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Full Length Research Paper

Immobilized lipase from potential lipolytic microbes for catalyzing biodiesel production using palm oil as feedstock

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Biodiesel has been regarded as a biodegradable and non-polluting fuel. Enzymatic transesterification reaction for manufacturing biodiesel from vegetable oils with alcohol is an attractive approach. However, the cost of enzyme remains a barrier for its industrial implementation. The aim of this research was the screening of lipase-producing microorganisms and the studies of potential lipase-mediated biodiesel production using palm oil as substrate. A total of 360 strains of bacteria, yeasts and fungi were isolated and screened from the samples of oil-contaminated soil and waste water. Among all the screened microbes, the potential lipolytic bacterium, *Staphylococcus warneri*, unicellular yeast, *Candida rugosa* and filamentous fungus, *Fusarium solani* were selected because of their high specific activities. The lipase-producing conditions were subsequently optimized by using palm oil as an inducer and lipase activities were compared for both hydrolytic and synthetic catalysis. *C. rugosa* lipase, which exhibited the highest potential for catalyzing the biodiesel production, was further purified and immobilized on various hydrophobic supports. The catalysis of transesterification between methanol and palm oil by the *C. rugosa* immobilized lipases revealed that immobilized lipase from *C. rugosa* on Sepabeads EC-OD was the most promising for further development as a biocatalyst for the application of enzyme-catalyzed biodiesel synthesis.

Key words: Screening, lipases, immobilization, biodiesel.

INTRODUCTION

Biodiesel is considered to be a sustainable energy substitute for petroleum based diesel derived from renewable sources, such as vegetable oils, animal fats and recycled or waste oils (Jeong et al., 2009; Patil and Deng, 2009). Palm oil is economically feasible for use as the feedstock for producing biodiesel due to availability and suitability from its characteristic as a fuel (Winayanuwattikun et al., 2008). Biodiesel production can be catalyzed by chemical and/or biological catalysts, such as acid, base and lipase. However, the biodiesel produced by chemical catalyst has several drawbacks such as difficulty in recovery of glycerol, removal of acid or base catalysts from product and the treatment of wastewater (Freeman et al., 1984; Mittelbach, 1990; Basri et al., 1997; Fukuda et al., 2001). On the contrary, the enzymatic reaction by lipase is a clean technology due to its non-toxic and environmental friendly nature. In addition, the process produces high purity of product and enables easy separation from the byproduct, glycerol (Iso et al., 2001; Kose et al., 2002; Shimada et al., 2002; Ranganathan et al., 2008). Lipases are one of the important enzymes used as industrial biocatalysts for a variety of biotechnological applications especially for biodiesel synthesis. There has been much research on overcoming problems such as enzyme inhibition by methanol, exhaustion of enzyme activity and

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high cost of enzymes, which may contribute to the global effort on industrial implementation of the enzymatic production of biodiesel in the near future. However, most studies have been performed by using commercial immobilized lipases (Nelson et al., 1996; Shimada et al., 1999; Dossat et al., 2002; Hsu et al., 2002; Du et al., 2005; Nie et al., 2006) but the cost of enzyme still remains a barrier for its industrial implementation. In this work, 285 bacterial strains, 5 yeast strains and 70 fungal strains were isolated from oil-contaminated soil samples and waste water. They were then screened for lipase-producing microorganisms. Potential lipolytic bacteria, yeast and filamentous fungi were subsequently identified and compared for the catalytic activities in transesterification using palm oil as substrate. As a consequence, *C. rugosa* lipase was further purified, immobilized and examined as a potential biocatalyst for biodiesel synthesis.

MATERIALS AND METHODS

Screening and isolation of potential lipolytic microorganisms

All the microorganisms used in this study were isolated from oil-contaminated soil samples and waste water. Serial dilutions of samples were made according to standard techniques (Nakayama, 1981). Preliminary screening of lipolytic bacteria, filamentous fungi and yeasts were carried out on nutrient agar plates supplemented with 10% (v/v) tributyrin or 0.001% (w/v) rhodamine B and 1% (w/v) palm oil (Kouker and Jaeger, 1987). Taxonomic identification, biochemical tests and gene-based sequencing were conducted to classify selected bacteria by 16s rRNA gene and yeasts and filamentous fungi by ITS1 gene.

Production of microbial lipases

Lipase-producers were cultured and conditions were optimized in liquid production media that contained 0.4% glucose, 0.6% $NH_4(SO_4)_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 3% palm oil for bacteria, 0.5% yeast extract, 3% bacto-peptone, 0.1% KH_2PO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4$ and 1% palm oil for filamentous fungi and YM medium that contained 0.4% glucose, 0.6% $NH_4(SO_4)_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 3% palm oil for yeast (Rapp and Backhaus, 1992; Cardenas et al., 2001; Fadiloglu and Erkmen, 2002). The cultures were sampled at intervals to determine the relationship between growth rate and lipase production.

Comparative studies of hydrolytic and synthetic activities

The hydrolytic reaction generally takes place at the oil-water interface. Therefore, the substrate reaction mixture; 50 mM Tris–HCl buffer, pH 8.0 and 50% oil emulsion (2.0% polyvinyl alcohol and palm oils) was sonicated and kept at 40°C for 5 min. Lipase was later added to the reaction mixture followed by continuous stirring at 300 rpm, at 40°C for 6 h. Then, 95% ethanol was immediately added to stop the reaction. Liberated free fatty acids were titrated with 50 mM NaOH using phenolphthalein as indicator (Yan et al., 2007). Moreover, the substrate specificity was spectrophotometrically determined using p-nitrophenyl esters (p-NP esters) of acetate (C_2), butyrate (C_4), caprylate (C_8), caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), and stearate (C_{18}) as substrates. The formation of p-nitrophenoxide ion was monitored at 410 nm. Specific activities were calculated using a molar extinction coefficient of 15,000 $M^{-1}cm^{-1}$ for p-nitrophenoxide anion (Maia et al., 2001). On the other hand, the synthetic activity (esterification) was analyzed using oleic acid and dodecanol as substrates. The reaction medium was magnetically stirred for 2 h at 40°C. Samples were then, taken and added to copper acetate reagent containing 5% copper acetate pH 6.1 as color indicator (Kwon and Rhee, 1986). The remaining oleic acid was spectrophotometrically quantified at 715 nm (Sandoval and Marty, 2007). In transesterification, the stepwise methanolysis of palm oil was conducted as follows; 1 g of palm oil was added with lipase and later mixed with 1:3 mole ratio of methanol (Shimada et al., 2002). The transesterification reactions were carried out by stirring the mixtures with magnetic stirrer for 48 h at 40°C. Samples were taken from the reaction mixture and later analyzed for the products by high performance liquid chromatography (Winayanuwattikun et al., 2008).

Purification of *C. rugosa* lipases

Under the optimized conditions, *C. rugosa* lipases were prepared by centrifugation at 10,000x g, at 4°C for 15 min and further concentrated by a 10 kDa cut-off polyethersulfone ultrafiltration. The filtrates were sequentially purified by DEAE HiTrap ion-exchange chromatography and phenylsepharose HiTrap hydrophobic interaction chromatography. The purity of lipase was determined by SDS-PAGE. Protein concentration was spectrophotometrically determined according to Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Immobilization of *C. rugosa* lipases on various hydrophobic supports

The purified *C. rugosa* lipases were immobilized on seven types of commercial hydrophobic supports namely, Amberlite XAD2, Amberlite XAD4, Amberlite XAD7, Amberlite XAD16, Amberlite XAD761, Sepabeads EC-BU and Sepabeads EC-OD under the same conditions. The supports were prepared by suspending 1 g of support powder in 3 ml methanol and washed 3 times with washing buffer (20 mM phosphate buffer pH 7.5). Then, 3 mg/ml of *C. rugosa* lipase was added and magnetically stirred at 350 rpm for 6 h at room temperature. The solution was removed from the immobilized enzyme and washed with washing buffer for 5 min until no enzyme activity was detected. The immobilized lipases were then left to dry at room temperature in a desiccator and finally, the activities from both the hydrolysis and transesterification of palm oil was assayed for.

RESULTS

Screening, optimization and lipase production

A total of 360 microorganisms, including 85 bacterial strains, 5 yeast strains and 70 fungal strains, were isolated from oil-contaminated soil samples and waste water. They were subsequently screened for potential lipase production in solid medium plates containing tributyrin and palm oil with rhodamine B as indicator. A total of 151 microorganisms, including 95 bacterial strains, 5 yeast strains and 51 fungal strains produced clear zones in the plates containing tributyrin. Among these, only 97 microorganisms; 55 bacterial strains, 4 yeast strains and

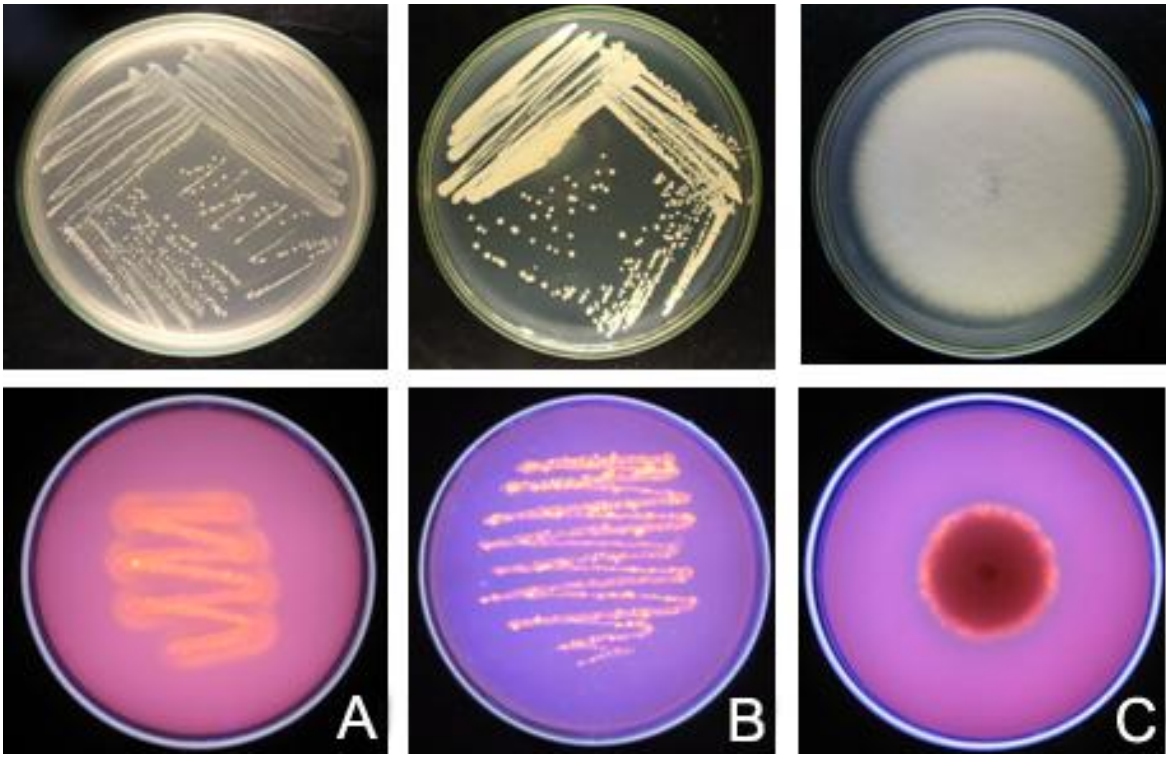

Figure 1. Potential lipolytic microorganisms (a) bacteria; *S. warneri*, (b) yeast; *C. rugosa* and (c) filamentous fungi; *F. solani* grown on NA, YM and PDA agar, respectively (upper panel) and on BYPO agar containing rhodamine B and palm oil (lower panel).

38 fungal strains showed hydrolysis on the palm oil plates indicating the presence of lipases acting on long chain triglycerides (Figure 1).

In order to select the best lipase producer, the selected 97 microorganisms were cultured in liquid medium by using palm oil as lipase inducer. After centrifugation of the culture media, lipase activity in the supernatants was spectrophotometrically determined for the hydrolysis of p-nitrophenyl palmitate (p-NPP), a chromogenic substrate. Taxonomic, biochemical tests and gene-based sequencing were used to identify the species of potential lipolytic bacteria, yeast and filamentous fungi with the highest lipase activity. The 16S rRNA gene sequence revealed a consistently 99% similarity with the bacterium, *Staphylococcus warneri* (Accession number: L37603.1) with activity of 1.29 ± 0.23 U/mg protein while the ITS1 gene sequence showed 99% identity with unicellular yeast, *C. rugosa* (Accession number: GQ376074) with 74.53 ± 2.12 U/mg protein and filamentous fungus, *Fusarium solani* (Accession number: AM412642.1) with 1.41 ± 0.08 U/mg protein. The result matches well with the conclusion from biochemical properties.

Lipase producers were cultured and the producing conditions were optimized in liquid production medium. Cell growth was determined by measuring A_{600} for S. *warneri* and *C. rugosa,* while the dry mass was measured for *F. solani*. Typical growth and lipase production curves are shown in Figure 2. The highest lipase production was found at the beginning of the stationary phase after 24, 120 and 72 h for S. *warneri*, *C. rugosa* and *F. solani*, respectively.

Comparative studies of hydrolytic and synthetic activities

Three different kinds of reactions; hydrolysis of palm oils, esterification of dodecyl oleate and transesterification of palm oil, were examined to select the most promising lipase for industrial application (Table 1). Beside the palm oil hydrolysis, the substrate specificity was determined with p-NP esters of different carbon chain lengths (Figure 3). *S. warneri* lipase had optimum activity for substrates with medium-chain length in the moderate range from 8 to 12. On the other hand, *C. rugosa* lipase showed broader range of substrate specificity, with high activity for the substrates from 4 to 16 carbon chain length. In contrast, lipase from the fungus, *F. solani* exhibited a narrow range of specificity towards only p-nitrophenyl laurate (C12). The esterification between oleic acid and dodecanol and transesterification of palm oil were used to verify the potential synthetic activity of the selected lipase. The results indicated that all the lipases can catlyze the synthetic reactions, but at different rates to the

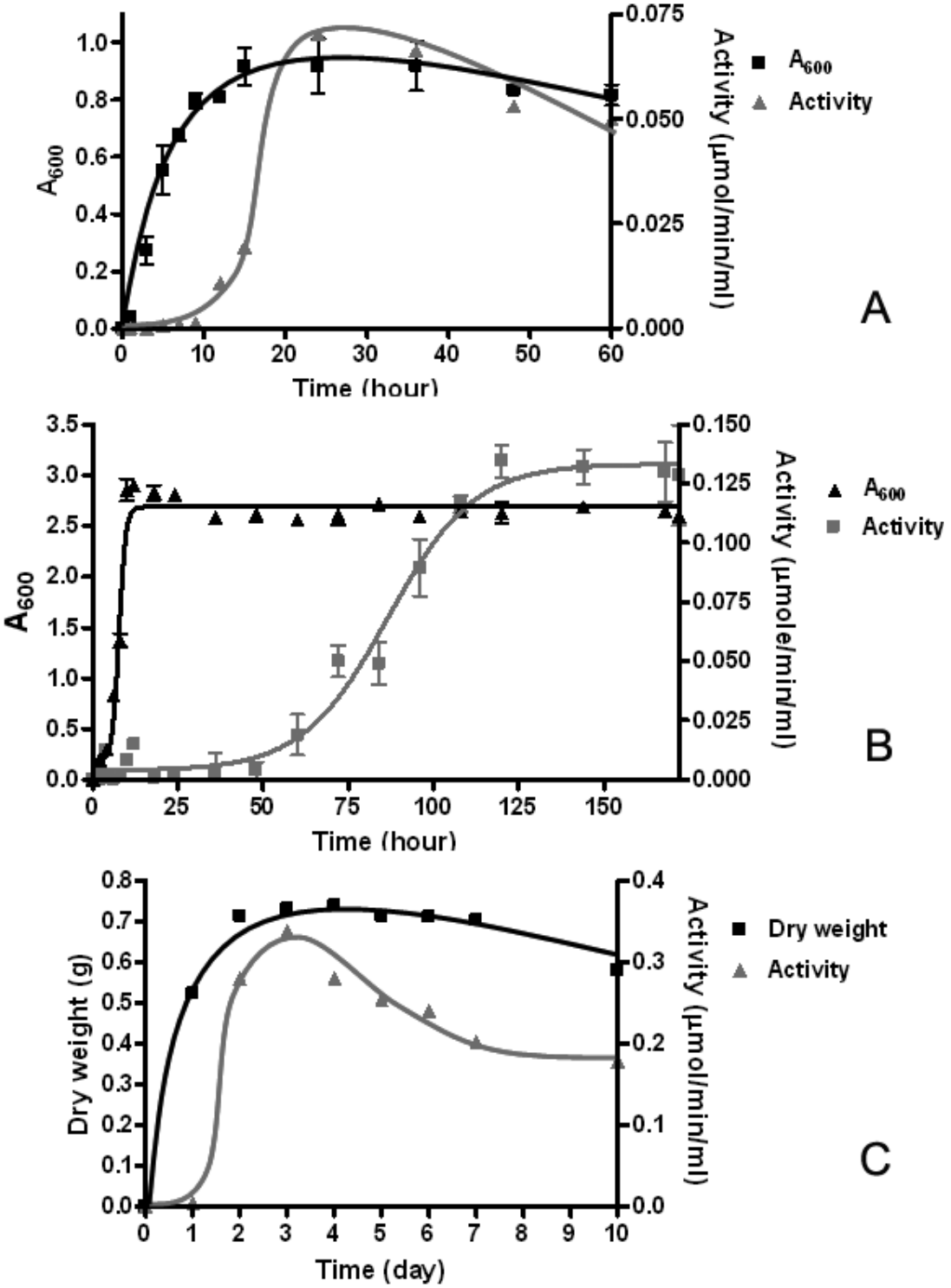

Figure 2. Growth curve and lipolytic activity of (a) bacteria, *S. warneri*, (b) yeast, *C. rugosa* and (c) filamentous fungi, *F. solani*. Lipolytic activity was measured in the growth medium at intervals using p-nitrophenyl-palmitate as substrate.

hydrolytic reactions. From the comparative results of the 3 studied microorganisms, the lipase from unicellular yeast, *C. rugosa* exhibited a broader range of specificities and also had the highest transesterification activities, suggesting that this microbial lipase is promising as a potential biocatalyst for production of biodiesel.

Table 1. The specific activities of lipases from *S. warneri*, *C. rugosa* and *F. solani* in the hydrolysis of palm oil, esterification of dodecyl oleate and transesterification of biodiesel from palm oil. The experiment was performed in triplicate, and data represent mean ± S.D.

Microorganism	Specific activity (µmole/min/mg protein)		
	Hydrolysis	Esterification	Transesterification
S. warneri	1.09 ± 0.26	12.69 ± 0.52	0.124 ± 0.01
C. rugosa	3.64 ± 0.12	33.33 ± 0.12	0.331 ± 0.03
F. solani	1.26 ± 0.18	2.4 ± 0.15	0.102 ± 0.01

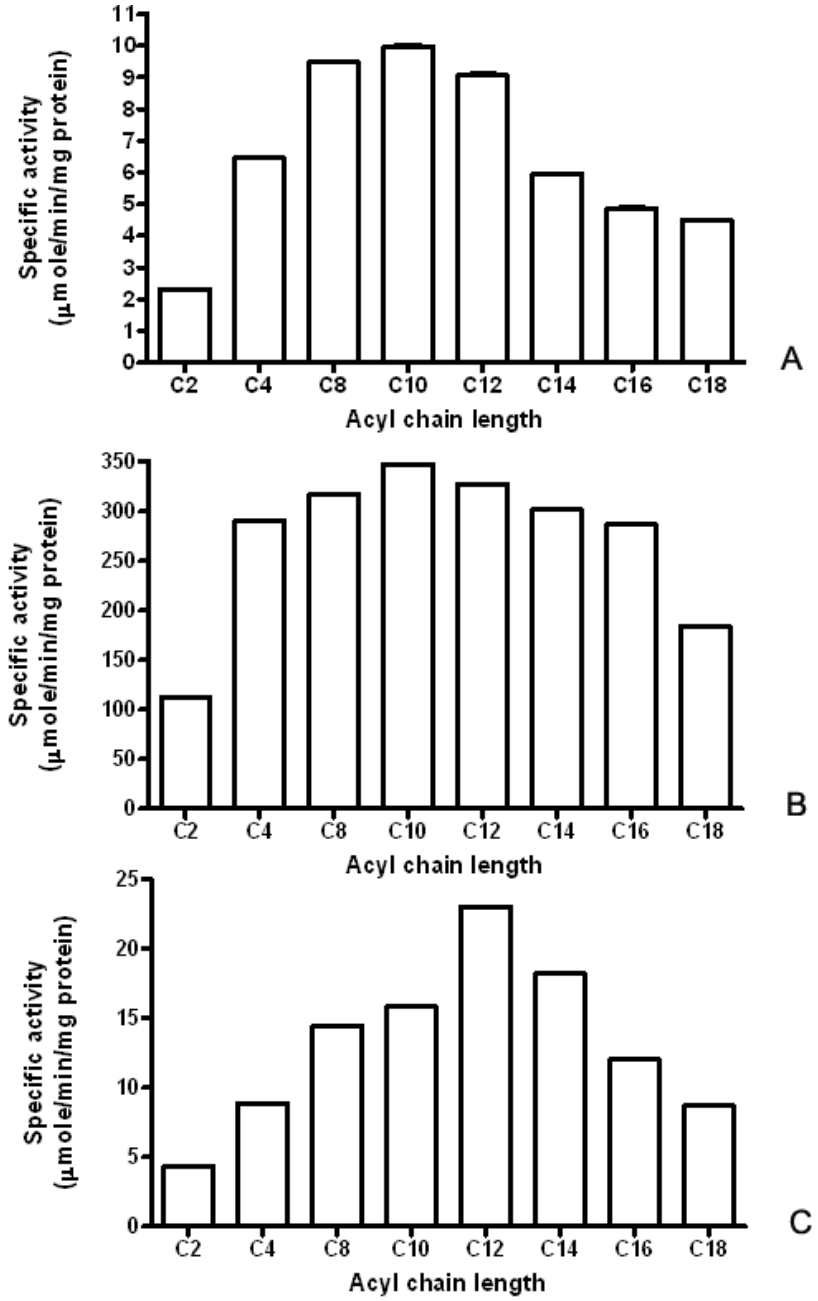

Figure 3. Substrate specificity of *S. warneri* (A); *C. rugosa* (B) and *F. solani* (C) lipases towards p-nitrophenyl esters**.** The experiment was performed in triplicate, and data represent mean ± S.D.

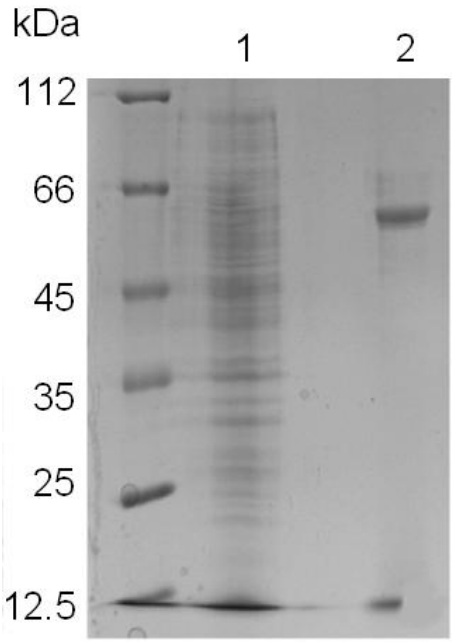

Figure 4. SDS-PAGE of purified *C. rugosa* lipase by DEAE HiTrap ion-exchange chromatography and phenylsepharose HiTrap hydrophobic interaction chromatography. Lane 1, crude lipase; Lane 2, purified *C. rugosa* lipase.

Purification of lipases

The concentrated cell-free supernatants from *C. rugosa* culture were purified by anion-exchange DEAE and followed by phenylsepharose chromatography. The cell-free crude preparation from culture broths was successfully purified with an overall yield of 46.4% and a purification fold of 4.4. The purity was confirmed by the presence of a single band with a relative molecular mass of 60 kDa in SDS-PAGE (Figure 4).

Immobilization of *C. rugosa* lipases on various hydrophobic supports

By virtue of the multiple advantages, seven types of commercial inorganic materials were selected as supports for lipase immobilization. The immobilized enzymes were primarily assayed for activities by hydrolysis of p-NPP. As a consequence, the immobilized enzymes with these supports were further examined for the catalysis of hydrolysis of palm oil and transesterification between methanol and palm oil. The results are shown in Figure 5, with the production of fatty acids and biodiesel expressed as percent conversion. The immobilized lipase on Amberlite XAD7 and Sepabeads EC-OD gave high hydrolytic activity, whereas only the immobilized lipase on Sepabeads EC-OD provided the highest transesterification activity approximately, 64%.

DISCUSSION

A total of 360 microorganisms; most active bacterium, *S. warneri*, most active unicellular yeast, *C. rugosa* and most active filamentous fungus, *F. solani* were optimized for the conditions of lipase production and compared for both hydrolytic and synthetic activities. Since the major fatty acids of palm oil are composed of 38.67% palmitic acid (16:0), 45.45% oleic acid (18:1) and 10.87% linoleic acid (18:2), the selection of lipase should therefore, be correlated to the oil feedstock in order to obtain the maximal yield. From comparative results, the lipase from unicellular yeast, *C. rugosa* was promising as a potential biocatalyst for the production of biodiesel toward palm oil as the feedstock due to the higher and broader range of specificities.

Moreover, the application of the lipase in industrial biodiesel synthesis normally requires the immobilized enzyme due to the greatly simplified design of the reactor, reduction of downstream operation and also to promote the recovery and reusablility of the enzyme. Therefore, *C. rugosa* lipases were immobilized by simpler and effective physical absorption method. This process is straightforward, easy and inexpensive. In addition, the regeneration is feasible from the reversible adsorption of the enzyme on the enzyme carriers (Palomo et al., 2003). The commercial inorganic materials were selected as supports from their properties such as high stability against physical, chemical and microbial degradation. Especially, highly porous polymer matrices with spherical beads and high hydrophobicity were appropriate for the application in lipase-catalyzed biodiesel production, in terms of greater capacity for enzyme loading and less negative effect from the by-product, glycerol adsorbed on the surface of the enzymes. The different physical properties in various types of supports such as functional groups, specific surface area, pores and particle sizes, significantly affect the accessibility and partitioning of substrates, products and water in the reaction mixture (Palomo et al., 2002; Lei et al., 2004; Panzavolta et al., 2005; Blanco et al., 2007; Ghiaci et al., 2009). Consequently, the catalytic efficiency of the immobilized enzyme can be influenced.

From comparative studies with two commercial immobilized lipases; Lipozyme RM IM (*Rhizomucor miehei* lipase) and Novozyme435 (*Candida antarctica* fraction B lipase), the percentage yield of immobilized lipase-catalyzed biodiesel production was slightly lower than in Lipozyme RM IM (72%) and Novozyme435 (75%). Such a result may have been from the fact that the conditions of immobilization and transesterification were not experimentally optimized. Further studies will be required to optimize the immobilization on the most potential support, Sepabeads EC-OD and the transesterification conditions

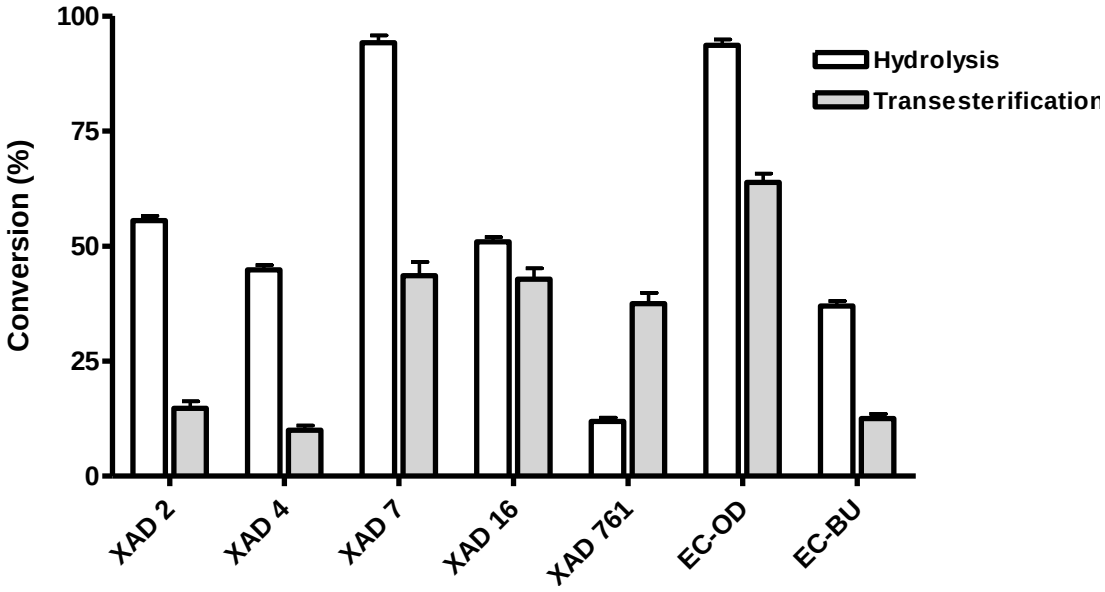

Figure 5. Percent conversion of palm oil in the hydrolysis and transesterification catalyzed by *C. rugosa* immobilized lipases on various hydrophobic supports.

for biodiesel synthesis from palm oil.

Conclusion

From the screening of lipase-producing microorganisms from oil-contaminated soil samples and waste water, *C. rugosa* showed the highest activity in both hydrolysis and synthesis. The lipases were purified by column chromatography and further immobilized on various hydrophobic supports. When the efficiency for the catalysis of transesterification from *C. rugosa* immobilized lipases was examined, the immobilized lipase on Sepabeads EC-OD displayed more potential as a biocatalyst for further development and application in enzyme-catalyzed biodiesel synthesis.

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