WALAILAK JOURNAL
http://wjst.wu.ac.th Agricultural Technology and Biological Sciences

Characterization of *Serratia nematodiphila* YM48 Lipase as a Biocatalyst in Fatty Acid Methyl Esters Production

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Received: 17 November 2015, Revised: 1 March 2016, Accepted: 15 April 2016

Abstract

Lipases are biocatalysts which have been used in transesterification for biodiesel production. The identification of a new lipase, *Serratia nematodiphila* YM48, necessitates exploration for its suitability in optimizing biodiesel production. *Serratia nematodiphila* YM48 is an obtained lipase gene similar to *Serratia marcescens* lipase. The properties of YM48 lipase included an optimal temperature and a pH of 40 °C and 8, respectively. The enzyme had increased stability compared with native enzyme when lyophilized with polyethylene glycol or α-cyclodextrin, and also when immobilized onto silica gel. It retained > 50 % activity after incubation at 40 °C, pH 8, for 48 h. Both lyophilized and immobilized lipase catalyzed reaction between palm oil and methanol (1: 6 molar ratio), resulting in an 84 - 88 % yield of fatty acid methyl esters (FAMEs). The approach of whole-cell culture immobilization resulted in the greatest amounts of FAME production, particularly fatty acids of methyl oleate, methyl linoleate, and methyl palmitate, and so has the potential for high quality biodiesel production.

Keywords: Immobilization, lipase, lyophilization, *Serratia nematodiphila*

Introduction

Biodiesel production is a process of mono-alkyl ester formation from oil and methanol, resulting in a mixture of fatty acid methyl esters (FAMEs). The resources required for biodiesel production via transesterification; microbial lipase, the type of substrate (oil), and the solvent (alcohol) - have been reported previously [1,2]. This process requires a catalyst; biocatalysts, such as lipases, offer benefits over chemical catalysts. The advantages of microbial catalysts are a short period of enzyme production, a high yield of substrate conversion into product, being more environmentally friendly, easy glycerol removal, and simplicity in genetic manipulation or enzyme technology. Incidences of microorganisms producing lipases have been reported in fungi, yeasts, bacteria, and Actinomycetes [3]; some strains were produced and applied in commercial productions. When used as biocatalysts, there are various processes for lipase preparation, particularly, immobilization techniques such as adsorption, covalent attachment, entrapment, cross-linked enzyme, and whole-cell biocatalysts. The immobilized lipases used in biodiesel are common in bacteria and fungi, which have different suitable methods [4,5].

Bacterial true lipase is classified in Family I, with 11 subfamilies [6]. For *Serratia* species, extracellular lipase is well documented in *Serratia marcescens* [7,8], but there are no comparable reports on *S. nematodiphila*. This study aimed to identify a new bacterial lipase source by investigating the

activity and stability of *S. nematodiphila* YM48 lipase, making comparisons between lyophilization and immobilization of lipase for FAME production via transesterification. As biodiesel continues to be an attractive fuel source, new resources are required to achieve high production and low costs for industry. Moreover, whole cell culture with immobilization was investigated for lipase preparation. This was studied because whole cell systems give benefits for lipase preparation, including cost reduction, greater convenience, increased cell yield, and recyclable biocatalysts, known in lipase of fungi and yeast [9].

Materials and methods

Bacterial cultivation for lipase production

The sample was taken from a pool of collected wastewater of at a modified chicken sausage processing factory (Khon Kaen, Thailand). Then, 25 ml of the sample were transferred into 225 ml YM medium containing (g/L): peptone 5, yeast extract 3, malt extract 3, glucose 10, and NaCl 1. Serial dilution of the sample was performed, and it was then spread on YM agar, supplemented with 1 % olive oil and 0.001 % rhodamine B. The plates were incubated at 30 °C for 48 h. The positive colonies, orange colonies under UV light (350 nm), were confirmed on YM agar supplemented with 1 % tributyrin incubated at 30 °C for 48 h, detecting lipase production of clear zone (hydrolysis) colonies. The YM48 strain was selected, and lipase activity was determined in YM medium supplemented with 1 % monosodium glutamate (MSG) and 1 % Tween 80, incubated by agitation at 150 rpm, 30 °C for 24 h. The supernatant collected by centrifugation 5000×*g*, 4 °C, for 10 min was used as crude lipase enzyme.

Bacterial strain identification

Strain YM48 was selected and grown in YM medium at 30 °C for 24 h; then, the cell pellet was collected by centrifugation. DNA was extracted using a Genomic DNA mini kit (Geneaid Biotech Ltd., Taiwan). The 16S rRNA gene was amplified using universal primers, 20F (5'-GAGTTTGATCCTGGCTCAG-3') and 1500R (5'-GTTACCTTGTTACGACTT-3') [10]. The initial denaturation step of polymerase chain reaction (PCR) was carried out at 94 °C for 3 min. It was followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 ° for 2 min, followed by a final amplification step at 72 °C for 3 min. The lipase gene was also amplified by PCR with lip_F (5'-CGGCTAACGTTGTTTCCCTG-3') and lip_R primers (5'-TGGGGTATGACGTGCCTTAA-3') which were designed based on a conserved sequence of bacterial lipase gene alignment. The PCR amplification was run as described above, with annealing temperature at 50 °C for 2 min. The PCR products were sequenced by 1st BASE; the nucleotide sequences were analyzed by the BLAST N and the tBLAST N.

Lipase assay

Lipolytic activity was determined by a colorimetric method, using ρ-nitrophenyl-palmitate (pNPP) as a substrate. The 0.1 ml sample of lipase was added into 2 ml of the reaction mixture (30 mg of pNPP in 10 ml of 2-propanol mixed with 207 mg of sodium deoxycholate, 100 mg of gum arabic in 90 ml of 5 mM phosphate buffer (pH 8.0)); the reaction was held at 37 °C for 15 min before it was stopped by adding 2.9 ml of sodium carbonate (21.18 mg in 100 ml of distill water). The absorbance was measured at 410 nm [11]. One unit (U) of lipase activity was defined as the amount of enzyme liberating 1 µmol of ρ-nitrophenol from ρ-nitrophenyl-palmitate per min per ml. The molar adsorption coefficient of ρ-nitrophenol was 15 L/mmol/cm. The protein content was detected by the Lowry method [12].

Characterization of lipase activity and stability

Strain YM48 was investigated for the optimum temperature and pH of lipase activity. The enzyme was combined with the substrate in phosphate buffer (pH 8) and incubated at various temperatures (30 - 80 °C) for 15 min; the reaction was measured for absorbance with lipase assay. The optimum pH was tested by mixing the substrate and enzyme in different pH buffers (0.05 M sodium citrate buffer for pH 4-5; 0.05 M phosphate buffer for pH 6 - 8; 0.05 M glycine-NaOH for pH 9; 0.05 M Na_2HPO_4-NaOH for pH 10), and incubated at 37 °C for 15 min of lipolytic activity. For determination of lipase stability, the

enzyme was incubated with phosphate buffer (pH 8) at 4 °C to measure pH stability, or at 40 °C to measure thermal stability. Every 2 h, a sample was taken to measure lipase activity. The enzyme was also determined in various distilled water/methanol ratios at 40 °C, pH 8, for methanol stability.

Lyophilization of lipase

The crude lipase enzyme of YM48 was concentrated by lyophilization by a method modified from Wang and Mei [13], using either polyethylene glycol (PEG 6000) or α-cyclodextrin as preservative agents. The additive, 30 g of PEG or 1.5 g of α-cyclodextrin, was combined with 275 ml of the crude enzyme and 15 ml of 1 M phosphate buffer (pH 8). The mixture was gently agitated with a magnetic stirrer, then transferred into a 1200 ml freeze flask in a shell-freeze incubator at −30 °C for 1 - 2 h and incubation continued at −60 °C for 2 h. The lipase mixture was completely coated onto the freeze flask; then, the mixture was lyophilized at −80 °C, vacuum 0.010 - 0.080 mbar, for 18 - 24 h. The lyophilized powder was kept at 4°C for further use.

Immobilization of lipase

All 3 sample types, whole cell culture (cells and supernatant), supernatant only, and powder of lyophilized PEG-lipase complex, were immobilized onto silica gel by adsorption and covalent bonding methods, according to Hwang *et al*. [14]. One hundred g of silica gel (70 - 230 mesh, pore diameter 0.063 - 0.200 mm, Merck) were prepared with 5 % (v/v) nitric acid at 90 °C for 1 h, and then incubated with 2 % (v/v) 3-aminopropyltriethoxysilane-acetone solution at 45 °C for 24 h. The silica gel was washed 5 times with distilled water and dried at 80 °C for 24 h. For lipase immobilization by the adsorption method, 5 ml of each lipase sample was mixed with 2 g of silica gel and gently agitated on a magnetic stirrer at 4 °C for 3 h. After incubation, the silica gel with the immobilized lipase was washed twice with 50 mM phosphate buffer (pH 10) and filtered through a 0.2 μm cellulose acetate filter. Surface modification of silica gel for the covalent bonding method was carried out by adding 0.1 % (v/v) of glutaraldehyde solution (pH 7) before incubation at room temperature for 3 h, followed by washing twice with 0.1 M phosphate buffer (pH 10) to remove non-bound glutaraldehyde. The silica gel-glutaraldehyde was used to immobilize lipase samples in the same way as in the adsorption method.

Analysis of fatty acid methyl esters (FAMEs)

Lyophilized lipase with PEG or α-cyclodextrin and immobilized lipase from supernatant or whole cell culture were used to catalyze transesterification between palm oil and methanol to generate FAMEs. Palm oil 8.43 g, lyophilized or immobilized lipase (1 U/ml) and methanol conducting reaction at oil/methanol molar ratios 1: 6 were performed using a 3-step addition methanol process by equally adding methanol at 0, 12, and 24 h of reactions. The reactions were carried out at 40 °C with agitation at 220 rpm. After 48 h incubation, 200 μl of reaction sample was collected, mixed with 1 ml of hexane, and centrifuged at room temperature at 10,000×*g* for 2 min. The upper layer of the sample was analyzed for FAMEs by gas chromatography (GC), using nitrogen as a carrier gas, with a flame ionization detector (FID).

Results and discussion

Identification of bacterial strain

A total of 70 isolates were purified from the wastewater of the modified chicken sausage industry using a qualitative plate assay on a rhodamine B agar plate. The positive colonies showed as orange colonies under UV light (350 nm) as a fluorescent rhodamine B indicator, indicating lipolytic bacteria. To confirm the potential lipolytic bacteria, lipase activity was determined on a tributyrin agar plate, resulting in a clear zone around the positive colonies for tributyrin hydrolysis. An effective strain, YM48, was selected for lipase production in YM medium supplemented with MSG and Tween 80, following recent reports that MSG and Tween 80 enhanced extracellular lipase production by many bacteria [15]. Strain YM48 was identified and showed 99.86 % similarity to *Serratia nemathodiphila* and was named *Serratia nematodiphila* YM48, with GenBank accession number of the 16S rRNA gene sequence KJ434944. The

lipase gene of strain YM48 was fully sequenced and consisted of 1845 bp encoding 614 amino acid residues (accession number KP325673). Comparison of YM48 lipase with the translated lipase gene of *S. marcescens* indicated 95 % identity, including a lipase consensus sequence (GHSLG) and no signal peptide (**Figure 1**). The lipase of *S. nematodiphila* YM48 might be secreted via the type I secretion system (Lip system) into culture medium in the same way as *S. marcescens* lipase [16,17], which has no N-terminal signal peptide. These results showed that *S. nematodiphila* YM48 is a new true lipase producing strain.

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Lip             MGIFSYKDLDENASKALFSDALAISTYAYHNIDNGFDEGYHQTGFGPGLPLTLITALIGS 60
YM48            MGIFSYKDLDENASKALFSDALAISTYAYHNIDNGFDEGYHQTGFGLGLPLTLITALIGS 60
D13253          MGIFSYKDLDENASKALFSDALAISTYAYHNIDNGFDEGYHQTGFGLGLPLTLITALIGS 60
                ********************************************* *************

Lip             TQSQGGLPGLPWNPDSEQAAQEAVNNAGWSVIDATQLGYAGKTDARGTYYGETAGYTTAQ 120
YM48            TQSQGGLPGLPWNPDSEQAAQEAVNNAGWSVIDATQLGYAGKTDARGTYYGETAGYTTAQ 120
D13253          TQSQGGLPGLPWNPDSEQAAQDAVNNAGWSVIDAAQLGYAGKTDARGTYYGETAGYTTAQ 120
                ********************:************:************************

Lip             AEVLGKYDSEGNLTAIGISFRGTSGPRESLIGDTIGDVINDLLAGFGPKGYADGYTLKAF 180
YM48            AEVLGKYDSEGNLTAIGISFRGTSGPRESLIGDTIGDVINDLLAGFGPKGYADGYTLKAF 180
D13253          AEVLGKYDSEGNLTAIGISFRGTSGPRESLIGDTIGDVINDLLAGFGPKAMRR-YTLKAF 179
                ************************************************.    ******

Lip             GNLLGDVAKFAQAHGLSGEDVVVSGHSLGGLAVNSMAAQSDANWGGFYAQSNYVAFASPT 240
YM48            GNLLGDVAKFAQAHGLSGEDVVVSGHSLGGLAVNSMAAQSDANWGGFYAQSNYVAFASPT 240
D13253          GNLLGDVAKFAQAHGLSGEDVVISGHSLGGLAVNSMAAQSDATWGGFYAQSNYVAFASPT 239
                **********************:******************.*****************

Lip             QYEAGGKVINIGYENDPVFRALDGTSLTLPSLGVHDAPHTSATNNIVNFNDHYASDAWNL 300
YM48            QYEAGGKVINIGYENDPVFRALDGTSLTLPSLGVHDAPHTSATNNIVNFNDHYASDAWNL 300
D13253          QYEAGGKVINIGYENDPVFRALDGTSLTLPSLGVHDAPHTSATNNIVNFNDHYASDAWNL 299
                ************************************************************

Lip             LPFSILNIPTWLSHLPFFYQDGLMRVLNSEFYSLTDKDSTIIVSNLSNVTRGSTWVEDLN 360
YM48            LPFSILNIPTWLSHLPFFYQDGLMRVLNSEFYSLTDKDSTIIVSNLSNVTRGSTWVEDLN 360
D13253          LPFSILNIPTWLSHLPFFYQDGLMRVLNSEFYSLTDKDSTIIVSNLSNVTRGSTWVEDLN 359
                ************************************************************

Lip             RNAETHSGPTFIIGSDGNDLIKGGKGNDYLEGRDGDDIFRDAGGYNLIAGGKGHNIFDTQ 420
YM48            RNAETHSGPTFIIGSDGNDLIKGGKGNDYLEGRDGDDIFRDAGGYNLIAGGKGHNIFDTQ 420
D13253          RNAETHSGPTFIIGSDGNDLIKGGKGNDYLEGRDGDDIFRDAGGYNLIAGGKGHNIFDTQ 419
                ************************************************************

Lip             QALKNTEVAYDGNTLYLRDAKGGITLADDISTLRSKETSWLIFNKEVDHQVTAAGLKSDS 480
YM48            QALKNTEVAYDGNTLYLRDAKGGITLADDISTLRSKETSWLIFNKEVDHQVTAAGLKSDS 480
D13253          QALKNTEVAYDGNTLYLRDAKGGITLADDISTLRSKETSWLIFSKEVDHQVTAAGLKSDS 479
                ****************************************.***************

Lip             GLKAYAAAATGGDGDDVLQARSHDAWLFGNAGNDTLIGHAGGNLTFVGGSGDDILKGVGN 540
YM48            GLKAYAAAATGGDGDDVLQARSHDAWLFGNAGNDTLIGHAGGNLTFVGGSGDDILKGVGN 540
D13253          GLKAYAAATTGGDGDDVLQARSHDAWLFGNAGNDTLIGHAGGNLTFVGGSGDDILKGVGN 539
                ********:***************************************************

Lip             GNTFLFSGDFGRDQLYGFNASDKLVFIGTEGASGNIRDYATQQNDDLVLAFGHSQVTLIG 600
YM48            GNTFLFSGDFGRDQLYGFNASDKLVFIGTEGASGNIRDYATQQNDDLVLAFGHSQVTLIG 600
D13253          GNTFLFSGDFGRDQLYGFNATDKLVFIGTEGASGNIRDYATQQNDDLVLAFGHSQVTLIG 599
                ********************:****************************************

Lip             VSLDHISTDQVVLA 614
YM48            VSLDHISTDQVVLA 614
D13253          VSLDHFNPDQVVLA 613
                *****:..******
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Figure 1 Alignments of lipase proteins to compare amino acids sequences of *Serratia marcescens* D13253 (BAA02519), *Serratia marcescens* WW4 (Lip; YP_007406058), and *Serratia nematodiphila* YM48 (KP325673), using ClustalW. The lipase consensus sequence is shown in the shaded box. The lipase enzyme of *S. marcescens* (D13253) was compared to represent the different amino acids of lipase in genus *Serratia*. The amino acid sequences of YM48 had 95 and 93 % similarity with WW4 and D13253 using tblastn analysis.

Activity and stability of YM48 lipase with respect to temperature, pH, and methanol

The optimum temperature and pH for lipase activity were 37 °C and pH 8, respectively (**Figure 2a**). YM48 lipase demonstrated activity at 40 °C, which is suitable for transesterification in biodiesel production. At higher temperature (50 - 70 °C), activity decreased markedly, and the enzyme was degraded in acidic conditions (pH 4 - 6), resulting in protein precipitation. Enzyme stability is an important attribute for a biocatalyst. **Figure 2b** shows the stability of YM48 lipase over time at 40 °C and at pH 8. Methanol is necessary for biodiesel production, but it can seriously impede lipase activity. It was shown previously that oil/methanol molar ratios of 1: 6 led to the highest levels of FAME production, but a molar oil:methanol ratio over 1: 10 slightly decreased FAME production [18]. Thus, a 1: 6 molar ratio was chosen to produce FAMEs using YM48 lipase, since the enzyme showed methanol stability over 6 h, with a relative activity of 78 % at this ratio at 40 °C (**Figure 3**).

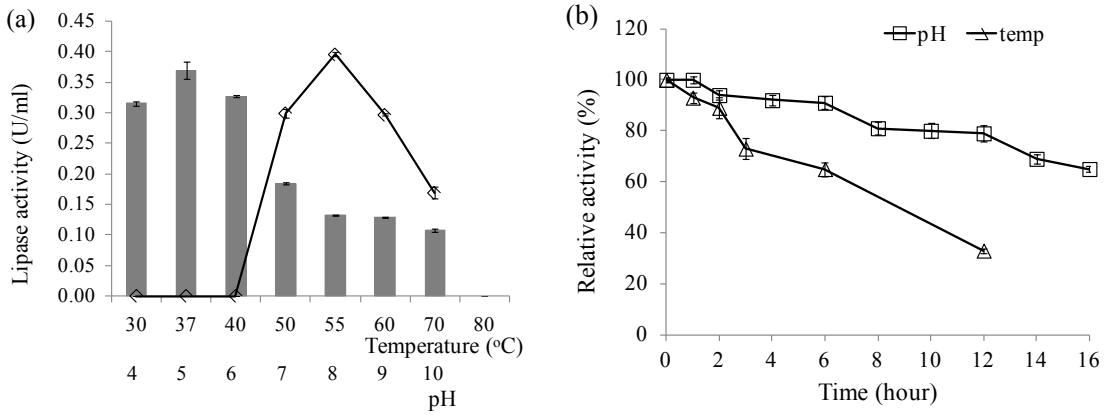

Figure 2 The property of YM 48 lipase for temperature and pH (a) effects of temperature (bar) and pH (line) on lipase activity shown that a range of temperature between 30 - 40 °C was a good condition for YM 48 lipase at pH 8.0; (b) thermal stability and pH stability at 40 °C and pH 8, during period of incubation. The results showed the percentage of residual activity which still obtained more than 60 % for pH stability after 16 h and for temperature stability after 6 h.

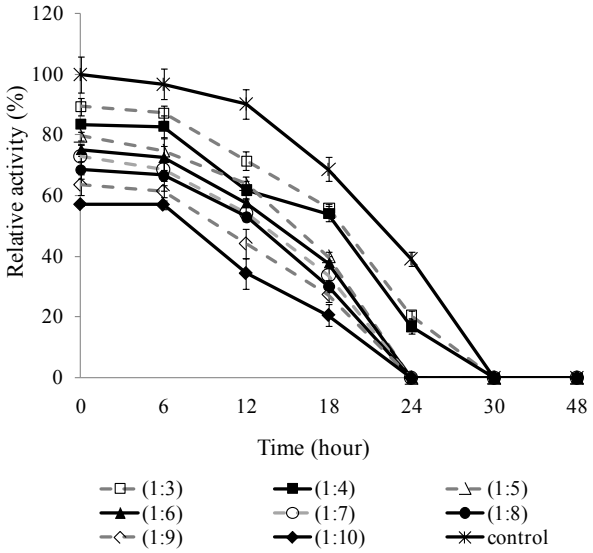

Figure 3 Relative lipase activity of YM 48 strain in water/methanol molar ratio of 1:3 - 1:10 at 40 °C during interval times compared with no methanol as a control. The higher methanol content the lower activity was observed; after 12 h, the lipase activity remained more than 60 % in the ratio of 1:3 - 1:8.

Lipase concentration using lyophilization and immobilization

Table 1 shows the lipase activity of enzyme lyophilized with PEG or α-cyclodextrin as preservative agents. Both preservative agents protected lipase from inactivation during the lyophilization step because of conformational changes to the enzyme on interaction with the preservative. Cyclodextrins bind to lipase, influencing the catalytic behavior of the enzyme. PEG molecules surround lipase creating a complex, resulting in micelle formation, which helps to increase catalytic activity [19]. PEG proved to be a good additive for YM48, as it is inexpensive and improved lipase activity.

Immobilization of lyophilized PEG-lipase complex powder did not increase the activity compared with lyophilized lipase with PEG (**Table 1**). This might be as a result of the PEG-lipase complex linking poorly with the silica gel, even via covalent bonding, because of size and conformational changes in the PEG-lipase micelle [19]. More probably, it was because of the hydration level of lipase (increased viscosity) affecting the hydrophilicity of the lipase in the adsorption process [20]. Culture supernatant and whole cell culture proved active when immobilized by either adsorption or covalent bonding methods. The adsorption method was more suitable for immobilizing all types of YM48 lipase than covalent bonding because of the effects of glutaraldehyde on the lipase in the covalent bonding method. The cells in culture did not interrupt extracellular lipase adsorption on silica gel, resulting in lipase preparation without centrifugation in the immobilization process. Adsorption also has the advantage of being an inexpensive process, and the adsorbed enzyme is easily removed from the reaction mixture.

Table 1 Lipase activity of native lipase, lyophilized lipase and immobilized lipase.

Sample	Method	Specific activity (U/mg)
Native lipase		0.28±0.03
Lyophilized lipase		
	with PEG	0.61±0.02
	with α-cyclodextrin	0.68±0.02
Immobilized lipase		
adsorption method	whole cell culture	1.20±0.05
	supernatant	1.25±0.03
	lyophilized PEG lipase	0.60±0.01
Covalent bonding	whole cell culture	0.94±0.01
method	supernatant	0.79±0.05
	lyophilized PEG lipase	0.46±0.09

The number is mean±SD; each sample was done in triplicate for measuring the lipase activity and protein content

Both lyophilized lipase and immobilized lipase were more thermostable than native lipase over a long period (**Figure 4**). Native lipase, lyophilized lipase, and immobilized lipase showed no significant differences in stability with respect to the molar ratio of water/methanol.

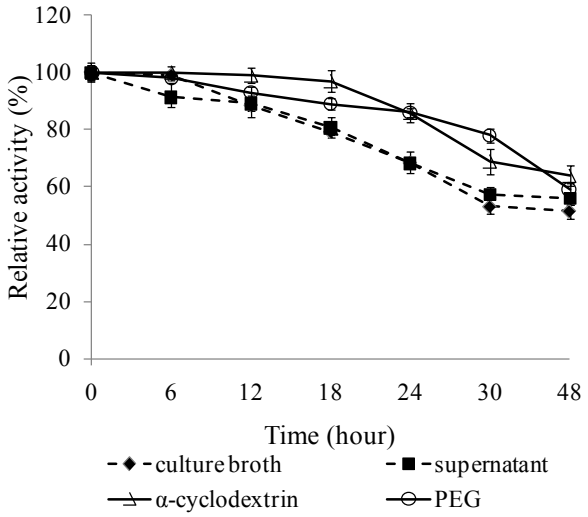

Figure 4 Thermal stability of lyophilized lipase (with α-cyclodextrin and PEG) and immobilized lipase (from whole culture broth and supernatant) at 40 °C over time. The lyophilized lipase with PEG and α-cyclodextrin exhibited 86 % activity in 24 h and still had the activity more than 50 % until 48 h. The immobilized lipase from supernatant and whole culture broth gave less activity at 24 h, 68 %; however, they still kept the activity until 48 h similar to lyophilized lipase.

Fatty acid methyl esters production by lyophilized and immobilized YM48 lipase

Table 2 shows the details of FAMEs generated via the transesterification of palm oil with methanol (in the final ratio 1: 6) using lyophilized and immobilized lipase. To maintain enzyme activity, a 3-step methanol addition process was used in the reaction, reducing the harmful effects of methanol. The immobilized enzyme was a highly efficient biocatalyst, able to convert fatty acids in palm oil to FAMEs in an 87 - 88 % yield, whereas the percentage yield of FAMEs from lyophilized and immobilized lipase varied slightly. The immobilized whole cell culture resulted in the highest level of FAME production and showed the greatest amounts of all FAMEs. This means that FAME yield, which was calculated from the weight of products, obtained mostly methyl ester derivatives from oil. YM48 whole cell culture was immobilized on silica gel without purification or centrifugation steps, and also has the advantage of biocatalyst reusability, meaning this approach may be carried out relatively quickly and may save costs. Because of its effectiveness, convenience, and cost benefits, YM48 lipase represents a new resource for biodiesel production. Moreover, *S. nematodiphila* YM48 was able to degrade used-palm oil and lard oil in preliminary studies with the same level of lipase activity as in palm oil, further indicating the suitability of YM48 lipase for waste oil biodiesel production.

Table 2 FAMEs produced in the reaction of a 1: 6 molar ratio of palm oil:methanol via transesterification using different biocatalysts.

Biocatalyst	Amount of fatty acid methyl ester (FAME) produced (unit of counts $\times 10^5$)					% Yield of FAMEs[b]
	Methyl palmitoleate (C16:1)	Methyl palmitate (C16:0)	Methyl stearate (C18:0)	Methyl oleate (C18:1)	Methyl linoleate (C18:2)	
[a] Standard FAME mix	23	10	47	41	30	
Lyophilized lipase with PEG	14	18	[c](-)	14	20	84
Lyophilized lipase with α-cyclodextrin	10	16	[c](-)	12	14	85
Immobilized lipase in supernatant	12	34	12	28	50	87
Immobilized lipase in whole cell culture	60	80	50	145	105	88

[a] A standard FAME mix (C14-C22; Sigma).
[b] The yield of FAMEs was calculated by weight of FAME products divided by the weight of palm oil, multiplied by 100.
[c] (-) indicates no clear detection

Conclusions

Serratia nematodiphila YM48 is a newly isolated lipase producing bacterium. Due to its favorable temperature, pH, and methanol tolerances, *S. nematodiphila* YM48 lipase proved suitable for use as a biocatalyst in transesterification. In both lyophilized and immobilized forms, the lipase showed increased stability relative to native lipase at 40 °C. PEG and α-cyclodextrin have the ability to increase the catalytic activity of lyophilized lipase in the conversion of fatty acids from palm oil to FAMEs. Adsorption successfully achieves YM48 lipase immobilization, particularly for whole cell culture. Immobilized YM48 whole cell culture operates as an effective biocatalyst for biodiesel production.

Acknowledgements

This research was financed by the National Research University Project of Thailand, Biofuel Cluster, Khon Kaen University, and partially supported by the Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Khon Kaen University, Thailand.

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