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Isolation, Characterization, and Production of Lipase from Indigenous Fungi for Enzymatic Interesterification Process

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Lipase catalyses hydrolysis and esterification of lipids. The aim of this study was to obtain lipase producing indigenous fungi, to identify the selected fungi, to study optimum temperature and pH of the enzyme activity, as well as the enzyme ability in interesterification reaction. The isolates used in the experiment were isolated from tempeh, oncom and BPPT laboratory culture collection. The results showed that three fungal isolate observed were positive produced lipase after qualitative assay using Rhodamine B, olive oil, and PVA. The morphology and molecular identification of the isolates, revealed that R isolate was *Aspergillus niger*, O isolate was *Neurospora* sp. and T isolate was *Rhizopus oligosporus*. Upon quantitative assay from determination of the media and time production, potato dextro broth (PDB) with 2% olive oil after 48 h fermentation showed the highest specific activity of the enzymes. Lipase produced from three isolates have the optimum at pH 4, temperatures at 40-45 °C, and stable in interesterification reaction (55 °C) for 30-40 min. HPLC analysis after interesterification enzymatic reaction in mixture palm kernel olein (PKOo) and palm stearin (POs) showed that the composition of triglycerides (TAG) did not change as compared to the commercial lipase (Lypozyme TL1M).

Key words : enzymes, fungi, interesterification, lipase

Lipase mengkatalisis hidrolisa dan esterifikasi lipid. Tujuan dari penelitian ini adalah mendapatkan lipase yang diproduksi oleh jamur asli Indonesia, mengidentifikasi jamur yang terpilih, mempelajari suhu optimum dan pH aktivitas enzim, serta untuk mengetahui kemampuan enzim dalam reaksi interesterifikasi. Isolat jamur yang digunakan dalam percobaan diisolasi dari tempe, oncom, dan koleksi kultur laboratorium BPPT. Hasil penelitian menunjukkan bahwa tiga isolat jamur yang diamati positif menghasilkan lipase setelah uji kualitatif menggunakan Rhodamine B, minyak zaitun, dan PVA. Identifikasi morfologi isolat tersebut, menunjukkan bahwa isolat R adalah *Aspergillus niger*, isolat O adalah *Neurospora* sp., dan isolat adalah *Rhizopus oligosporus.* Pada uji kuantitatif dari penentuan media dan produksi waktu, kaldu dekstro kentang (PDB) dengan minyak zaitun 2% setelah 48 jam fermentasi menunjukkan aktivitas enzim tertinggi. Lipase yang dihasilkan dari tiga isolat memiliki optimum pada pH 4, suhu 40-45 °C dan stabil dalam reaksi interesterifikasi (55 °C) selama 30-40 menit. Analisis HPLC setelah reaksi interesterifikasi enzimatik pada campuran palm kernel olein (PKOo) dan palm stearin (POs) menunjukkan bahwa komposisi trigliserida (TAG) tidak berubah dibandingkan dengan lipase komersial (Lypozyme TL1M).

Kata kunci : enzim, interesterifikasi, jamur, lipase

Advanced technology in oil palm based industries might change triglyceride's double bonds into single bond which is more stable than that of hydrogenation technique. The technique eliminates double bond in oil/fat by adding H_2 to change unsaturated oil to saturated one. The aim of the technique is to obtain saturated oil which has specific characteristic in flavour and texture by modifying solid fat content (SFC) and melting point (MP). The high temperature used in hydrogenation process tends to change cis form of double bonds in unsaturated fat into trans form. Partial hydrogenation in double bond produces trans-lipid acid. Several studies reported that trans form adversely affects health, i.e. increasing the risk of coronary heart disease (Mozaffian *et al.* 2006). To decrease the negative impact, the lipid can be modified using interesterification reaction.

Interesterification reaction can be carried out in two method, i.e. chemical interesterification *(*CIE) and enzymatic interesterification (EIE) (Rodriguez *et al.* 2009). Chemical interesterification usually uses sodium methoxide or sodium ethoxide as catalyst while the enzymatic one uses lipase (Amir *et al.* 2012). Lipase (triacylglycerol hidrolase) is biocatalyst with ability to catalyze lipid hydrolysis reaction into lipid acid and glycerol.

Lipase use in industrial's enzymatic bioconversion

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process, however, faces obstacles, *i.e.* the availability and the high price of imported lipase because lipase producers are only a few in the world (Suzuki *et al.*1988; Panji *et al.* 2008). The dependence of several food industries in Indonesia to use only imported lipase encourages enzyme experts or researchers to isolate lipase from indigenous microorganisms.

In microorganisms, lipase is produced by bacteria, yeast, and fungi (Faloni *et al.* 2006). The use of microbial lipase is considered cost-effective. Several lipase-producing bacteria are *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp., and *Staphylococcus* sp.; lipase-producing fungi are *Rhizhopus* sp., *Aspergillus* sp., *Geothricum* sp., *Mucor* sp., *Thricoderma reseei, Fusarium* sp., and *Rhzhomucor* sp.(Treichel *et al*. 2010); while lipase-producing yeasts are *Candida rugosa* (CRL) and *C. antarctica* (CAL) (Bussamara *et al.* 2010).

Fungi are potential lipase producing agents (Rajesh *et al.* 2010) and the best lipase producer (Ming *et al.* 1998). Several indigenous fungal species were studied to explore which fungal species capable on producing lipase. Indigenous fungi capable on producing lipase in high activity are necessary to carry out enzymatic interesterification technique to modify fat of margarine raw material. Lipase from *Rhizopus* sp. has been applied in interesterification of Amazonian patauá oil and palm stearin better than that of commercial *Thermomyces lanuginosus* lipase (Speranza *et al*. 2015).

This study aimed to select indigenous lipase producing fungi from several sources, to identify the selected isolates, to study the enzyme properties and determine the capability on enzymatic interesterification in the mixture of stearin and olein.

MATERIAL AND METHODS

Isolation, Screening of Lipolytic Fungus, and Morphological Identification. Lipase-producing fungi were isolated from soya-tempeh and *Oncom*. The screening of lipase-producing fungi was using petridish with Potato Dextrose Agar (PDA) in 39 g L^{-1} concentration. In addition, pure fungal isolate from BPPT-CC (Badan Pengkajian dan Penerapan Teknologi-Culture Collection) was also used.The morphological identification of macroscopic and microscopic fungal isolates was carried out following Gandjar *et al.* (1999) and Watanabe (1937).

Identification of the Fungal Isolates Based on Molecular Marker. Genomic DNA was extracted from the fungal isolates (R, O, and T) using PureLink® Genomic DNA Kits for purification of genomic DNA (Invitrogen, USA). PCR of 28S rRNA region was conducted using NL4 5'-GGTCCGTGTTTCAAG ACGG-3' and NL1 5'-GCATATCAATAAGCGGAGG AAAAG-3' as a primer pair (O'Donnell 1992), with the following cycles condition: 30 cycles of 94 °C 1 min, 55 °C 35 s, 72 °C 2 min, and followed by extension at 72 °C 10 min. The PCR product then purified and sequenced in 1st base sequencing service (Singapore). Sequencing results were compared to the data base from the NCBI website (http://www.ncbi.nlm.nih.gov) with the Basic Local Alignment Search Tool (BLAST). Nucleotide sequence alignment and phylogenetic tree construction were conducted using MEGA 5.2 software. Construction of a phylogenetic tree was conducted using the Neighbour Joining method.

Qualitative and Quantitative Enzyme Assay, Protein Content, and Specific Activity. Qualitative of lipolytic activity on solid media can be determined by using Olive Oil Rhodamine method (modifed from Kouker and Jaeger 1987) comprising of 39 g L^{-1} PDA, 0.01% Rhodamin, 1.5% (w/v) PVA, and 4% (v/v) olive oil substrate. There are other analysis using modified method from Silva (2005) and titration method (Yang *et al*. 2006). Silva's modified method (2005): 3 mg pNPP was dissolved in 1 mL 2-propanol (Solution A) and mixed with 10 mg stabiliser and 40 mg Triton X-100 to dissolution in 9 mL 50 mM buffer (Solution B). 0.1 mL enzyme sample mixed with 0.9 mL substrate solution and incubated at 40 °C for 30 min. The appearance of the sample can be monitored by reading the absorbance at 410 nm. Titration method (Yang *et al*. 2006): substrate contained 25% olive oil and 1.5% PVA in water, 1 mL crude extract enzyme, 5 mL substrate, and 4 mL 0.05 M pH 7 phospat buffer was incubated at 37 °C for 20 min shaker incubator and added 5 mL methanol (ethanol 100%) for stop the reaction. The liberated fatty acids were titrated with 0.05 M NaOH. All experiments were carried out in triplicate for the calculation of the mean value. Enzyme protein concentration was determined following Bradford's method (1976) using bovine serum albumin (BSA) standard solution. One unit enzyme activity was defined as the amount of enzyme that generated 1.0 mmol of fatty acid from a triglyceride in one min or the amount of enzyme that released 1 mmol of p-nitrophenol from pNPL in one minute.

Media Selection and Production Period of Enzyme. Cultivation media selected were (1) PDB and olive oil media consisted of 24 g L^{-1} PDB and 2%(v/v) olive oil; (2) PDB and Crude Palm Oil (CPO) media

consisted of 24 gL^{-1} PDB and 2%(v/v) Crude Palm Oil.

Effect of pH, Temperature, and Stability Enzyme. To evaluate the effect of reaction temperature and pH on lipase activity, the lipase activity was assayed at various pH 4,5 (citric acid buffer), 6,7 (phospat buffer), and 8 (Tris-HCl buffer) and also at various temperature (25, 30, 35, 40, 45, 50, and 55 °C). To determine the lipase stability assay, the lipase was conducted at interesterification reaction temperature of 55 °C up to 3 h. The analysis by sampling per 15 min after incubation for 1 h, 2 h, and 3 h.

Enzyme Production, Concentration, and Freeze Drying. Ten percent spore suspension was put into 1000 mL sterile production media in erlenmayer 2000 mL to incubated using shaking incubator at 30 °C for 150 rpm. After 48 h, the mycelia of the fungi were filtered using Whatman paper to separate from their cells. The result of filtration then centrifuged at 8000 rpm and 4 °C for 20 min to obtain crude extract enzyme. The enzyme was concentrated using polyetylene glycone (PEG) 20000 with dialysis tubing cellulose and Acetonein various concentrations, *i.e.* 20, 40, and 60% (v/v) as comparators. After that, the concentrated enzyme was freeze dried with the addition of 0.5% maltodextrin.

Interesterification Reaction. Material mixture followed Zainal and Yusoff (1999) consisted of palm kernel olein (PKOo): palm stearin(POs) in 75:25 (w/w) ratios. Ten percent enzyme (w/w) put in the melted PKOo-POs mixture. Interesterification reaction was conducted at 150 rpm and 55 °C (the result of enzyme stability) for 24 h. To stop the reaction, the sample mix was filtered through Whatman paper. Analysed of oils and interesterified products using High Performance Liquid Chromatography (HPLC) were determined by the rapid method of AOCS Official Method Ce 5b-89. The column used was RP C-18 (250 × 4 mm i.d.), with particle size of 5-µm (Merck, Darmstadt, Germany). Detector used was refractive index and aseton and asetonitril with ratio of 75:25 v/v) was used as mobile phase. The elution rate was 1 mL min^{-1}. Standard used was Mixture of Tri Acyl Glycerole (TAG) that consisted of gliseryl tridecanoate (CCC), glyseryl tridodecanoate (LaLaLa), glyseryl trimyristate (MMM), glyseryl trioctanoate (CaCaCa), dan glyseryl tripalmitin (PPP). Individual peaks was identified by comparing qualitative of retention times with those of pure TAG standards, negative control, palm stearin, palm kernel oils and the fat blend after interseterification, also the result of interesterification of commercial lipase (Lypozyme TL1M).

RESULT

Fungal Isolation, Lipase Qualitative Activity Assay and Morphological Identification. Fungal isolation from several sources after 2-3 d of incubation resulted in 3 pure fungal groups, *i.e*. isolate R from ferment (BPPT-CC), isolate T from tempeh, and isolate O from *Oncom*. The result of activity assay of lipase crude extract from the three fungal isolates under UV irradiation on Rhodamine B and olive oil media indicated that the three isolates positively produced lipase on the media assay. Lipid acid products released by enzyme from hydrolysis process were responsible for colour development around the colonies under UV irradiation (Kouker and Jaeger 1987). The three isolates were capable of producing orange fluorescence (Table 1) and forming clear zone on media and isolate O produced the highest fluorescence intensity (Fig 1).

Microscopic and macroscopic identification of the three isolates were matched with their respective fungal morphology guidance books which resulted in isolate R, T and O belonged to genus *Aspergillus*, *Mucor*, and *Neurospora* respectively (Fig 2). Based on BLAST and phylogenetic tree analyses, the fungal isolates R, O, and T belong to *Aspergillus niger*, *Nerospora* sp. and *Rhizopus oligosporus* species, respectively (Fig 3).

Table 1 The diameter of clear zone and the intesity of crude enzyme fluorescence of the three isolates under UV rays on Rhodamin Olive Oil Agar (ROA) media modified from Koeker and Jaeger (1987)

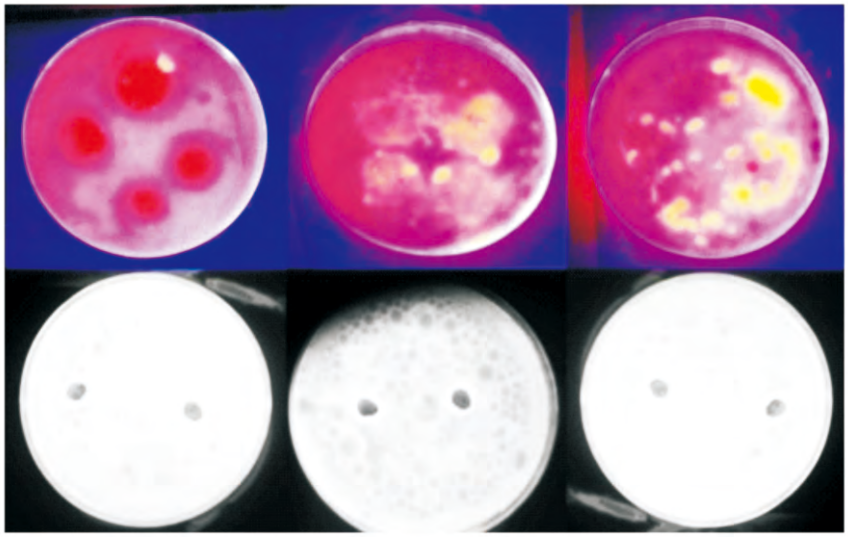
Variables	Isolate		
	R	T	O
Diameter of zona (cm)	0.2	0.3	0.3
Fluorescence	+	+++	++++

Information: [+] low intensity, [++] moderate intensity, [+++] high intensity, and [++++] very high intensity.

These fungal species are confirmed as GRAS for food product application.

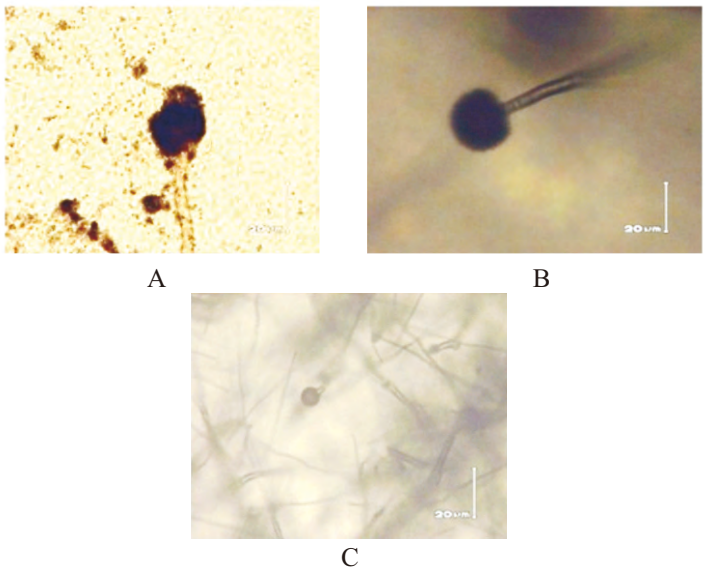
Enzyme Activity. Fungal growth of the three isolates experienced both increase and decrease during 6 days fermentation (Fig 4). On PDB and 2% olive oil media, enzyme specific activity of the three fungi reached the highest on day 2 of fermentation (isolate R88.96 U mg^{-1}, isolate T 84.53 U mg^{-1}, and isolate O 96.42 U mg^{-1}) before decreasing afterwards while on PDB and 2% crude palm oil occurred on day 1 of fermentation (isolate R 24.43 U mg^{-1}, isolate T 25.86 U mg^{-1}, and isolate O 20.92 U mg^{-1}) before also decreasing afterwards. This indicates that the three fungi produce lipase during their early growth period. However, the decrease in lipase produced by the three fungi was not followed with the decrease in biomass.

Media Selection and Production Period. The result of media selection and production period in Fig 5 indicated that media having the highest enzyme specific activity was PDB and Olive Oil media with optimum activity period occurred at 48 h. The highest lipase production by isolate R, T, and O was 43.963, 41.096, and 80.847 U mg^{-1}, respectively. PDB and Crude Palm Oil media produce the highest enzyme specific activity at 24 h, *i.e.* ranged from 20.918 to 25.868 U mg^{-1}. Enzyme specific activity of the three



A B C

Fig 1 Qualitative activity of lipase from three fungal isolates under UV on ROA media, modified from Koeker and Jaeger (1987), after 2 days incubation at room temperature, A) isolate R from BPPT-CC, B) isolate T from tempeh, and C) isolate O from *Oncom.*



A B C

Fig 2 The microscopic identification result of morphological characteristics of 2 days-pure fungal isolates using microscope, A) Isolate R, B) Isolate T, and C) Isolate O.

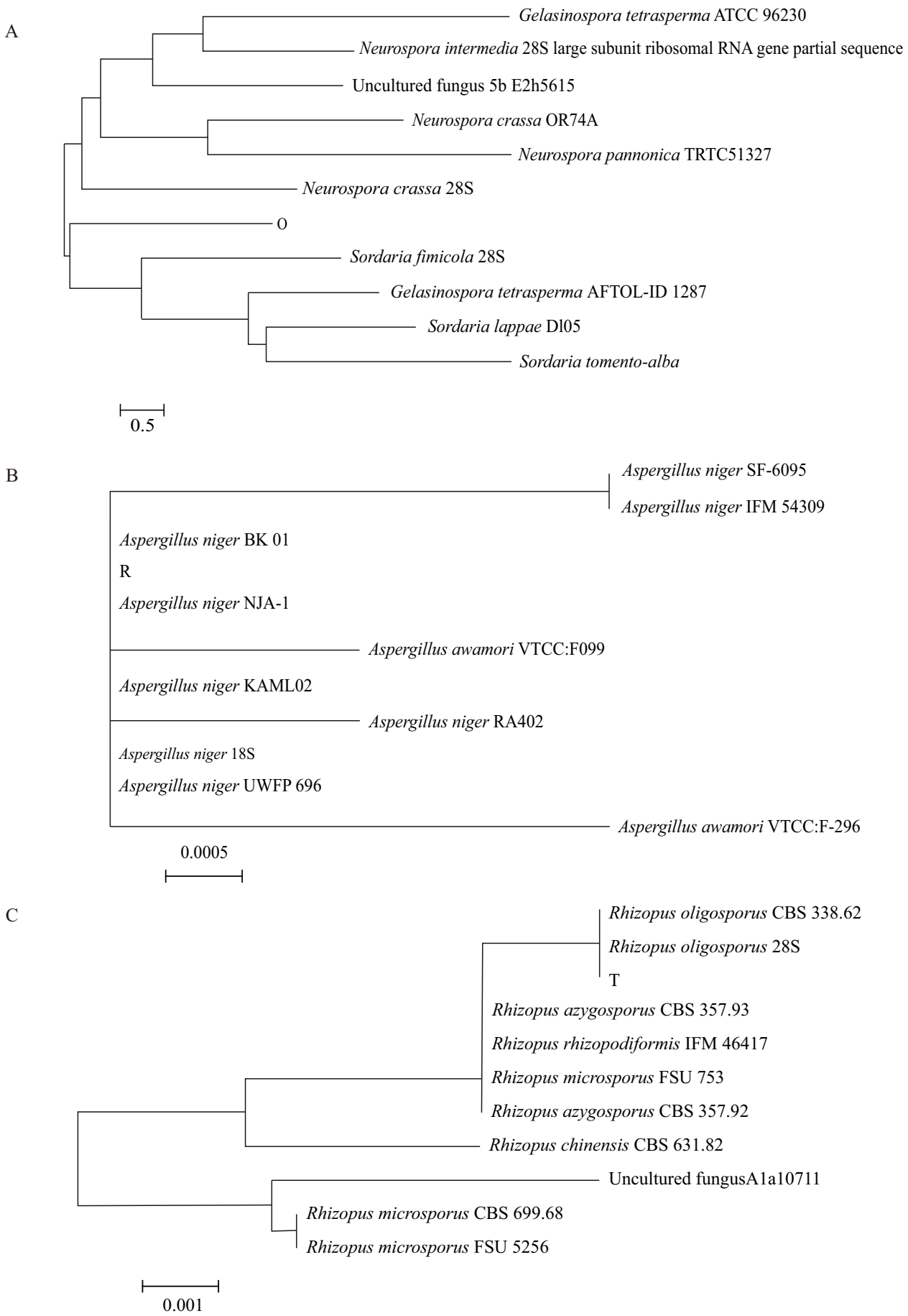


Fig 3 Phylogenetic tree analyses of 28S rRNA gene sequences of fungal isolates: O (A), R (B), and T (C). Construction of a phylogenetic tree was conducted using the Neighbour Joining method.

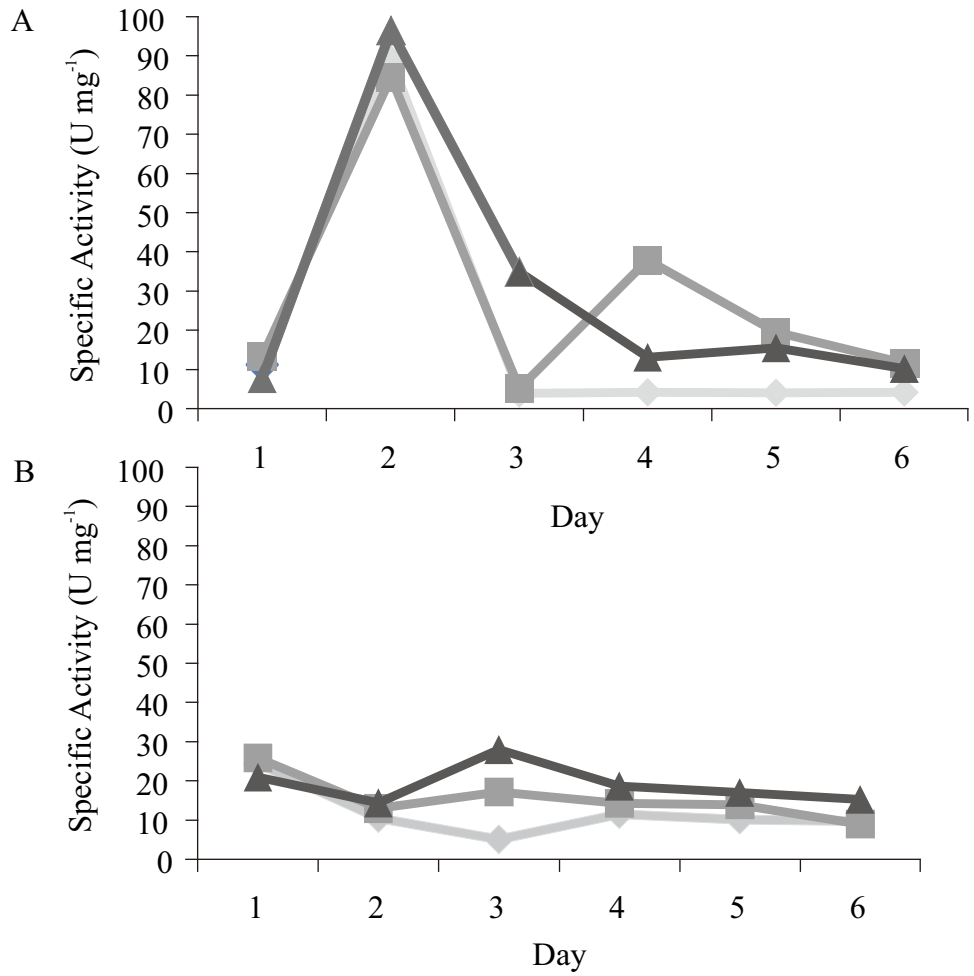


Fig 4 Comparison of enzyme specific activity of isolate R (—◆—), isolate T(—■—) , and isolate O (—▲—) during 6 days fermentation at room temperature, [A] PDB and 2% olive oil media, and [B] PDB and 2% crude palm oil media.

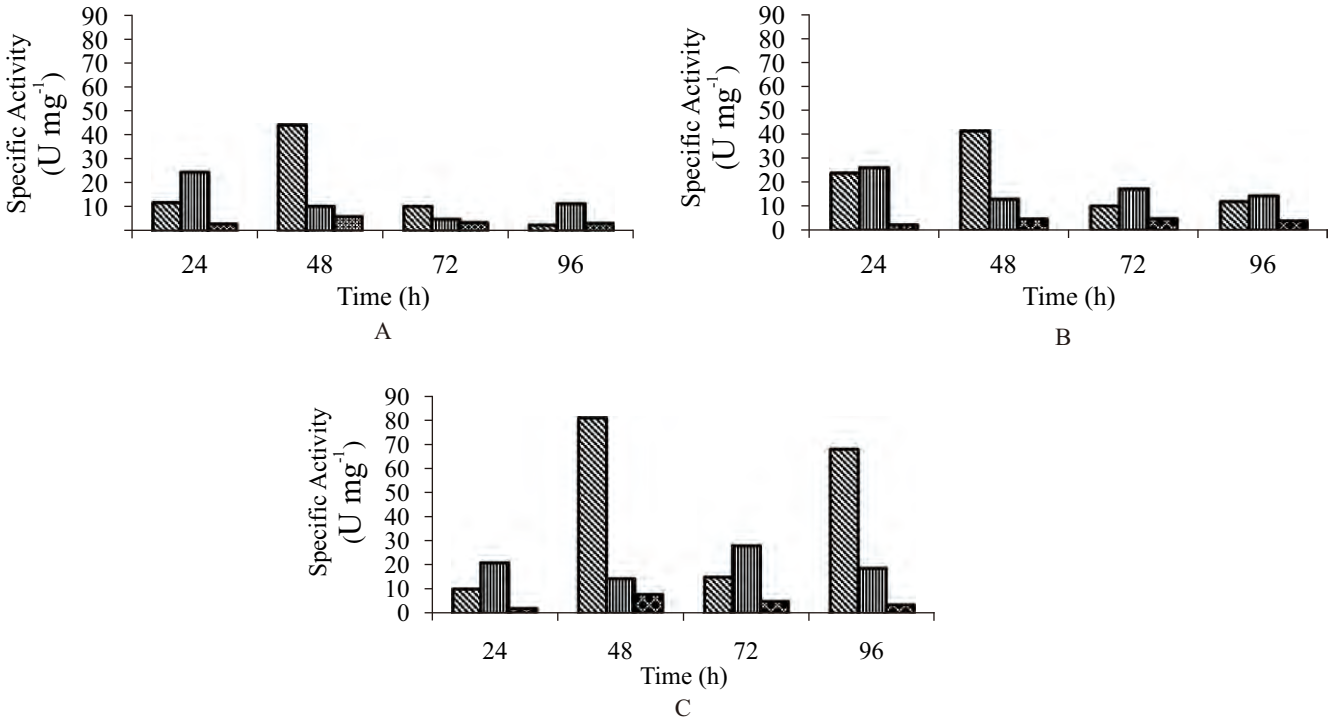


Fig 5 Enzyme specific activity of [A] isolate R, [B] isolate T, and [C] isolate O on PDB and olive oilmedia (▨), PDB and crude palm oil (▩), and Blain (▩).

isolates on Blain media, however, reached only 4.729-47.771 U mg^{-1} with 48 h as optimum production period.

The Effect of pH and Temperature on Enzyme's Activity and Stability. Based on the result of determining interesterification reaction temperature using enzyme stability test at 50 °C and 60 °C and compared to the enzyme activity at optimum temperature (40 °C), the activity of lipase at 50 °C decreased 26.67% in isolate R, 27.2% in isolate T and 22.63% in isolate O. Where as at 60 °C, the enzyme specific activity of isolate R decreased 64.44%, isolate T 57%, and isolate O 32.85% (Fig 6). Such decrease was considered not too significant and therefore the closest temperature (55 °C) was used for interesterification reaction. This is due to interesterification process in the mixture of margarine material consisting of palm kernel olein (PKOo) and palm stearin (POs) requires a rather high reaction temperature.

Temperature brings about significant effects, not only for enzyme activity but also enzyme stability. Lipase incubation at reaction temperature of 55 °C failed to maintain enzyme catalytic activity hence since minute 15 to 180 the enzyme's activity gradually decreased (Fig 7). Lipase enzyme activity of isolate R decreased from 0.368 U mL^{-1} to 0.193 U mL^{-1}, isolate T from 0. 318 U mL^{-1} to 0.024 U mL^{-1}, and isolate O from 0.424 U mL^{-1} to 0.031 U mL^{-1}. The result indicated that the longer the enzyme incubated at 55 °C, the lower the enzyme stability. The result of enzyme stabilty test was used as determinator of interesterification reaction period in the mixture of margarine material consisting of palm kernel olein (PKOo) and palm stearin (POs).

Enzyme concentration using polyethylene glycol (PEG). Enzyme concentration aimed to increase lipase activity before being used for enzymatic interesterification reaction. The result of comparison of enzyme specific activity before and after 10 times concentration (v/v) using PEG indicated that PEG was capable of increasing enzyme specific activity 2.9-5.1 times of before concentration (Fig 8).

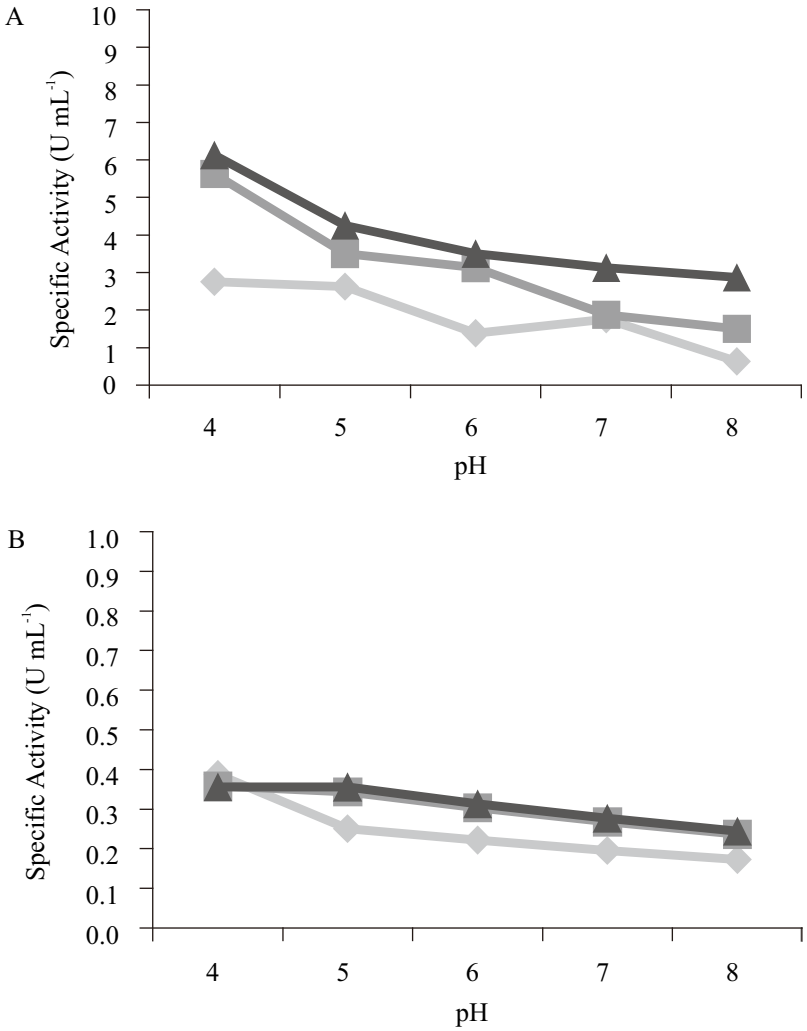


Fig 6 Enzyme activity of isolate R (—◆—), isolate T (—■—), and isolate O (—▲—) on several pH conditions using method of titration and Silva. (A) at 50 °C and (B) at 60 °C.

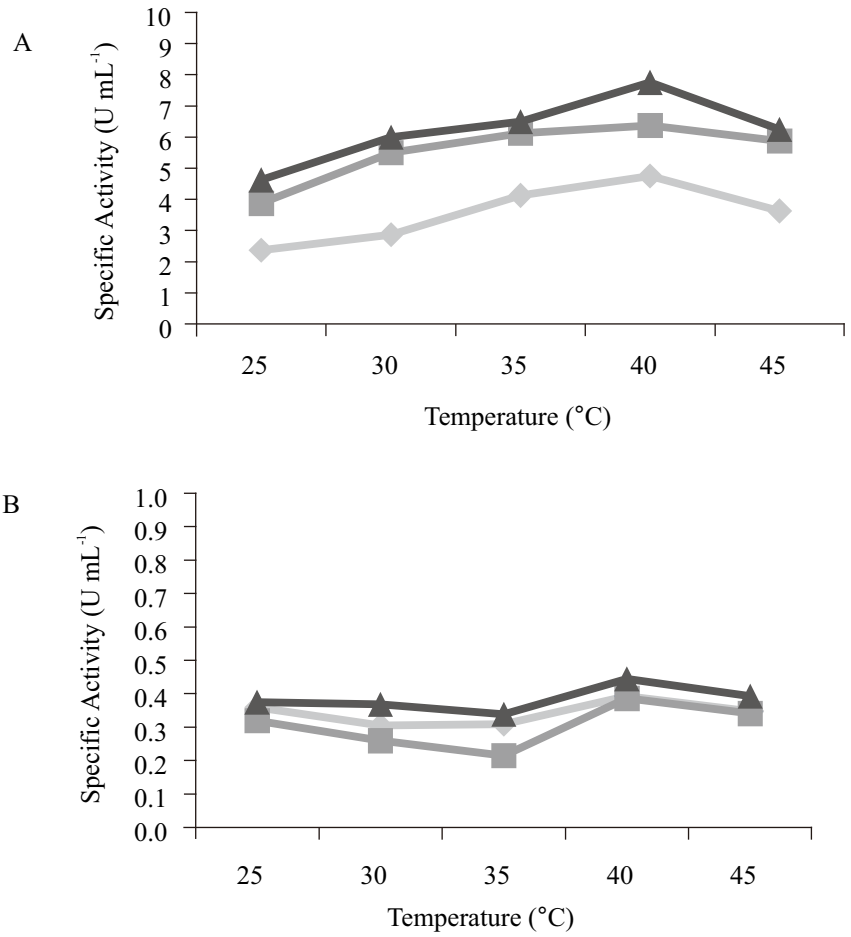


Fig 7 Enzyme specific activity of isolate R (—◆—), isolate T (—■—), and isolate O (—▲—) in several temperature conditions using method of titration (A) and Silva (B).

The highest increase (5.1 times) belonged to isolate T where the activity in isolate R increased 3.1 times and in isolate O 2.9 times.

Interesterification Reaction. The quality of margarine is usually can be determined based on the characteristic of raw materials and TAG composition (Pandiangan 2008). Based on the profil data of HPLC analysis result before and after enzymatic interesterification reaction using indigenous lipase of raw material formula consisting of palm kernel olein (PKOo) and palm stearin (POs), it was known that the result remained the same, or in another word there was no significant change in TAG composition while in commercial lipase, the TAG composition changed (Fig 9).

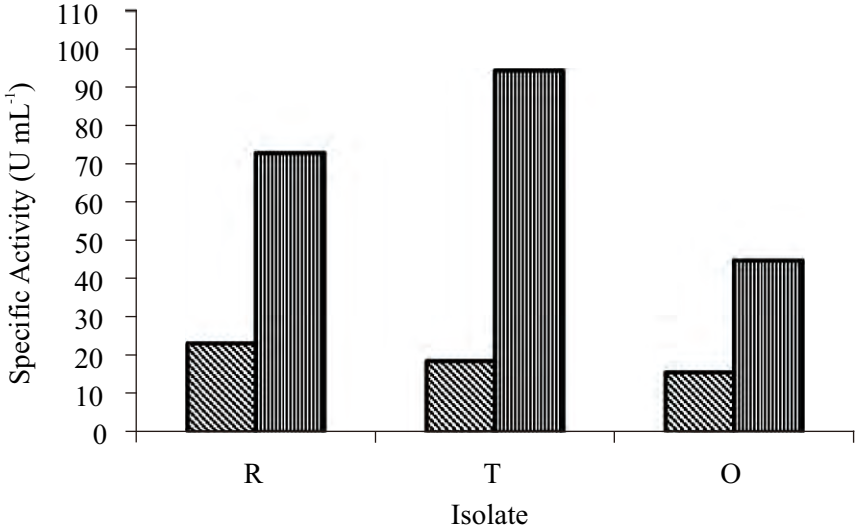


Fig 8 Enzyme specific activity of the three isolates before (▨) and after concentration (▥) using polyethylene glycole (PEG).

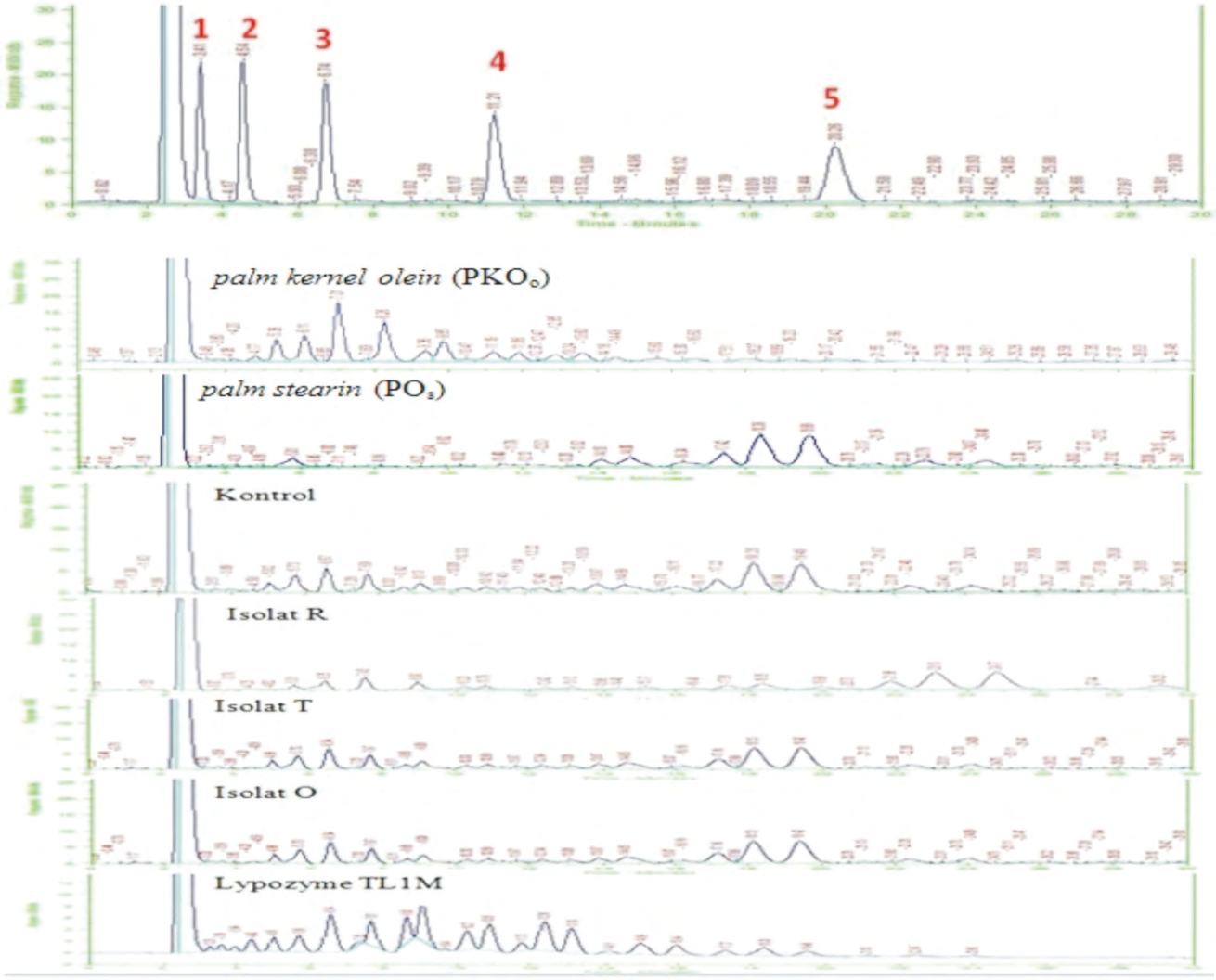


Fig 9 High Performance Liquid Chromatography (HPLC) analysis result. Triglyceride Mix Standart; Specification peak: [1]Glyceryl trioctanate; [2]Glyceryl tridecanoate; [3]Glyceryl tridodecanoate; [4] Glyceryl trimyristate; [5]Glycerol tripalmitate.

DISCUSSION

Lipase was used as biocatalyst on enzymatic interesterification reaction in the making of margarine raw material. The test of lipase activity of the three fungi was qualitatively carried out following the modification of Koeker and Jaeger's method (1987). This method could only confirm the lipase was produced but not for quantifying the enzyme activity. Media used was selective media with Rhodamin B and olive oil, and Polyvinyl Alcohol (PVA) as modification. Rhodamin B functions as the absorber of orange illuminating zone when lipase-producing fungi was placed under UV rays. Rhodamin B forms dimer complex with monoacylglycerol (MAG), diacylglycerol (DAG), and free fatty acid (FFA) (Koeker and Jaeger 1987). Olive oil functions as lipid source (Gupta *et al*. 2003), while PVA functions as emulsifier that can help stabilize oil and water soluble (Ota and Yamada 1966). Test result indicated that the three isolates were capable of producing orange fluorescence and forming clear zone on the media. According to Hou and Johnston (1992), fluorescence zone formed on selection media with CPO as lipid source also indicated that the isolate was capable of producing lipase which break triacylglycerol (TAG) in CPO into DAG, MAG, or FFA, and with Rhodamin B forming fluorescence compounds.Specific lipase hydrolyses ester bond at 1,3 position, producing fatty acid, monoacylglycerole, and diacylglycerole (Suharyanto *et al*. 2011).

Enzyme activity indicated the enzyme ability to catalyze the change of a substrate into product in unit. A unit of lipase activity was defined as the amount of lipase capable of releasing 1 μmol fatty acid per mL

min^{-1}, written in U mL^{-1}. Media selection and enzyme production period were carried out based on enzyme activity curve at hour 24 to 96 on various production media and inducers. A good fungal fermentation media to produce lipase contains carbon source such as fructose, nitrogen source such as peptone, and oil added such as palm oil and olive oil as inducer (Sharma *et al.* 2001). Inducer concentration highly affects enzyme activity. Too much oil as inducer in production media leads to low lipase activity due to low oxygen transfer into medium (Lima *et al.* 2003). Pramitasari *et al.* (2012) used 2% olive oils inducer to produce lipase and Suharyanto *et al.* (2011) used 2% crude palm oil (CPO) to produce 1.3 glyceride specific lipase from *Rhizopus oryzae* TP-2. Media selection and production period based on the comparison of enzyme specific activity (Fig 11) indicates that the optimum activity period of PDB and olive oil media was at 48 h.

In margarine raw material selection and making, POs can be mixed with PKOo which has shorter lipid chain to make the mixture better functional property such spread ability at room temperature (Noor *et al.* 2002). Interesterification using PKOo and palm oil stearin POswill likely have margarine free of trans fatty acid if the lipase produced by the three fungal isolates capable of performing interesterification reaction. Palm stearin (POs) is solid fraction from oil palm husks which has physical characteristic of easily solidified at room temperature. Therefore, interesterification reaction using the mixture of margarine material consisting of palm kernel olein (PKOo) and palm stearin (POs) requires high temperature, i.e. 60-70 °C. Zainal and Yusoff (1999) carried out interesterification reaction using commercial lipase from *Rhizomucor miehei* (Lipozyme IM 60) with the ratio of POs and PKOo of 30:70 at 60 °C.

Enzyme is protein sensitive to high temperature as denaturation can easily occurred along with temperature increase. Denatured protein adversely affects enzyme activity, and eventually decrease enzyme concentration and reaction rate. The optimum temperature of lipase produced from fungi isolated from tempeh, *oncom*, and BPPT-CC was 40 °C and then decreased along with the increasing incubation temperature. The decrease in lipase activity at 50 °C was 22.63%-27.2% while at 60 °C 32.85%-64.44%; such decrease is considered not highly significant. However, because of the low lipase activity of the three isolates compared to commercial lipase, it eventually decreased the effectiveness of lipase activity.

Qualitative analysis based on profile data from interesterification reaction using High performance liquid chromatography (HPLC) showed that TAG composition showed that there was not any change before and after the reaction using indigenous lipase and raw material consisting of palm kernel olein (PKOo) and palm stearin (POs), as compared to commercial lipase. This is allegedly because the activity of indigenous lipase was still lower than that of commercial one. Interesterification reaction requires lipase with high activity and thermostable characteristics under high temperature. Therefore before using lipase in interesterification reaction, optimization of enzyme and production media is necessary to obtain lipase with high productivity and that can be used for high temperature interesterification reaction.

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