Enhancement of lipase catalyzed-fatty acid methyl esters production from waste activated bleaching earth by nullification of lipase inhibitors

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This study sought to identify inhibitory factors of lipase catalyzed-fatty acid methyl esters (FAME) production from waste activated bleaching earth (wABE). During the vegetable oil refinery process, activated bleaching earth (ABE) is used for removing the impure compounds, but adsorbs vegetable oil up to 35-40% as on a weight basis, and then the wABE is discarded as waste material. The impurities were extracted from the wABE with methanol and evaluated by infra-red (IR)-spectroscopy, which revealed that some were chlorophyll-plant pigments. The chlorophylls inhibited the lipase during FAME conversion from wABE. The inhibition by a mixture of chlorophyll a and b was found to be competitive. The inhibition of the enzymatic hydrolysis of waste vegetable oil contained in wABE by chlorophyll a alone was competitive, while the inhibition by chlorophyll b alone was non-competitive. Furthermore, the addition of a small amount of alkali nullified this inhibitory effect and accelerated the FAME production rate. When 0.9% KOH (w/w wABE) was added to the transesterification reaction with only 0.05% lipase (w/w wABE), the maximum FAME production rate improved 120-fold, as compared to that without the addition of KOH. The alkali-combined lipase significantly enhanced the FAME production rate from wABE, in spite of the presence of the plant pigments, and even when a lower amount of lipase was used as a catalyst.
Keywords: Activated bleaching earth (ABE); Lipase; Chlorophyll; Fatty acid methyl ester (FAME); Biodiesel; Biorefinery.
1. Introduction

Due to the desires for worldwide environmental protection and the conservation of non-renewable natural resources, the use of biodiesel or fatty acid methyl esters (FAME) as replacements for fossil fuels has gained importance. The carbon present in biodiesel exhaust was originally fixed from the atmosphere, which makes it a bio-neutral fuel. Additionally, the levels of SO₂, CO, halogens and soot in the exhaust gas produced by the combustion of biodiesel are much lower than those produced by petroleum diesel (Ulusoy et al., 2009).

Biodiesel is produced by alcoholsysis of triglycerides with short chain alcohols, including methanol. The triglycerides are obtained from vegetable oils, such as soybean oil, rapeseed oil, palm oil, jatropha oil, sunflower oil, corn oil, peanut oil, canola oil and cotton seed oil (Fukuda et al., 2001). In 2006-2007, biodiesel production in the United States increased dramatically, by 80%, from 7.9 million tons in 2006 to an estimated 14.2 million tons in 2007 (Demirbas and Balat, 2006). Similarly, the overall biodiesel production in the European Union increased by 53%, from 3.2 million tons in 2005 to nearly 4.9 million tons in 2006 (Du et al., 2008). The EU has set a target for all-transport-related petrol and diesel to contain 5.75% biofuel by 2010.

Biodiesel is produced by either a chemical transesterification reaction or a biocatalyzed-transesterification conversion. Although chemical transesterification results in the high conversion of triglycerides to FAME in a short time, it requires
further downstream treatment, due to the formation of soap when alkali catalysts are used or the corrosion of equipment when acid catalysts are used. Furthermore, chemical transesterification is more energy-intensive, requiring a glycerol recovery process and the removal of the acidic or alkali catalysts from the FAME product, as well as alkaline waste-water treatment. Finally, free fatty acids and water can interfere with the reaction (Meher et al., 2006). In contrast to chemical transesterification, biocatalyzed-transesterification can convert the free fatty acids in used oil to FAME easily, under moderate conditions. Furthermore, the purification of the FAME product and the recovery of glycerol are much easier than those processes after a chemical transesterification reaction. However, the high cost of the biocatalyzed-transesterification process has hampered its industrial application (Fukuda et al., 2001).

Biodiesel fuel can be used in regular diesel vehicles without any engine modifications. Additionally, because biodiesel is oxygenated, it is a better lubricant than diesel fuel, which increases the life of the engine, and it is combusted more completely (Anastopoulus et al., 2001). Moreover, the higher flash point of biodiesel makes it a safer fuel to use, handle and store, which is particularly advantageous in sensitive environments, such as cities (Vasudevan and Briggs, 2008).

Activated bleaching earth (ABE) is a high capacity absorbent that is commonly used to absorb the dark color of crude oil, which is caused by chromophoric chloroplast-related materials, during the crude oil refinery process. Almost of all impure compounds
can be adsorbed by ABE, but vegetable oil is adsorbed by up to 35%-40% on a weight basis, and a large amount of wABE is discarded from vegetable oil-refinery industries (Kojima et al., 2004; Park et al., 2008). The ABE dosage is usually based on the reduction in color required during the vegetable oil refinery process. Currently, Japan discards more than 80,000 metric tons of wABE annually (Park et al., 2006). Therefore, it would be useful to utilize the oil contained in discarded wABE and convert it to more valuable products, such as biodiesel. The conversion of discarded oil in wABE to biodiesel has previously been reported (Lara and Park, 2004; Park and Mori, 2005; Pizzaro and Park, 2003). However, when wABE was used as a triglyceride source for FAME conversion, the FAME yield was not satisfactory, as compared to the use of ABE that contained vegetable oil. These findings suggest that the presence of some factors inhibited the lipase activity during the transesterification reaction. Therefore, in this study, the effects of the pigment components extracted from wABE on the transesterification reaction were investigated, as well as on the lipase activity. Furthermore, combining alkali with the lipase was found to enhance significantly the transesterification reaction in wABE.

2. Materials and methods

2.1. Materials
ABE and wABE were provided by Mizusawa Ind. Chem. Ltd. (Niigata, Japan).

Palm oil, hexane, methanol, acetone, propanol, and chlorophyll a and b were purchased from WAKO Pure Chem. Ltd. (Tokyo, Japan). QLM lipase was purchased from Meito Sangyo Ltd. (Nagoya, Japan). The artificial wABE was composed of ABE that had adsorbed 35% (w/w) crude palm oil.

2.2. Pigment extraction from wABE and purification

Palm oil was extracted from 10 g of wABE, by suspending the sample in 20 ml of hexane, shaking it for 1 min and then centrifuging the sample at 4000 × g for 5 min. This process was repeated three times. After centrifugation, the organic liquid phase was separated, and the hexane was evaporated by placing the sample in a heating block (80°C). The oil-extracted wABE that remained was then combined with 20 ml of methanol, shaken mildly for 1 min, and then centrifuged at 4000 × g for 5 min. This process was repeated twice. The supernatant containing the methanol was then evaporated, by placing the sample in a heating block at 80°C. The extracted pigment was then separated by partial column chromatography (18 mm × 100 mm), in a column packed with 22 ml of Silica gel 60 (40–63 μm), using a mixture of petroleum ether and acetone at a ratio of 3:1 as the eluent.

2.3. wABE-extracted pigment analysis
The pigment extracted from the wABE was analyzed by thin layer chromatography (TLC) and infra-red (IR) spectroscopy. For TLC analysis, the pigment fractions were diluted 10-fold with methanol. Pure chlorophyll a (0.37%, w/v) and b (0.28%, w/v) were diluted 3-fold with methanol and used as standards. Pure acetone was used as the solvent.

IR spectroscopy was conducted at wavelengths ranging from 450 to 800 nm. Pure chlorophyll a (1.1 mg/ml) and b (0.55 mg/ml) dissolved in methanol were used as standards for the IR spectroscopy.

The chlorophyll contained in the wABE was quantified by colorimetric determination. Briefly, 0.5 g of pigment-containing wABE were suspended in 3 ml of 80% acetone, vortexed, and then diluted to 5 ml with 80% acetone. The sample was vortexed again, and then was centrifuged for 5 min. The absorbances at 663.6 nm and 646.6 nm were used to measure the levels of chlorophyll a and b, respectively (Yang et al., 1998). Specifically, the contents of chlorophyll a and b were calculated as follows:

\[
\text{Chlorophyll a (ug/ml)} = 12.25A_{663.6} - 2.55A_{646.6}
\]

\[
\text{Chlorophyll b (ug/ml)} = 20.31A_{646.6} - 4.91A_{663.6}
\]

\[
\text{Chlorophyll a + b (ug/ml)} = 17.76A_{646.6} + 7.34A_{663.6}
\]

The weight of chlorophyll a, b or a and b per gram of sample (ug/g sample) was then obtained by multiplying the concentration by the appropriate dilution factor and dividing this value by the weight of the sample (g).
2.4. Enzyme stability in the reaction mixture without oil

Analytical grade chlorophyll a (370 μg/ml) and b (280 μg/ml) were added to hexane (60%, w/w), methanol (4.5%, w/w), and lipase (1.0%, w/w). The mixtures were then incubated for 4 h at 28°C and 120 rpm in a rotary shaker, during which time sampling was conducted every hour, and the organic solvent contained in the samples was evaporated. The resulting enzyme powder (2.5 mg) was then dissolved in 1 ml of water for the lipase assay.

2.5. Effects of lipase-catalyzed FAME conversion from wABE in the presence of plant pigments

The reaction was conducted in the presence of hexane (60%, w/w), vegetable oil (35%, w/w), methanol (4.5%, w/w), and lipase (1.0%, w/w). To investigate the inhibitory effect of pigments on lipase, two types of substrate were used: pure palm oil and palm oil extracted from wABE. In addition, pure chlorophyll a (0.37%, w/v) and b (0.28%, w/v) were used as the reference pigments, respectively. Sampling was conducted every 2 h, after which the lipase activity was measured. All reactions were conducted at 28°C and 120 rpm in a rotary shaker.

2.6. Kinetics of lipase inhibition during FAME conversion in the presence of plant pigments
To analyze the kinetic activity of the lipase in the presence of chlorophyll a and 
b, artificial wABE that adsorbed chlorophyll a and b at a similar ratio as in wABE was 
used. The effects of chlorophyll a alone and chlorophyll b alone were also measured.
The reaction conditions were as follows: ABE: 60 g; crude palm oil: various 
concentrations ranging from 0 M to 0.3 M; methanol: 4 moles to oil; hexane as the 
solvent: 100 ml; and lipase, 1% (w/w ABE). Various concentrations of pure chlorophyll 
dissolved in methanol (Table 1) were added this reaction condition and then, the 
reaction was conducted at 35°C. The maximum reaction velocity ($V_{\text{max}}$) and the 
Michaelis-Menten constant ($K_m$) were determined from a Lineweaver-Burk plot of the 
Michaelis-Menten kinetics, respectively.

The following equations were used to evaluate the inhibition kinetics:

\[
\frac{1}{V} = \frac{1}{K_m} \left(1 + \frac{I}{S}ight) + \frac{1}{V_{\text{max}}}
\]  
(1)

where $V$, $I$, $S$, $K_I$ denote the reaction velocity (conversion %/min), the concentrations of 
inhibitor and substrate, and the inhibition constant, respectively.

\[
K_{m \text{ obs}} = K_m \left(1 + \frac{I}{S}ight)
\]  
(2)

where $K_{m \text{ obs}}$ denotes the apparent Michaelis-Menten constant.
Rearranging equation (1),

\[ \frac{1}{v} = \frac{K_{m\text{obs}}}{K_m} \frac{1}{S} + \frac{1}{V_0} \]  

(3)

\( K_{m\text{obs}} \) was obtained from a Lineweaver-Burk plot of \( 1/V \) and \( 1/S \). From equation (2), the Michaelis-Menten constant and the inhibition constant were obtained.

2.7. Nullification of inhibition by using alkali-combined lipase as a catalyst in the presence of plant pigments and its kinetics

In order to nullify the inhibitory effect on FAME conversion, KOH (1%, w/w) was added into the reaction mixture containing hexane (60%, w/w), vegetable oil (35%, w/w), methanol (4.5%, w/w), pure chlorophyll a (0.37%, w/v) and b (0.28%, w/v) and lipase (1% or 0.05%, w/w). Sampling was performed every 3 h, and the lipase activity was measured. All reactions were conducted at 28°C and 120 rpm in a rotary shaker.

To investigate the effect of KOH addition on FAME production from the vegetable oil contained in wABE in the presence of plant pigments, the KOH concentration was varied from 0 to 1.5% (w/w ABE), with a fixed lipase concentration (0.05% w/w ABE). The reaction mixture contained ABE (60 g), crude palm oil (from 0 M to 0.3 M), pure chlorophyll a and b (0.37% and 0.28% w/v, respectively, dissolved in methanol), methanol (4 moles to oil), hexane as the solvent (100 ml), lipase (0.05% w/w
ABE), and KOH (0, 0.3, 0.6, 0.9, 1.2 and 1.5% w/w ABE). The reaction was conducted at 35°C. The maximum reaction velocity ($V_{\text{max}}$) and the apparent Michaelis-Menten constant ($K_{m \text{obs}}$) were determined from a Lineweaver-Burk plot of Michaelis-Menten kinetics, respectively.

2.8 Lipase assay

The lipase activity (in international units (IU)) was measured using a Lipase Kit S (Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan), according to the protocol specified by the supplier. Briefly, the reaction mixture was centrifuged at 4000 × g for 10 min, and the supernatant was then used for the enzyme assay. The supernatant was diluted with distilled water, and the reaction mixture was maintained at 30°C (Kurooka and Kitamura, 1978), while the optical density of the samples was measured at 412 nm. The activity was then determined as follows:

\[
\text{Lipase activity (IU/ml)} = 0.147 \times A_{412} \times \text{dilution factor}.
\]

3. Results

3.1 Extraction and analysis of pigments within wABE

A total of 3.5 g (35%, w/w) and 0.75 g (7.5%, w/w) of oil and pigment were extracted from 10 g of wABE, respectively. The extracted pigment was sticky and dark.
brown. After separation using partial column chromatography, 2 thick bands were observed in the silica column, but the components of the pigment could not be separated by TLC, due to poor mobility. This hindered mobility may have been a consequence of other impurities or the oil. Therefore, IR spectroscopy was used to identify the composition of the extracted pigment. The IR spectroscopic analysis revealed that the pigment extracted from wABE had spectra similar to those of chlorophyll a and b (data not shown). These results indicated that the pigments extracted from the wABE originated from plant seeds and were adsorbed on the ABE during the refinement of the vegetable oils. Additionally, the chlorophyll extracted from wABE containing soybean oil or rapeseed oil also had spectra similar to the control spectra of chlorophyll a and b. Furthermore, 788 μg of chlorophyll/100 g wABE were extracted from the wABE containing rapeseed oil, while 413 μg/100 g wABE were extracted from the wABE containing soybean oil. The chlorophyll a and b contents of 100 g of wABE containing palm oil were generally found to be 370 μg and 280 μg, respectively. Finally, since the extracted chlorophyll only represented a small portion of the total amount of extracted pigment, it seems that the extracted pigment from the vegetable oils within wABE contained not only chlorophyll, but also other impure pigments.

3.2. Enzyme stability in the presence of chlorophyll a and b without oil
When lipase was mixed with hexane and methanol without oil, it was dispersed properly throughout the mixture and resulted in the production of a powder; however, after the addition of chlorophyll, the enzyme coagulated and formed beads or gelatinous pellets (data not shown). Lipase QLM was stable in the presence of water, hexane, and methanol, but the activity of the lipase decreased to around 70% in the presence of chlorophyll (Fig. 1). These findings strongly support the idea that the low yield of biodiesel conversion from wABE is caused by the presence of plant pigments as impurities.

3.3. Effects of extracted pigments on the lipase-catalyzed FAME conversion from wABE

The effect of the pigment extracted from wABE on the lipase activity was investigated in the presence of hexane and methanol. The effects of hexane, methanol and biodiesel on the enzyme activity were negligible, as compared to the effects of chlorophyll and pigment extracted from wABE (Fig. 2). In the presence of chlorophyll, the enzyme activity was inhibited and gradually decreased to 60%. Moreover, in the presence of pigment extracted from wABE, the enzyme activity decreased drastically, suggesting that the pigment may have a strong inhibitory effect against the lipase activity.

3.4. Inhibitory effect of chlorophyll on lipase activity
To investigate the inhibitory effect of chlorophyll on the lipase activity, artificial wABE, containing chlorophyll a and b and vegetable oil, was used. To evaluate the lipase activity during the initial FAME conversion from wABE, the temperature was maintained at 35°C. The mixture of chlorophyll a and b was added, and then a double reciprocal plot of the initial reaction velocity and the substrate concentration was constructed (Fig. 3). The $V_{\text{max}}$, which was determined from Eq. 3, remained constant at 0.74 %/min. However, the $K_{\text{m obs}}$ value increased as the chlorophyll concentration increased (data not shown). These findings indicate that the affinity between the substrate and enzyme decreased as the concentration of the inhibitor increased. Based on the relationship between $K_{\text{m obs}}$ and the inhibitor, the $K_m$ and $K_I$ were determined to be 30.4 mM and 0.36 mg of chlorophyll a and b per liter, respectively. As indicated in Fig. 3, the type of inhibition induced by the chlorophyll a and b mixture was competitive reversible.

To investigate the individual effects of chlorophyll a and b, the experiments were conducted separately, using various concentrations of chlorophyll a and b (Table 1). When only chlorophyll a was added as an inhibitor, the type of inhibition was competitive reversible (Fig. 4A, Table 2). However, when only chlorophyll b was added (Fig. 4B), the $K_{\text{m obs}}$ values remained around 60.3 mM-68.3 mM, but the $V_{\text{max}}$ values decreased as the concentration of the inhibitor increased (Table 2). A comparison of the $K_{\text{m obs}}$ values of chlorophyll a and chlorophyll b (Table 2) indicated that the $K_{\text{m obs}}$ values
of chlorophyll a sharply increased with greater chlorophyll a concentrations, while the increase of $K_{m \text{obs}}$ of chlorophyll b was essentially constant. Thus, the inhibition induced by chlorophyll b was non-competitive reversible.

The $K_m$ and $K_I$ values were 40.5 mM and 0.52 mg/l, respectively, in the presence of chlorophyll a, while they were 57.12 mM and 24.91 mg/l, respectively, in the presence of chlorophyll b (Table 3). Chlorophyll a showed a higher inhibitory effect than chlorophyll b; therefore, it was assumed that chlorophyll a plays a dominant role in inhibiting the FAME conversion from wABE by QLM lipase.

3.5. Nullification of inhibitory effect by using alkali-combined lipase as a catalyst and its kinetics in the presence of chlorophylls

When 1% KOH (w/w wABE) was added into the reaction mixture containing 1% lipase as a catalyst, the FAME conversion was 100% complete within a 3 h reaction. Furthermore, when the lipase concentration was decreased to 0.05%, but 1% KOH was present in the reaction mixture, 45.5% of FAME conversion was achieved within 12 h, and 90.8% FAME conversion was acquired at 36 h (data not shown). The KOH-combined lipase significantly enhanced the FAME conversion, by nullifying the inhibitory effect of chlorophyll in wABE.

The enhanced effect of FAME conversion by alkali-combined lipase was analyzed. When 0.05% of lipase was used in the presence of various amounts of KOH
in the reaction, the $K_{m\text{obs}}$ and $V_{\text{max}}$ values were determined according to Eq. (3), as shown in Table 4. When the amount of KOH was 0.9%, the $K_{m\text{obs}}$ and $V_{\text{max}}$ values were 19.44 mM and 30.03%/min, respectively, whereas without KOH in the FAME reaction mixture, the $K_{m}$ and $V_{\text{max}}$ value were 0.16 mM and 0.25%/min, respectively (Table 4). Although the affinity decreased in the presence of 0.9% of KOH, the initial velocity increased 120-fold, as compared to that without KOH addition. Thus, KOH nullified the inhibitory effect of chlorophyll in the presence of wABE and worked as an enhancer for FAME conversion in wABE.

4. Discussion

Many studies have been conducted to evaluate the regeneration of wABE. Kalam and Joshi (1988) reported the regeneration of wABE in aqueous medium using hexane extraction and autoclaving at 235°C, but found that the adsorption capacity of the regenerated waste bleaching earth was decreased by up to 50% after the fourth cycle of regeneration. They also regenerated waste bleaching earth using wet oxidation (Zimmerman process) after hexane extraction and a thermal process at 200°C, using an oxygen intake pressure of 0.5 MPa, but obtained only 80-82% regeneration when a 35% slurry concentration was used.

Low et al. (1996) investigated carbonized and hexane-extracted bleaching earth that was used to remove organic dyes from aqueous medium. They found that the
maximum capacities to adsorb basic dyes were larger than those of acid dyes and reactive dyes, and that this was likely due to the negatively charged surface of the bleaching earth. Boukerroui and Ouali (2000) regenerated waste bleaching earth by thermal processing at 500°C, followed by washing with a hydrochloric acid solution. Additionally, Tsai et al. (2002, 2003) regenerated waste bleaching earth by pyrolysis in a rotary furnace at temperatures of 500-660°C, followed by chemical activation with chloride salts. Chang et al. (2006) regenerated waste bleaching earth by lye-extraction and thermal processing at 500°C and 800°C. Waldmann and Eggers (1991) regenerated waste bleaching clay by high-pressure extraction at 350-750 bars, while King et al. (1992) regenerated waste bleaching clay by using supercritical carbon oxide (SC-CO₂) extraction at 10,000-12,000 psig. All of the methods described above require a carbonizing method, a high temperature or a pressurized condition, which leads to increased costs.

Alternative utilization of wABE is to produce biodiesel from wABE without separating the oil from the wABE or any other pretreatment; therefore, high temperature and high pressure were not required. Unfortunately, some materials, which were mainly composed of pigments and residual gum, could not be saponified and remained in the wABE. These materials may cause increased viscosity and negatively influence the FAME conversion (Pizzaro and Park, 2003). Another limitation was that the cost of lipase is relatively high, making the industrial scale production of biodiesel unfeasible.
In this study, the pigment extracted from the wABE was analyzed, with the intent of breaking down the vegetable oil that remained in the wABE for biodiesel, using less lipase. The total amounts of oil and pigment extracted were 35% and 7.5% of the wABE, respectively, on a per weight basis, which indicates that the pigment content in the oil extracted from the wABE was up to 21.43%. Additionally, the pigments could not be separated by TLC; therefore, they were evaluated by IR spectroscopy. The results of the IR analysis revealed that the spectra of the extracted pigments were similar to those of chlorophyll a and b (data not shown). In addition, the results demonstrated that the extracts contained 3.7 and 2.8 mg of chlorophyll a and b per kilogram of wABE, respectively. These findings indicate that other components were also present in the pigment extracted from the wABE. Although the chlorophyll content in the wABE was low, the lipase activity was decreased by 30% in its presence. During FAME conversion, chlorophyll and pigment resulted in the lipase activity decreasing to 60% and 40%, respectively. Additionally, chlorophyll a was found to have a higher inhibitory effect than chlorophyll b. Although previous studies demonstrated that methanol showed dead-end inhibition in the Ping Pong Bi Bi mechanism of FAME conversion from waste cooking palm oil (Halim and Kamaruddin, 2008; Zuhair et al., 2007), the inhibitory effect of the chlorophyll is dominant in biodiesel production from wABE containing palm oil. Albertsson et al., (2007) reported that chloroplast membranes (thylakoids) inhibited the lipase/co-lipase-catalyzed hydrolysis of triacylglycerols. Specifically, they
found that the addition of chloroplast membranes to refined food resulted in the suppression of the food intake by rats, which caused a reduction in blood lipids.

In order to nullify the inhibitory effect of chlorophyll in the FAME conversion reaction several methods were tried. The extraction of chlorophyll using methanol, followed by evaporation (at $80^\circ C$), was still an impractical method and an expensive process on an industrial scale. Therefore, trapping the plant pigments using chemical agents may prevent the pigments from releasing from the wABE. For chlorophyll blocking agents, CaCO$_3$, SiO$_2$, ZnSO$_4$, NiSO$_4$, EDTA, lecithin, citric acid and polyethylene glycol (PEG) were tested. The addition of CaCO$_3$, SiO$_2$, ZnSO$_4$, EDTA, lecithin and citric acid did not yield any improvement of the FAME conversion, as compared to that without the addition of the blocking agents (data not shown). When 1% PEG (w/w wABE) was added to the FAME reaction mixture, the FAME conversion increased by 12% over that without the PEG addition. PEG was able to trap or to chelate chlorophyll, and formed a PEG-chlorophyll complex. However, when a lipase concentration of 0.05% (w/w wABE) was used, the PEG was unable to nullify the inhibitory effect of chlorophyll in the FAME reaction (unpublished data).

Surprisingly, the addition of 1% KOH (w/w wABE) to the reaction mixture facilitated 100% FAME conversion within only 3 h. Furthermore, when the lipase concentration was decreased to 1/20 of 1%, 91% FAME conversion could be achieved in a 36 h reaction. When 0.9% KOH was added, the $V_{\text{max}}$ value was 30.03%/min, which
was 120-fold higher than that without KOH addition. Thus, it concluded that KOH seems to enhance the FAME conversion by nullifying the inhibitory effect of chlorophyll in wABE. In this process, since the wABE adsorbs ionic compounds in the reaction mixture the amount of potassium ion in the FAME assumes very little. If the potassium ion is detected higher than the diesel oil standard, it should be removed. If it is possible to produce lipase in the presence of an organic solvent, then the FAME conversion reaction will be economized, because of the lower lipase cost. Many organic solvent-tolerant bacteria and yeast, such as Pseudomonas aeruginosa, which tolerates cyclo-hexane, and Bacillus sphaericus, Staphylococcus saprophyticus, Burkholderia cepacia, Burkholderia multivorans, and Candida cylindracea, which tolerate n-hexane solvents, are able to produce stable extracellular lipases (Dandavate et al., 2009; Fang et al., 2006; Hun et al., 2003; Ito et al., 2001, Shu et al., 2009; Warwel and Borgdorf, 2000). Candida cylindracea lipase showed a high activity in n-hexane, and produced more than 78±6% (w/w) biodiesel within the first 4 h of the reaction (Lara and Park, 2004). However, when Candida cylindracea cells were cultured in wABE-containing medium, it was difficult to grow cells in wABE medium, resulting in very low lipase activity in the culture broth (Mori et al., 2009). At present, immobilized lipases or immobilized whole cells have been utilized for biodiesel production in harmful organic solvents, but the cost of the final product is still too high, as compared to that of the chemical transesterification process (Akoh et al., 2007). However, the
wABE contains lipase inhibitors. To reduce the production cost, less lipase should be used for biodiesel production from wABE, by adding KOH to the reaction mixture. Only 0.05% lipase combined with 0.9% KOH was satisfactory for achieving higher than 90% FAME conversion within 36 h.

5. Conclusions

The presence of chlorophylls was shown to be responsible for the inhibition of lipase during lipase-catalyzed FAME production from wABE. The inhibition by chlorophylls was found to be competitive. Interestingly, the addition of a small amount of alkali could nullify this inhibitory effect and accelerate the FAME production rate. When 0.9% (w/w wABE) KOH was added to the transesterification reaction in the presence of only 0.05% lipase (w/w wABE), the maximum FAME production rate improved 120-fold, as compared to that without the addition of KOH. Alkali-combined lipase significantly enhanced the FAME production rate from wABE, even in the presence of the plant pigments.

Acknowledgements

This study was performed as a research project of the New Energy and Industrial Technology Development Organization (NEDO), Japan, in collaboration with Shizuoka University, Japan and Mizusawa Industrial Chemicals Ltd., Japan.
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Table 1. The concentrations of chlorophyll a and b used to evaluate their inhibitory effects on the lipase activity

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<th>Run no.</th>
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<td>13</td>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 2. $K_{m\,\text{obs}}$ and $V_{\text{max}}$ of lipase in the presence of various concentrations of chlorophyll a or b as an inhibitor during the initial FAME conversion from artificial wABE.

<table>
<thead>
<tr>
<th>Chlorophyll a or b (mg/l)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{m,\text{obs}}$ (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>55.55</td>
<td>113.18</td>
<td>187.72</td>
<td>248.74</td>
<td>377.99</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>55.55</td>
<td>62.83</td>
<td>61.61</td>
<td>60.26</td>
<td>68.30</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (%/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.74</td>
<td>0.68</td>
<td>0.73</td>
<td>0.73</td>
<td>0.74</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.74</td>
<td>0.36</td>
<td>0.33</td>
<td>0.33</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Table 3. $K_m$ and $K_i$ of QLM lipase in the presence of a mixture of chlorophyll a and b or chlorophyll a or b alone during the initial FAME conversion from artificial wABE

<table>
<thead>
<tr>
<th>Chlorophyll a and b mixture</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>30.40</td>
<td>40.5</td>
</tr>
<tr>
<td>$K_i$ (mg/l)</td>
<td>0.36</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Table 4. $K_{m\, obs}$ and $V_{max}$ of lipase with KOH in the reaction mixture during the initial FAME conversion from artificial wABE

<table>
<thead>
<tr>
<th>KOH (%, w/w)</th>
<th>0</th>
<th>0.3</th>
<th>0.6</th>
<th>0.9</th>
<th>1.2</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{m, obs}$ (mM)</td>
<td>0.16</td>
<td>0.25</td>
<td>0.44</td>
<td>19.44</td>
<td>2.35</td>
<td>0.30</td>
</tr>
<tr>
<td>$V_{max}$ (%/min)</td>
<td>0.25</td>
<td>0.39</td>
<td>0.67</td>
<td>30.03</td>
<td>3.60</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Figure legends

**Fig. 1.** Lipase stability in the solvent used in a FAME reaction without oil. QLM lipase (1%, w/w) was added to a mixture of hexane (60%, w/w) and methanol (4.5%, w/w) at 25°C and then shaken at 120 rpm for 4 h. Chlorophyll a (0.37%, w/v) and b (0.28%, w/v) were dissolved in methanol and added to the reaction, respectively. The mixtures were lipase and water (circles); lipase, chlorophyll a, hexane and methanol (solid triangles); lipase, chlorophyll b, hexane and methanol (solid squares); lipase, chlorophyll a and b, hexane and methanol (solid circles).

**Fig. 2.** Effects of reaction components on lipase stability during FAME conversion. The reaction solution contained hexane (60%, w/w), oil (35%, w/w), methanol (4.5%, w/w), QLM lipase (1%, w/w), chlorophyll a (0.37%, w/v) and chlorophyll b (0.28%, w/v). Reactions were conducted at 25°C and 120 rpm for 10 h. The reaction contents were lipase, pure palm oil and hexane (circles); lipase, pure palm oil, methanol and biodiesel (triangle); lipase, wABE extracted palm oil, hexane and methanol (solid circles); lipase, chlorophyll a, chlorophyll b, pure palm oil, hexane and methanol (squares); lipase, pure palm oil, wABE extracted pigment, hexane and methanol (solid triangles); and lipase, wABE extracted palm oil, wABE extracted pigment, hexane and methanol (solid squares).
Fig. 3. Lineweaver-Burk plots in the presence of mixtures of chlorophyll a and b as an inhibitor in artificial wABE. The inhibitory concentrations were 0 mg/l (rhombuses), 1 mg/l (squares), 2 mg/l (triangles), 3 mg/l (solid triangles), 4 mg/l (circles) and 5 mg/l (solid circles).

Fig. 4. Lineweaver-Burk plots in the presence of chlorophyll a (A) and b (B) as inhibitors in the artificial wABE. The inhibitor concentrations were 0 mg/l (rhombuses), 1 mg/l (squares), 2 mg/l (triangles), 3 mg/l (solid circles), and 4 mg/l (circles).
Dwiarti et al., Fig. 1

Residual activity (%) vs. Time (h)

Time (h)

Residual activity (%)