the fatty acid compositions were investigated. The results revealed that the accumulated lipid mainly consisted of 85% in C16:C18 fatty acids. In addition, yeast biodiesel properties were predicted and also provided a satisfactory property. It was suggested that lipid of *Psuedozyma* sp. was suitable to be an alternative feedstock for biodiesel manufacturing.**

***Index Terms*—oleaginous yeast, microbial biodiesel, response surface methodology, biodiesel property.**

I. Introduction

The diminishing of fossil fuels present highly effect with oil price in long term due to the increasing cost of exploring and extracting petroleum fuel [1]. The exhaust gases from petroleum oil also impact with environment pollution. Biodiesel has attracted attention as a renewable energy and environmentally friendly fuel because it can decrease the emission of sulfur, carbon monoxide, smoke and also hydrocarbon compounds during the combustion process of diesel engines [2], [3].

Biodiesel can be produced by tranesterification of triacylglycerols (TAGs) from plant oil or renewable biomass with short chain alcohol especially methanol. TAGs will be transformed to fatty acid methyl esters (FAMEs) and glycerol as a main product and by-product, respectively [4]. However, the cost of biodiesel is high due to the cost of the raw material (70-75% of total cost). An inexpensive raw material for biodiesel making could decrease the total production cost [5]. Microbial lipids that are produced by oleaginous microorganisms including microalgae, bacteria, fungi and yeasts are attracted interesting as promising potential feedstock for biodiesel production due to their similar fatty acid compositions to those of vegetable oils [6]. Moreover, microbial lipid production is not depended on weather and arable land when compared with the production of vegetable oils [7].

At present, oleaginous yeasts are applied to use as lipid producer for biodiesel production due to the growth and lipid production rate of yeast are higher than microalgae. Yeast does not require sunlight for photosynthesis. In addition, the cultivation of yeast can be operated with conventional bioreactor. Several kinds of inexpensive raw materials such as cellulosic material and agro-industrial waste can be applied as the raw materials for lipid production from oleaginous yeast [8], [9].

The conventional technique to optimize factors of process is one parameter variable at a time and keeping other remaining parameters constant. This technique also takes more time to experiment and has poor efficiency for optimizing a lot of factors [10]. This approach lacks the absoluteness to predict response variable under un-experimented trials of independent variables. In addition, the interaction amongst these variables is not investigated [11], it does not depict the complete effects of the parameters on the process, and might lead to incorrect conclusion. In order to overcome this problem, optimization studies can be carried out using response surface methodology (RSM) [12].

The objective of this work was to optimize the level of three important variables (glucose, $(NH_4)_2SO_4$, and KH_2PO_4) for lipid production by shaken cultures of an oleaginous yeast strain *Psudozyma* sp. using RSM designed with central composite design (CCD).

II. Materials and Methods

A. Microorganism and Inoculum Preparation

Psudozyma sp. in this work was screened from pomelo skin and long-term kept in 75% of glycerol at -80 ℃. Firstly, *Psudozyma* sp. was activated by cultivation in

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yeast extract-malt extract medium (YM) with shaking rate 150 rpm at 30 ℃ for 24 h. Subsequently, 2.5 mL of activated culture was inoculated to 50 mL seed medium containing (in g/L): glucose 20, $(NH_4)_2SO_4$ 5, KH_2PO_4 1, $MgSO_4$ $7H_2O$ 0.5, and yeast extract 0.5 and incubated under similar conditions for 20 h.

B. Lipid Production

Five milliliter of seed was transferred into 500 mL Erlenmeyer flask containing 95 mL of the lipid production medium containing (in g/L): $MgSO_4$ $7H_2O$ 1.5, Na_2HPO_4 2.0, and yeast extract, 1.0 while glucose (x_1), $(NH_4)_2SO_4$ (x_2), and KH_2PO_4 (x_3) concentration were varied to five levels as per the design of the experiment in Table I. The batch fermentation was carried out at 150 rpm and 30 ℃.

TABLE I. LEVEL OF THE FACTOR TESTED IN CCD

Factor	Symbol code	Level of factors				
		-1.682	-1	0	1	1.683
glucose (g/L)	(x_1)	20.0	40.3	70.0	99.7	120.0
$(NH_4)_2SO_4$ (g/L)	(x_2)	0.5	1.4	2.8	4.1	5.0
KH_2PO_4 (g/L)	(x_3)	0	1.4	3.5	5.6	7.0

C. Optimization by Response Surface Methodology

A CCD of RSM was operated to optimize the three important factors (glucose, $(NH_4)_2SO_4$, and KH_2PO_4) for enhancing lipid production efficiency of *Psudozyma* sp. The three independent factors were studied at five levels (-α, -1, 0, 1, α) (Table II). A total of 19 experiments were conducted. All factors were taken at a central code value, which was considered as zero. The minimum and maximum ranges of the factors were used. The biomass concentration, lipid concentration, lipid productivity, and lipid content were noted as response values (y), and each trial was the average of the triplicate.

D. Statistical Analysis and Modeling

The data obtained from RSM on lipid productivity were subjected analysis of variance (ANOVA). The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order polynomial equation (1):

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response, x_i and x_j are independent factors, β_o is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient. However, in this study, the independent variables were coded as x_1, x_2, and x_3. Thus, the second order polynomial equation can be presented as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (2)$$

Design-Expert, Version 7.0 trial version (STAT-EASE Inc., Minneapolis, USA) was used for the experimental design and statistical analysis of the experimental data. The ANOVA was used to estimate the statistical parameter. The Fisher's test was used to evaluate the statistical significant of the model equation and the model term. The quality of model fitting of the second-order polynomial equation was determined via the coefficient of determination (R^2). The fitted polynomial equation was presented in the form of contour plots to present the relationship between the responses and the experimental levels of each of the variables. The optimum level of each variable was expressed to explore the maximum response.

TABLE II. DESIGN AND RESPONSE OF THE CCD

Run	x_1	x_2	x_3	Responses			
				y_1	y_2	y_3	y_4
1	-1	-1	-1	8.58	3.97	0.79	46.21
2	1	-1	-1	9.66	4.36	0.87	45.13
3	-1	1	-1	9.48	4.55	0.91	47.94
4	1	1	-1	10.44	5.00	1.00	47.85
5	-1	-1	1	10.26	3.85	0.77	37.52
6	1	-1	1	10.82	4.82	0.96	44.50
7	-1	1	1	8.54	3.83	0.77	44.79
8	1	1	1	12.86	5.17	1.03	40.20
9	-1.682	0	0	6.96	2.74	0.55	39.30
10	1.682	0	0	12.02	5.56	1.11	46.26
11	0	-1.682	0	7.92	3.73	0.75	47.10
12	0	1.682	0	12.34	4.66	0.93	37.72
13	0	0	-1.682	6.64	4.12	0.82	61.97
14	0	0	1.682	11.84	5.26	1.05	44.38
15	0	0	0	8.22	3.49	0.70	42.46
16	0	0	0	8.46	3.64	0.73	42.97
17	0	0	0	8.64	3.75	0.75	43.35
18	0	0	0	8.04	3.45	0.69	42.85
19	0	0	0	8.74	3.75	0.74	42.51

E. Analytical Method

Culture medium was centrifuged at 10,000 rpm for 5 min. The supernatant was separated and kept at -20 ℃ for glucose analysis. The biomass was cleaned with water and centrifuged twice. Then, the washed cells were dried at 60 ℃ until weight was constant (usually after 24 h). The biomass was determined gravimetrically [13].

The total lipids within the cell were extracted with a mixture of chloroform and methanol according to modified Bligh and Dyer method [14]. Lipid content was expressed as gram lipid per gram dry biomass. The fatty acid compositions were determined by gas chromatography (GC) in fatty acid methyl esters (FAMEs) form.

F. Prediction of Cetane Number, Density, Kinematic, Viscosity, and Higher Heating Value of Biodiesel from Yeast

Cetane number, density, kinematic viscosity and higher heating value were calculated from the mass fraction of individual fatty acids using Equation of Reference [15].

III. RESULTS AND DISCUSSION

A. Optimization of Three Independent Variable Using Response Surface Methodology

The experimental runs and results for the CCD were shown in Table II. The 19 runs in a single block were used to study the effects of three factors on four response variables. For all combinations examined, biomass concentration ranged from 6.96 g/L to 12.34 g/L, lipid concentration varied from 2.74 g/L to 5.56 g/L, lipid productivity varied from 0.55 g/(L day) to 1.11 g/(L day), and lipid content differed from 37.52% to 61.97%.

TABLE III. ANALYSIS OF VARIANCE (ANOVA) FOR THE PARAMETERS OF RESPONSE SURFACE METHODOLOGY FITTED TO QUADRATIC MODEL

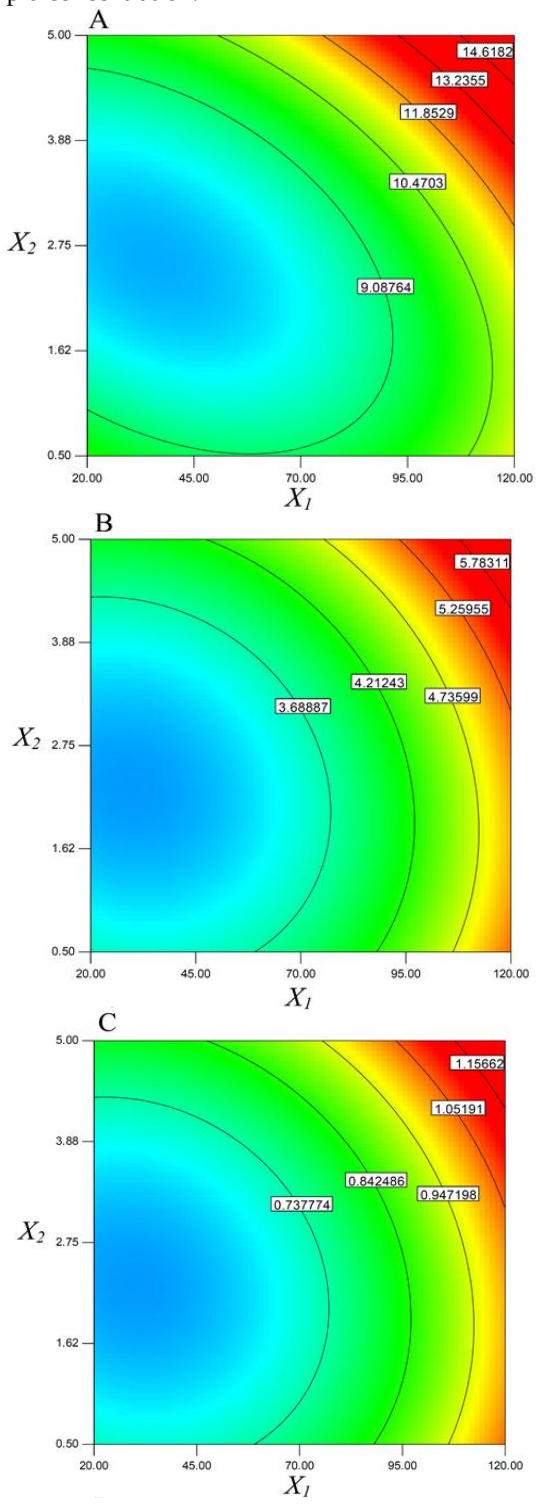
Coefficient	Responses			
	y_1	y_2	y_3	y_4
β_0	8.41[b]	3.60[a]	0.72[a]	42.90[b]
β_1	1.13[a]	0.58[a]	0.12[a]	0.95[c]
β_2	0.69[b]	0.23[b]	0.045[b]	-0.61[c]
β_3	0.96[b]	0.13[c]	0.025[c]	-3.64[a]
β_{12}	0.46[c]	0.054[c]	0.011[c]	-1.32[c]
β_{13}	0.36[c]	0.18[c]	0.037[c]	0.45[c]
β_{23}	-0.17[c]	-0.11[c]	-0.022[c]	-0.18[c]
β_{11}	0.46[c]	0.20[c]	0.041[c]	-0.45[c]
β_{22}	0.69[b]	0.22[c]	0.044[c]	-0.58[c]
β_{33}	0.37[c]	0.39[a]	0.079[a]	3.22[a]
R^2	0.81	0.88	0.88	0.77

Response: y_1 = biomass (g/L); y_2 = lipid (g/L); y_3 = lipid productivity (g/L day); y_4 (% lipid content) = $\left(\frac{y_2}{y_1}\right) \times 100$

[a]Highly significant (P<0.01).
[b]Significant (P<0.05).
[c]Not significant (P>0.05).

The application of response surface methodology yielded the following regression equation models which are empirical relationships between the biomass concentration, lipid concentration, lipid productivity, and cellular lipid content values and the test variables in coded units. The relation among the variables (as coded values) glucose(x_1), $(NH_4)_2SO_4$ (x_2), and KH_2PO_4 (x_3), were fitted by second-order polynomial of equations (1) and (2). The values of coefficient and regression models were presented in Table III. The regression models accurately described the experimental data, which indicated correlation among the three variables of lipid production process that affected the four response variables as discussed above. These were supported by the values of correlation coefficients of R^2, 0.81, 0.88, 0.88, and 0.77 for biomass concentration, lipid concentration, lipid productivity, and lipid content, respectively. These R^2 values suggested that the empirical models well represent the correlation between the experimental results and the theoretical values predicted by the model equation.

As shown in Fig. 1(A) to 1(C), it was observed that the response changed with glucose concentration and $(NH_4)_2SO_4$ concentration. Glucose concentration of about 100 g/L provided the maximum biomass concentration, lipid concentration, and lipid productivity at a given ammonium sulfate concentration. The reason would be that carbon source played the role as an energy generation and cellular biosynthesis [16]. Under optimal concentration of glucose, cell growth helps to enhance the accumulation of lipid in oleaginous yeast. Therefore, glucose is a key substrate to oleaginous yeast growth and lipid production. Moreover, it has been reported that nitrogen effected with the metabolism of protein formation. So it was identified as one of the important factors that induced the lipid production [10]. However, lipid productivity was also related with both of biomass and lipid concentration.



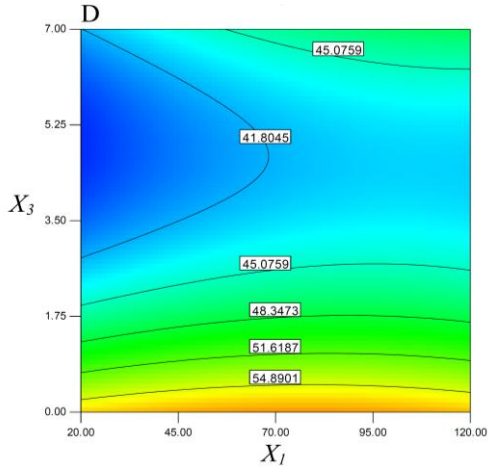


Figure 1. Contour plot diagrams for biomass (A), lipid (B), lipid productivity (C), and lipid content (D), plotted as a function of the significant variable; glucose (x_1), $(NH_4)_2SO_4$ (x_2), and KH_2PO_4 (x_3).

The effects of KH_2PO_4 concentration and glucose concentration on lipid content were shown in Figure 1(D). The result presented negative effect of KH_2PO_4 concentration on lipid content. It was indicated that the decreasing of KH_2PO_4 could increase lipid content of *Psuedozyma* sp. According to the literature, phosphorus limitation increased lipid production [17]. When oleaginous yeasts grew in the presence of excess glucose, cellular adenosine monophosphate (AMP) level dropped significantly due to the activation of AMP deaminase [18]. As the isocitrate dehydrogenases in mitochondria of oleaginous species were allosterically activated by AMP, a decreasing of AMP level led to building up citrate, which crossed mitochondrial membrane into the cytosol to fuel lipid accumulation. The phosphate exhaustion induced an AMP to release inorganic phosphate for other cellular processes. Thus, phosphorus limitation reduced isocitrate dehydrogenase activity in mitochondria due to depletion of the allosteric activator, AMP.

B. Validation of the Model

The validation of the statistical model and regression equation were conducted by taking of 99.73 g/L glucose, 4.09 g/L of $(NH_4)_2SO_4$, and 1.42 g/L of KH_2PO_4. Under these optimized conditions, the predicted response and the observed experimental value were shown in Table IV. These results confirmed that predicted value and experimented value were not significant different at α = 0.05. It was suggested that the statistical method of RSM was well applied to optimize the cultivation variable and study the effects of the test variables on lipid production.

C. Fatty acid Composition of Crude Lipid

The fatty acid compositions of crude lipids that were produced from *Psudozyma* sp. under optimum condition were shown in Table V. The results revealed that crude lipids of *Psudozyma* sp. mainly consisted of 85.0% in C16:C18 fatty acid approximately. The crude lipid was composed with 47.66% oleic acid, 18.67% palmitic acid, 11.40% linoleic acid, 4.34% palmitoleic acid and 2.93% stearic acid. These fatty acids were similar with fatty acid of oleaginous yeast strain (*Lipomyces starkeyi* and *Rhodosporidium toruloides*) and vegetable oils (jatropha and plam) which have been reported in biodiesel production. In a previous research, it is well known that fatty acid compositions influence the quality of biodiesel. Reference [19] reported that the suitable fatty acid compositions for biodiesel production are palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. Moreover, these fatty acids were identified as the most important fatty acids contained in biodiesel [20]. It was indicated that lipid produced by *Psudozyma* sp. has great potential as a feedstock for biodiesel production.

TABLE IV. DESIGN AND RESPONSE OF THE CCD

Parameter	Value	
	Predicted	Experimented*
Biomass concentration (g/L)	10.85	11.39 ±0.11^{ns}
Lipid concentration (g/L)	5.17	5.48 ±0.07^{ns}
Lipid productivity (g/(L day))	1.03	1.10 ±0.01^{ns}
Lipid content (%)	47.69	48.10 ±0.33^{ns}

*Data are means ± standard deviation from triplicate experiments.
^{ns}Not significant at α = 0.05.

TABLE V. RELATIVE FATTY ACID

Fatty acid (%)	Sources of lipid				
	Psuedozyma sp.	*L. starkeyi*[a]	*R. toruloides*[b]	Jatropha[c]	Palm[d]
C16:0	18.67	36.50	34.3	20.16	44.3
C16:1	4.34	3.6	1.90	1.32	-
C18:0	2.93	5.40	4.80	7.22	4.3
C18:1	47.66	52.80	46.20	39.77	39.3
C18:2	11.40	1.20	7.80	31.53	10.0

[a]Ref. [21], [b]Ref. [22], [c]Ref. [23], and [d]Ref. [24].

D. Biodiesel Properties Prediction

The determination of some of the biodiesel quality properties can be an expensive and sophistical process [25]. In order to reduce costs and analysis time, biodiesel properties were estimated numerically by using empirical correlation [15]. The predicted properties of biodiesel from yeast were shown in Table VI. It was found that cetane number (CN) of biodiesel of *Psuedozyma* sp. was higher than biodiesel from jatropha. Although, this CN value was lower than palm biodiesel. However, this value met both the standard value in ASTM and EN. The CN value is a dimensionless number for diesel and is associated with ignition delay time, i.e., the time between fuel injection and the beginning of ignition. The shorter the ignition time, higher is the cetane number [25]. In addition, the density (DN) and kinematic viscosity (KV) were in standard range. The DN and KV values have a direct effect on the atomization process during combustion. The higher heating value (HHV) of yeast biodiesel was slightly lower than value of jatropha and palm. HHV also known as the gross calorific value or gross energy is the amount of heat released during the combustion of one gram of fuel. Although, the HHV was not required in standard, this property was usually applied

to determine the energy content of fuels and thereby their efficiency [15].

TABLE VI. BIODIESEL PROPERTIES

Properties	Biodiesel			ASTM D6751	EN 14214
	Yeast*	Jatropha	Palm		
CN	56.48	55.7	61.9	min 47	min 51
DN (g/cm^3)	0.88	ND	ND	-	0.86-0.90
KV (mm^2/s)	3.83	4.75	4.61	1.9-6.0	3.5-5.0
HHV (Mj/kg)	37.78	40.7	40.6	-	-

**Psuedozyma* sp.
CN, cetane number; DN, density; HHV, higher heating value; KV, kinematic viscosity; IV, iodine values.
ND = No data found in literature

IV. CONCLUSION

The lipid producing condition of oleaginous yeast, *Psuedozyma* sp., was optimized in laboratory scale. The relationship between lipid production efficiency and cultivation process was investigated using RSM operated with CCD. The second-order polynomial regression equation provided a good coefficient of determination. The predicted model of lipid production was very close to the experimented values. Moreover, fatty acid compositions under optimum condition were similar with vegetable oils. It was suggested that it was suitable for using as a raw material for biodiesel production. Furthermore, the predicted biodiesel properties of yeast also reached in range of biodiesel standard. It was demonstrated that lipid produced from *Psuedozyma* sp. was appropriate to be an alternative feedstock for biodiesel production with offering good biodiesel properties.

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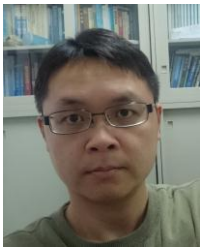

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