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

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ABSTRACT

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.

30 *Keywords:* Activated bleaching earth (ABE); Lipase; Chlorophyll; Fatty acid methyl

31 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 SO_2, 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 alcoholysis 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

52 further downstream treatment, due to the formation of soap when alkali catalysts are
53 used or the corrosion of equipment when acid catalysts are used. Furthermore, chemical
54 transesterification is more energy-intensive, requiring a glycerol recovery process and
55 the removal of the acidic or alkali catalysts from the FAME product, as well as alkaline
56 waste-water treatment. Finally, free fatty acids and water can interfere with the reaction
57 (Meher et al., 2006). In contrast to chemical transesterification, biocatalyzed-
58 transesterification can convert the free fatty acids in used oil to FAME easily, under
59 moderate conditions. Furthermore, the purification of the FAME product and the
60 recovery of glycerol are much easier than those processes after a chemical
61 transesterification reaction. However, the high cost of the biocatalyzed-
62 transesterification process has hampered its industrial application (Fukuda et al., 2001).
63 Biodiesel fuel can be used in regular diesel vehicles without any engine modifications.
64 Additionally, because biodiesel is oxygenated, it is a better lubricant than diesel fuel,
65 which increases the life of the engine, and it is combusted more completely
66 (Anastopoulus et al., 2001). Moreover, the higher flash point of biodiesel makes it a
67 safer fuel to use, handle and store, which is particularly advantageous in sensitive
68 environments, such as cities (Vasudevan and Briggs, 2008).
69 Activated bleaching earth (ABE) is a high capacity absorbent that is commonly
70 used to absorb the dark color of crude oil, which is caused by chromophoric chloroplast-
71 related materials, during the crude oil refinery process. Almost of all impure compounds

72 can be adsorbed by ABE, but vegetable oil is adsorbed by up to 35%-40% on a weight
73 basis, and a large amount of wABE is discarded from vegetable oil-refinery industries
74 (Kojima et al., 2004; Park et al., 2008). The ABE dosage is usually based on the
75 reduction in color required during the vegetable oil refinery process. Currently, Japan
76 discards more than 80,000 metric tons of wABE annually (Park et al., 2006). Therefore,
77 it would be useful to utilize the oil contained in discarded wABE and convert it to more
78 valuable products, such as biodiesel. The conversion of discarded oil in wABE to
79 biodiesel has previously been reported (Lara and Park, 2004; Park and Mori, 2005;
80 Pizzaro and Park, 2003). However, when wABE was used as a triglyceride source for
81 FAME conversion, the FAME yield was not satisfactory, as compared to the use of
82 ABE that contained vegetable oil. These findings suggest that the presence of some
83 factors inhibited the lipase activity during the transesterification reaction. Therefore, in
84 this study, the effects of the pigment components extracted from wABE on the
85 transesterification reaction were investigated, as well as on the lipase activity.
86 Furthermore, combining alkali with the lipase was found to enhance significantly the
87 transesterification reaction in wABE. .

88 **2. Materials and methods**

89 *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:

Chlorophyll a ($\mu g/ml$) = $12.25A_{663.6} - 2.55A_{646.6}$

Chlorophyll b ($\mu g/ml$) = $20.31A_{646.6} - 4.91A_{663.6}$

Chlorophyll a + b ($\mu g/ml$) = $17.76A_{646.6} + 7.34A_{663.6}$

The weight of chlorophyll a, b or a and b per gram of sample ($\mu g/g$ sample) was then obtained by multiplying the concentration by the appropriate dilution factor and dividing this value by the weight of the sample (g).

128 *2.4. Enzyme stability in the reaction mixture without oil*

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

135 *2.5. Effects of lipase-catalyzed FAME conversion from wABE in the presence of plant*
136 *pigments*

137 The reaction was conducted in the presence of hexane (60%, w/w), vegetable oil
138 (35%, w/w), methanol (4.5%, w/w), and lipase (1.0%, w/w). To investigate the
139 inhibitory effect of pigments on lipase, two types of substrate were used: pure palm oil
140 and palm oil extracted from wABE. In addition, pure chlorophyll a (0.37%, w/v) and b
141 (0.28%, w/v) were used as the reference pigments, respectively. Sampling was
142 conducted every 2 h, after which the lipase activity was measured. All reactions were
143 conducted at 28^{o}C and 120 rpm in a rotary shaker.

144 *2.6. Kinetics of lipase inhibition during FAME conversion in the presence of plant*
145 *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_{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:

$$\frac{1}{V}=\frac{K_m}{V_{max}}\left(1+\frac{I}{K_I}\right)\frac{1}{S}+\frac{1}{V_{max}} \qquad (1)$$

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

$$K_{m\ obs} = K_m\left(1+\frac{I}{K_I}\right) \qquad (2)$$

where $K_{m\ obs}$ denotes the apparent Michaelis-Menten constant.

166 Rearranging equation (1),

167

168 $$\frac{1}{V} = \frac{K_{m\,obs}}{K_{max}} \cdot \frac{1}{S} + \frac{1}{K_m} \qquad (3)$$

169

170 $K_{m\,obs}$ was obtained from a Lineweaver-Burk plot of 1/*V* and 1/*S*. From equation (2), the
171 Michaelis-Menten constant and the inhibition constant were obtained.

172 *2.7. Nullification of inhibition by using alkali-combined lipase as a catalyst in the*
173 *presence of plant pigments and its kinetics*

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

179 To investigate the effect of KOH addition on FAME production from the
180 vegetable oil contained in wABE in the presence of plant pigments, the KOH
181 concentration was varied from 0 to 1.5% (w/w ABE), with a fixed lipase concentration
182 (0.05% w/w ABE). The reaction mixture contained ABE (60 g), crude palm oil (from 0
183 M to 0.3 M), pure chlorophyll a and b (0.37% and 0.28% w/v, respectively, dissolved in
184 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_{max}) and the apparent Michaelis-Menten constant ($K_{m\ obs}$) 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:

Lipase activity (IU/ml) = $0.147 \times A_{412} \times$ 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

239 To investigate the inhibitory effect of chlorophyll on the lipase activity, artificial
240 wABE, containing chlorophyll a and b and vegetable oil, was used. To evaluate the
241 lipase activity during the initial FAME conversion from wABE, the temperature was
242 maintained at 35^{o}C. The mixture of chlorophyll a and b was added, and then a double
243 reciprocal plot of the initial reaction velocity and the substrate concentration was
244 constructed (Fig. 3). The V_{max}, which was determined from Eq. 3, remained constant at
245 0.74 %/min. However, the $K_{m\,obs}$ value increased as the chlorophyll concentration
246 increased (data not shown). These findings indicate that the affinity between the
247 substrate and enzyme decreased as the concentration of the inhibitor increased. Based
248 on the relationship between $K_{m\,obs}$ and the inhibitor, the K_m and K_I were determined to
249 be 30.4 mM and 0.36 mg of chlorophyll a and b per liter, respectively. As indicated in
250 Fig. 3, the type of inhibition induced by the chlorophyll a and b mixture was
251 competitive reversible.
252 To investigate the individual effects of chlorophyll a and b, the experiments
253 were conducted separately, using various concentrations of chlorophyll a and b (Table
254 1). When only chlorophyll a was added as an inhibitor, the type of inhibition was
255 competitive reversible (Fig. 4A, Table 2). However, when only chlorophyll b was added
256 (Fig. 4B), the $K_{m\,obs}$ values remained around 60.3 mM-68.3 mM, but the V_{max} values
257 decreased as the concentration of the inhibitor increased (Table 2). A comparison of the
258 $K_{m\,obs}$ values of chlorophyll a and chlorophyll b (Table 2) indicated that the $K_{m\,obs}$ values

of chlorophyll a sharply increased with greater chlorophyll a concentrations, while the increase of $K_{m\,obs}$ 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

278 in the reaction, the $K_{m\ obs}$ and V_{max} values were determined according to Eq. (3), as
279 shown in Table 4. When the amount of KOH was 0.9%, the $K_{m\ obs}$ and V_{max} values were
280 19.44 mM and 30.03%/min, respectively, whereas without KOH in the FAME reaction
281 mixture, the K_m and V_{max} value were 0.16 mM and 0.25%/min, respectively (Table 4).
282 Although the affinity decreased in the presence of 0.9% of KOH, the initial velocity
283 increased 120-fold, as compared to that without KOH addition. Thus, KOH nullified the
284 inhibitory effect of chlorophyll in the presence of wABE and worked as an enhancer for
285 FAME conversion in wABE.

286 **4. Discussion**

287 Many studies have been conducted to evaluate the regeneration of wABE.
288 Kalam and Joshi (1988) reported the regeneration of wABE in aqueous medium using
289 hexane extraction and autoclaving at 235^{o}C, but found that the adsorption capacity of
290 the regenerated waste bleaching earth was decreased by up to 50% after the fourth cycle
291 of regeneration. They also regenerated waste bleaching earth using wet oxidation
292 (Zimmerman process) after hexane extraction and a thermal process at 200^{o}C, using an
293 oxygen intake pressure of 0.5 MPa, but obtained only 80-82% regeneration when a 35%
294 slurry concentration was used.
295 Low et al. (1996) investigated carbonized and hexane-extracted bleaching earth
296 that was used to remove organic dyes from aqueous medium. They found that the

297 maximum capacities to adsorb basic dyes were larger than those of acid dyes and
298 reactive dyes, and that this was likely due to the negatively charged surface of the
299 bleaching earth. Boukerroui and Ouali (2000) regenerated waste bleaching earth by
300 thermal processing at 500^{o}C, followed by washing with a hydrochloric acid solution.
301 Additionally, Tsai et al. (2002, 2003) regenerated waste bleaching earth by pyrolysis in
302 a rotary furnace at temperatures of 500-660^{o}C, followed by chemical activation with
303 chloride salts. Chang et al. (2006) regenerated waste bleaching earth by lye-extraction
304 and thermal processing at 500^{o}C and 800^{o}C. Waldmann and Eggers (1991) regenerated
305 waste bleaching clay by high-pressure extraction at 350-750 bars, while King et al.
306 (1992) regenerated waste bleaching clay by using supercritical carbon oxide (SC-CO_2)
307 extraction at 10,000-12,000 psig. All of the methods described above require a
308 carbonizing method, a high temperature or a pressurized condition, which leads to
309 increased costs.

310 Alternative utilization of wABE is to produce biodiesel from wABE without
311 separating the oil from the wABE or any other pretreatment; therefore, high temperature
312 and high pressure were not required. Unfortunately, some materials, which were mainly
313 composed of pigments and residual gum, could not be saponified and remained in the
314 wABE. These materials may cause increased viscosity and negatively influence the
315 FAME conversion (Pizzaro and Park, 2003). Another limitation was that the cost of
316 lipase is relatively high, making the industrial scale production of biodiesel unfeasible.

317 In this study, the pigment extracted from the wABE was analyzed, with the intent of
318 breaking down the vegetable oil that remained in the wABE for biodiesel, using less
319 lipase. The total amounts of oil and pigment extracted were 35% and 7.5% of the wABE,
320 respectively, on a per weight basis, which indicates that the pigment content in the oil
321 extracted from the wABE was up to 21.43%. Additionally, the pigments could not be
322 separated by TLC; therefore, they were evaluated by IR spectroscopy. The results of the
323 IR analysis revealed that the spectra of the extracted pigments were similar to those of
324 chlorophyll a and b (data not shown). In addition, the results demonstrated that the
325 extracts contained 3.7 and 2.8 mg of chlorophyll a and b per kilogram of wABE,
326 respectively. These findings indicate that other components were also present in the
327 pigment extracted from the wABE. Although the chlorophyll content in the wABE was
328 low, the lipase activity was decreased by 30% in its presence. During FAME conversion,
329 chlorophyll and pigment resulted in the lipase activity decreasing to 60% and 40%,
330 respectively. Additionally, chlorophyll a was found to have a higher inhibitory effect
331 than chlorophyll b. Although previous studies demonstrated that methanol showed
332 dead-end inhibition in the Ping Pong Bi Bi mechanism of FAME conversion from waste
333 cooking palm oil (Halim and Kamaruddin, 2008; Zuhair et al., 2007), the inhibitory
334 effect of the chlorophyll is dominant in biodiesel production from wABE containing
335 palm oil. Albertsson et al., (2007) reported that chloroplast membranes (thylakoids)
336 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°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_{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

377 wABE contains lipase inhibitors. To reduce the production cost, less lipase should be
378 used for biodiesel production from wABE, by adding KOH to the reaction mixture.
379 Only 0.05% lipase combined with 0.9% KOH was satisfactory for achieving higher than
380 90% FAME conversion within 36 h.

381 **5. Conclusions**

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

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References

Akoh, C.C., Chang, S.W., Lee, G.C., Shaw, J.F., 2007. Enzymatic approach to biodiesel production. Journal of Agricultural and Food Chemistry 55, 8995-9005.

Albertsson, P.A., Kohnke, R., Emek, S.C., Mei, J., Rehfeld, J.F., Akerlund, H.E., Erlanson-Albertsson, C., 2007. Chloroplast membranes retard fat digestion and induce satiety: effect of biological membranes on pancreatic lipase/co-lipase. Biochemical Journal 401, 727-733.

Anastopoulus, G., Lois, E., Serdari, A., Zanikos, F., Stournas, S., Kalligeros, S., 2001. Lubrication properties of low-sulfur diesel fuels in the presence of specific types of fatty acid derivatives. Energy & Fuels 15: 106-112.

Boukerroui, A., Ouali, M.S., 2000. Regeneration of a spent bleaching earth and its reuse in the refining of an edible oil. Journal of Chemical Technology and Biotechnology 75, 773-776.

Chang, J.I., Tai H.S., Huang, T.H., 2006. Regeneration of spent bleaching earth by lye-extraction. Environmental Progress 25(4), 373-378.

Dandavate, V., Jinjala, J., Keharia, H., Madamwar, D., 2009. Production, partial purification and characterization of organic solvent tolerant lipase from *Burkholderia multivorans* V2 and its application for ester synthesis. Bioresource Technology 100, 3374-4481.

414 Demirbas, M.F., Balat, M., 2006. Recent advances on the production and utilization
415 trends of bio-fuels: A global perspective. Energy Conversion and Management 47,
416 2371-2381.

417 Du, W., Li, W., Sun, T., Chen, X., Liu, D., 2008. Perspectives for biotechnological
418 production of biodiesel and impacts. Applied Microbiology and Biotechnology 79,
419 331-337.

420 Fang, Y.W., Lu, Z.X., Lv, F.X., Bie, X.M, Liu, S., Ding, Z.Y., Xu, W.F., 2006. A newly
421 isolated organic solvent tolerant *Staphylococcus saprophyticus* M36 produced
422 organic solvent-stable lipase. Current Microbiology 53, 510-515.

423 Fukuda, H., Kondo, A., Noda, H., 2001. Biodiesel fuel production by transesterification
424 of oils. Journal of Bioscience and Bioengineering 92, 405-416.

425 Halim A.S.F., Komaruddin, A.H. 2008. Catalytic studies of lipase on FAME production
426 from waste cooking palm oil in *tert*-butanol system. Process Biochemistry 43,
427 1430-1439.

428 Hun, C.J., Rahman, R.N.A., Salleh, A.B., Basri, M., 2003. A newly isolated organic
429 solvent tolerant *Bacillus sphaericus* 205y producing organic solvent-stable
430 lipase. Biochemical Engineering Journal 15(2), 147-151.

431 Ito, T., Kikuta, H., Nagamori, E., Honda, H., Ogino, H., Ishikawa, H. Kobayashi, T.,
432 2001. Lipase production in two-step fed-batch culture of organic solvent-tolerant

Pseudomonas aeruginosa LST-03. Journal of Bioscience and Bioengineering 91(3), 245-250.

Kalam, A., Joshi, J.B., 1988. Regeneration of spent earth in aqueous medium. Journal of the American Oil Chemists' Society 65, 1917-1921.

King, J.W., List, G.R., Johnson, J.H., 1992. Supercritical carbon dioxide extraction of spent bleaching clays. The Journal of Supercritical Fluids 5, 38-41.

Kojima, S., Du, D., Sato, M., Park, E.Y., 2004. Efficient production of fatty acid methyl ester from waste activated bleaching earth using diesel oil as organic solvent. Journal of Bioscience and Bioengineering 98, 420-424.

Kurooka, S., Kitamura, T., 1978. Properties of serum lipase in patients with various pancreatic diseases. Analysis by a new serum lipase assay method (the BALB-DTNB method) in combination with gel-filtration and iso-electrofocusing techniques. The Journal Biochemistry 84, 1459-1466.

Lara, P.V., Park, E.Y., 2004. Potential application of waste activated bleaching earth on the production of fatty acid methyl esters using *Candida cylindracea* lipase in organic solvent system. Enzyme and Microbial Technology 34, 270-277.

Low, K.S., Lee, C.K., Wong, A.M., 1996. Carbonized spent bleaching earth as a sorbent for some organic dyes. The Journal of Environmental Science and Health 31(3), 673-685.

452 Meher, L.C., Sagar, D.V., Naik, S,N., 2006. Technical aspects of biodiesel production
453 by transesterification-a review. Renewable and Sustainable Energy Reviews 10,
454 248–268.
455 Mori, M., Ali, E., Du, D., Park, E.Y., 2009. Characterization and optimization of
456 extracellular alkaline lipase production by *Alcaligenes* sp. using stearic acid as
457 carbon source. Biotechnology and Bioprocess Engineering 14, 193-201.
458 Park, E.Y., Sato, M., Kojima, S., 2008. Lipase-catalyzed biodiesel production from
459 waste activated bleaching earth as raw material in a pilot plant. Bioresource
460 Technology 99, 3130-3135.
461 Park, E.Y., Mori, M., 2005. Kinetic study of esterification of rapeseed oil contained in
462 waste activated bleaching earth using *Candida rugosa* lipase in organic solvent
463 system. Journal of Molecular Catalysis B: Enzymatic 37, 95-100.
464 Park, E.Y., Sato, M., Kojima, S., 2006. Fatty acid methyl ester production using lipase-
465 immobilizing silica particles with different particle sizes and different specific surface
466 areas. Enzyme and Microbial Technology 39: 889-896.
467 Pizarro, A.V.L., Park, E.Y., 2003. Lipase-catalyzed production of biodiesel fuel from
468 vegetable oils contained in waste activated bleaching earth. Process Biochemistry 38,
469 1077-1082.
470 Shu, Z., Lin, R., Jiang, H., Zhang, Y., Wang, M., Huang, J., 2009. A rapid and efficient
471 method for directed screening of lipase-producing *Burkholderia cepacia* complex

472 strains with organic solvent tolerance from rhizosphere. Journal of Bioscience and
473 Bioengineering 107(6), 658-661.
474 Tsai, W.T., Chen, H.P., Hsieh, M.F., Sun, H.F., Chien, S.F., 2002. Regeneration of
475 spent bleaching earth by pyrolysis in a rotary furnace. Journal of Analytical and
476 Applied Pyrolysis 63, 157-170.
477 Tsai, W.T., Chen, H.P., Hsieh, M. F., Sun, H.F., Lai, C.W., 2003. Regeneration of
478 bleaching clay waste by chemical activation with chloride salts. The Journal of
479 Environmental Science and Health 38(4), 685-696.
480 Ulusoy, Y., Arslan, R., Kaplan, C., 2009. Emission characteristics of sunflower oil
481 methyl ester. Energy Sources, Part A: Recovery, Utilization, and Environmental
482 Effects 31, 906-910.
483 Vasudevan, P.T., Briggs, M., 2008. Biodiesel production-current state of the art and
484 challenges. Journal of Industrial Microbiology and Biotechnology 35, 421-430.
485 Waldmann, C., Eggers, R., 1991. De-oiling contaminated bleaching clay by high-
486 pressure extraction. Journal of the American Oil Chemists' Society 68(12), 922-
487 930.
488 Warwel, S., Borgdorf, R., 2000. Substrate selectivity of lipases in the esterification of
489 cis/trans-isomers and positional isomers of conjugated linoleic acid (CLA).
490 Biotechnology Letters 22, 1151-1155.

491 Yang, C.M., Chang, K.W., Yin, M.H., Huang, H.M., 1998. Methods for the
492 determination of the chlorophylls and their derivatives. Taiwania 43(2), 116-122.
493 Zuhair, S.A., Ling, F.W., Jun, L.S., 2007. Proposed kinetic mechanism of the
494 production of biodiesel from palm oil using lipase. Process Biochemistry 42, 951-
495 960.
496

497 Table 1. The concentrations of chlorophyll a and b used to evaluate their inhibitory

498 effects on the lipase activity

Run no.	Chlorophyll a (mg/l)	Chlorophyll b (mg/l)	Total concentration (mg/l)
1	2.85	2.15	5.00
2	2.28	1.72	4.00
3	1.71	1.29	3.00
4	1.14	0.86	2.00
5	0.57	0.43	1.00
6	4.00	0	4.00
7	3.00	0	3.00
8	2.00	0	2.00
9	1.00	0	1.00
10	0	4.00	4.00
11	0	3.00	3.00
12	0	2.00	2.00
13	0	1.00	1.00

499

500 Table 2. $K_{m\,obs}$ and V_{max} of lipase in the presence of various concentrations of

501 chlorophyll a or b as an inhibitor during the initial FAME conversion from artificial

502 wABE

Chlorophyll a or b (mg/l)		0	1	2	3	4
$K_{m\,obs}$ (mM)	Chlorophyll a	55.55	113.18	187.72	248.74	377.99
	Chlorophyll b	55.55	62.83	61.61	60.26	68.30
V_{max} (%/min)	Chlorophyll a	0.74	0.68	0.73	0.73	0.74
	Chlorophyll b	0.74	0.36	0.33	0.33	0.32

503

504 Table 3. K_m and K_I of QLM lipase in the presence of a mixture of chlorophyll a and b or

505 chlorophyll a or b alone during the initial FAME conversion from artificial wABE

	Chlorophyll a and b mixture	Chlorophyll a	Chlorophyll b
K_m (mM)	30.40	40.5	57.12
K_I (mg/l)	0.36	0.52	24.91

506

507

508 Table 4. $K_{m\,obs}$ and V_{max} of lipase with KOH in the reaction mixture during the initial

509 FAME conversion from artificial wABE

KOH (%, w/w)	0	0.3	0.6	0.9	1.2	1.5
$K_{m\,obs}$ (mM)	0.16	0.25	0.44	19.44	2.35	0.30
V_{max} (%/min)	0.25	0.39	0.67	30.03	3.60	0.45

510

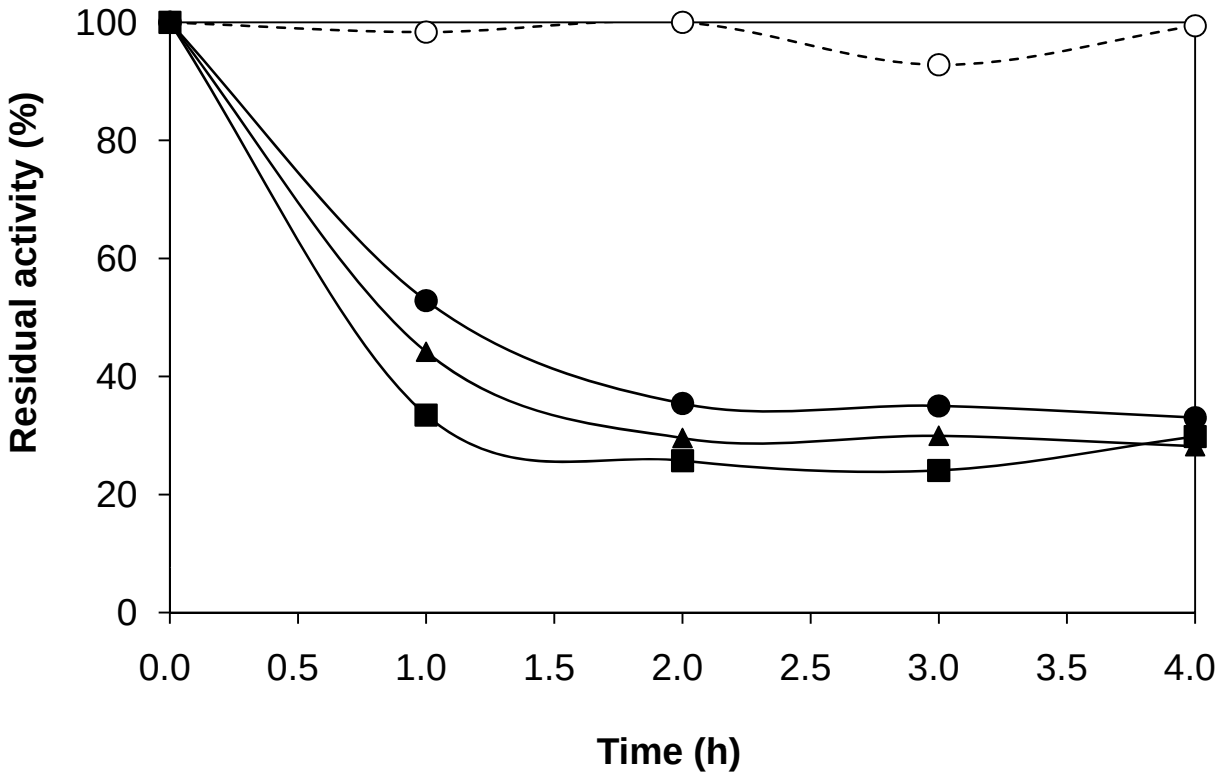
511 **Figure legends**

512 **Fig. 1.** Lipase stability in the solvent used in a FAME reaction without oil. QLM lipase
513 (1%, w/w) was added to a mixture of hexane (60%, w/w) and methanol (4.5%, w/w) at
514 25^{o}C and then shaken at 120 rpm for 4 h. Chlorophyll a (0.37%, w/v) and b (0.28%,
515 w/v) were dissolved in methanol and added to the reaction, respectively. The mixtures
516 were lipase and water (circles); lipase, chlorophyll a, hexane and methanol (solid
517 triangles); lipase, chlorophyll b, hexane and methanol (solid squares); lipase,
518 chlorophyll a and b, hexane and methanol (solid circles).

519 **Fig. 2.** Effects of reaction components on lipase stability during FAME conversion. The
520 reaction solution contained hexane (60%, w/w), oil (35%, w/w), methanol (4.5%, w/w),
521 QLM lipase (1%, w/w), chlorophyll a (0.37%, w/v) and chlorophyll b (0.28%, w/v).
522 Reactions were conducted at 25^{o}C and 120 rpm for 10 h. The reaction contents were
523 lipase, pure palm oil and hexane (circles); lipase, pure palm oil, methanol and biodiesel
524 (triangle); lipase, wABE extracted palm oil, hexane and methanol (solid circles); lipase,
525 chlorophyll a, chlorophyll b, pure palm oil, hexane and methanol (squares); lipase, pure
526 palm oil, wABE extracted pigment, hexane and methanol (solid triangles); and lipase,
527 wABE extracted palm oil, wABE extracted pigment, hexane and methanol (solid
528 squares).

529 **Fig. 3.** Lineweaver-Burk plots in the presence of mixtures of chlorophyll a and b as an
530 inhibitor in artificial wABE. The inhibitory concentrations were 0 mg/l (rhombuses), 1
531 mg/l (squares), 2 mg/l (triangles), 3 mg/l (solid triangles), 4 mg/l (circles) and 5 mg/l
532 (solid circles).

533 **Fig. 4.** Lineweaver-Burk plots in the presence of chlorophyll a (A) and b (B) as
534 inhibitors in the artificial wABE. The inhibitor concentrations were 0 mg/l (rhombuses),
535 1 mg/l (squares), 2 mg/l (triangles), 3 mg/l (solid circles), and 4 mg/l (circles).


Residual activity (%)
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80
60
40
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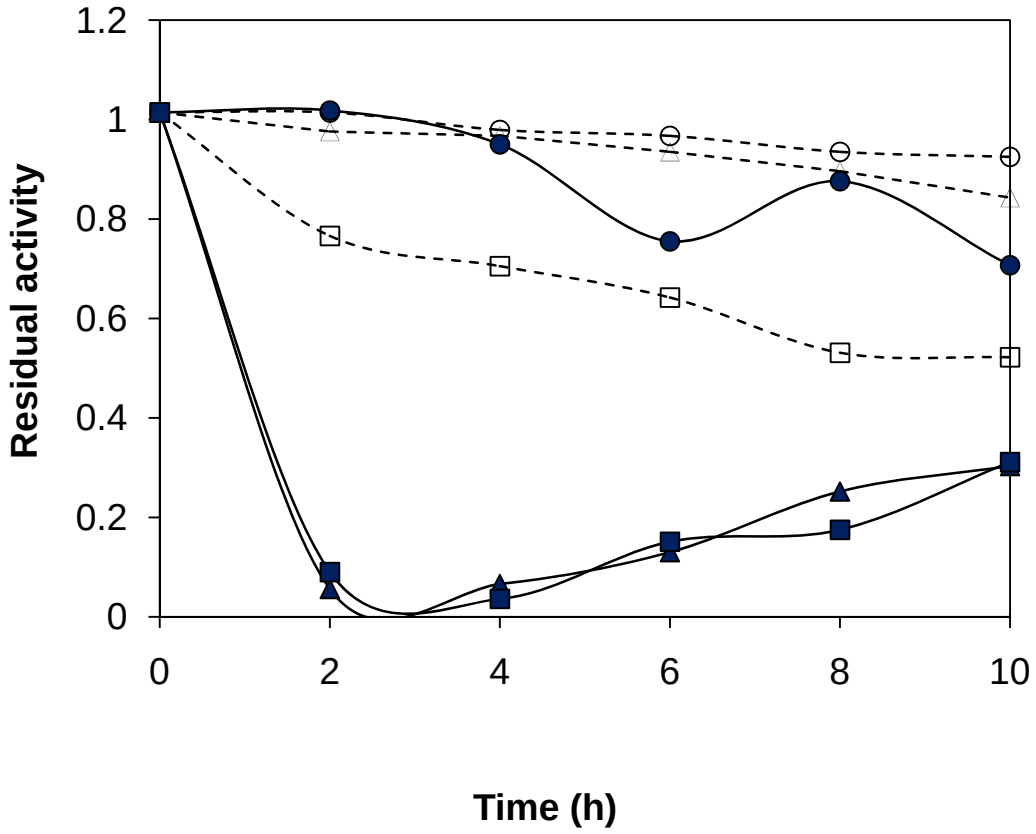
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Time (h)


Residual activity
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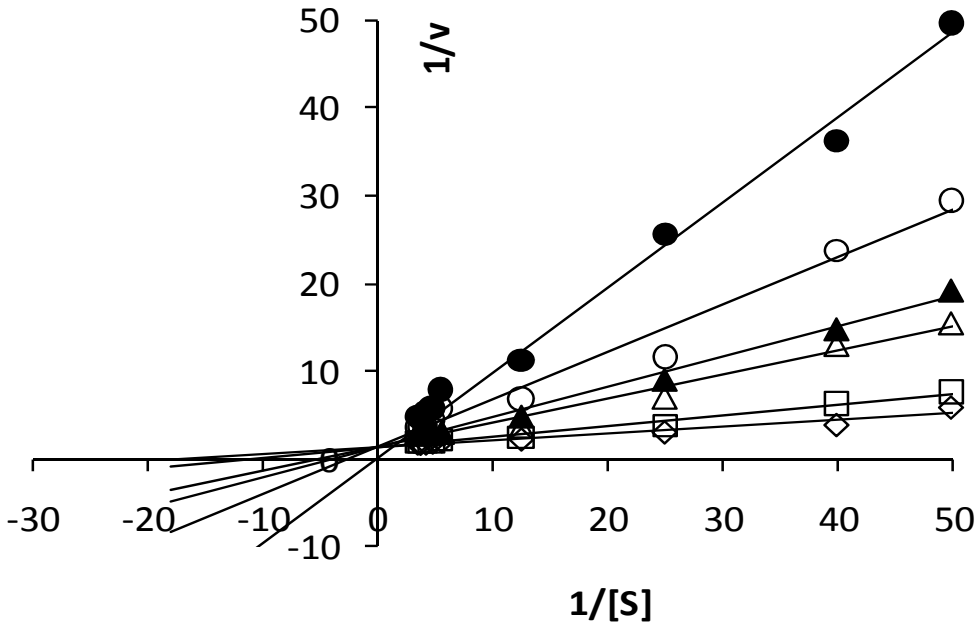
Time (h)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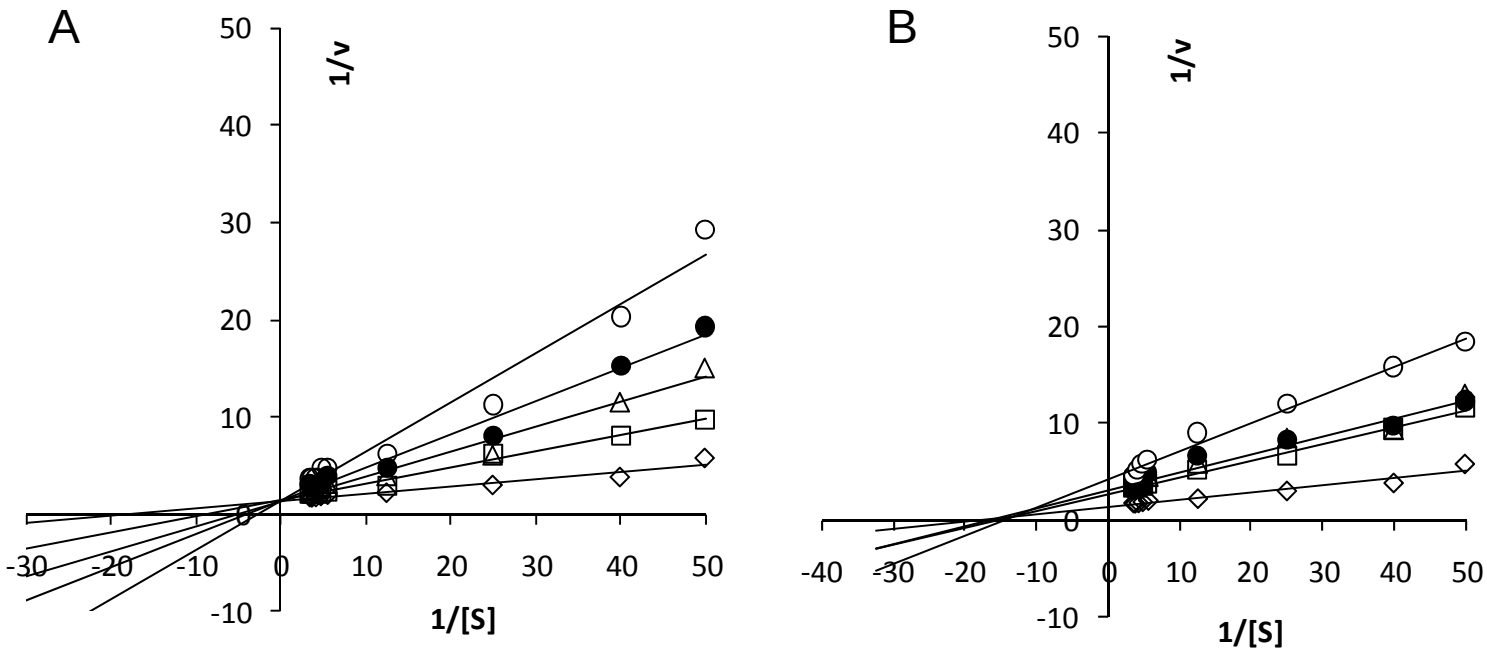
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