**Converting oils high in phospholipids to biodiesel using immobilized *Aspergillus oryzae*
whole-cell biocatalysts expressing *Fusarium heterosporum* lipase**

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Abstract

The presence of phospholipids in oil has been a major hurdle in the production of biodiesel using immobilized *Aspergillus oryzae* whole-cell biocatalysts. A density of phospholipids within the range of 10 to 30% could reduce both the rate of production and the final yield of biodiesel. Phospholipids in the oil leads to the formation of water-in-oil phospholipid-based reverse micelles. The water that activates the enzymatic process is observed to be trapped inside these reverse micelles. This has resulted in the inactivation of the reaction systems and has subsequently led to the deactivation of the immobilized lipase by the extended residence time of the added methanol. A reaction system involving gentle agitation and higher amount of water was found to reduce the reverse micelles formation. This simple technique improved the conversion efficiency by approximately 3-folds, producing a final biodiesel of more than 90 %, using immobilized *A. oryzae* whole cells expressing *Fusarium heterosporum* lipase. This demonstrates that, the above technique could be successfully applied to the enzymatic biodiesel conversion of oils containing high amounts of phospholipids such as that from microalgae.

Keywords

Biodiesel production; Bioconversion; whole-cell immobilized biocatalysts; Enzyme deactivation; Phospholipids; Reverse micelles

Introduction

Depleting oil reserves, increasing dependency on scarce energy and environmental deterioration from the use of fossil fuels has led to the search of cleaner and more sustainable energy. Recent advances in biotechnology are paving the way for the production of various fuels from biomass, and biodiesel has become a plausible substitute for petroleum diesel. Biodiesel is a mixture of Fatty Acid Methyl Esters (FAME) and can be directly used in existing infrastructure without modification. Biodiesel now accounts for a 78% share of the total biofuel consumption in Europe's transportation industry [1].

The cost of biodiesel production requires a cheap form of feedstock and this has shifted the feedstock to various sources [2]. Several microorganisms, such as microalgae, have been targeted as a highly promising feedstock for the commercial production of biodiesel [3,4].

Chemical conversion of microalgae oils to biodiesel has been successfully carried out by various researchers [5, 6]. However, the subsequent environmental issues and the high energy requirements associated with this method make it questionable [7, 8]. A more environmentally friendly and low-energy enzymatic method that has been successfully employed with various plant oils is thought to be a better alternative [9, 10, 11].

However, phospholipids represent a major setback in the application of enzymatic conversion methods to microalgae oil. Phospholipids constitute as much as 30% of the total lipid weight of the oil from these microorganisms, and are known to have a negative effect on these

enzymatic methods [12, 13]. The phospholipids in microbial oils are known to possess highly significant portions of C:16 and C:18 polyunsaturated fatty acids, which are the best of fatty acid chains required for a high-quality biodiesel [14, 15]. Watanabe et al. [16] reported that, even with of less than 1% phospholipid, the performance of immobilized *Candida antarctica* lipase was negatively affected, and that degumming of the plant oil was required for a successful conversion to biodiesel. Unfortunately, the process of degumming which has been suggested to remove phospholipids from plants oils may not be attractive for the high levels of phospholipids in microalgae, as it could lead to a great loss in feedstock – not to mention, the complete degumming of such oils may not possible [12]. Li et al. [17,18] have performed various studies on oils containing low amounts of phospholipids using free lipase, and found that the presence of phospholipids alone within the range investigated (2%) can accelerate the hydrolysis of oils, but that the co-existence of phospholipids and methanol leads to detrimental effects on free lipase.

Despite these findings, no report has documented the used of immobilized lipases for the production of biodiesel from high phospholipid-containing oils in a solvent-free system. In addition, the FAME production from oil containing amount of phospholipids that correspond to that of microbial oils using whole-cell immobilized lipases is yet to be investigated.

Immobilization of lipases offers the added advantages of easy product separation, reduced contamination of product and enzymes, rapid termination of the enzyme-substrate reaction, and immobilization may also alter the activity of the lipase [19, 20]. This points out a significant difference between free lipase and immobilized lipase, which suggests that applying the general conclusions from free lipase to immobilized lipases may not be warranted.

However, the industrial use of immobilized lipases has faced criticism because immobilization process often adds significant cost to the lipase, hence increasing the cost of this

process [21]. To this end, Hama et al. [22] developed immobilized recombinant *A. oryzae* whole-cell biocatalysts for the production of biodiesel. These whole-cell immobilized biocatalysts differ from other immobilized catalysts in that the enzyme cultivation step and the immobilization step are combined in a single unit process, which requires no further purification. This drastically reduces the cost and duration of the enzyme preparation. Due to the simplicity and cost effectiveness of this form of immobilized lipase, it would be advantageous if this could be successfully used to convert oils with high phospholipid content.

The present study investigated the effect that the high amount of phospholipids (30%) in microbial oils exerts on the enzymatic biodiesel production by immobilized *A. oryzae* whole cell biocatalyst expressing *Fusarium heterosporum* lipase. Two major factors were surveyed: agitation and water concentration. These factors were expected to significantly affect the reaction system. This allowed for the demonstration of a simple technique to overcome the low biodiesel conversion efficiency caused by the presence of high phospholipid content.

Methods

2.1 Materials and strains

The host strain, *A. oryzae* NS4 (niaD⁻, sC⁻), was derived from the wild-type strain [23]. Soybean refined oil and soybean phospholipids were obtained from Wako Pure Chemical industries (Osaka, Japan).

2.2 Construction of lipase expression vector and transformation of *A. oryzae*

pSENSU-FHL harboring a P-enolA142 and a 5'UTR of *Hsp12* with an FHL gene and an *sC* gene as a selectable marker was constructed as described in a previous study [24]. Briefly, the

gene encoding *F. heterosporum* lipase (FHL) was amplified from pNAN8142-FHL by PCR using FHL-F1 (5'-TCGCAAACATGATGCTCGTCCTATCTCTTC-3') and FHL-R1 (5'-GCTCTAGACTAAATCATCTGCTTAACAAAT-3') as primers. An amplified fragment was digested with *XbaI* and inserted into a pSENSU plasmid and was then digested again using *PmlI* and *XbaI*. The cells of *A. oryzae* transformant carrying pNAN8142-FHL were then transformed with pSENSU-FHL on a Czapek-Dox (CD)-NO₂-methionine selection plate [22]. The obtained lipase was then designated r-FHL.

A. oryzae biocatalyst expressing mono-and diglycerol lipase was prepared by isolating mdlB gene from *A. oryzae* niaD300 using two primers, fw-SalI (5'-tgGTCGACatgcgcttctctccgcttcgtttctgtt-3') and rv-SphI (5'-gtGCATGCttagcgcaatggcaatccaggacccttgca-3'). The fragments amplified by PCR were digested with *Sall* and *SphI* and inserted into the vector pSENSU. The constructed plasmid (pSENU-mdlB) was then integrated into *A. oryzae* cells. The obtained lipase was then designated r-mdlB.

2.3 Immobilized *A. oryzae* whole-cell biocatalyst preparation

The *A. oryzae* strains were initially cultured on a Czapek-Dox (CD) agar plate at 30 °C for 6 days. The spores were harvested with 5 ml of distilled water and aseptically inoculated into a 500 ml Sakaguchi flask containing approximately 850 mg of reticulated polyurethane foam BSPs (Bridgestone Corporation, Osaka, Japan) in 100 ml of DP medium. The BSPs used for the fungal cell immobilization had an average cuboid size of 6 mm × 3 mm × 3 mm and pore size of 50 pores per linear inch. The fungal strain was cultivated at 30 °C for 96 h on a reciprocal shaker at 150 oscillations per min. The cells were naturally immobilized in the pores of the BSPs during cultivation, and the immobilized whole cells were separated from the culture broth by simple

filtration. The BSP-immobilized whole cells were washed with distilled water, lyophilized for 48 h and used for biodiesel conversion.

2.4 Lipase-mediated FAME production

Soybean oil was spiked with various amounts of phospholipids to simulate the high phospholipid content of crude oils and subjected to sonication to ensure homogeneity. The lipase-catalyzed methanolysis was carried out in screw-capped cylindrical glass tubes. The reaction mixture consisted of oil (with or without phospholipids), distilled water, immobilized *A. oryzae* whole-cell biocatalysts, and 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 block rotator (Nissin Thermo Block Rotator SN-06BN) at 30 °C with a rotation speed of 7.5 (an arbitrary scale on the instrument) with or without pre-agitation. To avoid the general deactivation of the immobilized lipase by methanol, the remaining methanol was added step-wise at 24, 48 and 72 h (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 FAME production during the course of the reaction. For repeated use, the immobilized whole cells were either washed with tap water or reused directly without any treatment and applied on a fresh reaction mixture.

2.5 Gas chromatography analysis

Fatty Acid Methyl Esters produced during the course of the reaction 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 DB-5 capillary column (0.25 mm x 15 m) (J&W Scientific, USA), an auto-sampler and a flame

ionization detector. During the analysis, the temperatures of the injector and detector were set at 245 and 320 °C respectively. The column was configured at a temperature program with an initial temperature of 150 °C for 0.5 min, raised to 300 °C at 10 °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.

Results and discussion

3.1. Impact of Phospholipids on whole-cell biocatalyst performance

Two immobilized whole-cell biocatalysts with different lipase specificities were independently used for the conversion of high phospholipid-content oil into Fatty Acid Methyl Esters (FAMEs). *F. heterosporum* lipase-producing *A. oryzae* (r-FHL) is a solvent tolerant *sn* – 1, 3 regiospecific lipase with a high conversion efficiency of triglycerides, whereas mono- and diglyceride lipase-producing *A.oryzae* (r-mdlB) shows a high specificity for mono and diglycerides [25]. The presence of phospholipids in high quantity presents a heterogeneous substrate with different molecular conformations as such lipases of varying regiospecificities were used in this situation. The FAME yield after 96 h for the 2 sets of lipases varied greatly (Fig. 1) with r-FHL generally returning a higher yield than r-mdlB. In the absence of phospholipids, the final yields and reaction rates were significantly higher, with r-FHL and r-mdlB reaching 94.2 and 50.9% respectively, after 96 h. The higher yield for r-FHL was expected because although FHL is known to be *sn* – 1, 3 regiospecific lipase, it has the ability to enhance acyl migration from the *sn* – 2 position to either *sn* – 1 or *sn* – 3 positions just as other several lipases.

However, when these results were compared with those of the oil containing phospholipids, there was a great decrease in both the reaction rate and the final yield, which

agrees with the results previously reported as phospholipids have negative effects on enzymatic methanolysis [26]. The FAME yield for the refined oil at 24 h was higher than that of the oils containing phospholipids particularly for the lower phospholipid ranges (10 and 15%), after which there was no significant FAME production in these oils (Fig. 1). Since the major factor that was tempered with at 24 h was the concentration of methanol, further investigation on this factor and the initial reaction rate was carried out.

3.2. Effect of the initial reaction rate on enzymatic conversion efficiency

Lipases are generally deactivated by short-chain alcohols [19, 27]. To avoid this deactivation, methanol was step-wise added 4 times in 1:1 molar equivalents of the oil at a time interval of 24 h yielding a total methanol-to-oil molar ratio of 4:1. However, even with this step-wise strategy, deactivation occurred after 24 h in the phospholipid-containing oil reaction systems (Fig. 1). For this reason, the amount of methanol that remained in the reaction system at 24 h was investigated, and this was achieved based on the initial amount of methanol added and the amount of FAME produced at 24 h. Fig. 2 indicates that, in the absence of phospholipids almost all the added methanol was consumed by the methanolysis process, but in the presence of phospholipids significant amounts of methanol remained. The effect was higher for the oils with lower phospholipid content, with 10% phospholipid-containing oils retaining about half the initial methanol amount added. Although the amount of methanol remaining in oils containing 30% phospholipids was not very different from that of the refined oil, the average initial reaction rate for the refined oil was significantly higher than that of the oil containing phospholipids, suggesting that the residence time of methanol in the 30% phospholipid-containing oil was significantly higher than that of the refined oil. The extended time for the contact of lipase with

methanol in the oils containing phospholipids thus inhibited the lipase activity. The inhibited system was thus worsened by each addition of 1 molar equivalent of methanol, subsequently deactivating the lipase.

As the amount of phospholipids increased, the reaction rate increased slightly leading to a higher FAME yield at 24 h, which contrasted with using either free lipase [17] or Novozym 435 (*C. antarctica* lipase immobilized on acrylic resin) [16]. The pattern observed in the present work for both r-FHL and r-mdlB may have been due to the differing responses of lipases to different conditions as well as the nature of the immobilizing substrate because this can affect the activity of enzymes.

3.3. Water content and the formation phospholipid-based reverse micelles

A phospholipid molecule possesses a hydrophilic head and a hydrophobic tail, and this makes it an amphiphilic molecule. This amphiphilic property causes phospholipids to act as surfactants leading to the formation of water-in-oil reverse micelles. A Phospholipid-based reverse micelles mixture was observed, as shown in Fig. 3. In the presence of water, refined oil is seen as almost a clear solution with finely dispersed water droplets (Fig. 3b), while an intense cloudiness can be clearly seen in the oil containing phospholipid (Fig. 3e). Li et al. [17, 28] in their work with free lipase also reported that the presence of phospholipids leads to the formation of reverse micelles that solubilize both methanol and aqueous-phase lipase, leading to a high local concentration of methanol that gathers around the lipase in reverse micelles and subsequently deactivates it. Conversely, in the current work, since the lipase was immobilized, it was not solubilized in the reverse-micelles. However, the water in the system was entrapped in these reverse micelles and formed water pools. The dielectric constant and viscosity of these

water pools may be altered making their physico-chemical properties significantly different from that of bulk water [29, 30]. Furthermore, as clearly shown by the microscopic view in Fig. 3c, the surface of the water droplets in the refined oil was smooth providing the required aqueous-non-aqueous interfacial area for the activation of enzymatic catalysis. However, Fig. 3f shows a much distorted surface of the reverse micelles which likely consisted of a layer of phospholipids. The physical boundaries that were formed by the phospholipids around the water pools hindered the creation of the aqueous-non-aqueous interfacial area, which is a critical factor for the enzymatic methanolysis of most lipases including r-FHL. This phenomenon may lead to an inhibition of the reaction system and cause low conversion efficiency in the early stages of the reaction. Thus, the addition of more methanol to the inhibited system at 24 h may have caused lipase deactivation as observed in Fig. 1.

In the formation of reverse micelles, there always exists an amount of free surfactant, which is far from negligible [31]. With a fixed amount of water and an increase in phospholipids, more free surfactant remained in the system, and this free amphiphilic surfactant facilitated the mixing of the non-polar oil and polar methanol and slightly promoted methanolysis, as shown in Fig. 1. As the phospholipid amount increased, the conversion efficiency slightly increased. Furthermore, Fig. 4 shows that as the water concentration increased from 5 to 10%, both the initial and final FAME yield decreased, which indicates that the free surfactant was consumed in the formation of even more reverse micelles with an increased amount of water. Although the free surfactant slightly improved conversion efficiency by minimizing the mass transfer problems, the catalytic activity of the immobilized lipase was not distributed throughout the bulk of the mass of the reactants but on the interfacial area created by water. Therefore, the physico-

chemical properties of the water and this interfacial area must be maintained to ensure an efficient conversion.

3.4 Agitation effect and improvement of the conversion efficiency of r-FHL in oil containing high levels of phospholipids

Enzymatic methanolysis is an interfacial reaction, which necessitates the creation of aqueous-non-aqueous interfacial areas for the successful conversion of oils to biodiesel. To achieve this, water must be made available to the largest-possible portion of the oil, and a good measure of agitation is known to create a faster reaction and higher conversion yields [32, 33]. However, in the presence of surfactants (phospholipids) above a critical micelle concentration, agitation enhances the formation of reversed micelles. As shown in section 3.3, the formation of reverse micelles inhibits the enzymatic action of r-FHL. In order to avoid this formation of reverse micelles due to agitation, the reaction was performed without the usual pre-agitation in this study. Fig. 5 shows a significant enhancement of both the reaction rate and the final yield leading to a FAME conversion that was improved by approximately 50%. In addition, with the amount of water being a possible contributor to reverse micelle formation, the amount of water was reduced from 5% to 2%. Although the initial reaction rate did not change significantly, the FAME conversion yield was reduced by about 10%, which indicates that, the interfacial area might have been compromised by both inadequate agitation and low water concentration. To increase the interfacial area, the concentration of water was increased to 10% and this successfully improved the FAME conversion yield to approximately 90%. Although the initial reaction rate with 10% water was lower, which was likely to have been due to excessive methanol dilution, the final rate was higher leading to a higher final yield. The dilution of

methanol in the initial stages suppressed the inhibition of lipase. This helped the lipase to maintain its activity throughout the reaction. Also this amount of water was enough to assist the acyl migration from *sn*-2 to *sn*-1,3 [34] which facilitated an even better conversion.

The improved FAME conversion was slightly lower than that of refined oil, which showed that some formation of reverse-micelles in oil containing phospholipids is inevitable when using this technique. However, when comparing the results of the present study with those from previously reported works, this simple technique showed a significant improvement in the conversion of FAME from oils containing high phospholipids, which makes it quite suitable for the application of microalgal oils (Table 1).

3.5 Stability of Enzyme in reuse

For enzymes to become more economically attractive, its ability to maintain a high stability in subsequent reuse is important. Immobilization of the enzyme is one way to make this possible [20]. In this work, since the enzymes were immobilized, they could be easily separated from the reaction mixture by decanting. To reuse the enzymes, two different approaches were used. In the first case, oil containing 30% phospholipid was directly added to the used enzymes that have not undergone any treatment (Fig. 6). After the fourth batch, the potency of the enzyme dropped to about 85% of its original ability. However, in the second case when the used enzyme was washed twice with tap water before reuse, the FAME conversion after the fourth batch was still higher than 95% of its original conversion efficiency. This indicates that, there is likely to be some impurities that attaches to the BSPs possibly including left over methanol and the by-product, glycerol. Water being a universal solvent could then be successfully used to remove these impurities, which enables the immobilized lipase to retain a higher stability. The high

stability of whole cells immobilized on BSPs conforms to the results from previous reports [9, 22].

Conclusion

This work showed that the presence of phospholipids in oil leads to the formation of reverse micelles, which interferes with the aqueous-non-aqueous interfacial area required for the efficient conversion of oils to FAME by immobilized whole-cell biocatalysts. A significant factor – agitation, which in usual cases improves interfacial area, was found to assist in the formation of these undesirable reverse-micelles. This factor was therefore significantly reduced and compensated with an increase in water which helped to improve the interfacial area and a successful production of biodiesel.

Acknowledgements

This work was partly supported by Grants-in-Aid from the cross-ministerial strategic innovation promotion program (SIP) from the Japan Science and Technology Agency, Japan, and Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction, Kobe) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

[1] G. Koçar, N. Civaş, An overview of biofuels from energy crops: Current status and future prospects, *J. Renew. Sustain. Ener.* 28 (2013) 900–916.

- [2] A. Demirbas, Importance of biodiesel as transportation fuel, *Energ. Policy* 35 (2007) 4661–4670.
- [3] Y. Chisti, Biodiesel from microalgae, *Biotechnol. Adv.* 25 (2007) 294–306.
- [4] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: A review *Renew. Sust. Energ. Rev.* 14 (2010) 217–232.
- [5] E.S. Umdu, M. Tuncer, E. Seker, Transesterification of *Nannochloropsis oculata* microalga's lipid to biodiesel on Al₂O₃ supported CaO and MgO catalysts, *Bioresour. Technol.* 100 (2009) 2828–2831.
- [6] S.H. Ho, C.Y. Chen, J.S. Chang, Effect of light intensity and nitrogen starvation on CO₂ fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N, *Bioresour. Technol.* 113 (2012) 244–252.
- [7] L.C. Meher, D.V. Sagar, S.N. Naik, Technical aspects of biodiesel production by transesterification—a review, *Renew. Sust. Energ. Rev.* 10 (2006) 248–68.
- [8] M.K. Lam, K.T. Lee, A.R. Mohamed, Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review, *Biotechnol. Adv.* 28 (2010) 500–18.
- [9] D. Adachi, S. Hama, K. Nakashima, T. Bogaki, C. Ogino, A. Kondo, Production of biodiesel from plant oil hydrolysates using an *A. oryzae* whole-cell biocatalyst highly expressing *Candida antarctica* lipase B, *Bioresour. Technol.* 135 (2013) 410–416.
- [10] H. Fuduka, A. Kondo, H. Noda, Biodiesel fuel production by transesterification of oils, *J. Biosci. Bioeng.* 92 (2001) 405–16.

- [11] M.G. Kulkarni, A.K. Dalai, Waste cooking oil — an economical source for biodiesel: a review, *Ind. Eng. Chem. Res.* 45 (2006) 2901–13.
- [12] L. Chen, T. Liu, W. Zhang, X. Chen, J. Wang, Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion. *Bioresour. Technol.* 111 (2012), 208–214.
- [13] R.K. Balasubramanian, T.T. Yen Doan, J.P. Obbard, Factors affecting cellular lipid extraction from marine microalgae, *Chem. Eng. J.* 215 - 216 (2013) 929–936.
- [14] I.L. Olmstead, D.R.A. Hill, D.A. Dias, N.S. Jayasinghe, D.L. Callahan, S.E. Kentish, P.J. Scales, G.J. Martin, A quantitative analysis of microalgal lipids for optimization of biodiesel and omega-3 production, *Biotechnol. Bioeng.* 110 (2013) 2096-2104.
- [15] K. Servaes, M. Miranda, B. Prandi, S. Sforza, K. Elst, The polar lipid profile of *Nannochloropsis oculata* determined using a variety of lipid extraction procedures, *J. Agric. Food Chem.* (2015) DOI: 10.1021/acs.jafc.5b00241.
- [16] Y. Watanabe, Y. Shimada, A. Sugihara, Y. Tominaga, Conversion of degummed soybean oil to biodiesel fuel, *J. Mol. Catal. B: Enzym.* 17 (2002) 151–155.
- [17] Y. Li, W. Du, D. Liu, Exploration on the effect of phospholipids on free lipase-mediated biodiesel production, *J. Mol. Catal. B: Enzym.* 102 (2014) 88–93.
- [18] Y. Li, W. Du, D. Liu, Effect of phospholipids on free lipase-mediated methanolysis for biodiesel production, *J. Mol. Catal. B: Enzym.* 91 (2013) 61-71.
- [19] R. Maceiras, M. Vega, C. Costa, P. Ramos, M.C. Márquez, Enzyme deactivation during biodiesel production, *Chem. Eng. J.* 166 (2011) 358–361.

- [20] S. Raghuvanshi, R. Gupta, Advantages of the immobilization of lipase on porous supports over free enzyme, *Protein Pept. Lett.* 17 (2010) 1412–6.
- [21] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J.M. Woodley, Guidelines and Cost Analysis for Catalyst Production in Biocatalytic Processes, *Org. Proc. Res. Dev.* 15 (2011) 266–274.
- [22] S. Hama, S. Tamalampudi, Y. Suzuki, A. Yoshida, H. Fukuda, A. Kondo, Preparation and comparative characterization of immobilized *A. oryzae* expressing *F. heterosporum* lipase for enzymatic biodiesel production, *Appl. Microbiol. Biotechnol.* 81(2008) 637–645.
- [23] O. Yamada, B.R. Lee, K. Gomi, Transformation system for *A. oryzae* with double auxotrophic mutations, *niaD* and *sC*, *Biosci. Biotechnol. Biochem.* 61 (1997) 1367–1369.
- [24] T. Takaya, R. Koda, D. Adachi, K. Nakashima, J. Wada, T. Bogaki, C. Ogino, A. Kondo, Highly efficient biodiesel production by a whole-cell biocatalyst employing a system with high lipase expression in *A. oryzae*, *Appl. Microbiol. Biotechnol.* 90 (2011) 1171–1177.
- [25] J. Toida, K. Kondoh, M. Fukuzawa, K. Ohnishi, J. Sekiguchi, Purification and Characterization of a Lipase from *A. oryzae*, *Biosci. Biotechnol. Biochem.* 59 (1995) 1199–1203.
- [26] C.C. Lai, S. Zullaikah, S.R. Vali, Y.H. Ju, Lipase-catalyzed production of biodiesel from rice bran oil, *J. Chem. Technol. Biotechnol.* 80 (2005) 331–337.
- [27] M. Kaieda, T. Samukawa, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda, F. Nomoto, K. Ohtsuka, E. Izumoto, H. Fukuda, Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent, *J. Biosci. Bioeng.* 88 (1999) 627–631.

- [28] Y. Li, W. Du, D. Liu, Efficient biodiesel production from phospholipids-containing oil: Synchronous catalysis with phospholipase and lipase, *Biochem. Eng. J.* 94 (2015) 45–49.
- [29] F.M. Menger, J.A. Donohue, R.F. Williams, Catalysis in water pools, *J. Am. Chem. Soc.* 95 (1973) 286–288.
- [30] K. Tsujii, J. Sunamoto, J.H. Fendler, Microscopic Viscosity of the Interior Water Pool in Dodecylammonium Propionate Reversed Micelles, *Bull. Chem. Soc. Jpn.* 56 (1983) 2889–2893.
- [31] J.L. Lemyre, S. Lamarre, A. Beaupre, A.M. Ritcey, A New Approach for the Characterization of Reverse Micellar Systems by Dynamic Light Scattering, *Langmuir* 26 (2010) 10524–10531.
- [32] D. Surendhiran, M. Vijay, A.R. Sirajunnisa, Biodiesel production from marine microalga *Chlorella salina* using whole-cellyeast immobilized on sugarcane bagasse, *J. Environ. Chem. Eng.* 2 (2014) 1294–1300.
- [33] D.T. Tran, C.L. Chen, J.S. Chang, Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of *Chlorella vulgaris* ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst, *Bioresour. Technol.* 135 (2013) 213–221.
- [34] M. Oda, M. Kaieda, S. Hama, H. Yamaji, A. Kondo, E. Izumoto, H. Fukuda, Facilitatory effect of immobilized lipase-producing *Rhizopus oryzae* cells on acyl migration in biodiesel-fuel production, *Biochem. Eng. J.* 23 (2005) 45–51.

Figure captions

Fig. 1 FAME conversion of oils containing various percentages of phospholipids (PL) using r-FHL (a) and r-mdlB (b). Reaction conditions: 30 °C, 5% water 120 mg of immobilized lipase, 4:1 methanol:oil, 35 rpm

Fig. 2 Average reaction rate (% w/w oil h⁻¹) over the first 24 h of reaction for r-FHL catalyzed methanolysis, initial amount of methanol (mmols) added to the reactor and calculated amounts of methanol (mmols) remaining at 24 h.

Fig. 3 Observation of reverse micelle formation.

(a) Refined oil without agitation with water; (b) Refined oil after agitation with 5% water; (c) Microscopic view of refined oil after agitation with 5% water; (d) oil containing 30% phospholipids without agitation with water; (e) oil containing 30% phospholipids after agitation with 5% water; and (f) Microscopic view of oil containing 30% phospholipids after agitation with 5% water

Fig. 4 Effect of water concentration on the initial and final FAME yields of oils containing 30% phospholipids.

Reaction conditions: 30 °C, 120 mg of immobilized lipase, 4:1 methanol: oil, 30% phospholipid containing oil, 35 rpm, water concentration (5% - 10%)

Fig.5 FAME conversion of oil containing 30% phospholipids under the following conditions:

(diamond) pre-agitation with 5% water; (square) 2% water without pre-agitation; (triangle) 5%

water without pre-agitation; (circle) 10% without pre-agitation

Reaction conditions: 30 °C, 120 mg of immobilized lipase, methanol:oil = 4:1, 35 rpm

Fig.6 Stability of immobilized r-FHL in reuse expressed as a percentage of the FAME yield in the first

batch. Immobilized lipase were washed with tap water after each batch (diamond); immobilized lipase

were reused without washing (square)

Reaction conditions: 30 °C, 120 mg of immobilized lipase (r-FHL), 10 % water, 4:1 methanol:oil,

35 rpm, 96 h

Tables

Table 1

Improvement of FAME conversion of phospholipid containing oils by various techniques

Phospholipid content	Lipase	Technique	¹ Conversion improvement	Reference
1%	Novozym 435	Degumming	10.3 to 28.1%	[16]
1.4%	Novozym 435	Dewaxing/degumming	56 to 88%	[26]
10%	NS81006 free lipase	Synchronizing lipase with phospholipase	86.4 to 94.9%	[28]
30%	Immobilized whole-cell <i>A. oryzae</i> expressing FHL	Reduced agitation (reverse micelle reduction)	29.7 to 91.0%	This work

¹ Conversion improvement was based on the percentage of FAME conversion before and after the application of the mentioned technique

a

b

Fig. 1. FAME conversion of oils containing various percentages of phospholipids (PL) using r-FHL (a) and r-mdlB (b). Reaction conditions: 30 °C, 5% water 120 mg of immobilized lipase, 4:1 methanol:oil, 35 rpm

Fig. 2. Average reaction rate (% w/w oil h⁻¹) over the first 24 h of reaction for r-FHL catalyzed methanolysis, initial amount of methanol (mmols) added to the reactor and calculated amounts of methanol (mmols) remaining at 24 h. **Reaction conditions: 30 °C, 5% water, 120 mg of immobilized lipase, 35 rpm, 1:1 methanol:oil**

Fig. 3. Observation of reverse micelle formation.

(a) Refined oil without agitation with water; (b) Refined oil after agitation with 5% water; (c) Microscopic view of refined oil after agitation with 5% water; (d) oil containing 30% phospholipids without agitation with water; (e) oil containing 30% phospholipids after agitation with 5% water; and (f) Microscopic view of oil containing 30% phospholipids after agitation with 5% water

Fig. 4 Effect of water concentration on the initial and final FAME yields of oils containing 30% phospholipids.

Reaction conditions: 30 °C, 120 mg of immobilized lipase, 4:1 methanol: oil, 30% phospholipid containing oil, 35 rpm, water concentration (5% - 10%)

Fig.5 FAME conversion of oil containing 30% phospholipids under the following conditions: (diamond) pre-agitation with 5% water; (square) 2% water without pre-agitation; (triangle) 5% water without pre-agitation; (circle) 10% without pre-agitation

Reaction conditions: 30 °C, 120 mg of immobilized lipase (**r-FHL**), methanol:oil = 4:1, 35 rpm

Fig.6 Stability of immobilized r-FHL in reuse expressed as a percentage of the FAME yield in the first batch. Immobilized lipase were washed with tap water after each batch (diamond); immobilized lipase were reused without washing (square)

Reaction conditions: 30 °C, 120 mg of immobilized lipase (r-FHL), 10 % water, 4:1 methanol:oil, 35 rpm, 96 h