

Kinetic Study of Immobilized Whole-Cell Lipase *Rhizopus Oryzae* as Biocatalyst in Biodiesel Synthesis through Non-Alcohol Route

H. Hermansyah, M. B. Faiz, and R. Arbianti

Abstract—Utilizing whole-cell biocatalyst is a potential way to reduce catalyst cost in biodiesel production using lipase as catalyst. Whole-cell of *Rhizopus oryzae* was cultivated by one-step and two-step method and was immobilized on Biomass Support Particles (BSPs) and chitosan-TPP bead. Immobilized whole-cells on BSPs produce 11% (one-step) and 12% (two-step) FAME yield. While, FAME yield produced by immobilized whole-cell in chitosan-TPP beads are 23% (one-step) and 22% (two-step). Kinetic model based Michaelis-Menten used was found to fit fairly the substrate and product concentration profile. Value of K_m and V_{max} for *R. oryzae* whole-cell immobilized on BSP are 4 mole L⁻¹, 0.05 mole L⁻¹ h⁻¹ (one-step) and 3 mole L⁻¹, 0.04 mole L⁻¹ h⁻¹ (two-step). While, for immobilized whole-cell in chitosan-TPP bead, the values are 0.3 mole L⁻¹, 0.01 mole L⁻¹ h⁻¹ and 0.2 mole L⁻¹, 0.008 mole L⁻¹ h⁻¹ for single-step and two-step respectively.

Index Terms—Biodiesel, immobilization, Michaelis-Menten, whole-cell.

NOMENCLATURE

F-1	Suspended whole-cell <i>R. oryzae</i> cultivated by single-step cultivation method
F-2	Suspended whole-cell <i>R. oryzae</i> cultivated by two-step cultivation method
IB-1	Immobilized whole-cell <i>R. oryzae</i> on BSPs cultivated by single-step cultivation method
IB-2	Immobilized whole-cell <i>R. oryzae</i> on BSPs cultivated by two-step cultivation method
IK-1	Immobilized whole-cell <i>R. oryzae</i> in chitosan-TPP beads cultivated by single-step cultivation method
IK-2	Immobilized whole-cell <i>R. oryzae</i> in chitosan-TPP beads cultivated by two-step cultivation method
KZ	Immobilized commercially <i>C. rugosa</i> lipase on zeolite
KK	Immobilized commercially <i>C. rugosa</i> lipase in chitosan-TPP beads
[P]	FAME concentration (mole L ⁻¹)
[S]	Triglyceride concentration (mole L ⁻¹)
t	Time (h)
V_{max}	Maximum interesterification rate (mole L ⁻¹ h ⁻¹)
K_m	Michaelis constant (mole L ⁻¹)

I. INTRODUCTION

In the past decade, biodiesel has attracted considerable attention as a renewable, biodegradable and non-toxic fuel [1]. Current technology for biodiesel production, using an alkali or an acid catalyst, has several important limitations such as requires feedstock free of water, produce salts, generate glycerin as a low- grade by-product, etc [2]. In the other side, utilizing lipase as catalyst for biodiesel production over several advantage, such as require lower heating, works even in the presence of water, produces no salts [2]. Lipases have up to 74 times higher productivity than alkali catalyst [3].

Lipase is easy to be deactivated by alcohol, which is a reactant in biodiesel synthesis reaction. Therefore, we have developed new method to maintain the activity and stability of the biocatalyst during reaction by using methyl acetate as an acyl donor [4]. Unfortunately, the price of lipase is expensive due to purification procedures [2]. Haas *et al.* reported that catalyst cost in biodiesel production by using enzyme is 0.14US\$ per kg per kg ester [5]. It is much higher than by using NaOH which is 0.006 US\$ per kg ester [5].

In order to lower the catalyst cost, we used enzyme immobilization method in various support to make the lipase become reusable due to its easy recovery from the reaction mixture [6]–[9]. We have also developed kinetic model based on Michaelis-Menten and Ping Pong Bi Bi Mechanism describing the interesterification behavior of the triglyceride with methyl acetate to produce biodiesel using lipase [4], [6].

Although immobilization makes enzyme become reusable, but the process is time and cost consuming [6], [7]. Utilizing whole-cell overproducing intracellular lipase, such as *Rhizopus oryzae*, as biocatalyst in biodiesel synthesis is a potential way to reduce the biocatalyst cost [10]. The preparation of a biocatalyst requires no complex purification process, and the prepared whole-cells could be directly used as lipase containers [11].

Cells immobilization enables the whole-cell biocatalyst to be reused in subsequent reactions [2]. Whole-cells are frequently entrapped in biomass support particles (BSPs) [1], [10]–[15]. In this research, we also immobilized whole-cells of *R. oryzae* on BSP. Besides, the cells were immobilized by different method, i.e. encapsulation, in chitosan-TPP beads. *R. oryzae* was cultivated by different method, i.e. single-step and two-step cultivation.

Works which deal with modeling of enzymatic interesterification for whole-cell catalyzed biodiesel production are not often found in the literature. This work also presents a study of kinetics of enzymatic reaction of

Manuscript received May 7, 2014; revised July 17, 2014.

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cooking oil and methyl acetate for biodiesel production using immobilized *R. oryzae* whole-cell as biocatalyst. The kinetic was based on Michaelis-Menten mechanism.

II. MATERIALS AND METHODS

A. Materials

All experiments were carried out using *R. oryzae* obtained from School of Life Science and Technology, Bandung Institute of Technology, Indonesia. Potato Dextrose Broth (PDB) was obtained from BD Difco™ (USA). Trypton, NaNO₃, KH₂PO₄, MgSO₄·7H₂O, glutaraldehyde and methyl acetate were purchased from Merck (Darmstadt, Germany). Olive oil, cooking oil, BSPs, chitosan, acetic acid and tripolyphosphate (TPP) were obtained locally.

B. Suspended Whole-Cell Preparation

There are two different methods used for fungi cultivation, i.e. single-step and two-step method. One-step cultivation was carried out using 50ml of PDB medium at room temperature (25°C) for 24 hours on reciprocal shaker at 60 oscillations. Two-step method was worked following method of Jin and Bierma [2]. The method consisted of pre-incubation and incubation step. For pre-incubation, Erlenmeyer flask (100ml) containing 50ml of PDB were inoculated by aseptically transferring spores from a potato dextrose agar plate. For the incubation step, the filamentous fungi were aseptically transferred to other Erlenmeyer flask (100ml) containing 50ml of basal medium. The basal medium contained, in 1L of distilled water: 70g trypton, 1g NaNO₃, 1g KH₂PO₄, 0.5g MgSO₄·7H₂O and 30g olive oil. Each step in two-step method was conducted using the same operating condition as in single-step cultivation method.

C. Whole-Cell Immobilization on BSPs

Immobilization was carried out following method of Ban, *et al.* [12]. *R. oryzae* cells were immobilized on Biomass Support Particles (BSPs). The BSPs used for immobilization were 6mm cubes of polyurethane foam. Immobilization was effected by placing 1g BSPs inside a flask together with the medium. The cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask cultivation [12]. The immobilized whole-cell was separated from the culture broth by filtration. After washing with tap water for 1 min, they were treated with 0.1% glutaraldehyde solution for 1 hour.

D. Whole-Cell Immobilization in Chitosan-TPP Beads

Whole-cell encapsulation in chitosan-TPP bead was carried out after pre-incubation. Encapsulation process was started by mixing *R. oryzae* cells into chitosan (0.5g) dissolved in acetic acid 3% v/v (9ml). The mixture then was dripped into TPP solution 2% w/v. This process would support bead forming of chitosan-TPP. Such process was conducted for 4 hours.

E. Biodiesel Synthesis through Non-Alcohol Route

The interesterification reaction was carried out at 37°C in 250ml screw-cap bottle with incubation on a reciprocal shaker 150rpm). Cooking oil and methyl acetate were used

with 1:12 mole ratio. The whole-cell biocatalyst added was 10% of total reactant mass.”

F. Analysis

Reactant and product were analyzed using a HPLC system (L-7100, Hitachi, Ltd., Tokyo, Japan) equipped with an Inertsil ODS column (particle size 5.0×10⁻⁴ cm, i.d. 0.46 cm, length 25 cm, GL Science, Inc., Tokyo, Japan) and a UV detector (L-7400, Hitachi, Ltd., Tokyo, Japan) at 210 nm. Temperature of the column oven was 313 K. The mobile phase composition was methanol: acetone = 100:0 (v/v) for 20 min and then the acetone ratio increased up to 20 % (v/v) and the value was maintained from 21 min to 35 min. The flow rate of the mobile phase was 1.0 cm³ min⁻¹.

G. Kinetic Modelling

Michaelis-Menten kinetic (Eq. 1 and Eq. 2) based model was used to predict substrate and product concentration along reaction time.

$$\frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

$$\frac{d[S]}{dt} = \frac{V_{\max}[S]}{K_m + [S]} \quad (2)$$

III. RESULTS AND DISCUSSIONS

A. Catalytic Performance

1) Suspended whole-cell

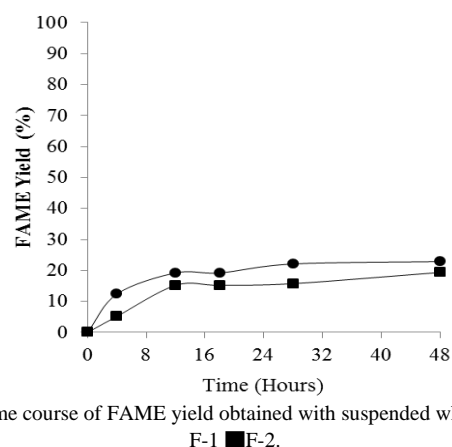


Fig. 1. Time course of FAME yield obtained with suspended whole-cell. ● F-1 ■ F-2.

To investigate the effect of cultivation method to catalytic performance of suspended whole-cell, we tried to use two different cultivation methods for fungi propagation. Fig. 1 shows the time course of FAME yield produced by reaction using suspended whole-cell cultivated by single-step and two-step method. When *R. oryzae* cells were cultivated by two-step method, the reaction produced lower FAME yield (19%) than when the cells were cultivated by single-step method (23%). Hama *et al.* reported that *R. oryzae* produces two type lipase bound to cell wall and cell membrane. In suspended cells, the amount of membrane-bound lipase decreased sharply with cultivation time. Furthermore, the

basal medium used in two-step cultivation method contained 30% olive oil which is consisted of 55-83% oleic acid. The unsaturated fatty acid existence in medium broth altered cell membrane permeability become more permeable [1] inducing lipase secretion.

2) Immobilized whole-cell on BSPs

In submerged cultures of filamentous fungi, cells form strands of interlocking hyphae. Agitation subjected to fungi and the medium growth increased oxygen transfer which allowed the fungal hyphae became entrapped inside the foam.

When the immobilized whole-cell cultivated by two-step method, the biocatalyst synthesized higher FAME yield (12%) than by single-step (11%). Cell morphology intensely affect enzyme productivity. Though the relationship of cell morphology and enzyme secretion depends on fungal strain and enzyme type, cell immobilization inducing pellet formation strongly inhibit *R. oryzae* lipase, particularly from the membrane. Moreover, immersed biocatalyst in glutaraldehyde solution induces intra- and inter-molecular cross-linking of lipase [11].

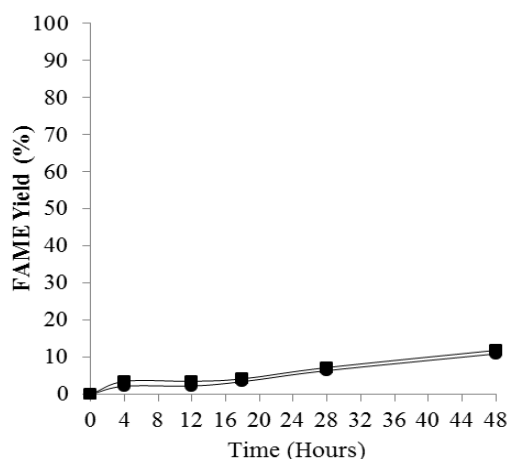


Fig. 2. Time course of FAME yield obtained with immobilized whole-cell on BSPs. ●IB-1 ■IB-2.

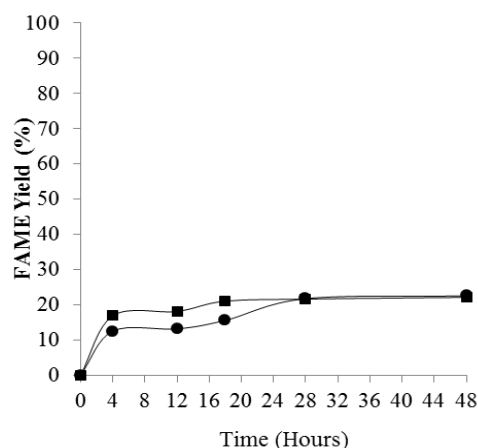


Fig. 3. Time course of FAME yield obtained with Immobilized whole-cell in Chitosan-TPP Beads. ●IK-1 ■IK-2.

It is noteworthy that the curve shape indicates that at 48th hour the biocatalysts have not achieved the maximum yield yet (Fig. 2). It might be due to the entrapment whole-cell on BSPs increases mass transfer resistance. Thereby, there is possibility to increase the methyl ester yield by prolonging

the reaction time.

3) Immobilized whole-cell in Chitosan-TPP

R. oryzae cells were encapsulated in chitosan-TPP beads. The beads were formed as ionic interaction between amino group of chitosan and anionic group of TPP.

Fig. 3 shows that the immobilized whole-cells cultivated by two-step methods, the FAME produced were lower than one-step. Such trend is exhibited by suspended whole-cell. It is indicated that the immobilization did not occurred intactly. Moreover, it was reported that chitosan-TPP beads have low mechanical stability.

4) Comparison with immobilized commercially lipase

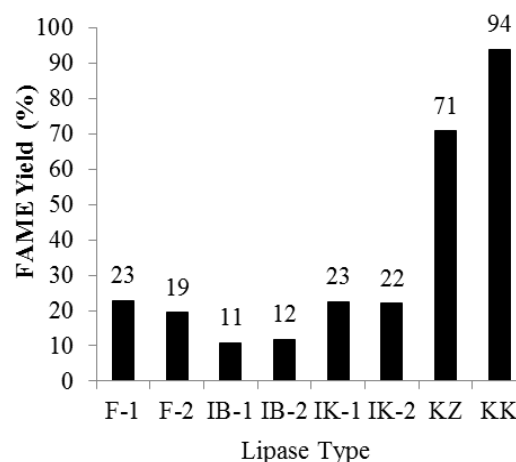
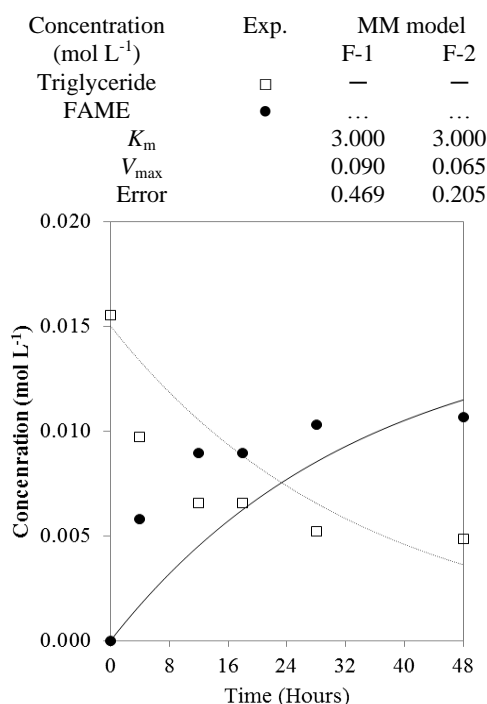


Fig. 4. FAME yield Comparison using different lipase type.

Comparisons of FAME yield obtained using whole-cell biocatalyst and immobilized commercial lipase are shown in Fig. 3. The data of immobilized commercially lipase was obtained from our previous experimental result. The reaction was carried out using 4% (w/w) biocatalyst and 50 hours reaction time. Fig. 3 shows that both immobilized commercial lipases exhibit remarkably higher FAME yield than suspended and immobilized whole-cell.



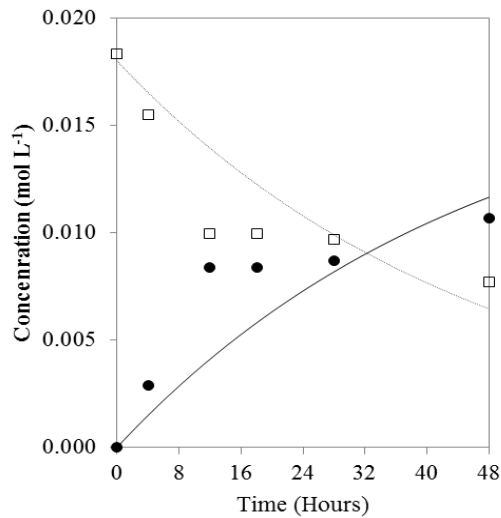


Fig. 5. Triglyceride and FAME profile using suspended whole-cell.

Concentration (mol L ⁻¹)	Exp.	MM model	
Triglyceride	□	IB-1	IB-2
FAME	●
K_m		4.000	3.000
V_{max}		0.050	0.040
Error		0.040	0.108

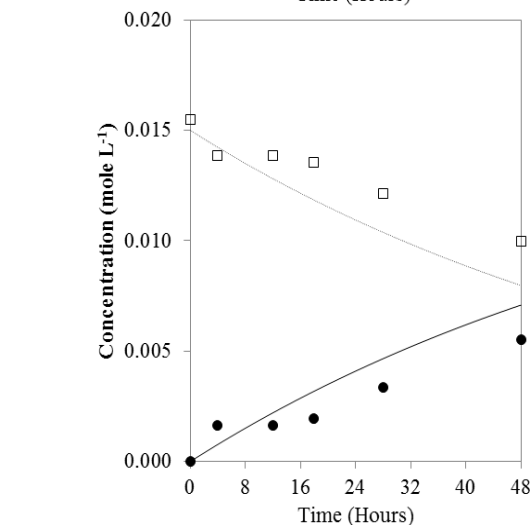
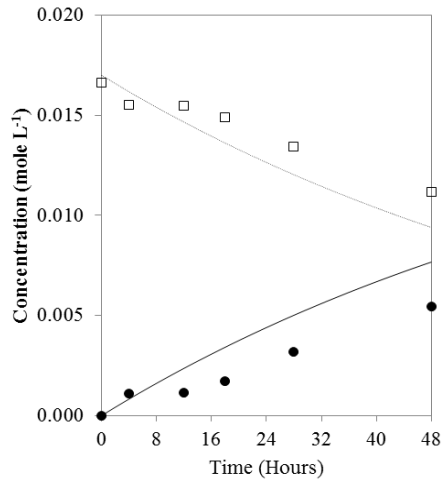


Fig. 6. Triglyceride and FAME profile using immobilized whole-cell on BSPs.

B. Model Verification

1) Suspended whole-cell

Fig. 5 shows that the model fit fairly with the experimental

data. F-1 has higher V_{max} than F-2. Thus, it is predicted that F-1 could achieve higher maximum interesterification rate than F-2. However, F-1 needs more substrate than F-2 to achieve half of its maximum reaction rate. This is indicated by K_m value.

2) Immobilized whole-cell on BSPs

Comparison of prediction and experimental data of triglyceride and methyl ester concentration is shown in Fig. 6. Both for single-step and two-step, the curve indicated that the reactions have not achieved their maximum rate yet. Therefore, we could predict that immobilized whole-cell could produce higher FAME yield by prolonging the reaction time.

Concentration (mol L ⁻¹)	Exp.	MM model	
Triglyceride	□	IK-1	IK-2
FAME	●
K_m		0.300	0.200
V_{max}		0.040	0.008
Error		0.108	0.879

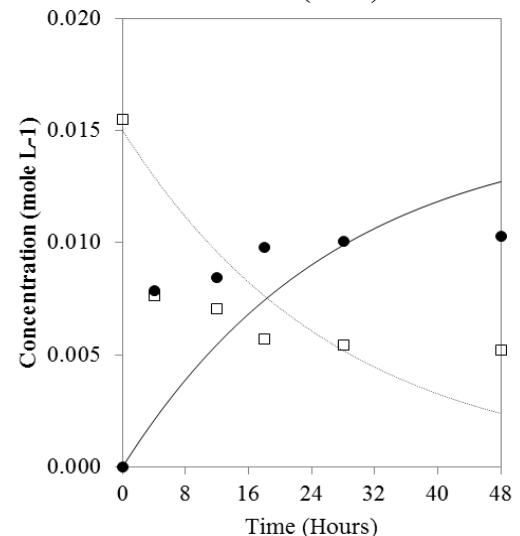
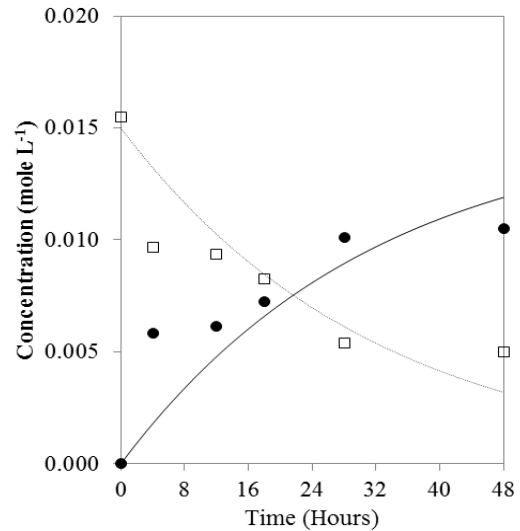


Fig. 7. Triglyceride and FAME profile using Immobilized whole-cell in Chitosan-TPP Beads.

3) Immobilized whole-cell in chitosan-TPP

Fig. 7 shows that the predicted result fit fairly with experimental result of immobilized whole-cell in chitosan-TPP beads. Utilizing two-step method causes this immobilized whole-cell has lower reaction rate and also

Michaelis constant value.

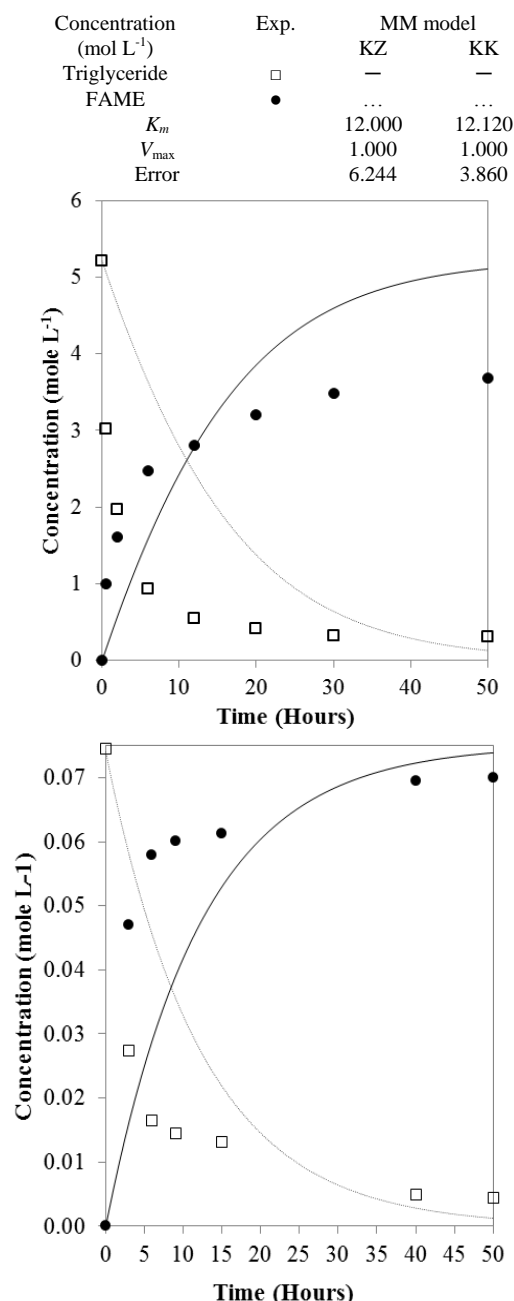


Fig. 8. Triglyceride and FAME profile using immobilized commercial lipase.

4) Immobilized commercially lipase and comparison with whole-cell

The experimental results using immobilized commercial lipase and the fitted results are shown in Fig. 8. Immobilized lipase on zeolite has same V_{max} value with immobilized lipase in chitosan-TPP beads. While, the K_m value is different. The V_{max} values of these immobilized commercially lipases are much higher than suspended and immobilized whole-cell. This is accordance with the experimental result which shows remarkable higher FAME yield using immobilized commercially lipase than whole-cell.

IV. CONCLUSION

We have demonstrated the interesterification of cooking oil and methyl acetate using suspended and immobilized

whole-cell of *R. oryzae*. The novel biodiesel production system using encapsulated whole-cell in chitosan-TPP beads here seems to be a promising alternative to the entrapped whole-cell on BSPs, where the FAME yield was higher.

Michaelis-Menten based kinetic model proposed in this work could predict the substrate and product concentration profile in whole cell-catalyzed biodiesel synthesis along the reaction time since it has a good agreement the experimental results.

ACKNOWLEDGEMENT

This work was supported by Indonesian Ministry of Research and Technology ("Sinergi Inovasi Nasional" research grant, RT-2013-1501).

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