Response Surface Methodology for Optimization of Biodiesel Production by *Acinetobacter baylyi*

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Abstract
Biodiesel is conventionally produced through the transesterification reaction of alcohol and triacylglycerols (TAG). TAGs are obtained from oilseed plants and animal fats, therefore the limited supply of TAG is the major bottleneck of the biodiesel production. In vivo esterification between fatty acid acyl-CoA and alcohol to produce fatty acid alkyl ester (FAAE) is an alternative choice for biodiesel production. In this study, FAAE production by Acinetobacter baylyi (A. baylyi) was optimized using response surface methodology. The optimization process employed a Box-Behnken design, where the investigated variables were carbon source content, nitrogen source content and shaking speed. Here, the value of the regression coefficient R^2 = 0.8255 could be explained by the model which is high to advocate the significance of the model. The maximum FAEE yield, 1,026.6 mg/L, was obtained. It could be concluded that in vivo FAEE synthesis using A. baylyi is a novel way for the biodiesel production.

Keywords: *Acinetobacter spp, Fatty acid acyl ester, Biodiesel, Response surface methodology*

1 Introduction

The continuous rise of energy demand from the human and industrial activities and the detrimental effects on global climate problem motivate the development of renewable biofuels. Biodiesel is one of biofuels used in a diesel engine. Biodiesel is composed of FAAE including fatty acid methyl ester (FAME) and fatty acid ethyl ester (FAEE). In general, FAME and FAEE are produced through transesterification reaction between alcohol and TAG. TAG is mostly available in oilseed plants and animal fats. Therefore, sustainable supply of TAG is a major bottleneck for biodiesel production since it is competitive with needs for human foods and animal feeds.

As an alternative option to produce biodiesel, *in vivo* esterification reaction between fatty acid acyl-CoA and alcohol has been developed by using diacylglycerol acyltransferase (DGAT) enzyme as a catalyst [1,2]. DGAT is the key enzyme for biosynthesis of storage lipids that found in eukaryotes and some prokaryotes, for example, *Acinetobacter* spp. DGAT catalyzes the final step of biosynthesis reaction of TAG and wax ester [3,4]. Therefore, biosynthetic of FAEE in *Acinetobacter* is possible and it has potential to utilize sugar instead of oily biomass-derived TAGs to produce biodiesel.

Via *in vivo* esterification, FAEE could be synthesized in sugar metabolic pathway. Previously, we studied the FAEE production in *Acinetobacter* spp. using carbon sources from rice straw hydrolysate containing reducing sugars and succinic acid [5,6]. Therefore, this study aims at optimizing culture condition to maximize the FAEE production using

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simple sugars as carbon source through a multivariate approach by using response surface methodology (RSM) via a Box-Behnken design.

2 Materials and Methods

2.1 *Culture condition of A. baylyi*

A. baylyi (courtesy provided by Assoc. Prof. Alisa Vangnai [7]) was streaked onto Nutrient Agar (NA) plates and incubated at 30°C for 16 h. Single colony was picked and inoculated into 3 ml of nutrient broth medium in a test tube and incubated at 30°C in a rotary shaker for 16 h. Then 1 ml of seed culture was inoculated in 100 ml modified minimal medium (containing 15 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4.7H_2O$ and 10 mM sucrose, pH 7.0 [4]). For optimization experiment, the amounts of $(NH_4)_2SO_4$ and sucrose (as a nitrogen source and a carbon source, respectively) were adjusted based on the design as shown in Table 1.

Table 1: Experimental design to test the effects of independent variables (shaking speed (X_1), nitrogen content (X_2) and carbon content (X_3)) on FAEE production (Y)

Run	X_1 (rpm)	X_2 (mM)	X_3 (mM)	Y (mg/L)
1	250 (+1)	10 (0)	2 (-1)	164 ± 1.5
2	250 (+1)	18 (+1)	10 (0)	380 ± 35.4
3	125 (0)	2 (-1)	18 (+1)	452 ± 3.4
4	125 (0)	2 (-1)	2 (-1)	160 ± 1.9
5	125 (0)	18 (+1)	18 (+1)	1166 ± 8.3
6	125 (0)	10 (0)	10 (0)	395 ± 31.6
7	125 (0)	10 (0)	10 (0)	361 ± 21.1
8	0 (-1)	10 (0)	2 (-1)	607 ± 1.5
9	250 (+1)	2 (-1)	10 (0)	413 ± 33.1
10	125 (0)	10 (0)	10 (0)	282 ± 28.6
11	0 (-1)	10 (0)	18 (+1)	254 ± 1.3
12	125 (0)	18 (+1)	2 (-1)	336 ± 4.3
13	125 (0)	10 (0)	10 (0)	161 ± 32.9
14	250 (+1)	10 (0)	18 (+1)	500 ± 20.0
15	0 (-1)	18 (+1)	10 (0)	180 ± 2.0
16	125 (0)	10 (0)	10 (0)	244 ± 28.5
17	0 (-1)	2 (-1)	10 (0)	336 ± 3.7

To collect *A. baylyi* culture for FAEE extraction, the cells were centrifuged at 5,000 rpm for 10 min and pellets were harvested. The FAEE was extracted for gas chromatography analysis (GC) based on standard method [8]. Bacterial pellet was mixed thoroughly with organic solvent containing chloroform and methanol (2:1 ratio by v/v). After dispersion, the whole mixture was agitated for 15 min in an orbital shaker at room temperature. The homogenate was centrifuged to recover the liquid phase and washed with 0.2 volume of 1% NaCl solution. The separated lower phase was recovered and concentrated using a rotory evaporator. Samples were analyzed by GC (Shimadzu GC-2010 Plus) using DB-wax column (Agilent, 30 m in length, with 0.25 mm ID and 25 μm film thickness). The following temperature program was applied: 1 min at 40°C, 15 min ramp to 280°C and constant at 280°C for 10 min. 0.1 mg of nonadecanoic acid methyl ester was added as an internal standard and the quantity of FAEE was calculated by reference to the internal standard. Each sample was analyzed in triplicate

2.2 *Experimental design*

Optimization of culturing condition for FAEE production (*Y*) was carried out using RSM with Box-Behnken design [9]. Three of independent variables were studied here including shaking speed, nitrogen content and carbon content. For each variables, three levels (max = +1, mid = 0, min = -1) was selected for the optimization, with a total of 17 runs. The range and levels of independent variables and code values were represented in Table 1. Experimental data were analyzed using the statistical software, Design-Expert software (version 7.0.0, STAT-EASE Inc., USA), to fit the second-order polynomial regression model:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

where Y is the response variable (FAEE production), β_0 is the constant, β_i is the linear coefficient, β_i is the quadratic coefficient and β_{ij} is the two factor interaction coefficient. The accuracy and general ability of the above polynomial

model could be evaluated by the coefficient of determination (R^2).

2.3 *DGAT gene amplification*

To confirm the existence of the dgat gene in *A. baylyi*, the gene amplification method from genomic DNA was selected. First, single colony of *A. baylyi* was inoculated in 3 ml of nutrient broth medium in a test tube and incubated at 30°C in a rotary shaker for 16 h. Cells were harvested by centrifugation at 5,000 rpm for 10 min. Then, genomic DNA was extracted by using Genomic DNA extraction kit (RBC Bioscience). Approximately 10^9 cells was harvested by centrifugation at 8,000 rpm for 5 min. 200 µl of GT buffer (supplied by the kit) was added into the cell pellets and mixed with vortexing. The mixture was incubated at room temperature for 5 min. Then 200 µl of GB buffer (supplied by the kit) was added and mixed with vortex for 5 min, incubated at 70°C for 10 min until the sample lysate became clear. To precipitate DNA, 200 µl of absolute ethanol was added and mixed with vortexing. All mixed sample was transferred into GD column (supplied by the kit), then GD column was centrifuged at 13,000 rpm for 2 min. DNA was washed by adding 400 µl of W1 (supplied by the kit) buffer into GD column and centrifuged again at 13,000 rpm for 2 min. 600 µl of washing buffer was additionally added to wash DNA. GD column was centrifuged and the flow-through was discarded. The DNA sample was eluted by adding 100 µl of sterile distilled water and GD column was centrifuged to collect DNA sample. The quantity of DNA sample was determined using a UV spectrometry at 260 nm.

Next, to amplify the dgat gene from genomic DNA, primers were designed specific to reference dgat gene sequence in NCBI database (NC_005966.1). The sequences of forward and reverse primers are 5' CACGACTGCAATGGTTCATC 3' and reverse primer is 5' AGTGAGGCAATCCACGCTATG 3', respectively. 50 ng of genomic DNA was used as a template. The components of 50 µl of polymerase chain reaction (PCR) (RBC Bioscience, containing 1X PCR reaction buffer, 0.1 µM dNTP mix, 0.2 µM primers, 1.25 units RBC Taq DNA polymerase) were mixed together. Then the sample was placed in thermal cycler machine (MS Major Science). Amplification was carried out using a thermal cycling protocol consisting of predenaturation for 10 min at 94°C followed by 30 cycles of 50 s at 94°C, 50 s at 57°C and 60 s at 72°C. The PCR product was separated in 1% agarose gel electrophoresis and detected under UV lamp after staining with ethidium bromide.

3 Results and Discussion

3.1 *Optimization of FAEE production*

Shaking speed of 0-250 rpm (X_1), nitrogen content of 2-18 mM (X_2) and carbon content of 2-18 mM (X_3) were tested to optimize the FAEE production. The experiment trials were designed for 17 runs with three different coded levels (high (+1), medium (0), low (-1)) based on Box-Behnken design as shown in Table 1. A response factor, FAEE content, was measured for each of the culturing conditions.

The statistic software package Design-Expert software version 7.0.0 was used for regression analysis of experimental data and to plot response surface. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters. The responses of Box-Behnken design were well fitted with the second-order polynomial equation,

$$Y = 872.76 + 1.55X_1 - 77.89X2 - 76.43X_3 + 3.04X_2X_3 - (8.98 \times 10^{-3})X_1^2 + 2.59X_2^2 + 3.21X_3^2$$

In this equation, Y is the FAEE content and X_1, X_2 and X_3 are the coded value of the test variables as shaking speed (rpm), nitrogen content (mM) and carbon content (mM), respectively. The statistical significance of the model was evaluated by the *F*-test for ANOVA (Table 2). ANOVA of the quadratic regresion model suggested that the model is significant with the value of "Prob>*F*" was < 0.05, which indicated that the computed model was statisticallysignificant with a confidence interval of 99.95%. The model *F*-value (6.05) implied that the model was significant and there was only a 0.78% chance that a "Model *F*-value" could occur because of noise. The "Lack of Fit" test also confirmed the statistical significance of the model because the "Prob>*F*" value was > 0.05.

Table 2: Results from regression analysis of the design

Source	Mean square	*F*-value	Prob>*F*
Model	1.085×10^5	6.05	0.0078
X_1	5.984×10^4	3.34	0.1000
X_2	9.786×10^3	0.55	0.4789
X_3	1.682×10^5	9.38	0.0135
X_2X_3	1.509×10^5	8.42	0.0176
X_1^2	8.286×10^5	4.62	0.0601
X_2^2	1.162×10^5	6.48	0.0314
X_3^2	1.777×10^5	9.91	0.0118
Lack of Fit	2.416×10^5	2.38	0.2104

The cut-off criteria of statistically significance with "Prob > *F* " with < 0.1 was applied to each "model term" and, in this case, X_1, X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 are significant model parameters. This suggests that all three variables and their interactions have direct relationships with the FAEE production. The coefficient of determination (R^2) of the model was 0.8255, which indicated that the model was suitable for representing the relationships among the selected variables and advocated a high significance of the model.

Response surface plots of the RSM as a function of two variables at a time are helpful in understanding both the main and the interaction effects of these variables (Figure 1). The maximal FAEE yield (1,026.6 mg/L) was obtained when 86.09 rpm of shaking speed, 18 mM of nitrogen content and 18 mM of carbon content were used, which is higher yield compared to other studies [4,10].

The effects of interactions between each variables on FAEE production are shown in Figure 1. At lower carbon content, it could be seen that FAEE production decreased when nitrogen content increased, while, at higher carbon content, it became further increased when the level of nitrogen increased (Figure 1A). The carbon content positively affected the FAEE production (Figures 1A and Figure 1C). The shaking speed effect was observed that the medium speed seems to be the optimal condition because the FAEE production decreased when the speed was either high or low (Figures 1B and Figure 1C).

The impact of variable factors in our study is also supported by related metabolic pathways, including lipid synthesis and glycolysis pathway. FAEE is the product of *in vivo* esterification reaction

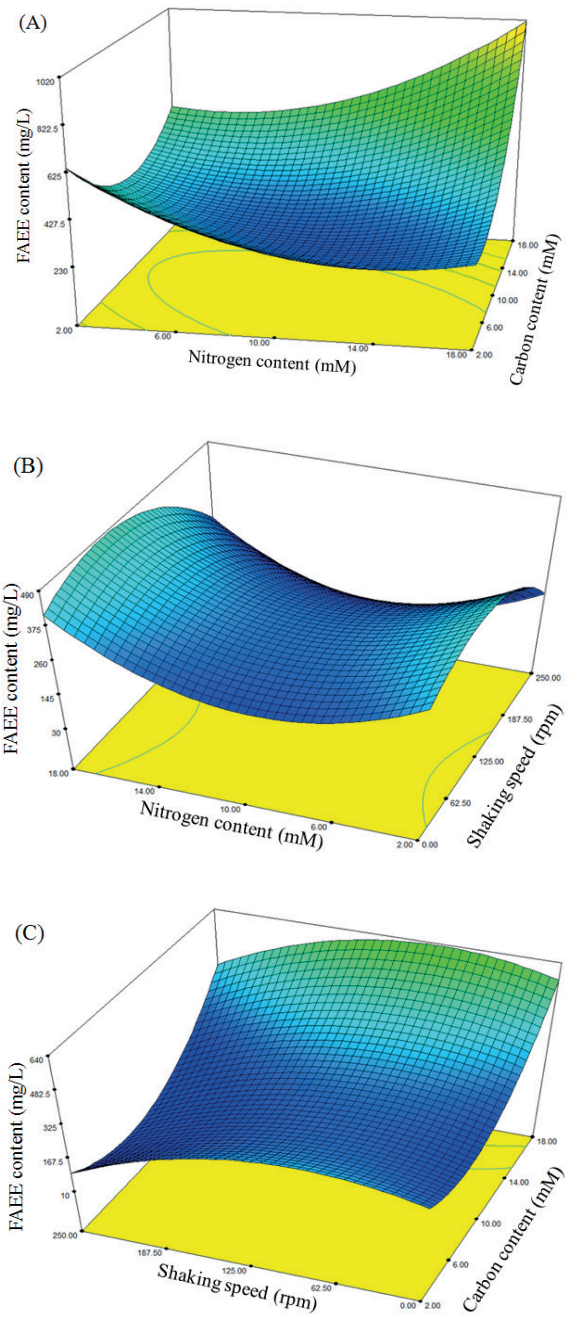

Figure 1: Response surface plots showing the interaction between the independent variables in the FAEE production. (A) Inteaction between nitrogen content and carbon content; (B) interaction between nitrogen content and shaking speed; (C) interaction between shaking speed and carbon content.

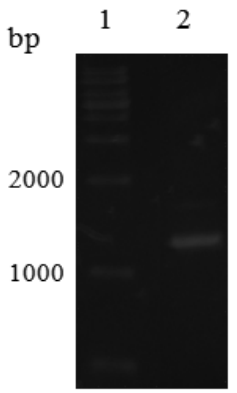

Figure 2: A PCR product that is amplified from genomic DNA of *A. baylyi*. Lane 1 is 1-kb DNA marker, lane 2 is a PCR product.

between fatty acid acyl-CoA and alcohol. Therefore, the pool of fatty acid acyl-CoA is controlled by the balance of carbon and nitrogen sources [4,10]. In such scenario, when nitrogen content is high, bacterial cells prone to undergo cell division process, which reduce the accumulation of lipid. Otherwise, when carbon content is low, cells do not have enough material to produce storage lipid. Additionally alcohol production is preferred under anaerobic condition. However, to accumulate cell biomass, cells require oxygen (as shaking speed) to produce energy. These interactions suggest that we should find the optimal balance point of these factors to culture bacteria to maximize FAEE production and to avoid the negative interaction effect from the interacting factors.

3.2 *Dgat gene amplification*

To validate the existence of the dgat gene in *A. baylyi*, genomic DNA of *A. baylyi* was extracted and used as a template for the dgat gene amplification. As described in the method, primers specific to the dgat gene of *Acinetobacter* spp bacteria were designed. After performing PCR, the product was analyzed by gel electrophoresis as shown in Figure 2. It is clear that a band with approximate 1.3 kbp size could be observed, which is the expected size of PCR product of the dgat gene. So, we can conclude that *A. baylyi* has the dgat gene. However, further validation may be needed, for example, sequence comparison of this PCR product to dgat gene sequence in database.

4 Conclusions

A. baylyi was cultured in the flask-shake scale over a range of different shaking speed, carbon and nitrogen contents to determine the optimal condition for FAEE production. The culturing condition was successfully modeled by the polynomial mathematic equation based on experimental data. We believe that the optimization experiment and modelling in this study is the primary step to achieve the ultimate goal for biodiesel production from sugars derived from hydrolyzed lignocellulosic biomass in the future.

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