https://doi.org/10.48048/tis.2021.45

Purification and Characterization of an Extracellular Lipase Produced by *Aspergillus oryzae* ST11 as a Potential Catalyst for an Organic Synthesis

Pattarapon Paitaid[1,*], Jirayu Buatong[2], Souwalak Phongpaichit[2] and Aran H-kittikun[1]

[1]*Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand*
[2]*Department of Microbiology and Natural Products Center of Excellence, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand*

([*]Corresponding author's e-mail: aw_54321@hotmail.com)

Received: 1 July 2020, Revised: 5 May 2021, Accepted: 11 May 2021

Abstract

The lipase producing *Aspergillus* sp. ST11 was identified by molecular and morphological methods. The primers ITS1/ITS4 were used for amplifying the ITS region. It showed that the strain was grouped with *Aspergillus oryzae* and *Aspergillus flavus* (98 % bootstrap value). The colony morphology of *Aspergillus* sp. ST11 on malt extract agar and Czapek yeast agar showed a characteristic of *A. oryzae*. Therefore, it was identified as *Aspergillus oryzae* ST11. The lipase produced by the strain was purified and characterized. The purification steps involved precipitation with chilled acetone and separation by column chromatography, with HiTrap® Q HP and Toyopearl Butyl-650M, respectively. After purification, the lipase activity was increased 13 fold and with 7.9 % yield. Its molecular mass was 25 kDa. The purified lipase was stable at a pH between 5.0 - 8.0 and had optimum activity at pH 7.5. It was stable at 30 °C and had optimum activity at 37 °C. Its activity was promoted in the presence of Mg^{2+} but it was greatly decreased in the presence of Co^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+}. Surfactants (Triton X-100, Tween-80, Tween-20, arabic gum, and sodium dodecyl sulfate) showed negative effects on lipase activity, while inhibitors (PMSF, EDTA, and β-mercaptoethanol) did not reduce the activity significantly. Polar solvents, such as methanol and ethanol, had much negative effect on lipase activity compared to non-polar solvents, such as hexane and isooctane. The concentrated lipase from *A. oryzae* ST11 was used to catalyze the transesterification and gave the highest bioconversion (90 %) after 24 h.

Keywords: *Aspergillus*, Biodiesel, Characterization, Lipase, Purification

Introduction

Triacylglycerol hydrolases, or lipases (E.C. 3.1.1.3), are groups of hydrolytic enzymes that break down the molecules of triglycerides and release free fatty acids and glycerol. They are also able to synthesize ester compounds. Lipases are obtained from various sources of living organisms, including plants, animals, and microorganisms [1,2]. Most of the lipases used in the biotechnological application are from bacteria and fungi, which are cultivated in submerged and solid-state fermentation [3-5]. The prominent points of using microbial lipase for industrial application are stability, substrate specificity, and production cost. They are more stable compared to lipases derived from animals or plants. Moreover, microbial lipases could be produced in a large-scale process, which is very important to many industries such as pharmaceuticals, detergents, and food [6-8]. Among lipase-producing microorganisms, filamentous fungi have become the best source. They can produce an extracellular enzyme that is easy for harvest and purification compared to bacterial lipases [7]. Moreover, fungal lipases are easily produced in large-scale production, which is more attractive for industrial applications [9]. The utilization of lipases in biofuel production has drawn attention from researchers due to their ability to catalyze with a wide range of oils, including low-grade oil containing a high amount of free fatty acids [10]. Xiao *et al.* [11] reported biodiesel production using a whole-cell biocatalyst from *Aspergillus niger*, which gave a high biodiesel yield (> 90 %) after 72 h. Li *et al.* [12] reported the utilization of *Rhizopus oryzae* lipase expressed in

Pichia pastoris for enzymatic biodiesel production. Their study showed a high biodiesel production (> 90 %) after 72 h.

The development of polymerase chain reaction (PCR), DNA sequencing, and molecular techniques has become the standard discipline to identify unknown organisms [13]. The identification of fungal strains is very important for strain selection. The safety of fungal use has been a concern for many applications. Some fungi produce mycotoxins, which affect the health of both humans and animals. *Aspergillus* spp., such as *A. niger*, *A. flavus*, *A. fumigatus* and *A. terreus*, are known as mycotoxin producers. Hence, the identification of fungal strains at the species level with morphological and molecular techniques is required. The internal transcribed spacer (ITS) is amplified by using ITS1 and ITS4 primers [14]. Kantak *et al.* [15] identified the isolate JK-1 using ITS1 and ITS4 primers and found that this isolate belonged to *Rhizopus oryzae*. Durairaj *et al.* [16] isolated the pathogenic fungal strains from ginseng root rot. The ITS1 and ITS2 primers were used for amplifying ITS region and showed pathogenic strains such as *Cladosporium*, *Ilyonectria liliigena*, *Neonectria* sp., and *Fusarium* sp. However, some species of *Aspergillus* could not be distinguished by using the only molecular technique. Morphological study is required to reliably identify the species level of *Aspergillus* [14].

Lipases produced by living organisms have been extensively studied by the purification scheme to understand their characteristics. Generally, the common steps of lipase purification consist of ion-exchange, hydrophobic, and gel filtration chromatographies. The purification of lipase from *Aspergillus* sp. is normally started from the ammonium sulfate precipitation of crude lipase, followed by applying it to ion-exchange chromatography [17]. However, some studies have had a slightly different scheme for lipase purification, by using a hydrophobic interaction column after ammonium sulfate precipitation [18,19].

This research aims to find the capability of an extracellular lipase produced by this fungus, which is expected to offer the flexibility of enzymatic use in many applications over the use of whole-cell biocatalyst that is widely studied. Moreover, with the versatility of lipase, it would be expected to apply it to a process that requires high specificity towards a substrate and give a high quality of product. To achieve this objective, the selected strain of lipase-producing fungus was identified and used to produce lipase by cultivating it in submerged fermentation. The produced extracellular lipase was then purified and characterized to study its properties. The concentrated crude lipase was used for biodiesel production to study the possibility of organic synthesis.

Materials and methods

Substrates and chemicals

Palm oil, rice bran oil, coconut oil, sunflower oil, soybean oil, corn oil, and olive oil were purchased from a local market. The metal ions; $FeCl_3$, $CoCl_3$, $CuCl_2$, $MgCl_2$, $HgCl_2$, $CaCl_2$, $ZnCl_2$, KCl, NaCl and $AlCl_3$, and the surfactants; Triton X-100, Tween 80, Tween 20, gum arabic, and sodium dodecyl sulfate (SDS) were purchased from Analytical Univar Reagent (Auckland, New Zealand). The enzyme inhibitors: ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, USA).

Analytical methods

Lipase activity

The hydrolytic activity of lipase was measured in a 2-phase system according to the modified colorimetric method [20], using palm oil as a substrate in isooctane. 0.1 mL of the crude enzyme was added to 0.5 mL of 10 % w/v palm oil in isooctane. After that, the mixture was incubated in a Thermomixer (TAITEC, Japan) at 37 °C and 1,200 rpm for 30 min. The reaction was eventually stopped by adding 0.15 mL of 6 N HCl. 0.1 mL of the upper organic phase was withdrawn and mixed with 0.9 mL of iso-octane. The solution was then mixed thoroughly with 0.2 mL of cupric acetate solution. The upper phase was measured for absorbance at 715 nm using isooctane as a blank. Lipase activity was determined by measuring the amount of fatty acids liberated as palmitic acid. One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per min at the specified conditions [21].

Protein determination

The protein of the samples in the purification steps was determined using Folin-Ciocalteu reagent according to Lowry's method [22].

Analysis of biodiesel production using thin-layer chromatography with flame-ionized detector (TLC-FID analyzer)

Transesterification products were analyzed by IATROSCAN™ MK-5 TLC-FID analyzer (Iatron Laboratories, Inc. Tokyo, Japan). The sample was spotted on the Chromarod-SIII. The spotted Chromarods were developed by the 2-solvent system. The 1st chamber contained a solvent mixture of n-hexane/diethyl ether/formic acid (50:20:0.3 v/v/v), and the 2nd chamber contained a solvent mixture between benzene/n-hexane (1:1 v/v). The spotted Chromarods were developed in the 1st chamber for 15 min and subsequently transferred to the 2nd chamber with a development time of 30 min. The Chromarods were dried in a hot air oven at 105 °C for 10 min. The dried Chromarods were scanned by a TLC-FID analyzer (2,000 mL/min air flow rate, 160 mL/min of H2 flow rate, and 30 s/scan of scanning speed). The peak area ratio was calculated with i-ChromStar software. Biodiesel production was reported as the percentage of the area compared to all peak areas of transesterification reaction [23].

Molecular and morphological identification

Aspergillus sp. isolate ST11 was obtained from the stock culture of the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University. Hat Yai, Thailand. The internal transcribed spacer (ITS) region was amplified using primers ITS1/ITS4 [24]. The universal primers used for fungal amplification were ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). Reactions involved 1 cycle of initial denaturation at 94 °C for 5 min, followed by 35 cycles with a denaturation step at 94 °C for 1 min, an annealing step at 58 °C for 1 min, and an extension step at 72 °C for 1 min, followed by 1 cycle at 72 °C for 10 mins. The ClutalWin Bioedit was used for aligning the consensus sequence amplified by ITS1/ITS4 primers [25]. The sequence was then compared (BLAST) against the GenBank database. Phylogenetic analysis was performed using MEGA software version 6.0 [26] by applying a neighbor-joining (NJ) method on ITS sequence.

For morphological identification, the isolate ST11 was cultured on malt extract agar (MEA) and Czapek yeast agar (CYA) and incubated at 25 and 37 °C for 7 days. The colony color and microscopic morphology were observed [14].

Production and purification of lipase

The fungal spore was inoculated on potato dextrose agar and incubated at 37 °C for 5 days. The spores were collected and adjusted to 1×10^7 spore/mL using a hemacytometer, and 1 mL of inoculum was inoculated into the optimized medium (100 mL) containing 1 % olive oil, 2 % peptone, 0.2 % $NaNO_3$, 1 % KH_2PO_4, 0.05 % $MgSO_4$, and 0.5 % lactose (w/v), and the pH adjusted to 6.0. The culture medium was shaken at 150 rpm and 37 °C for 4 days. Purification of the lipase was carried out with the steps of solvent precipitation (chilled acetone), an anion-exchange column chromatography (HiTrap Q HP 5 mL), and a hydrophobic column chromatography (Toyopearl Butyl-650M).

The culture broth was separated from the mycelium by filtration with Whatman no.1 filter paper. The crude enzyme was precipitated from the culture broth by adding 3 volumes of cold acetone per 1 volume of the culture broth and being kept at 4 °C overnight. The solution was centrifuged (8,000 rpm at 4 °C for 10 min) and the precipitate was dissolved with a small amount of 50 mM Tris-HCl buffer pH 7.5 and transferred to a dialysis bag (3,500 Da). The sample was dialyzed against the same buffer at 4 °C overnight. The expanded volume of the sample was reduced using the adsorption of carboxymethyl cellulose powder (CMC). The concentrated lipase was collected for purification and characterization.

The lipase solution (2 mL) was applied to a HiTrap Q HP column by using an ÄKTA Prime chromatography system (Amersham Pharmacia Biotechnique Group, Sweden). The process was carried out with 2 buffer systems (50 mM Tris-HCl buffer pH 7.5) that contained no NaCl and with 1 N NaCl. The concentration of NaCl was increased from 0 - 5, 5 - 15 and 15 - 100 %. All fractions were monitored for absorbance at 280 nm and lipase activity was assayed. The active fractions were combined and used for the next purification step.

The enzyme obtained from the anion exchange chromatography step was applied to the Toyopearl Butyl 650M column. The column was equilibrated with 50 mM Tris-HCl buffer pH 7.5 with 50 % ammonium sulfate. After applying enzyme solution to the column, the column was washed with equilibrate buffer and the concentration of salt was gradually decreased from 50 to 0 %. The fractions showing absorbance (280 nm) were collected and the lipase activity was assayed. The active fractions were pooled and dialyzed against the same buffer without ammonium sulfate at 4 °C overnight and concentrated by adsorption with CMC. The concentrated enzyme solution was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were mixed in sample buffer and boiled in the water bath at 90 °C for 10 min. Samples were applied to a 5.0 % gel system concentrator and a 10 % separator. Electrophoresis was performed at 50 V for 30 min and 100 V for 60

min. After electrophoresis, the separated proteins were stained with Coommassie Blue R-250 reagent. The molecular weight of the purified ST11 lipase was compared to the standard marker (Pink Plus Prestained Protein ladder with sizes ranging from 10 - 175 kDa).

Characterization of purified lipase from *Aspergillus oryzae* ST11

Effect of pH and pH stability

The optimum pH for lipase ST11 activity was studied; the lipase was prepared in different buffers with the same concentration (50 mM) and was determined for activity at 37 °C. The buffers used in the study were acetate buffer (pH 4.0 - 6.0), phosphate buffer (pH 6.0 - 7.0), and Tris-HCl buffer (pH 7.0-9.0). In the case of pH stability, the purified lipase was mixed with the above buffers (1:3, v/v), and the sample was incubated at 30 °C for 2 h. The residual activity of the purified lipase ST11 was determined at pH 7.5.

Effect of temperature and thermostability

The purified lipase activity was assayed at temperatures ranging from 30 - 65 °C in 50 mM Tris-HCl buffer, pH 7.5. For effect of temperature on lipase stability, the purified lipase was incubated at different temperatures from 30 - 65 °C for 2 h before determining activity at 37 °C.

Effect of metal ions

Different metal ions (Fe^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Hg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Na^{+} and Al^{3+}) were used for testing their effects on the activity of the purified ST11 lipase. The enzyme was added into the reaction mixture containing 50 mM Tris-HCl buffer pH 7.5 and metal ions at 1.0 and 10.0 mM. The reaction was carried out at 30 °C for 30 min. The lipase activity was assayed and compared to the control without the addition of metal ions.

Effect of inhibitors and surfactants

Different inhibitors and surfactants were determined for their effects on the lipase ST11 activity. The purified ST11 lipase was incubated at 30 °C for 30 min in 50 mM Tris-HCl buffer pH 7.5 containing different inhibitors (1.0 mM): β-mercaptoethanol, phenyl methyl sulphonyl fluoride (PMSF), and ethylene diamine tetra-acetic acid (EDTA), and surfactants (1.0 %, w/v): gum arabic, Triton X-100, Tween 20, Tween 80, and sodium dodecyl sulfate (SDS). The lipase activity was assayed and compared to the control without the addition of inhibitors or surfactants.

Effect of organic solvents

The effects of different organic solvents (ethanol, methanol, acetone, ethyl acetate, isopropanol, xylene, toluene, isooctane, and hexane) on the lipase activity was determined. The organic solvent (0.15 mL) was added to purified lipase solution (0.45 mL) in a microcentrifuge tube. The mixture was incubated in a Thermomixer shaker (TAITEC, Japan) at 30 °C and 500 rpm for 1 h. The lipase activity was then measured and compared to the control.

Substrate specificity

The substrate specificity of the purified ST11 lipase was studied by using different vegetable oils as a substrate, including palm oil, rice bran oil, coconut oil, sunflower oil, soybean oil, corn oil and olive oil. Each kind of oil was used to replace the palm oil in the reaction mixture for lipase activity determination using the cupric acetate method.

Biodiesel production catalyzed by concentrated ST11 lipase

The concentrated *A. oryzae* ST1 lipase (100 µL) from the chilled acetone precipitation step was mixed with 2 mL of 10 % palm oil in isooctane and methanol at a molar ratio of methanol/palm oil at 3:1. The reaction mixture was incubated at 37 °C in a Thermomixer with 1,200 rpm for 24 h. The sample (50 µL) was withdrawn and mixed with isooctane (50 µL). The biodiesel produced was analyzed by the TLC-FID analyzer.

Statistical analysis

The experimental data were expressed as mean ± standard deviation from triplicates. One-way analysis of variance (ANOVA) was carried out, and comparisons of means were done by Duncan's new multiple range tests, with a significance threshold $p < 0.05$. Statistical analyses were performed with the statistical program SPSS (version 16.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Molecular and morphological identifications

According to neighbor-joining (NJ) phylogenetic tree analysis of the ITS sequence, *Aspergillus* sp. ST11 was grouped with *A. flavus* and *A. oryzae* (98 % bootstrap value). This strain showed sequence similarity to *A. flavus* UOA/HCPF 10017 (FJ878656) and *A. oryzae* A-4 (GU120193) with 100 % (**Figure 1**). Molecular identification using ITS sequence cannot be used to differentiate *A. flavus* and *A. oryzae*. Therefore, the morphology of the strain ST11 on MEA and CYA was further investigated. For morphological identification, the strain ST11 grew well on CYA and MEA. The colony on CYA was yellow-green (**Figures 2(a) - 2(d)**) and the colony on MEA was olive green (**Figures 2(e) - 2(h)**). Conidiophores were 0.08 - 0.09 µm in length, colorless, and roughened (**Figures 3(a)** and **3(b)**). Conidia were 3.2 - 4.1 µm in diameter, globose to the ellipsoidal, and had smooth walls (**Figure 3(c)**). The results from this study were well related to the morphology of the *A. oryzae* colony on CYA and MEA [14]. Therefore, the isolate ST11 was designated as *A. oryzae* ST11 using molecular identification based on ITS regions and morphological characters (GenBank accession number MK784896).

Purification of lipase

The purification table is summarized in **Table 1**. The yield of lipase after chilled acetone precipitation was 56 %, and there was a 7.7-fold increase in activity. The sample was then applied to the ÄKTA Prime chromatography system connected with a HiTrap Q HP column. It was found that 15 % NaCl could elute the target enzyme from the column (**Figure 4(a)**). At this step, 28.9 % of lipase yield and a 10.2-fold increase of activity were obtained. For Toyopearl Butyl-650M column chromatography, the active fractions were obtained after 50 mM Tris-HCl buffer pH 7.5 with 0.4 % ammonium sulfate was applied to the column (**Figure 4(b)**). The yield of lipase at this step was 7.9 %, and the purification fold was 13.0. The decrease of the lipase yield after the lipase concentration step was similar to the study of Toida *et al.* [27] that reported a decrease of lipase yield of *A. oryzae* to 54 % after ammonium sulfate precipitation and gave s yield of only 5.5 % after the final purification step. Mehta *et al.* [18] studied the purification of lipase from *A. fumigatus* using Octyl Sepharose column chromatography (hydrophobic column) and found that the lipase yield was 11.03 % and the purification fold was 6.96.

The molecular mass of the purified lipase was determined by using the SDS-PAGE technique. The purity of the *A. oryzae* ST11 lipase was examined and confirmed with a single band of protein with 25 kDa molecular mass (**Figure 5**). The molecular mass of the lipase from *A. oryzae* ST11 in this study was similar to the size (27 kDa) of lipase from *A. oryzae* isolated from waste cooking oily soil [28]. However, the study of Toida *et al.* [27] showed that the molecular mass of lipase from *A. oryzae* was 41 kDa.

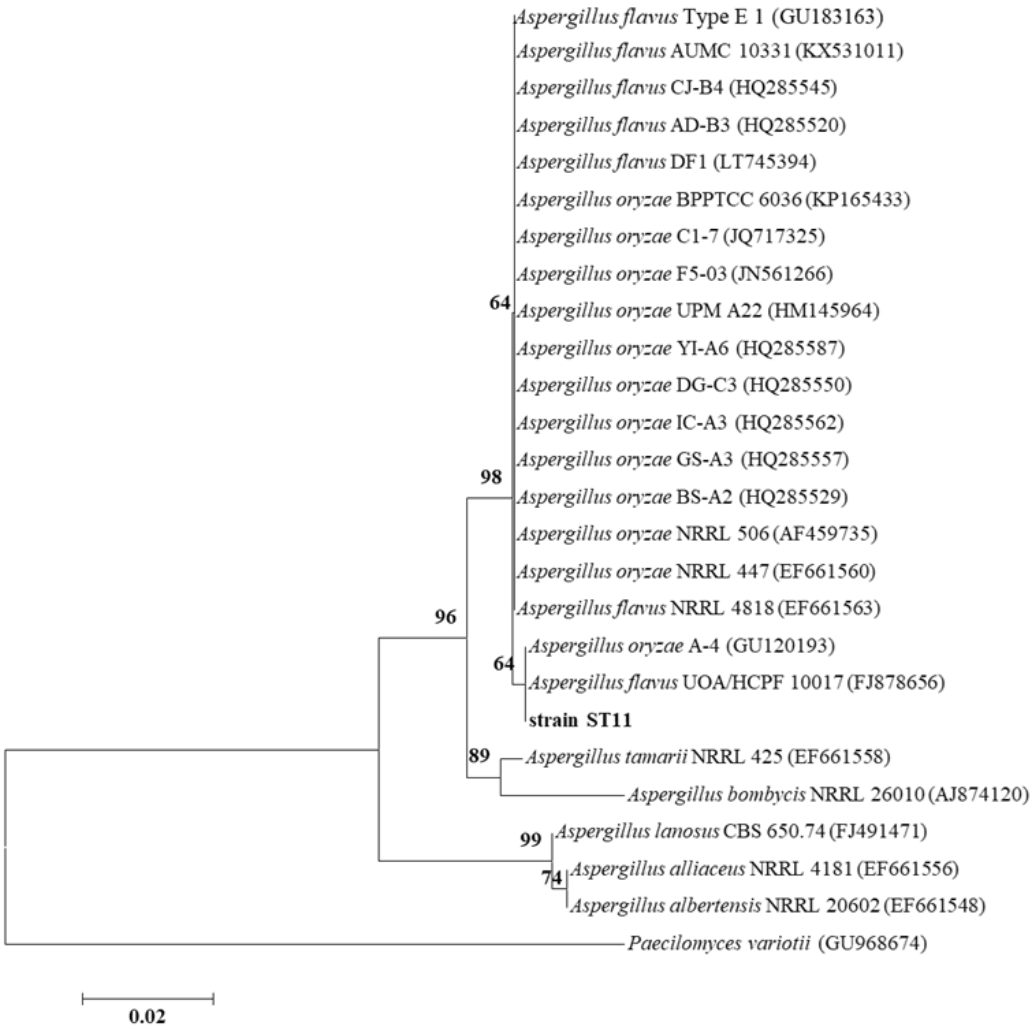

Figure 1 The Neighbor-joining phylogenetic tree obtained from ITS rDNA sequence analysis based on the Tamura Nei model, calculated using MEGA 6.0. The scale bar denotes 0.02 substitutions per position. *Paecilimyces variotii* was used as the root of tree.

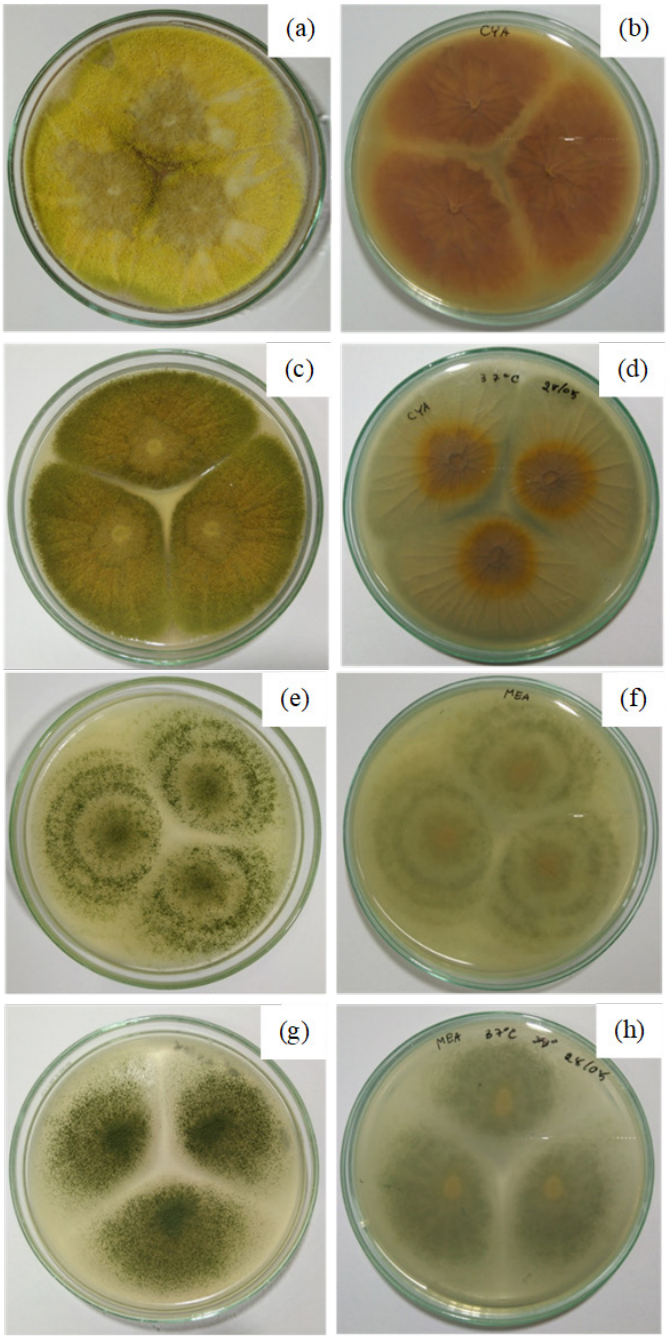

Figure 2 Colony morphology of *Aspergillus* sp. ST11 on Czapek's yeast agar at 25 °C (a, b) and 37 °C (c, d) and on malt extract agar at 25 °C (e, f) and 37 °C (g, h) for 7 days.

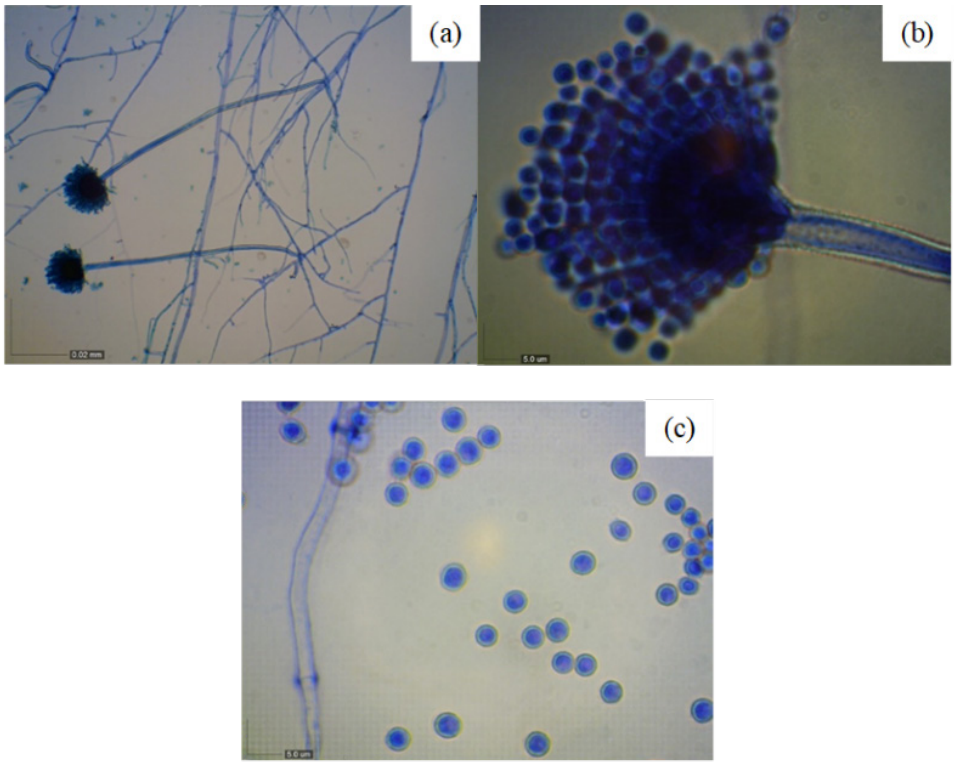

Figure 3 Conidiophores at 100× (a) and 400× (b), and conidia at 400× (c), of *Aspergillus* sp. ST11.

Table 1 Purification table of lipase from *Aspergillus oryzae* ST11.

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Yield (%)	Purification fold (fold)
Supernatant	1,300	16,000	12.3	100	1.0
Acetone precipitation	95	8,960	94.3	56	7.7
Q-HP column chromatography	37	4,635	125.3	28.9	10.2
Butyl-Toyo pearl column chromatography	8	1,278	159.8	7.9	13.0

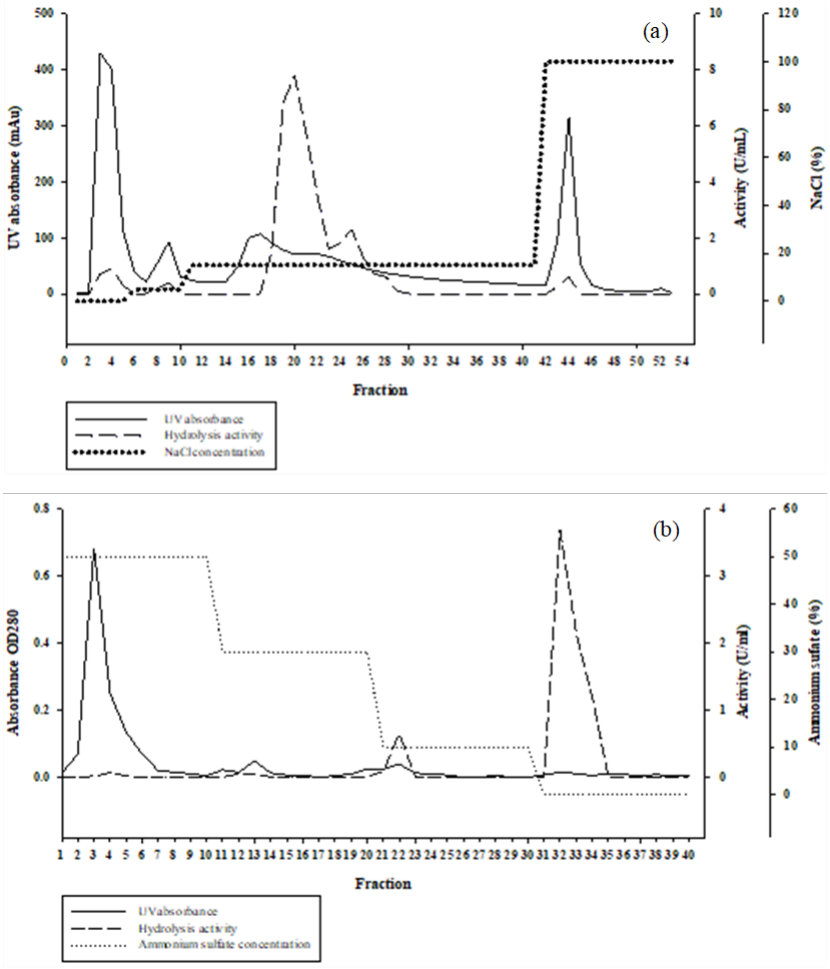

Figure 4 Purification of *Aspergillus oryzae* ST11 lipase carried out by ion exchange chromatography (a) and hydrophobic chromatography (b).

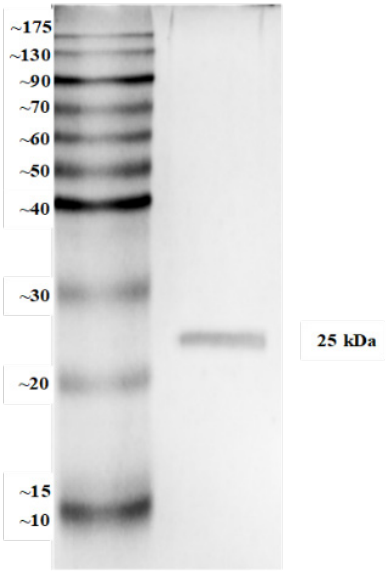

Figure 5 SDS-PAGE of purified lipase from *Aspergillus oryzae* ST11.

Characterization of purified lipase from *Aspergillus oryzae* ST11

Effect of pH on activity and stability

The purified *A. oryzae* ST11 lipase had an optimum pH for lipase activity at pH 7.5 (**Figure 6(a)**). This still gave lipase activity of more than 80 % in the pH range between 6.5 - 8.0 and was 58 % at pH 9.0. The optimum pH for the activity of the purified lipase of *A. oryzae* studied by Toida *et al.* [27] was at pH 7.0. However, the optimum pH for the activity of the lipase from *A. oryzae* CJLU-31 was pH 4.0, which was considered an acidic lipase [28]. For other *Aspergillus* species, *A. fumigatus* lipase had an optimum pH at 9.0 [14], and *A. carneus* lipase had an optimum pH at 8.5 [19], whereas *A. niger* lipase had an optimum pH at 7.0 [29]. It could be concluded that the lipase produced by *Aspergillus* species had a wide range of optimum pH.

pH stability was the desired characteristic of enzyme application. After incubation of the purified lipase in a wide range of pH buffers, the purified *A. oryzae* ST11 lipase retained an activity of more than 80 % in the buffers between pH 5.0 - 8.0. The relative activity of purified lipase greatly decreased after incubation in buffer pH of lower than 5.0 and of higher than 8.0. In comparison, the activity of *A. oryzae* lipase was low at acidic pH and was stable in a pH range from 6.0 - 9.0 at 25 °C [27]. Another report showed that the purified lipase from *A. niger* NCIM 1207 was stable at pH 8.0 - 11.0 but retained activity at 10 % in acidic pH [30].

Effect of temperature on activity and stability

Figure 6(b) shows the effect of temperature on the activity and stability of *A. oryzae* ST11 lipase. The purified lipase from *A. oryzae* ST11 showed an optimum temperature for lipase activity at 37 °C. The results showed that it still had high activity in a range of temperatures between 30 and 45 °C. However, when the temperature of the system was higher than 55 °C, the activity was decreased drastically and retained only 23 % at 65 °C.

For thermostability, after incubation of the purified lipase from *A. oryzae* ST11 at 30 - 65 °C for 2 h, the remaining activities of lipase at 30 and 37 °C were higher than 90 %. The thermal stability was decreased rapidly when the temperature was higher than 45 °C, and the activity was 32 % at 65 °C. However, its stability was different from the lipase of *A. oryzae* studied by Toida *et al*. [27], which was stable at 30 °C, but was greatly reduced at 40 °C, with 10 % of the original activity.

Effect of metal ions

Different metal ions were used to study the effect on the activity of the purified *A. oryzae* ST11 lipase, with concentrations at 1.0 and 10.0 mM compared with the control of lipase solution in Tris-HCl buffer without the addition of metal ions (**Table 2**). The results showed that the activity of lipase was slightly enhanced when Ca^{2+}, K^{+} or Mg^{2+} (1.0 mM) was presented in the reaction mixture. However, when the concentration of metal ions was increased to 10.0 mM, only the addition of Mg^{2+} showed a significant enhancement of activity compared to other metal ions. The increase of lipase activity in the presence of Mg^{2+} was also supported by the report of other studies with the lipases from *A. niger* NRRL3 [31] and *A. terreus* var. africanus (CBS 130.55) [32]. However, the report of Toida *et al.* [27] showed the opposite effect; Mg^{2+} (10.0 mM) slightly decreased the lipase activity of *A. oryzae*. The lipase activity of *A. oryzae* ST11 was drastically suppressed by Hg^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} at both 1.0 and 10.0 mM. The negative effects of Hg^{2+}, Zn^{2+} and Cu^{2+} were also reported in the study of other purified *A. oryzae* lipases [27,32]. The negative effects of Zn^{2+} and Cu^{2+} on lipase activity might be due to the interactions of those ions on the enzyme surface charge. It made the change of enzyme conformation, leading it to be less stable. As well as the effect of Hg^{2+}, which had an inhibitory effect on the lipase activity with participation of the SH group in the enzyme structure, this indicates that thiol-containing amino acid residues might be important to enzymatic function [32,33].

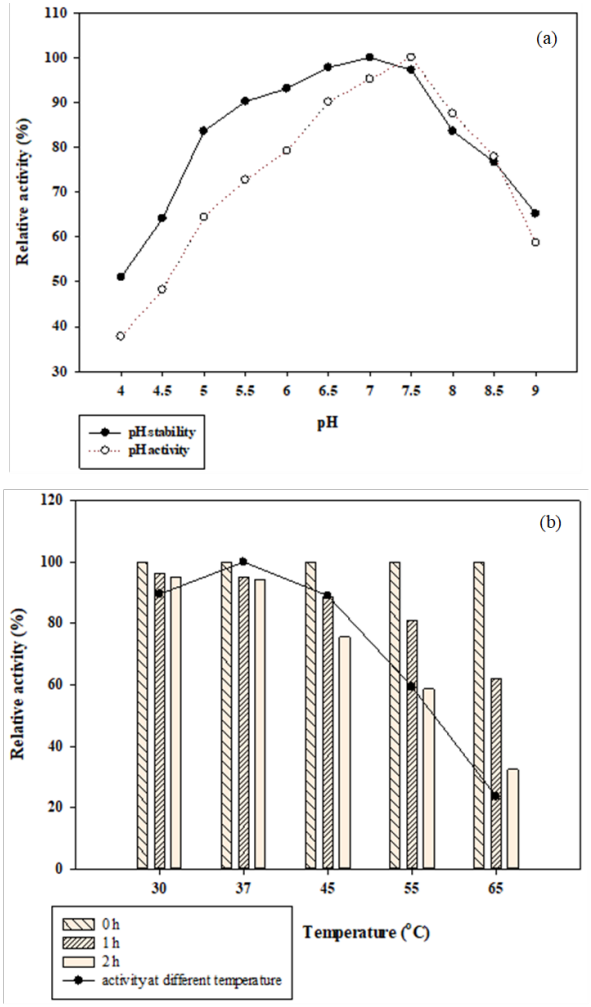

Figure 6 Effects of pH (a) and temperature (b) on the activity and stability of the purified lipase from *Aspergillus oryzae* ST11.

Table 2 Effect of metal ions on the stability of the purified *Aspergillus oryzae* ST11 lipase.

Effect of metal ions	Relative activity (%)	
	1.0 mM	**10.0 mM**
Control*	100.0 ± 4.5^{b}	100.0 ± 4.5^{b}
$FeCl_3$	102.5 ± 6.6^{ab}	83.5 ± 8.0^{c}
$CoCl_3$	82.8 ± 2.6^{c}	14.3 ± 0.9^{h}
$CuCl_2$	32.5 ± 5.4^{d}	33.5 ± 4.4^{f}
$MgCl_2$	103.9 ± 2.3^{ab}	122.7 ± 1.4^{a}
$HgCl_2$	14.6 ± 2.6^{e}	14.1 ± 1.4^{h}
$CaCl_2$	109.3 ± 1.2^{a}	66.7 ± 1.4^{d}
$ZnCl_2$	18.7 ± 0.3^{e}	19.4 ± 1.6^{h}
KCl	106.3 ± 1.9^{ab}	84.1 ± 6.2^{c}
NaCl	101.9 ± 1.3^{ab}	88.9 ± 9.0^{c}
$AlCl_3$	87.2 ± 5.9^{c}	48.2 ± 5.1^{e}

*Lipase solution in 50 mM Tris-HCl buffer pH 7.5 without the addition of metal ions

Effects of inhibitors and surfactants

Many surfactants and inhibitors were investigated for their effects on the lipase activity of *A. oryzae* ST11. In general, surfactants are used to enhance the catalysis of lipase by increasing the lipid water

interfacial area [2]. However, in this study, most surfactants showed a negative effect on the lipase activity of *A. oryzae* ST11 (**Table 3**). The negative effect of surfactants was observed in the presence of sodium dodecyl sulfate (SDS), Tween-80, Tween-20, and arabic gum with the activity of 21.1, 21.6, 23.7 and 72.1 %, respectively. Triton X-100 severely reduced the activity of the purified lipase, and the activity was retained at only 6 %. A reduction of lipase activity after the addition of surfactants also occurred in the study by Zhou *et al.* [28]. The activity of purified lipase from *A. oryzae* CJLU-31 was decreased in the presence of Tween-40, Tween-80, and SDS, and retained activity at 20.0, 11.0 and 22.0 %, respectively [28]. Saxena *et al.* [19] found that anionic detergent, such as SDS, had a negative effect on lipase activity. The strong inhibitory effects of Tween-20 and Tween-80 were most likely due to their competitive attaching to the lipase molecule or the change in protein conformation; this leads to the blocking of the active form of protein, which negatively influences lipase catalysis [18,34,35].

The inhibitors (EDTA, PMSF, and β-mercaptoethanol) were used for studying the effect on the lipase activity at 1.0 and 10.0 mM. There was no significant loss of the lipase activity of *A. oryzae* ST11 in the presence of these inhibitors (**Table 3**). The presence of β-mercaptoethanol showed a slight effect on lipase activity. This meant there was no disulfide bond in the structure of lipase. In the case of EDTA addition, the activity of *A. oryzae* ST11 lipase was slightly higher than that of the control condition. It also implied that purified *A. oryzae* ST11 lipase was a non-metalloenzyme [2]. The PMSF which was used as serine inhibitors did not reduce the *A. oryzae* ST11 lipase activity. A similar result was observed from the study of Toida *et al.* [27], which revealed a non-significant reduction of *A. oryzae* lipase activity after incubation with PMSF. However, PMSF affected negatively the activity of purified *A. carneus* lipase [19].

Table 3 Effects of surfactants and inhibitors on the stability of the purified *Aspergillus oryzae* ST11 lipase.

Effects of surfactants and inhibitors	Relative activity (%)
Control*	100.0 ± 0.02^{a}
Surfactants	
Triton X-100 (1 %)	6.0 ± 0.5^{d}
Tween-80 (1 %)	21.6 ± 9.8^{c}
Tween-20 (1 %)	23.7 ± 1.0^{c}
Gum Arabic (1 %)	72.1 ± 8.5^{b}
SDS (1 %)	21.1 ± 4.7^{c}
Inhibitors	
EDTA (1.0 mM)	101.9 ± 5.2^{a}
EDTA (10.0 mM)	92.3 ± 1.6^{a}
β-mercaptoethanol (1.0 mM)	92.7 ± 2.3^{a}
β-mercaptoethanol (10.0 mM)	92.5 ± 2.9^{a}
PMSF (1.0 mM)	97.1 ± 4.1^{a}

* Lipase solution in 50 mM Tris-HCl buffer pH 7.5 without the addition of surfactants or inhibitors

Effect of organic solvents

The hydrolysis reaction catalyzed by lipases of water-insoluble substrates takes place at the interface. The use of organic solvents can encourage the hydrolysis reaction of water-insoluble substrates by the lipase [1]. Hence, the high stability and the activity of lipase in the solvent system are of concern for the application of the enzyme. Investigation of the organic solvent effect on the stability of the purified *A. oryzae* ST11 lipase was conducted. The different polar and non-polar organic solvents (polarity index 0.0 - 5.2) were studied (**Table 4**). The activity of purified lipase was decreased after 1 h of incubation in all organic solvents compared to the control condition containing 50 mM Tris-HCl buffer pH 7.5. The highest relative activity was obtained in the presence of isooctane and hexane, which are hydrophobic solvents. The high stability of *A. oryzae* ST11 lipase in non-polar solvents was supported by the many reports showing that the lipase was stable in the presence of hydrophobic solvents, while the activity was reduced in systems containing polar solvents. This was caused by the promotion of hydrophilicity of the solvent, leading to the removal of water from enzyme molecules [36-39].

Table 4 Effects of organic solvents on the lipase activity of the purified *Aspergillus oryzae* ST11 lipase.

Organic solvents (25 %)	Polarity index	Relative activity (%)
Ethanol	5.2	3.7 ± 0.4^{i}
Methanol	5.1	2.6 ± 1.1^{i}
Acetone	5.1	26.3 ± 2.8^{f}
Ethyl acetate	4.4	48.0 ± 2.1^{e}
Isopropanol	3.9	56.0 ± 2.9^{d}
Xylene	2.5	13.3 ± 1.1^{h}
Toluene	2.4	19.0 ± 1.4^{g}
Iso-Octane	0.1	70.0 ± 1.8^{b}
Hexane	0.1	62.0 ± 2.8^{c}
50 mM Tris-HCl buffer pH 7.5	-	100.0 ± 3.5^{a}

Substrate specificity

The different natural oils were tested to determine the substrate specificity of the purified *A. oryzae* ST11 lipase. The results are shown in **Figure 7**. A hydrolysis reaction using palm oil as a substrate was controlled as a base value at 100 % relative activity, and was measured at pH 7.5 and 37 °C. Among nine natural oils, it was found that olive oil showed the highest activity, at 110 % relative activity higher than that of palm oil. The rice bran oil and coconut oil had relative activities of lipase of more than 70 %, followed by linseed oil, for which the activity was around 50 %. In contrast, sunflower oil, soybean oil, canola oil, and corn oil showed lower activities of below 40 %. Considering the fatty acid compositions in each type of oil, the substrate specificity of the lipase was enhanced when the content of C18:n in the oil increased corresponding to oleic acid (C18:1) content existing largely in olive oil [40]. Shu *et al*. [41] also studied the substrate specificity of natural oils on the activity of *A. niger* F044 lipase. The results showed that *A. niger* F044 lipase had a broad range utilization of oil, and that olive oil gave the highest relative activity, while the lowest relative activity was obtained from castor oil.

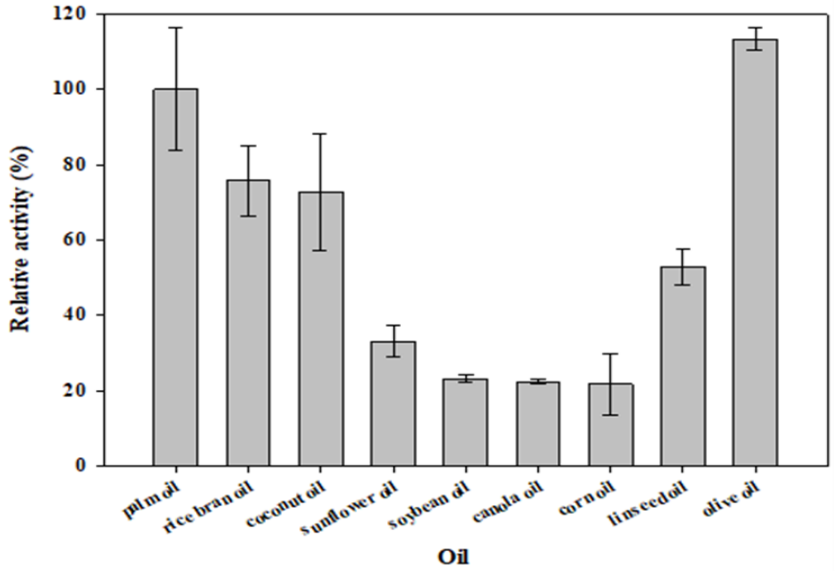

Figure 7 Effects of oil types on the activity of the purified lipase from *Aspergillus oryzae* ST11.

Biodiesel production catalyzed by concentrated *A. oryzae* ST11 lipase

Concentrated *A. oryzae* ST11 lipase was obtained from chilled acetone precipitation and used as a liquid enzyme for biodiesel conversion from palm oil and methanol. **Figure 8** shows the highest biodiesel conversion (90 %) at 24 h with one-step addition of methanol (3 moles of methanol per 1 mole of palm oil). This result was obtained under the condition that water content in the system was 50 % compared with the

weight of palm oil in isooctane. Even though water was crucial for stabilizing the protein structure, the excess amount of water content from the lipase solution led to the presence of free fatty acid in the final product, which was caused by the promotion of hydrolysis reaction (**Figure 9**). The existence of free fatty acids in a system containing high water content was supported by the study of Andrade *et al.* [42] to produce biodiesel from Castor oil. The addition of water enhanced the biodiesel production providing the water-lipid phase for lipase catalysis. Water hydrolyzed the one mole of triglyceride into 3 moles of free fatty acid and 1 mole of glycerol. The 3-fatty acids were subsequently reacted with an alcohol to form biodiesel. However, increasing the water beyond the optimal point led to a negative effect on the biodiesel production, with the reverse reaction to break down the biodiesel molecule and gave a lower yield of biodiesel. Nevertheless, the suitable water content for biodiesel production might be different depending on the type and the condition of the reaction. Chen *et al.* [43] reported that biodiesel production from waste cooking oil using immobilized *Candida* lipase can be achieved at 91.08 % with water content at 25 %, and that the yield of biodiesel was decreased when the water content applied to the system was higher than 25 %. In contrast, Baloch *et al.* [44] reported that biodiesel production from Jatropha oil by immobilized *Magnusiomyces capitatus* A4C extracellular lipase was enhanced when the water content was increased in the system, and it was achieved at 89.7 % with water content at 100 %.

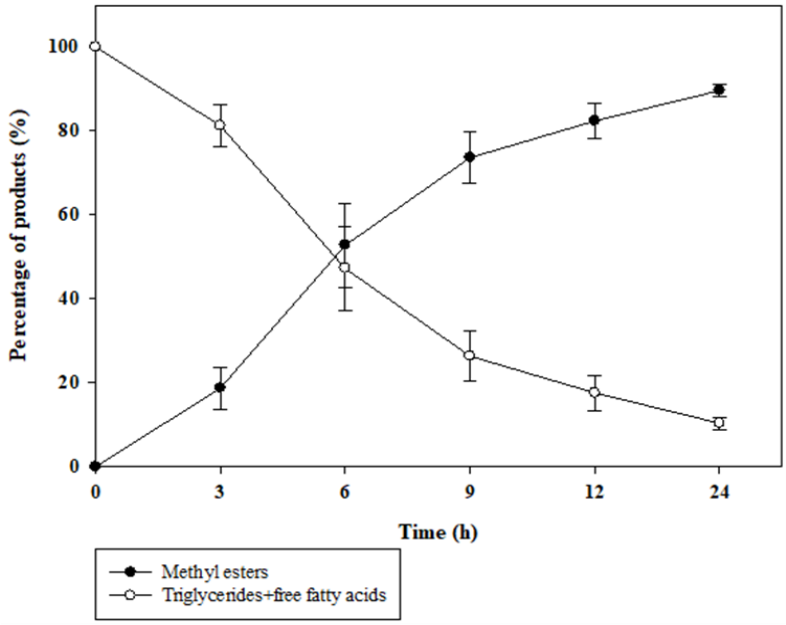

Figure 8 Time course of biodiesel production from palm oil catalyzed by concentrated *A. oryzae* ST11 lipase. The reaction mixture contained palm oil to methanol (1:3 mol/mol) and 100 µL of the concentrated lipase. The reaction was carried out in a Thermomixer at 37 °C and 1,200 rpm for 24 h.

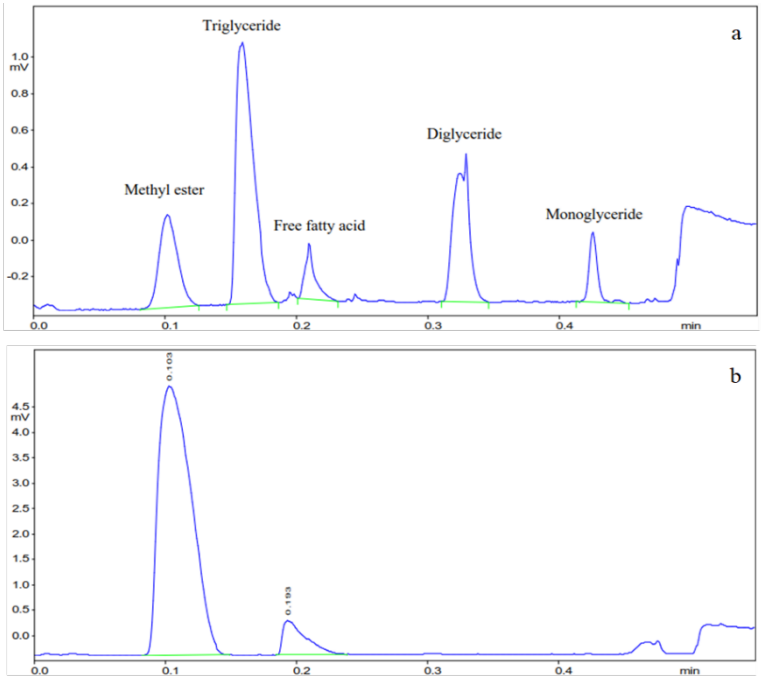

Figure 9 The transesterification profile analyzed by a TLC-FID analyzer with different standard compounds containing methyl ester, triglyceride, free fatty acid, diglyceride and monoglyceride, respectively (a) and the final product profile of transesterification reaction after 24 h (b).

Conclusions

The selected fungus was identified as *Aspergillus oryzae* ST11 and showed its capability to produce an extracellular lipase. This offered more flexibility of enzyme to be used in many industrial applications, compared with the use of whole-cell biocatalysts which might be limited in some applications. Moreover, the purified lipase from *A. oryzae* ST11 also presented the desired characteristics, with good stability at different pH and temperature. Even the use of the partially purified form of enzyme still had the ability to catalyze the transesterification reaction and yielded a high biodiesel conversion. Thus, these capabilities of lipase promise the biotechnological potential to be further studied and to be used in industrial applications.

Acknowledgements

This research was financially supported by the joint research fund between Prince of Songkla University and the Thailand Research Fund through the Royal Golden Jubilee Scholarship Ph.D. Program (Grant No. PHD/0321/2552).

References

[1] T Chaiyaso, P Seesuriyachan, W Zimmermann and A H-Kittikun. Purification and characterization of lipase from newly isolated *Burkholderia multivorans* PSU-AH130 and its application for biodiesel production. *Ann. Microbiol*. 2012; **62**, 1615-24.

[2] P Ungcharoenwiwat and A H-Kittikun. Purification and characterization of lipase from *Burkholderia* sp. EQ3 isolated from wastewater from a canned fish factory and its application for the synthesis of wax esters. *J. Mol. Catal. B Enzym.* 2015; **115**, 96-104.

[3] H Treichel, D de Oliveira, MA Mazutti, MD Luccio and JV Oliveira. A review on microbial lipases production. *Food Bioprocess Tech*. 2010; **3**, 182-96.

[4] A Marini, N Imelio, G Picó, D Romanini and B Farruggia. Isolation of a *Aspergillus niger* lipase from a solid culture medium with aqueous two-phase systems. *J. Chromatogr. B*. 2011; **879**, 2135-41.

[5] LN Nguyen, TT Dao, T Živković, M Fehrholz, W Schäfer and S Salomon. Enzymatic properties and expression patterns of five extracellular lipases of *Fusarium graminearum in vitro*. *Enzyme Microb. Tech*. 2010; **46**, 479-86.

[6] FJ Contesini, DB Lopes, GA Macedo, MDG Nascimento and PDO Carvalho. *Aspergillus* sp. lipase: Potential biocatalyst for industrial use. *J. Mol. Catal. B. Enzym.* 2010; **67**, 163-71.
[7] K Geoffry and RN Achur. Screening and production of lipase from fungal organisms. *Biocatal. Agr. Biotechnol*. 2018; **14**, 241-53.
[8] A Bajaj, P Lohan, PN Jha and R Mehrotra. Biodiesel production through lipase catalyzed transesterification: An overview. *J. Mol. Catal. B Enzym*. 2010; **62**, 9-14.
[9] AK Singh and M Mukhopadhyay. Overview of fungal lipase: A review. *Appl. Biochem. Biotechnol*. 2012; **166**, 486-52.
[10] OV Okoro, ZF Sun and EJ Birch. *Lipases for biofuel production*. *In*: R Aluko, EJ Birch, D Larsen, L Melton, M Rogers, F Shahidi, R Stadler, DS Waterhouse and P Varelis (Eds.). Encyclopedia of food chemistry. Elsevier, Amsterdam, 2018, p. 150-7.
[11] M Xiao, C Qi and JP Obbard. Biodiesel production using *Aspergillus niger* as a whole-cell biocatalyst in a packed-bed reactor. *GCB Bioenerg*. 2011; **3**, 293-8.
[12] Z Li, X Li, Y Wang, F Wang and J Jiang. Expression and characterization of recombinant *Rhizopus oryzae* lipase for enzymatic biodiesel production. *Bioresour. Tech*. 2011; **102**, 9810-13.
[13] Y Chen, BA Prior, G Shi and Z Wang. A rapid PCR-based approach for molecular identification of filamentous fungi. *J. Microbiol.* 2011; **49**, 675-9.
[14] NA Zulkifli and L Zakaria. Morphological and molecular diversity of *Aspergillus* from corn grain used as livestock feed. *HAYATI J. Biosci*. 2017; **24**, 26-34.
[15] JB Kantak, AV Bagade, SA Mahajan, SP Pawar and AA Prabhune. Isolation, identification and optimization of a new extracellular lipase producing strain of *Rhizopus* sp. *Appl. Biochem. Biotechnol*. 2011; **164**, 969-78.
[16] K Durairaj, P Velmurugan, KA Vedhanayakisri, WS Chang, P Senthilkumar, KM Choi, JH Lee and BT Oh. Molecular and phenotypic characterization of pathogenic fungal strains isolated from ginseng root rot. *Physiol. Mol. Plant Pathol*. 2018; **104**, 141-6.
[17] SM Basheer, S Chellappan, PS Beena, RK Sukumaran, KK Elyas and M Chandrasekaran. Lipase from marine *Aspergillus awamori* BTMFW032: Production, partial purification and application in oil effluent treatment. *N. Biotechnol*. 2011; **28**, 627-38.
[18] A Mehta, C Grover and R Gupta. Purification of lipase from *Aspergillus fumigatus* using Octyl Sepharose column chromatography and its characterization. *J. Basic Microbiol*. 2018; **58**, 857-66.
[19] RK Saxena, WS Davidson, A Sheoran, and B Giri. Purification and characterization of an alkaline thermostable lipase from *Aspergillus carneus*. *Process Biochem*. 2003; **39**, 239-47.
[20] SY Lee and JS Rhee. Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzym. Microb. Tech*. 1993; **15**, 617-23.
[21] P Ungcharoenwiwat, B Canyuk and A H-Kittikun. Synthesis of jatropha oil based wax esters using an immobilized lipase from *Burkholderia* sp. EQ3 and Lipozyme RM IM. *Process Biochem*. 2016; **51**, 392-8.
[22] OH Lowry, NJ Rosebrough, AL Farr and RJ Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem*. 1951; **1**, 265-75.
[23] N Rakchai, A H-Kittikun and W Zimmermann. Production of whole-cell lipase from *Aspergillus nomius* ST57 and optimization of methyl esters synthesis from palm oil in one step. *Chiang Mai J. Sci*. 2018; **45**, 746-61.
[24] MA Innis, DH Gelfand, JJ Sninsky and TJ White. *PCR protocols - a guide to methods and applications.* Academic Press, California, 1990.
[25] TA Hall. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser*. 1999; **41**, 95-8.
[26] K Tamura, G Stecher, D Peterson, A Filipski and S Kumar. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*. 2013; **30**, 2725-9.
[27] J Toida, K Kondoh, M Fukuzawa, K Ohnishi and J Sekiguchi. Purification and characterization of a lipase from *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem*. 1995; **59**, 1199-203.
[28] J Zhou, WW Chen, ZB Jia, GR Huang, Y Hong, JJ Tao and XB Luo. Purification and characterization of lipase produced by *Aspergillus oryzae* CJLU-31 isolated from waste cooking oily soil. *Am. J. Food Tech*. 2012; **7**, 596-608.
[29] D Pokorny, A Cimerman and W Steiner. *Aspergillus niger* lipases: Induction, isolation and characterization of two lipases from a MZKI A116 strain. *J. Mol. Catal. B Enzym*. 1997; **2**, 215-22.
[30] N Mhetras, S Patil and D Gokhale. Lipase of *Aspergillus niger* NCIM 1207: A potential biocatalyst for synthesis of isoamyl acetate. *Indian J. Microbiol*. 2010; **50**, 432-7.

[31] NZ Adham and EM Ahmed. Extracellular lipase of *Aspergillus niger* NRRL3; production, partial purification, and properties. *Indian J. Microbiol.* 2009, **49**, 77-83.
[32] HS Hamdy and MA Abo-Tahon. Extracellular lipase of *Aspergillus terreus* var. africanus (CBS 130.55): Production, purification and characterisation. *Ann. Microbiol*. 2012; **62**, 1723-36.
[33] Z Liu, Z Chi, L Wang and J Li. Production, purification and characterization of an extracellular lipase from *Aureobasidium pullulans* HN2.3 with potential application for the hydrolysis of edible oils. *Biochem. Eng. J.* 2008; **40**, 445-51.
[34] S Sharma and SS Kanwar. Purification and bio-chemical characterization of a solvent-tolerant and highly thermostable lipase of *Bacillus licheniformis* strain SCD11501. *Proc. Natl. Acad. Sci. India Sect. B. Biol. Sci.* 2017; **87**, 411-9.
[35] V Delorme, R Dhouib, S Canaan, F Fotiadu, F Carrière and JF Cavalier. Effects of surfactants on lipase structure, activity, and inhibition. *Pharm. Res.* 2011; **28**, 1831-42.
[36] DS Dheeman, S Antony-Babu, JM Frías and GTM Henehan. Purification and characterization of an extracellular lipase from a novel strain *Penicillium* sp. DS-39 (DSM 23773). *J. Mol. Catal. B. Enzym*. 2011; **72**, 256-62.
[37] B Ayaz, A Ugur and R Boran. Purification and characterization of organic solvent-tolerant lipase from *Streptomyces* sp. OC119-7 for biodiesel production. *Biocatal. Agric. Biotechnol.* 2015; **4**, 103-8.
[38] A Hiol, MD Jonzo, N Rugani, D Druet, L Sarda and LC Comeau. Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme Microb. Technol*. 2000; **26**, 421-30.
[39] B Venkatesagowda, E Ponugupaty, AM Barbosa-Dekker and RFH Dekker. Purification and characterization of lipases from *Lasiodiplodia theobromae*, and their immobilization and use for biodiesel production from coconut oil. *Appl. Biochem. Biotechnol*. 2018; **185**, 619-40.
[40] BS Lakshmi, P Kangueane, B Abraham and G Pennathur. Effect of vegetable oils in the secretion of lipase from *Candida rugosa* (DSM 2031). *Lett. Appl. Microbiol*. 1999; **29**, 66-70.
[41] ZY Shu, JK Yang and YJ Yan. Purification and characterization of a lipase from *Aspergillus niger* F044. *Chin. J. Biotechnol*. 2007; **23**, 96-100.
[42] TA Andrade, M Errico and KV Christensen. Influence of the reaction conditions on the enzyme catalyzed transesterification of castor oil: A possible step in biodiesel production. *Bioresour. Technol*. 2017; **243**, 366-74.
[43] Y Chen, B Xiao, J Chang, Y Fu, P Lv and X Wang. Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor. *Energy Convers. Manag.* 2009; **50**, 668-73.
[44] KA Baloch, A Upaichit and B Cheirsilp. Multilayered nano-entrapment of lipase through organic-inorganic hybrid formation and the application in cost-effective biodiesel production. *Appl. Biochem. Biotechnol.* 2021; **193**, 165-87.