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Production and Optimization of Oleic Acid Ethyl Ester Synthesis Using Lipase From Rice Bran (*Oryza sativa* L.) and Germinated Jatropha Seeds (*Jatropha curcas* L.) by Response Surface Methodology

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Abstract

Recently, the fatty acid ethyl ester has been synthesized in place of fatty acid methyl ester since ethanol has been more renewable. In this research, oleic acid ethyl ester (OAEE) was synthesized using germinated jatropha seeds (*Jatropha curcas*.L) and rice bran **(***Oryza sativa*) as source of lipase. The objective of the research was to optimize the synthesis conditions using *Response Surface Methodology*. Factors, such as crude enzyme concentration, molar ratio of oleic acid to ethanol, and the reaction time, were evaluated. The results show that lipase from germinated jatropha seeds had the hydrolitic and esterification activity about 6.73 U/g and 298.07 U/g, respectively. Lipase from rice bran had the hydrolitic and esterification activity about 10.57 U/g and 324.03 U/g, respectively. The optimum conditions of esterification reaction using germinated jatropha seed lipase as biocatalyst were crude enzyme concentration of 0.31 g/ml, molar ratio of oleic acid to ethanol of 1 : 1.81, and reaction time of 50.9 min. The optimum conditions of esterification reaction using rice bran lipase were crude enzyme concentration of 0.29 g/ml, molar ratio of oleic acid to ethanol of 1 : 2.05, and reaction time of 58.61 min. The obtained amounts of OAEE were 810.77 µmole and 626.92 µmole for lipases from rice bran and germinated jatropha seed, respectively.

Keywords : Oleic acid ethyl ester, lipase, germinated jatropha seeds, rice bran, *Response Surface Methodology*.

Introduction.

Fatty Acid Alkyl Ester (FAAE) is an alternative fuel that may be produced by esterification of vegetable or animal oils using chemical catalyst or lipase as biocatalyst (Zhou and Boocock 2006a; Oliveira *et al*. 2006 and Salis *et al*. 2008). Most synthesized FAAE are fatty acid methyl ester (FAME), in which methanol has been used as alkyl source (Stavarache *et al*., 2003 and 2008; Mao *et al*., 2004; Mahajan *et al*., 2006 and 2007; Zhou and Boocock, 2006). However, methanol in the synthesis of FAME is not renewable. Recently, ethanol has been used in place of methanol for synthesis of FAAE (Dalla Rosa *et al*, 2009; Joshi *et al,* 2008; Hamad *et al*, 2008) because it is renewable. The product is a fatty acid ethyl ester (FAEE).

In enzymatic esterification, microbial lipases have been used as biocatalyst (Ganesan *et al*., 2009; Bisen *et al*., 2010; Watanabe *et al*., 2007; Salis *et al*., 2008). On the other hand, the exploration of local inexpensive sources of oilseeds, such as *Jatropha curcas*, *Pentaclethra macrophylla* Benth, *Nigella sativa* L., *Umbellularia californica* and rice bran were reported for preparation of lipases (Abigor

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et al., 2002; Enujiugha *et al*., 2004; Tuter *et al*., 2003; Haas *et al*., 2001; Natarajan *et al*, 2010; Chuang *et al*, 2010; Bhardwaj *et al*, 2001). However, the unique substrate specificity of plant lipases may still have low consideration for industrial application, especially for the synthesis of fatty acid ethyl ester.

In order to obtain the optimum yield of the reaction, the optimum conditions of the enzymatic process have to be optimized. One of the optimization methods is Response Surface Methodology (RSM). The excellences of RSM are to construct the model of optimization that involving interactions among all variables (factors) in the reaction and the interactions among one variable to another variable can be analyzed easily (Myers *et al*, 2009). RSM has previously been used for the optimization of some processes and reactions. Manohar and Divakar (2004) used RSM to optimize the lipase-catalyzed esterification using methanol. RSM has also been used to optimize the lipase-catalysed synthesis of flavours (Nogales *et al*., 2005).

In this research, the plant lipases from germinated jatropha seeds and rice bran obtained from local resources for the synthesis of oleic acid ethyl ester (OAEE) were evaluated. Factors, such as concentration of crude enzyme, molar ratio of oleic acid to alcohol, and reaction time were evaluated. Response Surface Methodology (RSM) was used to obtain the optimum conditions.

Materials and Methods

The jatropha seeds were obtained from Forestry Department Office of Gunung Kidul Regency, Daerah Istimewa Yogyakarta Province. Rice bran variety IR4 was obtained from local supplier. Olive oil was obtained from Sigma Co., (St. Louis, MO, USA). Pyridin, oleic acid, acetone, isooctane, $NaH_2PO_4.H_2O$, $NaH_2PO_4.2H_2O$, ethanol and cupri-acetate were obtained from Merck KGaA (Darmstadt, Germany). Fungicide was obtained from local supplier.

Crude enzyme preparation

The jatropha seeds were selected and the seeds were further soaked in 0.1 M phosphate buffer pH 6. Fungicide (1.5 g/l) was then added. Seeds were incubated for 12 h at the ambient temperature. The excess water was removed and the seeds were aerated for 1 h. Then, the seeds were re-hydrated at relative humidity of 90% for 24 h. The seeds were germinated in incubator at ambient temperature. The harvesting of germinated seeds was carried out if the length of shoots had reached 2 – 2.5 cm. The germinated jatropha seeds were stored at – 20 °C directly after harvesting. While, the fresh rice bran which obtained from local supplier were also stored at – 20 °C.

Twenty gram of peeled germinated jatropha seeds and rice bran were homogenized and further defatted in Soxhlet using 35 ml cold acetone (-20 °C). The defatted germinated jatropha seeds and rice bran were dried at ambient temperature, and further stored at – 20 °C until used.

The effect of crude enzyme concentration on the synthesis of oleic acid ethyl ester

Ethanol was added into 1 ml oleic acid, in which the molar ratio was 1 : 1. Various amount of crude enzyme (0.1 g, 0.2 g, 0.3 g, 0.4 g, and 0.5 g) were added into the mixture. The reactions were carried out at 30 °C for 40 min. The concentration of oleic acid was further determined according to Hidayat *et al*., (2008). The forming of OAEE was calculated as the amount of oleic acid that reacted with ethanol.

The effect of molar ratio of oleic acid to ethanol on the synthesis of oleic acid ethyl ester

Crude enzyme (0.3 g) was added into the various mixture of substrates (oleic acid-ethanol molar ratios : 1 : 1, 1 : 2, 1 : 3, 1 : 4, 1 : 5, 1 : 6, to 1 : 7). The reaction was carried out at 30 °C for 40 min. The concentration of oleic acid was determined according to Hidayat *et al*., (2008). The forming of OAEE

was calculated as the amount of oleic acid that reacted with ethanol.

The effect of reaction time on the synthesis of oleic acid ethyl ester

Ethanol was added into 1 ml oleic acid, in which the molar ratio was 1 : 2. Crude enzyme (0.3 g) was added into the mixture of substrates. The reaction was performed at 30 °C for various time (20, 40, 60, 80 and 100 min). The concentration of oleic acid was determined according to Hidayat *et al*., (2008). The forming of OAEE was calculated as the amount of oleic acid that reacted with ethanol.

Determination of the optimum condition on the synthesis of oleic acid ethyl ester

The optimum condition was evaluated using Box-Behnken design as shown in Table 1.

Table 1. Experimental combinations following on Box-Behnken design used in the response surface methodology in terms of actual and coded value.

No	X1 (Concentration of Crude Enzyme)	X2 (Molar Ratio of Oleic Acid to Ethanol)	X3 (Reaction Time)
1	0 (0.3 g/ml)	1 (1 : 3)	1 (80 min)
2	0 (0.3 g/ml)	1 (1 : 3)	-1 (40 min)
3	0(0.3 g/ml)	-1 (1 : 1)	1 (80 min)
4	0(0.3 g/ml)	-1 (1 : 1)	-1 (40 min)
5	1 (0.4 g/ml)	0 (1 : 2)	1 (80 min)
6	1 (0.4 g/ml)	0 (1 : 2)	-1 (40 min)
7	-1 (0.2 g/ml)	0 (1 : 2)	1 (80 min)
8	-1 (0.2 g/ml)	0 (1 : 2)	-1 (40 min)
9	1 (0.4 g/ml)	1 (1 : 3)	0 (60 min)
10	1 (0.4 g/ml)	-1 (1 : 1)	0 (60 min)
11	-1 (0.2 g/ml)	1 (1 : 3)	0 (60 min)
12	-1 (0.2 g/ml)	-1 (1 : 1)	0 (60 min)
13	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)
14	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)
15	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)

Hydrolytic activity of lipase

Hydrolytic activity was determined according to Hidayat *et al*., (2008). Crude enzyme (0.1 g) was added into 2 ml of 60% solution containing olive oil in isooctane. The mixture was incubated in water bath shaker at 30 °C, 120 rpm for 20 min. The reaction was terminated by cooling the reaction mixture in an ice bath. Free fatty acid (FFA) was further determined. Unit activity was expressed as the amount of free fatty acid that produced from the hydrolysis of olive oil per min.

Esterification activity of lipase

The esterification activity was determined according to Hidayat *et al*., (2008). Crude enzyme (0.1 g) was added into 2 ml of solution containing of oleic acid (0,5 M) and ethanol (0,5 M). The mixture was incubated in agitated water bath shaker at 30 °C, 120 rpm for 20 min. The reaction was terminated by cooling the reaction mixture in an ice bath. FFA was further determined. Unit activity was expressed as the amount of free fatty acid that reacted with ethanol to form OAEE per min.

Free fatty acid assay

Sample (200 µl) was added into the mixture of isooctane (1.8 ml) and cupri-acetate pyridin (0.4 ml). The mixture was incubated at 30 °C for 10 min, and the absorbance was determined at 715 nm. Free fatty acid was determined by difference.

Results and Discussion.

Characterization of defatted germinated jatropha seeds and rice bran

The characteristic of defatted germinated jatropha seeds and rice bran are shown in Table 2. The recovery of germinated jatropha seeds after the defatting process was 2.7 times lower comparing with rice bran, since jatropha seeds had high concentration of oil. The releasing of oil during defatting process resulted in lower recovery of defatted germinated jatropha seeds. In general, lipase from defatted rice bran had better enzyme activity than lipase from defatted germinated jatropha seeds. Hydrolytic activity of lipase from defatted rice bran was 1.5 times higher than lipase from defatted germinated jatropha seeds. However, the esterification activity was not significant different. The esterification activity of crude

Table 2. Defatted recovery, hydrolysis activity and esterification activity of lipases.

Source of Lipases	Defatted Recovery (%)	Hydrolytic Activity (U/g)	Esterification Activity (U/g)
Germinated Jatropha Seeds	29,41	6.73	298.07
Rice Bran	80	10.57	324.03

enzymes was 44 and 31 times higher than hydrolytic activity for lipase from defatted germinated jatropha seeds and lipase from defatted rice bran, respectively.

The effect of crude enzyme concentration on the synthesis of oleic acid ethyl ester

Figure 1 shows that the amount of OAEE increased with an increase in crude enzyme concentration from both the defatted germinated jatropha seeds and rice bran. The amount of OAEE increased about 3.42 and 3.22 times in an increased concentration of crude enzyme from 0.1 g/ml to 0.3 g/ml for defatted germinated seeds and rice bran, respectively. High concentration of lipase might result in an increase in the frequency of substrates to interact with enzyme. As a consequence, the reaction may increase. The highest amount of OAEE was obtained when the crude enzyme concentration of both enzyme sources were 0.3 g/ml. The amounts of OAEE were 613.84 µmole and 412.30 µmole for rice bran and germinated jatropha seeds, respectively. Further increase in crude enzyme concentration resulted in a decrease in the amount of OAEE. The decrease in the amount of OAEE may be caused by hydrodynamic limitation since enzyme powder may increase the viscosity of the system. The crude enzyme was employed in this research since the activity of lipase is unstable after extraction (unpublished data) (Hidayat *et al*, 2008).

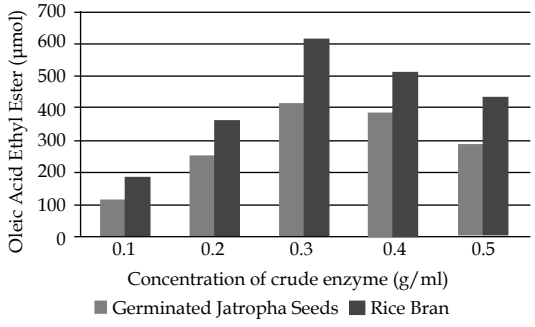

Figure 1. The effect of crude enzyme concentration towards the amount of produced oleic acid ethyl ester which was carried out at 30°C, ratio of oleic acid to ethanol of 1 : 1, for 40 min.

The effect of molar ratio of oleic acid to ethanol on the synthesis of oleic acid ethyl ester

Figure 2 shows that an increase in molar ratio of oleic acid to ethanol from 1 : 1 to 1 : 2 resulted in an increase in the amount of OAEE about 1.58 and 1.69 times for defatted germinated jatropha seeds and rice bran, respectively. Further increase in substrate molar ratio resulted in a decrease in the forming of OAEE. The lowest amount of OAEE was obtained in the molar ratio of 1 : 7 for both enzyme sources. It is suggested that the presence of alcohol at high concentration may cause water inside the lipase structure diffuse into the outside of lipase structure (Ophart, 2003). As consequence of this condition, the protein denaturation may occur and therefore, lipase becomes inactive. The highest amount of OAEE was obtained when the molar ratio of oleic acid to ethanol was 1 : 2 for both enzyme sources. The obtained OAEE were 563.84 µmole and 528.46 µmole for rice bran and germinated jatropha seeds, respectively.

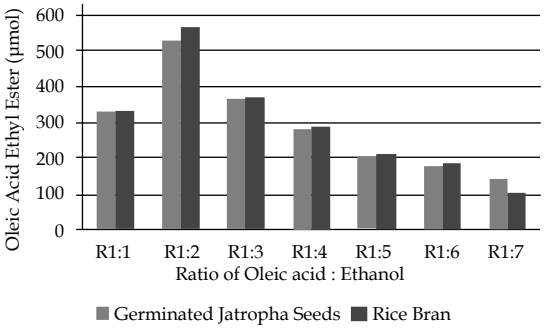

Figure 2. The effect of molar ratio of oleic acid to ethanol towards the amount of produced oleic acid ethyl ester which was carried out at 30°C, using crude enzyme concentration of 0.3 g/ml, for 40 min.

The effect of reaction time on the synthesis of oleic acid ethyl ester

Figure 3 shows that an increase in reaction time from 20 min to 60 min resulted in an increase in the amount of OAEE about 4.53 and 4.67 times for defatted germinated jatropha seeds and rice bran, respectively. Further increase in reaction time resulted in a decrease in the forming of OAEE. It is suggested that a decrease in OAEE due to the forming of water during esterification process since the reaction between 1 mole of oleic acid and ethanol produced 1 mole of water. An increase in water concentration caused the hydrolysis of the formed OAEE. The optimum amount of OAEE was obtained when the esterification reaction was 60 min for both of enzyme sources, in which the amount of OAEE were 871.53 and 701.53 µmole for rice bran and germinated jatropha seeds, respectively.

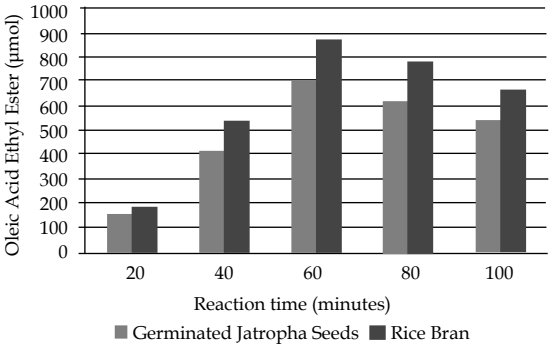

Figure 3. The effect of reaction time towards the amount of produced oleic acid ethyl ester which was carried out at 30 °C, ratio of oleic acid to ethanol of 1 : 2, using crude enzyme concentration of 0.3 g/ml.

Optimization of enzymatic oleic acid ethyl ester synthesis using lipase from rice bran

The effect of crude enzyme concentration, molar ratio of oleic acid to ethanol and reaction time on the forming of OAEE were optimized using lipase from rice bran. The un-coded points for -1, 0 and 1 were chosen according to Figure 1, 2 and 3. Box-behnken design was used to analyze the data as shown in Table 3. The result was then plotted in Figure 4. The mutual interaction between the reaction time and crude enzyme concentration, in the range of 51 - 69 min and 0.27 g/ml – 0.345 g/ml, respectively, resulted in high amount of OAEE when the reaction was carried out at molar ratio of ethanol to oleic acid fixed at 2 as shown in Figure 4A. The high amount of OAEE was obtained when the interaction between molar ratio of ethanol to oleic acid and the reaction time in the range of 1.4 – 2.2 and 51 - 69 min, respectively at enzyme concentration of 0.3 g/ml (defatted rice bran) as shown in Figure 4B. While, the reaction was carried out for 60

Table 3. Box-Behnken design in terms of un-coded and coded value for lipase from rice bran, experimental data for 3-level-3-factors contour plot analysis

No	Concentration of Crude Enzyme (X1)	Molar Ratio of Oleic Acid to Ethanol (X2)	Reaction Time (X3)	Yield (µmol)
1	0 (0.3 g/ml)	-1 (1 : 1)	-1 (40 min)	613.84
2	0 (0.3 g/ml)	1 (1 : 3)	1 (80 min)	428.46
3	0 (0.3 g/ml)	-1 (1 : 1)	1 (80 min)	446.15
4	0 (0.3 g/ml)	1 (1 : 3)	-1 (40 min)	371.53
5	-1 (0.2 g/ml)	0 (1 : 2)	-1 (40 min)	412.31
6	1 (0.4 g/ml)	0 (1 : 2)	1 (80 min)	573.85
7	-1 (0.2 g/ml)	0 (1 : 2)	1 (80 min)	346.92
8	1 (0.4 g/ml)	0 (1 : 2)	-1 (40 min)	396.92
9	-1 (0.2 g/ml)	-1 (1 : 1)	0 (60 min)	452.31
10	1 (0.4 g/ml)	1 (1 : 3)	0 (60 min)	408.46
11	-1 (0.2 g/ml)	1 (1 : 3)	0 (60 min)	304.62
12	1 (0.4 g/ml)	-1 (1 : 1)	0 (60 min)	346.92
13	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)	871.53
14	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)	820.00
15	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)	811.54

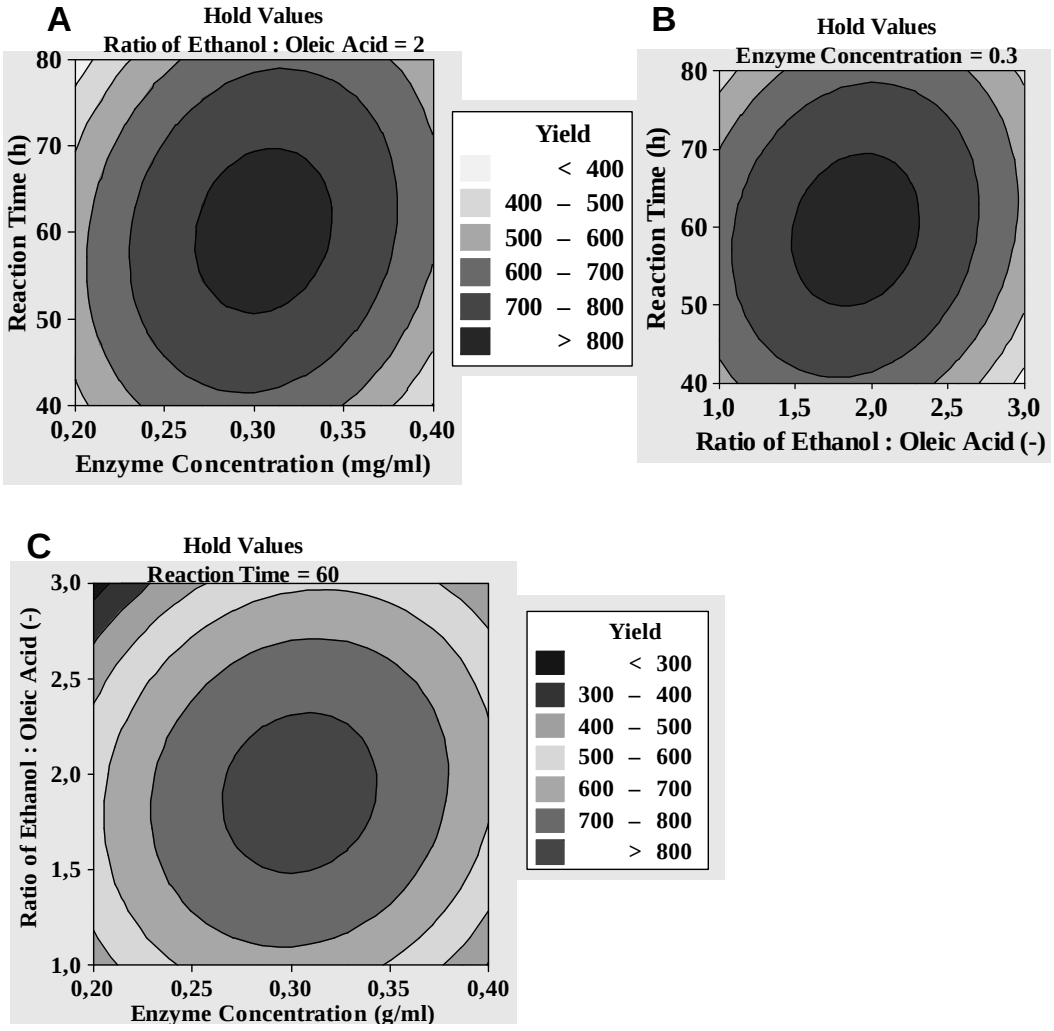

Figure 4 (A) Contour plots showing the effect of rice bran crude enzyme concentration, reaction time, and their mutual interaction on oleic acid ethyl ester synthesis at ethanol : oleic acid molar ratio fixed at 2. (B) Contour plots showing the effect of ethanol : oleic acid molar ratio, reaction time, and their mutual interaction on oleic acid ethyl ester synthesis using 0.3 mg/ml rice bran. (C) Contour plots showing the effect of rice bran crude enzyme concentration, ethanol : oleic acid molar ratio, and their mutual interaction on oleic acid ethyl ester synthesis at reaction time of 60 min.

min, the mutual interaction between molar ratio of ethanol to oleic acid and crude enzyme concentration in range of 1.4 – 2.2 and 0.27 g/ml – 0.345 g/ml, respectively, resulted in high amount of OAEE as shown in Figure 4C.

Optimization of enzymatic oleic acid ethyl ester synthesis using lipase from germinated jatropha seeds.

The effect of crude enzyme concentration, molar ratio of oleic acid to ethanol and the reaction time on the forming of OAEE were also optimized using lipase from germinated jatropha seeds. Box-behnken design using the un-coded points for -1, 0 and 1 was used to analyze the data as shown in Table 4 and the result was then plotted in Figure 5.

The mutual interaction between molar ratio of ethanol to oleic acid and crude enzyme concentration, in range of 1.3 – 2.5 and 0.25 g/ml – 0.35 g/ml, respectively, resulted high amount of OAEE when the reaction was

Table 4. Box-Behnken design in terms of un-coded and coded value for lipase from germinated jatropha seeds, experimental data for 3-level-3-factors contour plot analysis

No	Concentration of Crude Enzyme (X1)	Molar Ratio of Oleic Acid to Ethanol (X2)	Reaction Time (X3)	*Yield*/etil oleat(µmole)
1	0 (0.3 g/ml)	-1 (1 : 1)	-1 (40 min)	412.31
2	0 (0.3 g/ml)	1 (1 : 3)	1 (80 min)	364.62
3	0 (0.3 g/ml)	-1 (1 : 1)	1 (80 min)	317.69
4	0 (0.3 g/ml)	1 (1 : 3)	-1 (40 min)	367.69
5	-1 (0.2 g/ml)	0 (1 : 2)	-1 (40 min)	333.08
6	1 (0.4 g/ml)	0 (1 : 2)	1 (80 min)	206.15
7	-1 (0.2 g/ml)	0 (1 : 2)	1 (80 min)	346.92
8	1 (0.4 g/ml)	0 (1 : 2)	-1 (40 min)	369.23
9	-1 (0.2 g/ml)	-1 (1 : 1)	0 (60 min)	368.46
10	1 (0.4 g/ml)	1 (1 : 3)	0 (60 min)	380.77
11	-1 (0.2 g/ml)	1 (1 : 3)	0 (60 min)	218.46
12	1 (0.4 g/ml)	-1 (1 : 1)	0 (60 min)	288.46
13	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)	701.53
14	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)	628.46
15	0 (0.3 g/ml)	0 (1 : 2)	0 (60 min)	632.31

carried out for 60 min, as shown in Figure 5A. The mutual interaction between the reaction time and crude enzyme concentration, in the range of 45.5 - 70 min and 0.25 g/ml – 0.35 g/ml, respectively, resulted high amount of OAEE when the reaction was carried out at molar ratio of ethanol to oleic acid fixed at 2 as shown in Figure 5B. Figure 5C shows that the high amount of OAEE could be obtained, when the interacted molar ratio of ethanol to oleic acid and the reaction time in the range of 1.7 – 2.2 and 54 - 61 min, respectively, at enzyme concentration of 0.3 g/ml (defatted rice bran). It can be concluded that the mutual interaction between one variable to another variable influenced the amount of the formed OAEE for both enzyme sources.

Table 5 shows the optimum condition of reaction. The optimum concentration of crude enzyme, molar ratio of oleic acid to ethanol and the reaction time for lipase from rice bran were 0.29 g/ml, 1 : 2.05 and 58.61 min, respectively. While, the optimum concentration of crude enzyme, molar ratio of oleic acid to ethanol and the reaction time for lipase from germinated jatropha seeds were 0.31 g/ml, 1 : 1.81 and 50.90 min, respectively. The computational prediction of the formed OAEE for lipase from rice bran and germinated jatropha seeds using RSM was 829.99 µmole and 635.00 µmole, respectively. The validation experiment was carried out in order to assess the validity of the constructed model of RSM. The result was 810.77 µmole and 626.92 µmole for lipase from rice bran and lipases from germinated jatropha seeds, respectively, as summarized in Table 5.

By comparing the yields of the obtained OAEE computationally with the yields of the obtained OAEE experimentally

Table 5. The optimum conditions of reaction, the yield obtained computationally (prediction) and yield obtained experimentally (Validation) for lipases from rice bran and germinated jatropha seeds.

No	Source of Enzyme	Concentration of Crude Enzyme (g/ml)	Molar Ratio of Oleic Acid to Ethanol	Reaction Time (Min)	Prediction (µmole)	Validation (µmole)
1	Rice Bran	0.29	1 : 2.05	58.61	829.99	810.77
2	Germinated Jatropha Seeds	0.31	1 : 1.81	50.90	635.00	626.92

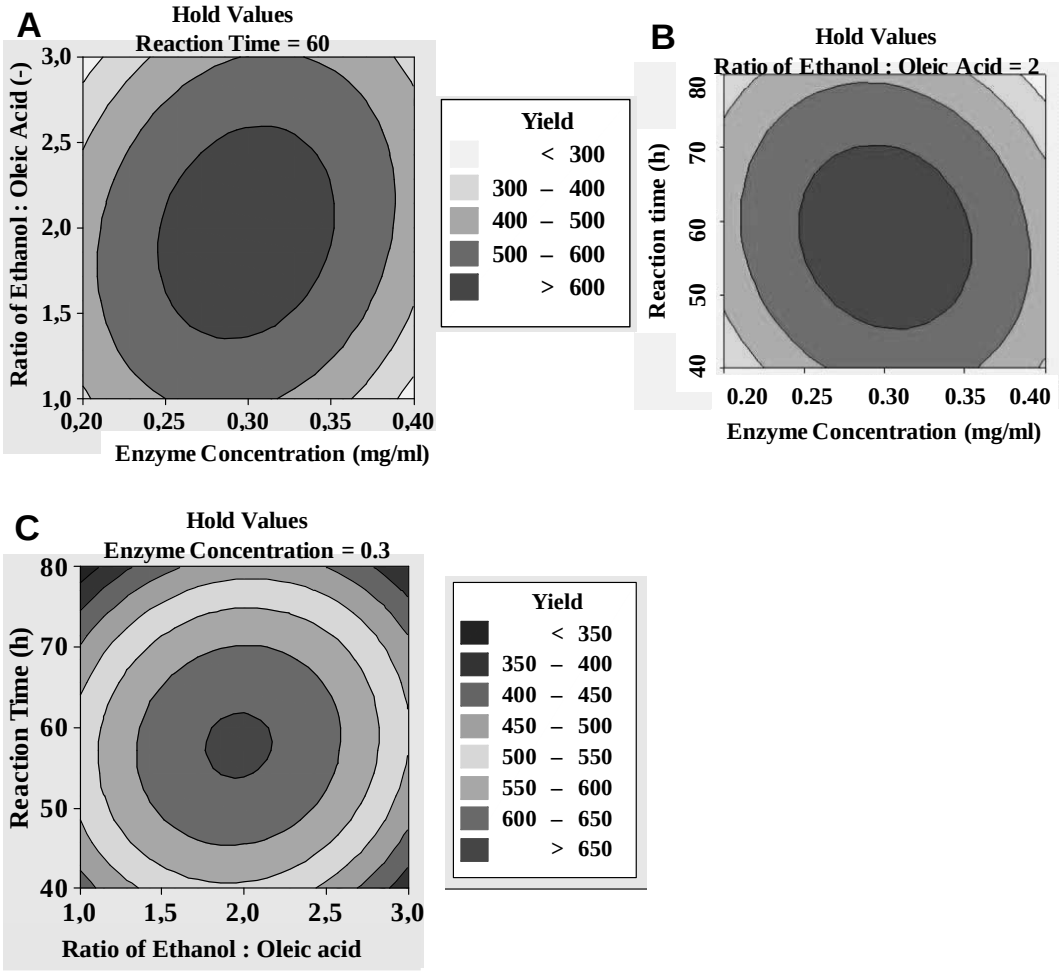

Figure 5. (A) Contour plots showing the effect of germinated jatropha seeds crude enzyme concentration, ethanol : oleic acid molar ratio and their mutual interaction on oleic acid ethyl ester synthesis at reaction time of 60 min. (B) Contour plots showing the effect of germinated jatropha seeds crude enzyme concentration, the reaction time and their mutual interaction on oleic acid ethyl ester synthesis at ethanol : oleic acid molar ratio fixed at 2. (C) Contour plots showing the effect of ethanol : oleic acid molar ratio, reaction time and their mutual interaction on oleic acid ethyl ester synthesis using 0.3 mg/ml lipase from germinated jatropha seeds.

(validation experiment) as described in Table 5, it is concluded that the yields among two different approaches were not much different for both of enzyme sources.

In this research, lipase from germinated jatropha seeds had the hydrolitic and esterification activity about 6.73 U/g and 298.07 U/g, respectively. Lipase from rice bran had the hydrolitic and esterification activity about 10.57 U/g and 324.03 U/g, respectively. The optimum conditions of reaction using lipase from rice bran were obtained when concentration of crude enzyme, molar ratio of oleic acid to ethanol and the reaction time were 0.29 g/ml, 1 : 2.05 and 58.61 min for respectively. For lipase from germinated jatropha seeds, the optimum condition of reaction were obtained when concentration of crude enzyme, molar ratio of oleic acid to ethanol and the reaction time were 0.31 g/ml, 1 : 1.81 and 50.90 min, respectively. The amounts of OAEE were 810.77 µmole and 626.92 µmole for lipase from rice bran and germinated jatropha seeds, respectively.

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