1 **Lipase cocktail for efficient conversion of oils containing phospholipids to biodiesel**

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16

17 **Abstract**

18 The presence of phospholipid has been a challenge in liquid enzymatic biodiesel production.
19 Among six lipases that were screened, lipase AY had the highest hydrolysis activity and a
20 competitive transesterification activity. However, it yielded only 21.1 % FAME from oil
21 containing phospholipids. By replacing portions of these lipases with a more robust
22 bioFAME lipase, CalT, the combination of lipase AY – CalT gave the highest FAME yield
23 with the least amounts of free fatty acids and partial glycerides. A higher methanol addition
24 rate reduced FAME yields for lipase DF – CalT and A10D – CalT combinations while that of
25 lipase AY – CalT combination improved. Optimizing the methanol addition rate for lipase
26 AY – CalT resulted in a FAME yield of 88.1 % at 2 h and more than 95 % at 6 h. This
27 effective use of lipases could be applied for the rapid and economic conversion of unrefined
28 oils to biodiesel.

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31 **Keywords**

32 Biodiesel; Enzyme biocatalysis; Mixed lipase; Transesterification; Phospholipid

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35 **Introduction**

36 Biodiesel, an alternative to fossil fuel keeps gaining attention in research due to its
37 environmentally friendly nature among other advantages. It is a mixture of Fatty Acid Methyl
38 Esters (FAME) that has conventionally been produced from the alkaline transesterification of
39 animal and vegetable oils (Vyas et al., 2010). In advanced researches, a two – step method of
40 esterifying high free fatty acid containing oil with acid catalysts before transesterification
41 with the alkaline catalyst has been developed (Chen et al., 2012; Hancsok et al., 2004; Wang
42 et al., 2006). This is to prevent the formation of soap. Other heterogeneous alkaline and acid
43 catalysts such as $\text{CaO}/\text{Al}_2\text{O}_3$ and $\text{Li-CaOFe}_2(\text{SO}_4)_3$ have been developed over the past years
44 for the production of biodiesel (Umdu et al., 2009; Endalew et al., 2011). Although these
45 catalysts may offer some advantages over the conventional ones, the environmentally benign
46 purpose of substituting fossil fuel with biodiesel is undermined. These chemicals cause
47 corrosion to reactors and the post-processing needed to finalize the biodiesel production
48 causes an equally environmental hazardous situation. To this end, current research in
49 biodiesel production has been channeled to the use of EC 3.1.1.3 lipases.

50 EC 3.1.1.3 lipases are hydrolases that act on ester bonds in carboxylic esters. There
51 are hundreds of these lipases and many of them from various microorganisms have been
52 known to be applicable in the industrial production of biodiesel (Li et al., 2011; Ghaly et al.,
53 2010). A well-known bacterium for lipase production is *Burkholderia glumae* (Jaeger et al.,
54 1998). *Thermomyces lanuginous* and *Rhizopus oryzae* are popular lipases from fungi and
55 *Candida rugosa* is a common yeast for lipase production (Nordblad et al., 2014; Oda et al.,
56 2005; Chang et al., 1994). These lipases can either be used in the immobilized form or in the
57 liquid form. The use of immobilized lipases offers an advantage of easy product separation
58 and reuse of lipase. Unfortunately, on the industrial scale, the immobilization of purified
59 lipase adds significant cost to the biodiesel production and the immobilization of lipases often

60 alters the desired specificity of the lipase (Guldhe et al., 2015; Christopher et al., 2014).
61 Furthermore, immobilizing lipase leads to an increase in mass transfer resistance due to the
62 solid nature of the enzyme and the liquid nature of the other reactants and this often leads to
63 slower reaction times. This gives liquid lipases an urge over immobilized lipases.

64 The drift of attention towards the enzymatic biodiesel production is due to the fact
65 that, enzymes can convert oils to biodiesel under very mild conditions of temperature and
66 pressure, they are highly environmentally friendly and due to the unique specificities of
67 lipases, the production of unwanted products are eliminated. Although the regiospecific
68 nature of lipases eliminates the production of unwanted products, this specificity may also
69 lead to low product yield as most of the oil substrate consist of a wide range of compounds.
70 Generally, most oil substrates are made of triglycerides, diglycerides, monoglycerides and
71 free fatty acids of various chain lengths. Some lipases are known to be **sn-1,3 regiospecific**
72 which can cleave the ester at positions 1 and 3 of the triglyceride alone (Du et al., 2005;
73 Hama et al., 2008; Guan et al., 2010). Others are known to act on di and monoglycerides only
74 where some are only effective on free fatty acids (Hama et al., 2009; Adachi et al., 2011).
75 Furthermore, with the aim of reducing the cost of feedstock, the use of unrefined oils are
76 being considered for biodiesel production and this often contains high levels of free fatty
77 acids as well as phospholipids, making the oil substrate even more complicated.

78 Phospholipids have been known to accelerate lipase inhibition with the formation of
79 reverse micelles which alters the nature of the reaction mixture (Amoah et al., 2016; Li et al.,
80 2014). Degumming of crude oil has been proposed for curbing this problem however, this
81 adds additional cost, increases processing steps and causes loss of feedstock. Various
82 common lipases have been used for the conversion of refined vegetable oil to biodiesel but
83 their successful application in oils containing phospholipids is yet to be investigated. **Adachi**
84 **et al. (2013)** reported the successful use of *C. rugosa* lipase for the hydrolysis of soybean oil

for a two – step biodiesel conversion. Oda et al. (2005) also reported the use of an sn – 1,3 regiospecific lipase where acyl migration was facilitated by an increased water content resulting in an efficient FAME production.

Due to the unique properties of individual lipases, the concept of lipase cocktail which involves the combination of two or more lipase has led to interesting results. Lee et al. (2011) found out that a combination of *C. rugosa* and *R. oryzae* lipases in supercritical carbon dioxide gave a better yield than the use of a single lipase. A combination of CalT, RML and CalB improved the conversion yield from olive oil to 95 % in 18 h, up from the 50 % for the best individual lipase (CalB) where as a combination of CalT and RML improved the conversion from palm oil to 80 % (Poppe et al., 2015). In the ethanolysis of used palm oil, the combined use lipase AY and lipase AK gave a higher yield of biodiesel than using AK alone (Tongboriboon et al., 2010).

In this work, the concept of lipase cocktail is employed to reduce total lipase loading in order to reduce the cost of lipase. The first part deals with screening of 6 lipases commonly used in the food industry, and their applicability in biodiesel production from oil containing phospholipid is explored. Following this, a comparative analysis of several lipase cocktails is carried out and the best lipase combination based on reaction rate, final FAME yield and residual partial glyceride composition selected. Ultimately, the improvement of reaction rate for an efficient production of FAME from oil containing phospholipid is demonstrated.

2. Materials and Methods

2.1 Materials and lipase strains

Callera Trans L, a liquid formulation of *Thermomyces lanuginosus* lipase (CalT) was obtained from Novozymes (Bagsvaerd, Denmark). Powdered lipases DF and MER from

Rhizopus oryzae, AY from *Candida cylindracea* (formerly known as *Candida rugosa*), R from *Penicillium roqueforti* and A from *Aspergillus niger* were obtained from Amano (Nagoya, Japan). Powdered lipase A-10D from *Rhizopus* sp, was obtained from Nagase Enzymes (Kyoto, Japan). Refined soybean oil and soybean phospholipids were obtained from Wako Pure Chemical industries (Osaka, Japan).

2.2 Lipase solution preparation

1 g each of 6 different powdered lipases was dissolved separately in 10 ml pH 7 phosphate buffer. They were set on Bioshaker at 30 °C and 200 rpm for 30 min. The activities of the obtained lipase solutions were tested and were further used for the transesterification reaction.

2.3 Lipase activity assay

The activity assays of the lipase for both transesterification and hydrolysis were carried out. The transesterification activities were tested using p-nitrophenyl butyrate (pNPB) as a chromogenic substrate. A stock solution was prepared by dissolving 5 µl of pNPB in 250 µl of ethanol and further diluting to 50 ml with distilled water. The lipase-hydrolytic activities at 35 °C of the various lipases were carried out by incubating for 10 min in a Bioshaker, Taitec, Japan. After incubation, 5% of trichloroacetate was added to terminate the reaction. The absorbance of the produced para nitrophenol (pNP) was measured at 400nm (UV-Vis spectrophotometer, Shimadzu). 1 unit (U₂) of lipase activity was defined as the amount of lipase that liberates 1 µmol of pNP from pNPB per minute.

For hydrolysis, the reaction mixture contained 2 g of olive oil, 9 ml of 0.1M acetate buffer (pH 5.6) and 1 ml of 0.05 M CaCl₂ in a 50 ml screw-cap bottle. It was stirred in a water bath at 250 rpm at 30 °C and the reaction was terminated by the addition of ethanol.

The obtained emulsion was titrated against 0.1 NaOH till a pH of 10. This hydrolytic activity (U_1) is defined as the the amount of lipase that liberates 1 μ mol of FFA from olive oil at pH 10 and 37 °C.

2.4 Lipase-mediated methanolysis

Soybean oil was spiked with 5% (w/w) soybean phospholipid to simulate the presence of phospholipid in low quality crude oils and subjected to sonication to ensure homogeneity. The lipase-catalyzed methanolysis was performed in a cylindrical glass tubes with rubber silicon stopper. The reaction mixture consisted of 10 g oil (with or without 5 % phospholipids) and a pre-mixed liquid lipase and distilled water. In this work, the aqueous content expressed in percentage (w/w) represents the total amount of the lipase solution and the additional distilled water. The reaction was initiated via the addition of an initial amount of methanol (1:1 molar ratio of the oil). The reaction proceeded in a Chemistation (Eyela chemistation, PPW-20A) with a stirrer at varying rotation rates and operated at various reaction temperatures. Generally, the remaining methanol was added step-wise in three steps (1:1 molar ratios of the oil at each time leading to a total of 1:4). Samples were taken at specified times to determine the amount of remaining triglycerides and produced partial glycerides, free fatty acids and fatty acid methyl esters over the course of the reaction.

2.5 Gas chromatography analysis

Fatty Acid Methyl Esters (FAMES), Free Fatty Acids (FFA), Monoglycerides (MAG) and Diglycerides (DAG) produced during the course of the reaction as well as Triglycerides (TAG) were measured via gas chromatography. Samples taken at specified times were centrifuged at 12,000 g for 5 min at 4 °C, and the upper layer was analyzed using GC – 17A (Shimadzu, Kyoto, Japan) equipped with a ZB-5HT capillary column (0.25 mm x 15 m)

(Phenomenex, USA), an auto-sampler and a flame ionization detector. During the analysis, the temperatures of the injector and detector were set at 320 and 380 °C respectively using Helium as carrier gas at a flow rate of 58 ml/min. The column was configured at a temperature program with an initial temperature of 130 °C for 2 min, raised to 350 °C at 10 °C/min, then to 370 °C at 7 °C/min, and maintained at this temperature for 10 min. The FAME composition in each reaction mixture was reported as the percentage of the oil in the reaction mixture using tricaprylin as an internal standard.

3. Results and discussion

3.1. Evaluation of lipase activity

In methanolysis reactions involving lipases, some enzymes are known to hydrolyze the triglycerides into free fatty acids (FFA) which is then followed by the esterification of the free fatty acids into fatty acid methyl esters (FAME). In other cases, the fatty acids on the triglycerides are directly transesterified to fatty acid methyl esters. This shows that, some lipases have higher activity towards hydrolysis than transesterification and vice versa. In order to verify this for the 6 lipases, two different methods for lipase activity determination were employed (Table 1). In the first activity test, p-nitrophenyl butyrate was used as the substrate for transesterification. The activity of the lipase depends on its ability to break the ester bond in this substrate and this allows for an unbiased evaluation where the efficiencies of the lipases are not limited by their regiospecificities or chain lengths. With this method, lipase A is found to be the most efficient in cleaving the ester bond amongst the other lipases under study with an activity of 19400 U/ml. Lipases from different sources have different structural properties which affects their activity, and the unique structure of lipase A might be the reason for its high activity (Christopher et al., 2014). The activities of 4 lipases, DF, A –

10D, R and AY were found to be almost the same, whereas MER showed the least, with an activity less than half of the average of the other lipases.

The second method involves the hydrolytic activity using olive oil as substrate where the extent of hydrolysis by the individual lipases was determined. Lipase AY from *Candida cylindracea* clearly shows a high extent of hydrolysis which is more than 2 times higher than lipase DF, the next best candidate. This is in conformity with other researchers who reported the high hydrolysis effectiveness of lipase AY. More than 90 % FFA acid has been produced in 24 h using *C. cylindracea* (Adachi et al., 2013; Talukder et al., 2010). The rest of the lipases showed relatively poor activity for hydrolysis despite their high transesterification activity. This explains the high preference of transesterification over hydrolysis for these lipases. With a very high activity towards hydrolysis and a very comparative activity towards transesterification, lipase AY tends to be a versatile lipase in enzymatic biodiesel conversion.

3.2 Lipase screening

Although the lipase activities have been determined, the performance of the lipase on an actual biodiesel's feedstock substrate may vary due to the varying regiospecificities of lipases. Refined soybean oil which has been known to be a high quality substrate for biodiesel production was then used to screen the lipases for production of biodiesel (Fig. 1). The results from this screening clearly show the higher preference for hydrolysis over transesterification for AY in the initial stages of the reaction. Within the first 3h, FAME production reached only 29 % whereas the FFA production reached above 45 %. This is an indication that, under the reaction conditions, lipase AY greatly promotes the preference of water uptake than methanol leading to the formation of more FFA rather than FAME. Theoretically, 1 molar equivalent of methanol produces a maximum of 33 % FAME. Under

the given reaction conditions, there is a possibility that, after most of the initially added methanol has been used up for FAME production, lipase AY with a high activity for hydrolysis continues to convert the triglycerides to FFA. The produced FFA was then quickly converted to FAME at 6 h doubling the amount of FAME to about 60 % which is also the highest among the tested lipases. Most lipases have been known to exhibit greater performance in FFA-rich substrates such as waste cooking oils than in TAG-rich oils (No, 2011). This enabled a quicker conversion to FAME reaching about 60 % by 6 h. However, at the end of the reaction (24 h), the amount of FFA remaining in the reaction system was above 20 %. This indicates the lipase AY's limitation to effectively convert all the produced FFA to FAME. Fig. 1a also shows that partial glycerides remained significantly low throughout the reaction with AY indicating a rapid liberation of fatty acids from the glycerol backbone.

Lipase DF which is known to be a sn – 1,3 regiospecific lipase also showed a relatively good FAME production rate. It had the highest initial reaction rate among the tested lipases reaching about 33 % at 3 h. However, this rate reduced after the next three hours. This was expected because, the amount of fatty acids at positions 1 and 3 in the reaction mixture had reduced fairly and with a reduced reactant concentration, the reaction rate decreased. Fig. 1b shows an initial production of a small amount of free fatty acid which signifies that, although lipase DF has some hydrolysis effect, it is more favorable towards transesterification. With this, significant amounts of partial glycerides could be observed along the progress of reaction with a plateau from 9 h which can be as a result of limitations to regiospecificity. A similar pattern was observed for lipases A – 10D and MER with a slightly lower reaction rate and final FAME yield.

Lipase R being an α – specific lipase has a similar regiospecificity as lipase MER. It is however observed that, lipase MER has a significantly better performance than lipase R, although lipase R shows a higher activity for transesterification (Table 1). This further shows

the effect the chain lengths of the fatty acids have on the effectiveness of lipases. Lipase MER effectively hydrolyzes long, medium and short chain fatty acids and this allows it to act on a broader range of chain lengths compared to lipase R which is specific only for medium and short fatty acid chains. Since biodiesel feedstock have varying fatty acid chain lengths including C16 and C18, it is more effective to use lipases that have the ability to cleave bonds of varying chain length of fatty acids. Although from Table 1 lipase A had the highest hydrolytic activity, it produced almost no yield throughout the reaction. This is may be due to lipase A's poor methanol tolerance under the given reaction conditions.

3.3. Effect of PL on lipases

In order to apply cheap lipases on cheap and unrefined feedstock, the best 3 lipase candidates from the screening experiments were used to convert soybean oil containing phospholipids. The selected lipases included AY, DF and A 10D. Surprisingly, from Fig. 2 the order of performance changed significantly with DF producing as high as 69 % FAME at 9 h. The performance of A10 – D also improved slightly compared to the case of the refined oil. A similar improvement in FAME production using liquid lipase in 2 % phospholipid has been reported earlier by (Li et al., 2013). The amphipatic property of phospholipid was thought of to contribute to the total emulsification of the reaction mixture which consequently boosted the catalytic performance of the free lipase.

Unfortunately, AY which gave a faster reaction rate and a higher FAME yield produced only a maximum of 21 % FAME. This indicates a quick deactivation of lipase AY in phospholipid containing oil contrarily to DF and A – 10D. The poor performance of *C. cylindracea* (lipase AY) in the presence of high amounts of polar compounds has been reported by various researchers (Moreira et al., 2007; Pereira et al., 2003). High concentration

of phospholipids in the feedstock leads to the formation of phospholipid reverse micelles which then causes both the liquid lipase and methanol to solubilize inside the reverse micelles (Li et al., 2014). This increases the methanol concentration around the locality of lipase AY. The poor tolerance of polar compound methanol, by lipase AY causes great loss in the transesterification activity thus, resulting the decrease of FAME yield after the subsequent addition of methanol.

3.4. Lipase cocktail for conversion of phospholipid – containing oil

The best candidate among the cheap lipases for the transesterification of refined oil showed a very poor performance in the presence of phospholipids. For this reason, the concept of lipase cocktail was employed to remediate this problem. In this system, a small portion of a more robust lipase for biodiesel production was added to the various lipases separately. In the first stage of the experiments, the process of using this industrialized lipase, Callera Trans L (CalT), a liquid formulation of *T. lanuginosus* lipase, was optimized. The results of the optimization have been summarized in Table 2. Although 0.5 % (w/w of refined oil) of the lipase yielded more than 90 % FAME, the optimization was carried out using 0.3 % lipase loading in order to reduce the amount of lipase for economic reasons. It can be observed that, by varying the experimental conditions, the conversion efficiency could not be significantly improved using 0.3 % CalT. The use of liquid lipase is associated with an inevitable presence of an aqueous phase, which is also required for the creation of an aqueous – non – aqueous interfacial area needed for lipase activation. An efficient agitation is a co-factor for the creation of this interfacial. Although lower concentrations of water have been reported for *T. lanuginosus*, the use of a more efficient agitation may be the reason for an allowable higher water concentration. Due to higher cost of this lipase, the possibility of

280 using even a lower amount of this lipase and combining with the other cheaper lipases for a
281 more economic but effective lipase usage was therefore explored. In the use of the cheaper
282 lipases, 0.4 % (w/w of refined oil) of lipase AY was found to be the highest lipase loading
283 that was unable to effectively assimilate the initial 1:1 molar equivalent of methanol to oil in
284 the conversion of the oil to FAME (supplementary fig. 1). Hence, further experiments were
285 carried out by combining 0.1 % CalT with 0.4 % of the other individual lipases (Fig. 3).

286 Under this condition of lipase cocktail and reduced total lipase loading, the FAME
287 yields and reaction rates were generally higher. Interestingly, the poor performance of AY in
288 phospholipid containing oil has improved with the addition of CalT. The improvement in
289 FAME production by mixing lipase AY with CalT is likely due to CalT's ability to
290 effectively convert triglycerides into FAMEs even in the presence of phospholipids. CalT is
291 known to have a very high initial conversion rate of the sn – 1,3 fatty acid which is associated
292 with a rapid consumption of methanol (Nordblad et al., 2014). This results in a quicker
293 reduction of methanol which is solubilized inside the phospholipid reverse micelles. With the
294 rapid reduction of polar methanol in the locality of lipase AY inside the reverse micelles, the
295 risk of methanol inhibition is consequently decreased. This elimination of inhibition thus
296 enables lipase AY to contribute to the FAME production process leading to an improvement
297 over using lipase AY alone in a high polar reaction medium.

298 Furthermore, lipase AY – CalT cocktail resulted in a drastic reduction of residual
299 partial glycerides and free fatty acids (Fig. 3b). The high hydrolysis activity and non –
300 specificity of lipase AY may have contributed to the efficient liberation of fatty acids leading
301 to the absence of di- and mono-glycerides which rather persisted in the other combinations or
302 CalT alone. Although di – and triglycerides are good substrate for the conversion by CalT,
303 the low loading of CalT may be the reason for persisting higher amount of residual di – and
304 triglycerides at 24 h for 0.1 % CalT alone. High amounts of FFA, MAG, DAG and TAG

were also found in the products from DF – CalT cocktail and lipase A10D – CalT cocktail. These were however lower than the case of 0.1 % CalT because the presence of lipase DF or A10D further contributed to the total conversion of partial glycerides and free fatty acids to FAME. The performance of DF – CalT cocktail was slightly better than A10D – CalT which is in correspondence to the higher activity of lipase DF than lipase A10D. Although the specificity of lipase A10D is not well known, this results suggests that it might be a sn – 1,3 specific lipase due to the high amounts of remaining MAG similarly to lipase DF. DAGs and MAGs are hygroscopic molecules which absorbs moisture from the environments. Their presence in biodiesel increases the moisture content of the biodiesel which is an undesirable property (Jakeria et al., 2014; Prasad et al., 2000). Concentrations of less than 0.2 % and 0.8 % respectively have been recommended. The absence of these components in the conversion using lipase AY – CalT is an added advantage of using this cocktail.

3.5 Impact of methanol addition rate on lipase cocktail

Although in biodiesel production short chain alcohols are essential for the methylation of the fatty acids, high concentration of these alcohols possess negative effect on most lipases. Lipase mediated methanolysis of triglycerides is known to progress with a nucleophilic addition reaction to form an enzyme-substrate complex. Methanol then attaches itself to this acyl enzyme intermediate and through a series of electron transfers, FAME is produced. High alcohol concentration is known to cause competitive inhibition where an alcohol molecule directly reacts with the enzyme to produce a dead-end enzyme-alcohol complex instead of the alcohol reacting with an enzyme-fatty acid intermediate (Al-Zuhair et al., 2007). Most lipases have very low tolerance for methanol as such a strategy of step-wise addition of methanol has been used to successfully convert triglycerides to FAMEs. High methanol addition rate often

leads to high concentration of methanol which subsequently leads to lipase deactivation whereas low methanol addition often leads to extended reaction times. [Fig. 3c](#) shows the performance of the 3 lipase cocktails under a higher methanol addition rate. The usual rate of 1 molar equivalent added at 3 h intervals was increased to 1 molar equivalent at 2 h intervals. It can be observed that although the initial reaction rates for all 3 lipase systems were similar, the rate began to slow down significantly after 6 h except that of AY – CalT cocktail. Lipase A10D – CalT cocktail does not show any significant change compared with the low addition rate and thus, the general low reaction rate and yield were not improved by the increase in methanol addition rate. Unfortunately, lipase DF – CalT cocktail which had a comparable yield to lipase AY – CalT under low methanol addition rate was significantly retarded under higher methanol addition rate yielding only 73 % at 24 h. This indicates lipase DF's limitation to higher methanol concentration within this range. Interestingly, for AY-CalT cocktail, the reaction continued to progress at a high rate reaching more than 90 % FAME by 12 h. The usual decrease in the reaction rate after the addition of 3 and 4 molar equivalents of methanol was not observed in this case. This happens because CalT is known to have high activity for transesterification and esterification but a slower conversion of monoglycerides. With the combination of two lipases which have different reaction pathways, a high level of transesterification and high level of hydrolysis occur simultaneously. Both methanolysis and hydrolysis have been explained using the ping-pong bi bi mechanism where methanolysis is usually preferred over hydrolysis in the presence of sufficient amount of methanol. In the reaction system where both CalT and AY exists, even at the point where methanol concentration is low, AY having a high activity for hydrolysis perpetuates the progress of the reaction leading to the production of high level of FFA. CalT is known to function better under lower water concentration ([Table 2](#)). By the formation of FFA, the concentration of water in the mixture reduces, which creates a better environment for both esterification of the

produced FFA and transesterification of other remaining glycerides by the CalT. This esterification also liberates the water that was used up in the hydrolysis which again supports the hydrolysis activity of lipase AY. The elimination of the acyl migration rate limiting step by lipase AY, coupled with the constant production of FFA which is more miscible with methanol compared with triglycerides, a more efficient consumption of methanol is achieved leading to an allowable faster methanol addition rate and a faster reaction.

3.6 Optimization of methanol addition rate

As the combination of lipase AY – CalT showed very low residual glycerides and a better tolerance to high methanol addition rate, the rate of methanol addition was therefore optimized for this combination. The range tested for in the set of experiments was 4 times methanol addition of 1:1 molar equivalent of the oil at time intervals of 120 min, 60 min, 30 min, 10 min as well as the 1 time addition of 1:4 molar equivalent of the oil at 0 min. In [Fig. 4](#) increasing the methanol addition rates from 120 min intervals to 30 min intervals a consistent increase in the reaction rate can be observed. Although both the addition rates of 60 min intervals and 30 mins intervals reached over 90 % at 6 h, the additions at 30 mins interval yielded 88.1 % at 2 h compared to only 57.8 % for the additions at 60 mins intervals. This is due to the availability of methanol and enough reaction time. In the additions at 30 mins intervals, a total of 1:4 molar equivalent of methanol could be successfully added by 1.5 h and due to the high presence of FFA resulting from the hydrolysis by lipase AY, 0.5 h was sufficient for the fast conversion to FAME by CalT at 2 h. However, for the additions at 60 mins intervals, the addition of only 1:2 molar equivalents is attained at 2 h. Theoretically, a minimum of 1:3 molar equivalents of methanol is required for the complete FAME production but since the reaction is reversible, an excess of methanol is usually needed. With

an insufficient amount of methanol at this addition interval, only less than 60 % could be produced.

Further increase in the addition rate to 1:1 molar equivalent at 10 min intervals shows a significant decrease in the reaction rate especially towards the later part. This is likely due to the accumulation of methanol beyond the tolerance of this lipase system. The concentration of methanol at this point maybe so high that, even though CalT effectively transesterifies fatty acids at positions 1 and 3, the amount of methanol is still enough to deactivate lipase AY. This is further revealed in the later part of the reaction where although the reaction proceeds but at a very slow rate. The theoretical yield for sn – 1,3 regiospecific lipases is 66.7 %, but the ability of some of these lipases to induce acyl migration ensures higher yield but at a slower pace (Du et al., 2005; Oda et al., 2005). Since the non – specific activity of the lipase cocktail was lost in the early part of the reaction, the inevitable rate – limiting step of acyl migration was carried out by CalT leading to a slower FAME production after over 55 % FAME has been produced. The 1 time addition of 1:4 molar equivalents further shows slower reaction rate leading to a FAME production of only 66.9 % at 24 h. This is an indication that, within this range of methanol concentration, CalT also faces some inhibition by methanol and reduces its effectiveness in the initial stages of the reaction and the acyl migration. The optimum methanol addition rate for lipase AY – CalT combination is therefore 4 times addition of 1:1 molar equivalent to the oil at 30 min intervals.

Conclusion

The presence of 5 % phospholipid in oil inhibits lipase AY. A combination of 0.4 % lipase AY and 0.1 % CalT led to an improvement in biodiesel production. This resulted from CalT's high initial reaction rate and lipase AY's high hydrolysis activity and its non – regiospecificity. The different reaction pathways of the individual lipases led to efficient methanol assimilation and reduce lipases deactivation. 88.1 % FAME was achieved in 2 h with the addition of 1:1 molar equivalent of methanol to oil at 0.5 h intervals. This cheap lipase system could be useful for the conversion of unrefined oils.

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509

Figure caption

Fig. 1 Lipase screening for biodiesel conversion from refined soybean oil

(a) = AY, (b) = DF, (c) = A10D, (d) = MER, (e) = R, (f) = A

Reaction conditions: 1% lipase loading (0.1 g) based on oil weight (10 g); 800 rpm; 1:4 oil methanol ratio (0.37 g each at 0, 3, 6, 9 h); 35 °C; 10 % aqueous portion (1 g) based on oil weight

Fig. 2 FAME production from 5 % phospholipid containing oil

Reaction conditions: 1% lipase loading (0.1 g) based on oil weight; 800 rpm; 1:4 oil methanol ratio (0.37 g each at 0, 3, 6, 9 h); 35°C; 10 % aqueous portion (1 g) based on oil weight

Fig. 3.

(a) FAME production by various cocktail lipases

(b) FFA, MAG, DAG and TAG residues at 24 h from 5 % phospholipid containing oil

Reaction conditions: 0.1% CalT and 0.4% lipase (other) based on oil weight; 800 rpm; 1:4 oil methanol ratio (0.37 g each at 0, 3, 6, 9 h); 35°C; 10 % aqueous portion based on oil weight
(c) Effect of increased methanol addition rate lipase cocktail on FAME conversion. Addition of 1:4 oil methanol ratio of methanol (0.37 g each at 0, 2, 4, 6 h)

Fig. 4. Optimization of methanol addition rate for AY – CalT lipase combination

Reaction conditions: 0.1% CalT (10 mg) and 0.4% AY (40 mg) based on oil weight (10 g); 800 rpm; 35 °C; 10 % (1 g) aqueous portion based on oil weight

1 **Tables**

2 Table 1

3 Activities and specificity of various lipases

Lipase	Transesterification activity (U/ml)	Hydrolysis activity (U/ml)	Specificity
A	19400 ± 96.8	7750 ± 283.0	α – position of medium and long chain fatty acid
R	16200 ± 569.0	6500 ± 318.0	α – position of medium and short chain
MER	6640 ± 814.0	9250 ± 226.0	α – position of long, medium and short chain fatty acid
A10D	15900 ± 269.0	5500 ± 248.0	–
DF	15300 ± 411.0	24300 ± 328.0	sn – 1,3 of medium and long chain fatty acid
AY	16200 ± 569.0	62400 ± 354.0	Non – specific

4

5

6 Table 2

7 Summary of CalT test results

(a) Various lipase loading			
		Conversion yield at 9h	Conversion yield at 24h
		(%)	(%)
Lipase loading (%)	0.05	66.5 ± 1.2	48.2 ± 2.9
	0.1	71.3 ± 0.6	79.3 ± 2.6
	0.3	70.6 ± 0.6	86.7 ± 1.5
	0.5	82.8 ± 3.1	95.6 ± 1.4
(b) 0.3 % lipase loading			
Temperature (°C)	30	72.7 ± 1.9	81.6 ± 1.4
	35	70.6 ± 0.6	86.7 ± 1.5
	40	63.1 ± 0.6	66.3 ± 0.5
Stir rate (rpm)	400	58.6 ± 1.2	57 ± 1.4
	800	71.9 ± 1.1	87.7 ± 2.1
	1200	70.6 ± 0.5	86.7 ± 1.5
Aqueous percentage (%)	2	25.8 ± 0.8	25.1 ± 0.8
	4.5	70.6 ± 0.3	86.7 ± 0.7
	8.5	71.8 ± 0.4	88.6 ± 1.9
	10	62.7 ± 0.7	82.0 ± 2.1

8 (a) The use of various loadings of CalT. Reaction conditions: 1200 rpm; 1:4 oil methanol

9 ratio (0.37 g each at 0, 3, 6, 9 h); 35 °C; 4.5 % aqueous portion based on oil weight

10 (b) Optimization of CalT for FAME production in refined oil using 0.3 % lipase loading.

11

Fig. 1. Lipase screening for biodiesel conversion from refined soybean oil

(a) = AY, (b) = DF, (c) = A10D, (d) = MER, (e) = R, (f) = A

Reaction conditions: 1% lipase loading (0.1 g) based on oil weight (10 g); 800 rpm; 1:4 oil methanol ratio (0.37 g each at 0, 3, 6, 9 h); 35 °C; 10 % aqueous portion (1 g) based on oil weight

Fig. 2. FAME production from 5 % phospholipid containing oil
Reaction conditions: 1% lipase loading (0.1 g) based on oil weight; 800 rpm; 1:4 oil methanol ratio (0.37 g each at 0, 3, 6, 9 h); 35°C; 10 % aqueous portion (1 g) based on oil weight

Fig. 3. (a) FAME production by various cocktail lipases; (b) FFA, MAG, DAG and TAG residues at 24 h from 5 % phospholipid containing oil; Reaction conditions: 0.1% CalT and 0.4% lipase (other) based on oil weight; 800 rpm; 1:4 oil methanol ratio (0.37 g each at 0, 3, 6, 9 h); 35°C; 10 % aqueous portion based on oil weight

(c) Effect of increased methanol addition rate lipase cocktail on FAME conversion. Addition of 1:4 oil methanol ratio of methanol (0.37 g each at 0, 2, 4, 6 h)

Fig. 4. Optimization of methanol addition rate for AY – CalT lipase combination
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 800 rpm; 35 °C; 10 % (1 g) aqueous portion based on oil weight

Step-wise
Methanol
addition

Step-wise
Methanol
addition