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Immobilized thermophilic lipases for the interesterification of soybean oil

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Abstract

Vegetable oils are a major source of healthy dietary lipids. However, the physicochemical properties of vegetable oils are varied, which affects their application in foods. Interesterification is a method often used to modify the physicochemical characteristics of oils and fats. Enzymatic interesterification is a process that uses enzymes to exchange the acyl groups among triacylglycerols (TAG); an immobilized lipase is one such enzyme that can be used for interesterification. This study immobilized lipases from two strains of thermophilic bacteria (*Geobacillus stearothermophilus* (GS) and *Anoxybacillus flavithermus* (AF)) onto silica beads. These immobilized lipases were used to interesterify soybean oil (SBO) with palmitic acid (PA) to change the physicochemical properties of the oil. High-performance liquid chromatography (HPLC) was used to determine the TAG composition and free fatty acids (FFA) after interesterification. The melting point was measured using differential scanning calorimetry (DSC). Optimum reaction conditions were observed at 70°C, and 24 hrs for all reaction ratios of SBO to PA (1:0.1, 1:0.25, and 1:0.5 (v/v)). The PA significantly decreased after 24 hrs at all reaction ratios, and the TAG concentration was significantly different after interesterification. FFA concentration changed after SBO interesterification, and the melting points of the samples increased from 4°C to 40°C. The results suggest that both immobilized lipases have suitable properties for oil interesterification and industrial applications.

1. Introduction

Vegetable oils are used in a wide range of industries, including the food industry. In order to enhance the functional value of oils, oil manufacturers modify vegetable oils to improve their physical and chemical properties. Oils and fats can be modified chemically by hydrogenation or interesterification. Additionally, oils can be enzymatically interesterified (Fattahi-far *et al*., 2006). Soy bean oil (SBO) is a commonly consumed vegetable oil around the world. Currently, the U.S. consumes 95% of its domestic production of soybean oil. The U.S. ranks first in soybean oil production (8.5 million tonnes), followed by China, Argentina, Brazil, and India (Wilson, 2008). It has considerable economic value and high functionality and is a particularly interesting raw material for producing specialty fats. SBO is ideal for interesterification because it is a low-cost oil (Zeitoun *et al*., 1993).

Interesterification is employed in the food industry to modify the physical and chemical properties of natural oils and fats (Kavadia *et al*., 2019). Lower production cost, maximum yield, and enzyme activity are all important factors for lipase-catalyzed interesterification reactions (Utama *et al*., 2020). The interesterification reaction involves an exchange of acyl groups among triacylglycerides (TAGs) molecules to produce new combinations of TAG molecules in the final product. This change in FFA distribution leads to a substantial change in melting properties that alter physical properties (plasticity, crystalline structure, and thermal stability), and can enhance the nutritional value of the original starting mixture (Akoh, 2017). Interesterification reactions can be performed by either chemical or enzymatic reactions and both reactions change the acyl groups in the TAG. However, one produces randomized positional changes (chemical interesterification) and enzymatic interesterification can produce specific positional changes (Amir *et al*., 2012).

Lipases are hydrolases that catalyze the synthesis of soluble or insoluble carboxylic acid esters and other compounds with ester bond analogs or amide bonds. These enzymes have the ability to interesterify oils and

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produce modified oils with different properties (Samoylova *et al*., 2017). Lipases isolated from thermophilic bacteria are often used commercially for interesterification because of their high thermostability and activity. In general, commercial lipases used for interesterification in the oil industry are isolated from *Thermomyces lanuginosus, Rhizomucor meihei,* and *Candida antarctic*, which have thermostable activity (Lee *et al*., 2011; Calero *et al*., 2014).

In addition, lipases from the genus *Geobacillus* are promising biocatalysts due to their high thermal and operational stability (Samoylova *et al*., 2017). Immobilization is a technique used to cross-link an enzyme to a solid support material, which will reduce the cost of the enzyme used in the industry, the enzyme is not part of the final product, and immobilized enzymes are generally more thermostable than soluble enzymes (Barbosa *et al*., 2015). Chemical enzyme immobilization is more advantageous than physical adsorption because in chemical immobilization the enzymes are fixed into the support and do not leach into the food system (Saponji *et al*., 2010). The selection of an immobilization method also depends on enzyme properties such as specificity, stability, and activity.

In the present work, lipases were enriched from two thermostable bacteria, *Geobacillus stearothermophilus* (GS) and *Anoxybacillus flavithermus* (AF) cell lysates, and the enzyme activity was compared to that of a commercial thermophilic immobilized lipase from *Thermomyces lanuginosus*. In the second part of the study, the chemical composition and melting properties of SBO were modified using immobilized lipase-catalyzed interesterification. Palmatic acid (PA) was used as the substrate for SBO interesterification with three different reaction ratios (SBO:PA of 1:0.1, 1:0.25, and 1:0.5 (v/v)). The degree of interesterification was monitored by recording the changes in the chemical (TAG composition and free fatty acid (FFA) composition) and physical (differential scanning calorimetry (DSC) melting profiles) properties.

2. Materials and methods

2.1 Experimental design

For the first part of the study, crude cell lysates were harvested from GS and AF and used as enzyme sources. Silica beads were derivatized and used to immobilize the proteins from the cell lysates using glutaraldehyde crosslinking. The immobilized lipase activity was evaluated and compared with a commercial immobilized lipase.

For the second part of the study, the immobilized lipases were then used for the interesterification of SBO with free PA at three different molar ratios. The time course of SBO and PA interesterification reactions was evaluated at 70°C from 0 to 36 hrs to study the reaction time. Enzymatic interesterification products of SBO were studied with respect to their chemical composition and melting point properties. TAG composition and FFA composition of interesterified products for each sample were analyzed by HPLC. DSC was used to determine the melting point properties of interesterified samples after removing FFA. All experiments were performed in triplicate, analyzed in triplicate, and average values were reported.

2.2 Growth of microorganisms

GS culture was prepared by adding 0.1 mL of a stock solution obtained from NAMSA *G. stearothermophilus* spore suspension (2.4×10^6 spores/0.1 mL, VWR, Atlanta GA. USA) to 10 mL of sterile water. This diluted stock was incubated in a water bath (ISOTEMP 210, Fisher Scientific, Dexter, MI. USA) for 10 mins at 80°C. One mL of this was added to 25 mL of tryptic soy broth (TSB, VWR, Atlanta, GA. USA) media in a sterile 250 mL Erlenmeyer flask. The flask was covered with sterile foil and the sample was incubated at 55°C for 24 hrs in a shaker at 100 rpm (New Brunswick Scientific Series 25, Incubator Shaker, NJ. USA). The optical density (OD) of bacterial growth was measured using a spectrophotometer (Shimadzu, BioSpec-1601, Kyoto, Japan) at 600 nm (Beatty and Walsh, 2016). *Anoxybacillus flavithermus* TNO-09.006 culture was a gift from Remco Kort (Vrije University, Amsterdam, Netherlands) in the form of slant tubes. One loop from the slant tube was used to make a streak plate on tryptic soy agar (TSA: VWR, Atlanta, GA. USA) and was incubated at 55°C for 48 hrs. A subculture was grown by inoculating 25 mL of TSB with one loop of the culture grown on the streak plate in a sterile 250 mL Erlenmeyer flask covered with sterile foil. Cells were grown with the aerobic condition at 55°C in a shaker at 100 rpm for 16 – 18 hrs. The growth of AF was confirmed by measuring OD 600 using a spectrophotometer (Saw *et al*., 2008).

Frozen stocks were prepared by inoculating 2 mL of overnight culture into 20 mL of a 30% glycerol solution (Mallinckrodt Specialty Chemicals Co, St. Louis, MO. USA) (glycerol/TSB, w/v) and stored in 2.0 mL cyro-vials at -20°C. Frozen stocks (0.1 mL) of each organism were added to 25 mL TSB in a sterile 250 mL Erlenmeyer flask, then covered with sterile foil and incubated at 55°C for 16-18 hrs in a shaker at 100 rpm (Rueckert *et al*., 2005). The 25 mL growth of bacteria was added to 1 L of TSB and cells were grown at 55°C at 100 rpm until the OD at 600 nm was between 0.7 to 0.9.

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The bacteria were separated from the media using Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Company, Newtown, CT. USA) at 5,000×*g* for 10 min at 10°C. The cells were washed in 10 mL 50 mM Tris-HCl (Tris hydrochloride, Fisher Scientific, Waltham, MA. USA) buffer pH 7.5 and centrifuged again as described above. After centrifugation, the cells were mixed with 5-10 mL 50 mM Tris–HCl, pH 7.5, and this mixture was sonicated at 40% amplitude for 1 min using a 4.5 mm microtip and a Qsonica Sonicator power source (500 W power; QSonica Q500, Newtown, CT. USA). The sonicated solution was centrifuged at 10,000 rpm at 0°C for 20 mins and the lysate was filtered using a microfilter of 1.0 µm (Glassfiber Prefilter, Tullagreen, Carrigtwohill Co. Ireland). The crude protein lysates were used for further experiments.

2.3 Heat treatment

The lipases from cell lysates of GS and AF were partially purified using heat treatment to precipitate non-lipase proteins (Lee *et al*., 2001). The cell lysates from both organisms were heated at (70°C) for 10 mins in a water bath and the temperature was monitored every 25 s to ensure the required temperature was maintained using a precision digital thermometer (Fisherbrand (Traceable) Total-Range Thermometer, Fisher Scientific, TX. USA). Samples were placed in an ice bath after heat treatments to reduce the temperature. The solutions were centrifuged at 10,000×*g* for 30 mins at 4°C to precipitate denatured proteins and the supernatants were saved. The supernatants were assayed for lipase activity and protein content using the standard lipase assay and protein determination kit as described below.

2.4 Total protein and enzyme activity

Crude cell lysates and lysate fractions were assayed using p-nitrophenyl acetate (PNPC2, Sigma-Aldrich Chemical Co. St. Louis, MO. USA) as the substrate. Five mL of substrate stock solutions (20 mM of PNPC2) were prepared using an acetonitrile and isopropanol (Fisher Chemicals, Waltham, MA. USA) mixture (1:4 v/v) as described by (Glogauer *et al*., 2011). One mL of the substrate stock was diluted with 20 mL of assay buffer (50 mM Tris HCl, pH 7.5 containing 1mM $CaCl_2$ (calcium chloride, Thermo Fisher Scientific, Waltham, MA. USA) and 0.3% Triton X100 (Mallinckrodt Specialty Chemicals Co, St. Louis, MO. USA) for a final concentration of 0.055 mM substrate solution. For the standard lipase assay, 900 µL of substrate solution was pipetted into a 1.5 mL centrifuge tube (VWR, Atlanta GA, USA) and mixed with 100 µL of assay buffer for a final volume of 1 mL. After a 1 min reaction at 20°C, the sample was transferred to 1.5 mL two-sided disposable plastic cuvettes polystyrene (PS) (VWR Atlanta, GA. USA). Absorbance at 410 nm was recorded every 5 s over 1 min using a duel beam spectrophotometer (Shimadzu, BioSpec-1601, Kyoto, Japan) at room temperature with the bank value being subtracted from the sample. One unit of lipase activity (U) was defined as 1 µmoL substrate hydrolyzed per minute under the assay conditions. This is referred to as the standard lipase assay. The protein content was determined using a Pierce BCA protein assay kit (BCA kit #23227, Thermo Scientific, Rockford, IL.USA).

2.5 Preparation of silica beads

To clean the silica beads (wide pore, 150 angstroms - 100 +200 Mesh, S.A. 350-400 m/g, Alfa Aesar, Haverhill, MA. USA), 25 g of beads were soaked in 50 ml distilled water and degassed for 15 min in a 250 mL vacuum flask. One hundred millilitres of 20% nitric acid (Fisher Chemicals, Waltham, MA. USA) was added to the beads before placing them in a water bath at 100°C for 60 min. The silica beads were then thoroughly washed with 2000 mL DI water to completely remove all nitric acid. Finally, beads were suspended in 50 mL water for the next step (Janolino and Swaisgood, 1997).

Clean silica beads were mixed with 3 volumes of a 10% 3-aminopropyltriethoxysilane (98%, Beantown Chemical, BTC, Hudson, NH. USA) solution (pH 4.0) before degassing for 15 mins in a 250 mL vacuum flask. The mixture was then incubated in a water bath with occasional swirling for 3 hrs at 70°C. The mixture was diluted with distilled water and washed with 1 L DI water to remove excess reagent and the wet silica beads were dry in an oven at 80 – 100°C overnight to complete the polymerization. Silica beads were washed again with distilled water (2 L) on a fitted glass filter under suction and dried again in an oven at 80°C for 12 hrs (Baytas *et al*., 2004; Li *et al*., 2014).

The presence of amino groups on the aminopropyl silica beads was determined using 2,4,6-trinitrobenzene sulfonic acid (TNBS, Thermo Scientific, Rockford, IL. USA). After the synthesis of aminopropyl in silica beads, the TNBS test yielded a visible yellow colour compared to control acid-washed beads which were white. A 100 µL of silica beads was added to 1 mL of 20 mM sodium tetraborate (Sigma-Aldrich Chemical Co. St. Louis, MO. USA) and 2 drops of 1% TNBS were added and allowed to stay at 20°C for 1 min. The mixture was washed four times with distilled water, and the colour of the silica beads was observed. Beads with white colour indicate no amino groups, while yellow beads indicate the presence of amino groups (Antoni *et al*., 1983).

Glutaraldehyde (25%, Alfa Aesar, Haverhill, MA. USA) was used as a crosslinking agent between amino

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groups in the enzymes and amino groups in the aminopropyl silica beads (Nazari *et al*., 2016). Aminopropyl beads were washed with 2 volumes of distilled water, and 10 g of the beads were added to 20 mL of 0.2 M phosphate buffer (pH 7.0) (monobasic/ dibasic sodium phosphate, Fisher Chemicals, Waltham, MA. USA) containing 15% (v/v) glutaraldehyde in a vacuum flask and degassed for 10 min. After the addition of glutaraldehyde to the aminopropyl beads, the colour changed from white to brown due to the activation of glutaraldehyde. The slurry was rocked at room temperature for 16 hrs and then washed with 25 mM phosphate buffer pH 7.0 with a volume of at least 5 times the volume of the beads. Then the mixture was washed with distilled water to remove all glutaraldehyde so no free glutaraldehyde remained (Li *et al*., 2014).

2.6 Immobilization of lipases

The silica beads were activated by washing with 25 mM sodium phosphate buffer (pH 7.0). The activated silica beads were added to 50 mM Tris-HCl (pH 7.5) buffer (1:10 [w/v] silica beads to buffer) containing heat-treated cell lysate solution containing 3 mg protein (lysed cells from two different bacteria, GS and AF) /ml buffer. The lipase activity of silica beads was determined after 2, 4 and 6 hours to select a high point of lipase activity using the standard assay. After a 1 min initial reaction time, the matrix was centrifuged at 1000×*g* at 10°C for 3 mins.

The resulting supernatant was added to a cuvette and the absorbance at 410 nm was recorded using a spectrophotometer. The highest activity point of lipase from GS was after 4 hrs and AF was after 6 hrs. The silica beads which had the activity of the high lipase were filtered using 110 mm grade 4 circles filter paper (Whatman International lid Maidstone, England) under suction and dried at room temperature for 5-6 hrs. The lipases beads were subsequently washed with 10 mM glycine solution (Mallinckrodt Specialty Chemicals Co. St. Louis, MO. USA) in 25 mM sodium phosphate buffer pH 7.0 at 4°C overnight to block the remaining glutaraldehyde active sites on the beads. The damp lipases beads were collected and dried in an oven at 60° C overnight (Li *et al*., 2014).

2.7 Interesterification reactions

Before the interesterification, silica beads with immobilized lipases from lysate from GS (ILGS) and AF (ILAF) and the commercial lipase IMMOZYME TLL-T2-150 (IMT2, recombinant lipase from *Thermomyces lanuginrsa,* ChiralVision, Netherlands) was adjusted to 0.75 water activity overnight by placing in a water activity chamber (saturated NaCl solution with water activity 0.7547 at 20°C).

Interesterification samples were prepared by mixing 6 g of SBO (purchased from a local grocery store) in a 15 mL glass beaker with PA (Alfa Aesar, Haverhill, MA. USA) at molar ratios of 1:0.1, 1:0.3, and 1:0.5 (v/v). The blending resulted in blends between SBO and PA of 90:10, 75:25, and 50:50%. After complete melting of the mixtures at 70 – 80°C for 15 mins under magnetic stirring, mixtures were stored at -20°C for further use.

The interesterification reaction volume for each blend was 1 g of SBO and PA blends. For example, a 1:0.1 reaction ratio was 900 mg SBO and 100 mg PA which is 90% SBO and 10% PA. The interesterification mixing was performed by adding in an amber glass vial, 200 mg molecular sieves (3A molecular sieves 3-5 beads, Alfa Aesar, Haverhill, MA. USA) which is 2% of the total reaction volume. The lipase interesterification was performed in three different enzyme systems: IMT2, ILGS, and ILAF. IMT2, 100 mg, was added to each reaction (1% of IMT2 of the total reaction volumes), and 300 mg of ILGS and ILAF were added to each reaction (3% of the total reaction volumes) and incubated at 70°C in a shaker at 200 rpm.

A time course of the reactions was conducted in amber glass vials and individual samples were pulled at 4, 8, 12, 24, and 36 hrs. The products of each reaction were collected after the required time and stopped by adding 1 mL (95%) ethanol (Fisher Chemicals, Waltham, MA. USA). The progress of the time course reaction was followed by measuring the consumption of free PA over time. The time at which free PA was completely absent was deemed appropriate for performing the reaction in a scale-up system.

TAG analysis was stopped after 24 hrs by adding 1 mL (95%) ethanol. Aliquots were collected and diluted with 1× chloroform (Fisher Chemicals, Waltham, MA. USA) to improve the solubilities of the samples (Solaesa *et al*., 2014). The remaining interesterified blend was filtered using a 0.2 µm HPLC syringe filter (Acrodisc LC PVDF, Gelman Sciences, Ann Arbor, MI. USA) and stored at -20°C until further analysis.

TAG standards; trilinolein (LLL); trimyristin (MMM); triolein (OOO); tripalmitin (PPP); trilinolenin (LnLnLn); of purity greater than 98% were purchased from NU-CHEK-PREP, INC. (Elysian, Minnesota, USA.). The code letters used for the fatty acids are PA, palmitic; M, myristic; S, stearic; L, linoleic; O, oleic; Po, and Ln, linolenic. A 5 mg/ml stock solution of TAGs was prepared by dissolving 25 mg of each LnLnLn, LLL, OOO, MMM, and PPP in 5 ml of chloroform. Five different concentrations of TAGs were prepared using

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linear-gradient 3.33, 1.66, 0.83, 0.41, and 0.2 mg/mL and 20 µL of each concentration were injected into the HPLC. The standard curve of TAG concentration was graphed to get an equation that could be used to quantitate the amount of TAG. Samples were dissolved in 1x chloroform and injected into the mobile phase of HPLC, samples were soluble in chloroform rather than in the mobile phase (Solaesa *et al*., 2014). Six TAGs were identified and quantified with the following coefficients of determination: 0.995 LnLnLn, 0.993 LLL, 0.997 OOO, 0.995 MMM, and 0.996 PPP, in the range studied (0.13 – 5 mg/mL).

FFA content in samples was done by separating the FFA from TAG after the interesterified reactions, 3 samples of each ratio were washed two times with 1:10 (v/v) 95% ethanol. One mL of sample was washed with 10 mL (95%) ethanol in a 15 mL test tube and vortex for 1 min, the tubes were centrifuged at 7000×*g* (Marathon Micro-A model AR Centrifuge, Fisher Scientific, Austin, TX. USA) for 5 min. After centrifugation, the tubes were cooled to -20°C for 5 mins until the sample inside the tubes became two layers. The top layer of the sample after cooling was liquid and its FFA with ethanol (supernatants) and the bottom layer of the sample after cooling was solid because of its content, solid TAG. The supernatant was collected and concentrated using a SpeedVac (Automated Environmental, ThermoSavant, Holbrook, New York, USA) and stored at -20°C and the pellets was collected and stored at -20°C. For FFA analysis, the samples were dissolved in 1 mL acetonitrile and injected into the mobile phase of the HPLC. FFA standards, lauric (C12), myristic (C14), palmitic (C16), linoleic (C18:2), oleic (C18:1), and stearic (C18:0) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO. USA).

2.8 HPLC-ELSD

Interesterification time course and TAGs were determined using high-performance liquid chromatography (HPLC) (Beckman System Gold 125 Solvent Module, Ontario, Canada) system with an evaporative light scattering detector (ELSD) (Agilent Technologies, Santa Clara, CA, USA) and Vydac reverse phase C18, 4.6 × 250 mm, cat no 201TP54 column (Grace Vydac, Hesperia, CA. USA). The effluent was monitored with the following settings: evaporator temperature of 40°C and a gas pressure of 3.55 bar. The time course and TAG used a gradient of 95% distilled water with 5% acetonitrile (A) and 95% acetonitrile with 5% distilled water (B). Elution was performed at a solvent flow rate of 1 mL/min with a linear gradient as follows: 0-2 min 0% B, and a gradient of up to 100% B over 50 min, keeping these conditions for 55 min and returning to the initial conditions within 5 mins.

The FFA analysis used a gradient of 95% acetone with 5% acetonitrile, containing 0.1% formic acid (A) and 95% acetonitrile containing 0.1% formic acid, and 5% acetone (B). Elution was performed at a solvent flow rate of 1 mL/min with a linear gradient as follows: 0 to 5 min 0 to 60% B, 5-15 min 60 to 80% B, 15 to 20 min 80 to 85% B, and 20 to 45 min 85 to100% B. The HPLC column was a SynChrom C8, 4.6 × 250 mm, cat No. 253326 (SynChrom, Lafayette, ID. USA). The final conditions were maintained for 45 min before returning to the initial conditions. The column temperature was maintained at 40°C using a column heater (Bio-Rad 125-0425 HPLC Column Heater, Redmond, WA. USA) to sustain sample solubility and obtain sharp peaks. The compounds were detected by an ELSD at 40°C with 3.55 bar of nitrogen gas. HPLC grade acetonitrile with 0.1% formic acid and acetone was obtained from Fisher Chemicals (Waltham, MA. USA). A volume of 20 µL of each sample was injected after heating to 40°C for 2 min in a water bath.

2.9 Determination of kinetics for immobilized enzymes

The kinetic parameters including maximum reaction rate (Vmax) and Michaelis-Menten constant (Km) were calculated using SBO and PA as substrate. Initial rate measurements with immobilized lipases at a constant temperature of 70°C in a shaker at 200 rpm with increasing substrate concentrations (40, 80, to 200 mM), were performed to determine the kinetic parameters. Lineweaver-Burk plots were used to determine the reaction rates and Michaelis-Menten constants by linear regression analysis for each experiment using Microsoft Excel 2016 Office 365. The reciprocal of V was graphed vs the reciprocal of [S] and the best-fit line was obtained. Values of Vmax and Km were determined using the equation:

$$\frac{1}{v} = \frac{Km}{Vmax[S]} = + \frac{1}{Vmax}$$

2.10 Thermal behavior

The thermal behavior of the interesterification reactions was analyzed by differential scanning calorimetry (DSC, TA Instruments, New Castle, DE, USA) using an empty pan as a reference. The DSC was calibrated with indium and nitrogen was used as the carrier gas. An aliquot (10 - 20 mg) of the sample was placed in a DSC pan, covered with a lid, sealed, and placed in the DSC oven stabilized at T_c 25ºC. The starting temperature of the DSC was set to 25ºC. The sample was heated from 25°C to 80°C at a rate of 5°C/ min. Then, the sample was cooled from 80 to -20ºC to ensure complete crystallization of the sample (Lee et al., 2020). Melting parameters were evaluated: onset

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temperature (T_{on}), peak temperature (T_p), and the change in enthalpy associated with the melting process. The analysis was replicated and the melting point was reported as the average of the results from the two runs. DSC diagrams before and after removing FFA from interesterification samples.

2.11 Statistical analysis

Analysis of variance t-tests was performed to test for statistical significance ($\alpha = 0.05$) using SAS Studio (SAS Institute Inc., Cary, NC, USA) and Excel statistics (Excel Office 365). The statistical significance of differences between enzyme activity measurements was tested using t-tests. Statistical significance of differences between TAG, FFA, and a melting point of SBO before and after the interesterification reaction was tested using t-tests.

3. Results and discussion

3.1 Determination of immobilized enzyme activity and kinetics

In the present study, a commercial immobilized lipase (IMT2) was used along with lipases from AF and GS which were immobilized onto silica beads in two stages. In the first stage, silica beads were silanized with 3-aminopropyltriethoxy silane, and amino groups were covalently attached to the support. In the second stage, beads were treated with glutaraldehyde, and the enzymes were immobilized to the supports (Nazari *et al*., 2016). After immobilization, both silica beads had lipase activity (Table 1). The enzyme hydrolysis activity using PNPC2 for IMT2 was ten times higher than the activity for ILAF and ILGS per gram beads. The enzyme activity was similar between ILAF and ILGS (Table 1). Differences are presumed to be due to the amount of lipase immobilized.

Table 1. Enzyme activity and kinetic of immobilized lipases from *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* and a commercial lipase IMT2.

Enzyme	Activity (U/mg)	Vmax (mM/min/mg)	Km (mM)
IMT2	0.100±0.024	0.513±0.031	483.000±0.012
ILGS	0.011±0.002	0.342±0.021	306.032±0.033
ILAF	0.010±0.002	0.390±0.002	359.922±0.025

Values are presented as mean±SD.
p-nitrophenyl acetate as a substrate to determine immobilized enzyme activity.
Lineweaver-Burk plots were used to determine the kinetics of the immobilized enzymes.
Soybean oil and palmitic acid were used as substrates to determine the lipase kinetics.
U/mg = U of immobilized enzyme per mg of silica beads, IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus*

Kinetic constants of interesterification reactions using IMT2, ILGS and ILAF were determined with a different concentration of SBO and PA blends (Table 1). The Michaelis – Menten kinetic constants of Km and Vmax were determined using Lineweaver – Burk plots. The maximum reaction rate (Vmax) of interesterification reaction using IMT2 was 0.51 mM/min/mg and the Km was 483 mM. ILGS showed a Vmax of interesterification reaction of 0.34 mM/min/mg and the Km was 306.03 mM. While the Vmax of interesterification reaction using ILAF was 0.39 mM/min/mg and the Km was 359.92 mM. Based on the results, the Michaelis – Menten kinetic constants (Vmax and Km) of interesterification reactions were similar using IMT2, ILGS and ILAF as the biocatalysts. (Table 1).

These results indicated the possibility of using IMT2, ILGS, and ILAF to interesterify SBO and PA blends. Under immobilization conditions, we found that thermophilic lipases isolated from GS and AF have a stable activity that may help to increase lipase-specific activity after immobilization based on the kinetic results. The increase in specific activity of immobilized lipases has been previously reported (Malcata *et al*., 1992; Garcia *et al*., 1995), and the authors propose that this activity increase is caused by a strong interaction of hydrophobic areas of lipases near or on the lid with the support surface, which leads to conformational changes that result in a highly active open form.

3.2 Time course of interesterification reactions

The time course reaction is an important characteristic of the immobilized enzymes and determines the extent of interesterification. The optimal time for maximum PA mol% incorporated was determined using a time course. Changes in free PA levels during interesterification with SBO are shown in Figures 1 A, B, and C. In each immobilized enzyme and blending ratios system, approximately 50% of the free PA substrate was consumed within the first 12 hrs of the reaction when the substrate ratio was 1:0.25 or 1:0.5. Results show (Figure 1) that there was no significant difference between total PA incorporated for 24 and 36 hrs for all reactions. After PA was almost exhausted, the formation of interesterification products did not improve significantly with additional reaction time.

Figure 1A shows the course time of IMT2. After a 12 hrs reaction, 12 mM% of the free PA was incorporated into the TAGs when the substrate ratio was 1:0.1 and 88 mM% of the total PA for substrate molar ratios 1:0.1 remained. Approximately 50 – 58 mM% of PA was incorporated into the TAGs after 12 hrs when the substrate ratio was 1:0.25 and 1:0.5, respectively. In

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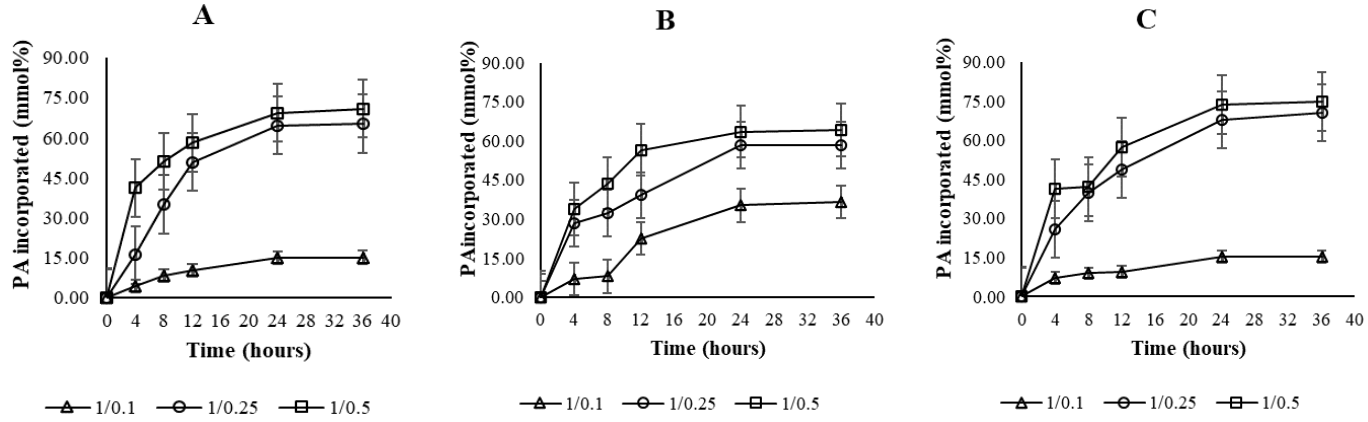

Figure 1. Time course reaction of palmitic acid (PA) and all enzymatic reactions, where (A) incorporation of PA using IMT2 as the biocatalyst, (B) incorporation of PA using ILGS as the biocatalyst, (C) incorporation of PA using ILAF as the biocatalyst. Note: the reaction conditions for PA substrate blends ratios of soybean oil to PA of 1:0.1, 1:0.25, and 1:0.5 at 70°C with 200 rpm shaking, enzyme load of 1% (w/w) ITM2, 3% ILGS and, 3% ILAF. IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus.* Standard deviations of the values are given.

addition, there were no significant differences between all reaction ratios of IMT2 for free PA after 24 hrs and 36 hrs.

However, ILGS incorporated 22 mM% of PA into the TAGs after a 12 hrs reaction when the substrate ratio was 1:0.1 (Figure 1B). Also, ILGS incorporated 58 mM% of PA into the TAGs after 24 hrs and 36 hrs when the substrate ratio was 1:0.25 and 63 – 64 mM% with a 1:0.5 substrate ratio after 24 hrs and 36 hrs reactions. Approximately 10, 48.5, and 57 mM% of PA were consumed within 12 hrs of the reaction using ILAF when the substrate ratio was 1:0.1, 1:0.25, and 1:0.5, respectively (Figure 1C). ILAF incorporated 68 - 70 mM% of PA into the TAGs after 24 hrs and 36 hrs when the substrate ratio was 1:0.25 and 73 – 74 mM% with 1:0.5 substrate ratio after 24 hrs and 36 hrs reactions. After 24 hrs and 36 hrs reactions, total PA mM% incorporation into TAGs was not significant with all reaction ratios.

In each case, 70 - 75% of the substrate was consumed within the first 24 hrs of the reaction, after which the reaction gradually reached equilibrium. After palmitic acid was almost exhausted, the formation of interesterified products did not improve significantly with additional reaction time, although FFA levels increased gradually. Therefore, the reaction time selected for the large-scale reactions was 24 hrs with ratios of 1:0.1, 1:0.25, and 1:0.5 (v/v) of SBO: PA.

3.3 Triacylglycerol composition of interesterification products

TAG composition was determined via HPLC with ELSD. The HPLC separation was dependent on the fatty acid chain length and the total number of double bonds in the TAG molecules (Andrikopoulos, 2002). The ELSD detector had advantages for TAG analysis: it allows direct detection of nonvolatile compounds, it is not sensitive to room temperature fluctuation, it can be used with gradient elution, and it has a higher sensitivity than a RI detector (Kohler *et al*., 1997).

The TAG composition was determined before and after the interesterification reactions. The TAG peak areas were determined and converted to concentration units using linear regression for each standard. As shown in Table 2, the TAG composition of SBO before interesterification was LLL (14.18 µmoL/mL), OOO (2.84 µmol/mL), and MMM (18.94 µmoL/mL). With no detection of LnLnLn and PPP, this result is similar to (Cunha and Oliveira, 2006; Silva *et al*., 2009; Da Silva *et al*., 2011). After interesterification with IMT2 with a 1:0.1 (v/v) ratio MMM and PPP were significantly different from the SBO sample before interesterification (Table 2).

IMT2 changed the TAG composition by increasing the molar concentration of LnLnLn, LLL, OOO, and PPP after 24 hrs with a 1:0.25 and 1:0.5 (v/v) reaction ratios (Table 2). LnLnLn was increased from 0 to 1.60 µmol/mL after 24 hrs with a 1:0.25 and 1:0.5 (v/v) reaction ratios. LLL was increased from 14.18 to 20.87 µmol/mL with a 1:0.25 (v/v) ratio and 29.36 µmol/mL with a 1:0.5 (v/v) reaction ratio. OOO was increased from 2.84 to 15.21 µmol/mL with a 1:0.25 (v/v) ratio and 25.11 µmol/mL with a 1:0.5 (v/v) reaction ratio. PPP was found in all reactions with IMT2 with a gradual increase of concentration from 0 to 5.45 µmol/mL with a 1:0.25 (v/v) ratio and 6.18 µmol/mL with 1:0.5 (v/v) ratio.

The TAG composition was changed using ILGS for all reaction ratios. LnLnLn was increased from 0 to 1.58

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Table 2. Triacylglycerol composition in soybean oil and their blends after lipase catalyzed interesterification.

Samples	TAG (μmol/mL)				
	LnLnLn	LLL	OOO	MMM	PPP
IMT2					
SB	0.00±0.00^{b}	14.18±3.16^{ab}	2.84±0.02^{b}	18.94±2.36^{b}	0.00±0.00^{b}
SP 1:0.1	0.00±0.00^{b}	10.24±8.85^{ab}	8.97±2.10^{b}	42.49±6.86^{a}	5.33±1.17^{a}
SP 1:0.25	1.60±0.03^{a}	20.87±9.95^{ab}	15.21±3.31^{a}	24.57±4.15^{ab}	5.45±0.27^{a}
SP 1:0.5	1.60±0.06^{a}	29.36±0.58^{a}	25.11±1.51^{a}	16.71±2.43^{b}	6.18±1.22^{a}
ILGS					
SB	0.00±0.00^{b}	14.18±3.16^{ab}	2.84±0.02^{b}	18.94±2.36^{b}	0.00±0.00^{b}
SP 1:0.1	1.58±0.10^{a}	6.06±0.22^{b}	3.07±0.53^{b}	33.89±9.70^{ab}	0.00±0.00^{b}
SP 1:0.25	1.56±0.03^{a}	23.30±0.65^{ab}	9.27±0.03^{b}	29.96±0.52^{ab}	0.00±0.00^{b}
SP 1:0.5	1.66±0.01^{a}	29.46±9.42^{a}	17.72±9.66^{a}	20.66±7.63^{b}	0.00±0.00^{b}
ILAF					
SB	0.00±0.00^{b}	14.18±3.16^{ab}	2.84±0.02^{b}	18.94±2.36^{b}	0.00±0.00^{b}
SP 1:0.1	1.83±0.07^{a}	4.28±0.09^{b}	4.05±0.57^{b}	24.81±1.08^{ab}	3.64±0.71^{a}
SP 1:0.25	1.56±0.09^{a}	5.80±2.15^{b}	4.39±1.78^{b}	24.33±7.86^{ab}	5.96±1.11^{a}
SP 1:0.5	1.78±0.22^{a}	10.56±2.37^{ab}	7.60±0.77^{b}	17.97±0.52^{b}	5.87±1.32^{a}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (α = 0.05) within each enzyme system.

SB: Soybean oil, SP: SB mix with palmitic acid at 24-hour reaction with three different blends ratios (1:0.1, 1:0.25, and 1:0.5 v/v), TAG: triacylglycerol, LnLnLn: trilinolenin, LLL: trilinolein, OOO: triolein, MMM: trimyristein, PPP: tripalmitin, IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus*

μmol/mL and MMM was increased from 18.94 to 33.89 μmol/mL with a 1:0.1 (v/v) ratio using ILGS (Table 2). On the other hand, LnLnLn was increased from 0 to 1.56 μmol/mL after a 24 hrs reaction with a 1:0.25 and 1.66 μmol/mL with a 1:0.5 (v/v) ratio using ILGS. LLL was increased from 14.18 to 23.30 μmol/mL with a 1:0.25 (v/v) ratio and 29.46 μmol/mL with a 1:0.5 (v/v) ratio. MMM was increased from 18.90 to 29.96 μmol/mL with a 1:0.25 (v/v) ratio and no significant difference with a 1:0.5 (v/v) ratio. OOO was increased from 2.84 to 17.72 μmol/mL with a 1:0.5 (v/v) ratio and no significant difference with a 1:0.1 and 1:0.25 (v/v) after 24 hrs reaction. In addition, no significant difference between SBO before and after interesterification using ILGS with PPP after 24 hrs reaction.

The composition of PPP and LnLnLn, were significantly different using ILAF after 24 hrs. (Table 2). With ILAF, the composition of MMM increased from 18.94 to 24.81 μmol/mL after 24 hrs with a 1:0.1 and 24.33 μmol/mL with a 1:0.25 (v/v) ratio. LLL composition was decreased from 14.18 to 4.28 μmol/mL after 24 hrs with a 1:0.1 and 5.80 μmol/mL with a 1:0.25 (v/v) ratios using ILAF.

Comparing the enzyme reaction activity between IMT2, ILGS, and ILAF, IMT2 increased tripalmitin (PPP) for all reaction ratios. ILGS changed some of the TAG by increasing or decreasing the concentrations of LnLnLn, LLL, and MMM. In addition, ILAF changed the TAG composition, it was similar to IMT2 activity by increasing PPP concentrations. According to these results, each immobilized enzyme changed the composition of FFA on TAGs. Interesterification products were produced with each immobilized enzyme used, at all reaction ratios, by changing TAG composition in SBO after interesterification. IMT2 and ILAF produced similar changes in the TAGs of SBO, especially with increasing tripalmitin and trilinolenin. This is due to the enzyme specificity towards changing the FFA on glycerol. Studies indicated that the fatty acids originally located at the sn-2 position should largely remain in this position even though some degree of acyl migration into the sn-1,3 position might occur (Zhang *et al*., 2001). This indicates that palmitic acid after enzymatic interesterification would be more commonly found in the sn-1,3 position. Conversely, ILGS was less active with palmitic acid and changed the FFA location at sn-1,3. Also, previous studies indicated that commercial immobilized lipase from *Thermomyces lanuginosus* specifically acts at the *sn*-1,3 positions (Torres *et al*., 2002; Utama *et al*., 2020).

3.4 Free fatty acid composition of interesterification products

The separation of FFA generated from interesterification reactions was achieved using HPLC with an ELSD detector. After the interesterification reaction was complete, samples for all reaction ratios were washed with ethanol to extract the FFA and then compared with SBO. Table 3 shows the total FFA

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compositions mg/g SBO and those of the interesterified blends obtained from the reactions with the three different immobilized enzymes. The SBO sample shows no FFA before blending, which indicates that all FFA were anchored to the TAG molecules (Table 3).

IMT2 was used as a biocatalyst to change FFA compositions at different reaction ratios. After a 24 hrs reaction, C14:0, C16:0, C18:1, and C18:2 after interesterification were significantly different than before interesterification (Table 3). Using IMT2 with a 1:0.1 (v/v) ratio, C14:0 was increased from 0 to 4.33, C16:0 was increased from 0 to 17.4, C18:1 was increased from 0 to 32.43, and C18:2 was increased from 0 to 82.65 with all values in mg/g SBO. On the other hand, using IMT2 with a 1:0.25 and 1:0.5 (v/v) ratio, C14:0 was increased from 0 to 4.88 and 4.58, C16:0 was increased from 0 to 57.66 and 92.82, C18:0 was increased from 0 to 1.24 and 10.92, C18:1 was increased from 0 to 35.72 and 43.08, and C18:2 was increased from 0 to 88.95 and 98.09 with all values in mg/g SBO (Table 3).

The composition of C14:0, C16:0, C18:0, C18:1 and C18:2 with a 1:0.1 (v/v) ratio after 24 hrs interesterification reaction was changed using ILGS. Using ILGS with a 1:0.1 (v/v) ratio, C14:0 was increased from 0 to 1.24, C16:0 was increased from 0 to 47.70, C18:0 was increased from 0 to 0.82, C18:1 was increased from 0 to 5.20, and C18:2 was increased from 0 to 13.34 with all values in mg/g SBO (Table 3). After the interesterification reaction of SBO, the composition of C16:0 was 94.66 mg/g with a 1:0.5 (v/v) ratio of SBO to PA using ILAF. Also, C18:0 with a 1:0.25 and 1:0.5 (v/v) ratio of SBO to PA after 24 hrs interesterification reaction was not produced using ILAF (Table 3). ILAF produced higher linoleic acid than ILGS, while C18:2 was similar to IMT2 and ILAF (Table 3). Oleic acid C18:1 was the lowest using ILGS while it was highest when using ILAF and IMT2. PA values were high with all three lipases presumably because PA was used as a substrate for the interesterification reactions.

Based on the results, the major FFA of SBO after interesterification with a 1:0.5 (v/v) ratio was C18:2, which accounted for 98.09 using IMT2, 113.58 using ILAF, and 30.65 using ILGS with all values in mg/g SBO. The next most abundant FFA was C16:0: 92.82 using IMT2, 94.66 using ILAF, and 114.77 using ILGS with all values in mg/g SBO. This value may have been high due to it being added to the reactions. Finally, the last was C18:1: 43.08 using IMT2, 39.66 using ILAF, and 10.87 using ILGS with all values in mg/g SBO (Table 3). These results were close to or similar to previous studies (Ribeiro *et al*., 2009; Silva *et al*., 2009).

Interesterification reactions with all the immobilized enzymes used in this study were achieved through the rearrangement of FFA and results in the formation of TAGs with varying compositions compared to SBO. The specificity of the enzyme was different between IMT2, ILGS, and ILAF. IMT2 and ILAF produced more myristic, oleic, and linoleic acid than ILGS. Palmitic

Table 3. Free fatty acid compositions (mg/g) in soybean oil and blends after lipase-catalyzed interesterification.

Samples	Fatty acid compositions (mg of free fatty acid/g of soybean oil)				
	C14:0	C16:0	C18:0	C18:1	C18:2
IMT2					
SB	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{d}
SP 1:0.1	4.33±0.92^{ab}	17.41±3.24^{cde}	0.00±0.00^{b}	32.43±7.98^{a}	82.65±6.16^{ab}
SP 1:0.25	4.88±0.60^{ab}	57.66±1.92^{bc}	1.24±0.33^{b}	35.72±2.39^{a}	88.95±8.98^{a}
SP 1:0.5	4.58±0.02^{ab}	92.82±8.31^{ab}	10.92±2.19^{a}	43.08±10.38^{a}	98.09±6.05^{a}
ILGS					
SB	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{d}
SP 1:0.1	1.24±0.27^{bc}	47.70±5.82^{bcd}	0.82 ±0.05^{b}	5.20±1.79^{bc}	13.34±4.29^{cd}
SP 1:0.25	2.26±0.15^{bc}	89.86±8.07^{ab}	1.31±0.09^{b}	8.04±0.40^{bc}	24.85±6.18^{cd}
SP 1:0.5	2.45±0.02^{bc}	114.77±2.54^{a}	1.66±0.11^{b}	10.87±0.06^{bc}	30.65±0.10^{cd}
ILAF					
SB	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{d}
SP 1:0.1	1.98±0.07^{bc}	3.03±0.43^{de}	0.00±0.00^{b}	11.64±0.60^{bc}	24.52±2.29^{cd}
SP 1:0.25	3.45±0.29^{bc}	27.01±3.83^{cde}	0.00±0.00^{b}	23.83±2.09^{ab}	58.26±7.15^{abc}
SP 1:0.5	7.95±3.13^{a}	94.66±9.27^{ab}	1.00±0.03^{b}	39.66±1.92^{a}	113.58±4.57^{a}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (α = 0.05) within each enzyme system.

SB: Soybean oil, SP: SB mix with palmitic acid at 24-hour reaction with three different blends ratios (1:0.1, 1:0.25, and 1:0.5 v/v), C12:0: lauric, C14:0: myristic, C16:0: palmitic, C18:0: stearic, C18:1: oleic, C18:2: linoleic, IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus*

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acid was still present following the reaction because levels were saturated during the interesterification. Based on the results of Table 2 and Table 3, ILAF was more active and substrate-specific to change the TAG and FFA content of SBO compared to ILGS. ILAF had the ability to change the FFA in the sn-1,3 position and potentially change the sn-2 position on the glycerol backbone.

3.5 Thermal behaviours of interesterification products

Products of interesterification reactions will affect physical properties including the crystallization point and melting point of oils and lipids (Karabulut *et al*., 2004). Thermal attributes were assessed using DSC analysis to determine the melting point of interesterified samples before and after removing FFA. The different melting parameters calculated from these thermograms include the onset melting temperature (T_{on}), peak melting temperature (T_p), and melting enthalpy (ΔH).

DSC values are tabulated in Table 4, for the SBO samples after interesterification and before removing FFA. The T_{on}, T_p, and ΔH values of SBO samples after interesterification and after removing FFA are tabulated in Table 5. Melting peaks were significantly different between SBO samples before and after the interesterification reactions. Analysis of SBO samples, with all reaction ratios, three melting peaks were observed after interesterification reactions (Tables 4 and 5). The onset melting temperature (T_{on}), peak melting temperature (T_p), and melting enthalpy (ΔH) changed with all sample peaks samples after interesterification. The melting parameters of SBO after enzyme-catalyzed interesterification and after removing FFA (Table 5) were lower than the melting parameters of SBO before FFA removal (Table 4). Rearrangement or randomization of acyl residues in TAGs has provided fats or oils with new physical properties (Zeitoun *et al*., 1993).

The highest melting parameters were selected to compare enzyme interesterification samples before and after FFA removal (Table 6). There was a decrease in the peak melting temperatures (T_p) of the IMT2 with a 1:0.1 reaction ratio from 29.97°C to 4.15°C. However, the T_p of IMT2 was not different with a 1:0.25 and 1:0.5 (v/v) ratio: 33.35°C to 37.12°C and 40.65°C to 40.30°C, respectively (Table 6). The peak melting temperatures (T_p) before and after removing FFA were different for all reaction ratios with ILGS (Table 6). Similar results were found using ILAF to decrease the peak melting temperature (T_p) after removing FFA.

Based on the results of Table 3, increased C16:0 was found with an increase in the reaction ratios, which caused changes in the melting temperature, since palmitic acid was used as a substrate with SBO. Also, increased oleic and linoleic (Table 6) will have a large effect on changes in the SBO profile and changes in the melting point. In addition, the specificity of the enzyme was different between IMT2, ILGS, and ILAF. IMT2 and ILAF were active during the interesterification reactions to change the positions of FA and produce FFA which changed the melting point of SBO. The results showed that all immobilized enzymes changed the melting temperature of SBO after interesterification. This corresponds well with the change in TAG and FFA compositions of SBO after interesterification with IMT2, ILAF, and ILGS. These changes in the vegetable oil profile would increase or decrease the melting point from the original (25°C), which has great potential for applications in the food industry, such as with confections (Yassin *et al*., 2003).

4. Conclusion

Commercial immobilized lipases are available for industrial enzymatic interesterification to produce fats and oils. In recent years, the development of cost-effective immobilized lipases is improving the economic viability of the enzymatic interesterification process (Ifeduba *et al*., 2016). Lipases from GS and AF were successfully immobilized on solid supports. Further, the enzyme activities of ILGS and ILAF can be used for interesterification. ILGS and ILAF were compared with IMT2 for the ability to change the profile of SBO. The catalytic performance of the enzymes was evaluated by determining the changes in FFA and TAG composition and concentrations. This study demonstrated that enzymatic interesterification significantly changed the TAG and FFA composition, and consequently modified the melting point of the SBO blends with PA. All reaction ratios of SBO and PA changed the TAG concentration and produced FFA as a by-product. The melting temperature of SBO after interesterification was changed before and after removing FFA with a temperature range of 4°C to 40°C. IMT2 and ILAF changed the melting point of SBO. This SBO profile with new physical properties produced from interesterification can be used for example, in making chocolate substitutes. The interesterification processing of oil is safe, easy, and cost-effective and can be used for application in the food industry.

Conflict of interest

The authors declare no conflict of interest.

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Table 4. Onset temperature (T_{on}, °C), peak temperature (T_p, °C), and melting enthalpy (ΔH, J/g) of soybean oil after lipase-catalyzed interesterification before removing free fatty acids.

Sample	Peak 1		Peak 2			Peak 3		
	Tp (°C)	ΔH(J/g)	Ton (°C)	Tp (°C)	ΔH(J/g)	Ton (°C)	Tp (°C)	ΔH(J/g)
IMT2								
SB	-10.4±0.0^{a}	6.3±1.0^{cd}	ND	ND	ND	ND	ND	ND
SP 1:0.1	-12.1±1.0^{a}	3.0±1.5^{d}	-4.0±0.1^{e}	2.5±0.0^{f}	9.8±3.0^{bc}	11.1±1.0^{d}	29.9±0.7^{abc}	0.6±0.0^{d}
SP 1:0.25	-13.8±0.2^{a}	7.5±2.0^{cd}	-2.5±0.0^{e}	5.2±0.1^{de}	10.8±0.1^{b}	19.6±0.0^{bc}	33.3±0.4^{ab}	17.2±0.3^{b}
SP 1:0.5	-14.4±0.1^{a}	10.1±1.4^{bc}	3.7±0.6^{d}	7.6±0.1^{d}	1.3±0.2^{ed}	27.8±0.5^{a}	40.6±0.0^{a}	39.5±2.2^{a}
ILGS								
SB	-10.4±0.0^{a}	6.3±1.0^{cd}	ND	ND	ND	ND	ND	ND
SP 1:0.1	-12.5±1.9^{a}	9.5±3.1^{bc}	3.4±2.2^{d}	5.9±1.9^{de}	0.4±0.0^{e}	14.6±3.3^{cd}	20.7±0.5^{bc}	0.5±0.0^{d}
SP 1:0.25	-12.9±1.0^{a}	10.6±2.0^{bcd}	17.8±4.2^{c}	38.5±0.1^{c}	8.8±0.7^{bc}	ND	ND	ND
SP 1:0.5	-14.5±0.0^{a}	14.4±0.5^{b}	36.7±0.0^{a}	49.7±0.1^{a}	38.1±0.3^{a}	ND	ND	ND
ILAF								
SB	-10.4±0.0^{a}	6.3±1.0^{cd}	ND	ND	ND	ND	ND	ND
SP 1:0.1	-12.6±0.7^{a}	7.2±0.4^{cd}	-2.9±1.0^{e}	2.2±0.3^{f}	5.4±1.2^{cd}	9.5±0.5^{d}	12.7±0.6^{c}	0.5±0.0^{d}
SP 1:0.25	-16.1±0.1^{a}	5.8±2.8^{cd}	-3.5±0.8^{e}	4.5±0.4^{ef}	9.1±1.9^{bc}	20.3±1.3^{b}	30.7±2.4^{abc}	9.4±4.9^{c}
SP 1:0.5	-10.5±5.2^{a}	32.4±1.0^{a}	23.4± 0.3^{b}	44.3±0.0^{b}	34.8±0.4^{a}	ND	ND	ND

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (α = 0.05) within each enzyme system.

SB: Soybean oil, SP: SB mix with palmitic acid at 24-hour reaction with three different blends ratios (1:0.1, 1:0.25, and 1:0.5 v/v), IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus,* ND: Not detected.

Table 5. Onset temperature (T_{on}, °C), peak temperature (T_p, °C), and melting enthalpy (ΔH, J/g) of soybean oil after lipase-catalyzed interesterification after removing free fatty acids.

Sample	Peak 1		Peak 2			Peak 3		
	Tp (°C)	ΔH(J/g)	Ton (°C)	Tp (°C)	ΔH(J/g)	Ton (°C)	Tp (°C)	ΔH(J/g)
IMT2								
SB	-10.4±0.0^{b}	6.3±1.0^{cd}	ND	ND	ND	ND	ND	ND
SP 1:0.1	-12.8±0.1^{cde}	9.9±1.7^{bc}	-1.4± 0.1^{ef}	4.1±0.4^{b}	15.0±0.0^{a}	ND	ND	ND
SP 1:0.25	-12.5±0.5^{cd}	14.2±1.1^{b}	2.6± 0.9^{bc}	5.5±1.1^{b}	1.4±0.0^{d}	11.1±2.8^{ab}	37.1±0.7^{a}	14.8±8.4^{b}
SP 1:0.5	-2.4±0.2^{a}	19.0±1.2^{a}	6.2± 0.2^{a}	10.4±0.2^{a}	5.1±0.0^{c}	10.4±0.2^{ab}	40.3±0.3^{a}	13.9±0.0^{bc}
ILGS								
SB	-10.4±0.0^{a}	6.3±1.0^{cd}	ND	ND	ND	ND	ND	ND
SP 1:0.1	-11.7±0.4^{c}	10.4±0.7^{bc}	1.1± 0.0^{cd}	3.7±0.0^{b}	1.1±0.3^{d}	9.3±0.5^{b}	13.7±0.3^{c}	1.0±0.2^{d}
SP 1:0.25	-13.9±0.2^{def}	13.2±1.7^{b}	1.2± 0.1^{cd}	5.7±1.4^{b}	1.2±0.0^{d}	12.1±2.4^{ab}	22.4±2.3^{b}	1.1±0.1^{d}
SP 1:0.5	-14.5±0.0^{f}	12.8±1.6^{b}	3.8± 0.0^{b}	8.8±1.2^{a}	0.7±0.2^{d}	12.0±0.2^{ab}	26.1±1.0^{b}	2.2±0.3^{cd}
ILAF								
SB	-10.4±0.0^{a}	6.3±1.0^{cd}	ND	ND	ND	ND	ND	ND
SP 1:0.1	-14.1±0.8^{ef}	7.6±0.0^{c}	-2.5± 1.1^{f}	3.5±0.4^{b}	8.6±0.6^{b}	8.9±0.3^{b}	13.3±0.3^{c}	33.8±4.5^{a}
SP 1:0.25	-12.6±0.5^{cde}	10.0±1.5^{bc}	-0.1± 0.0^{def}	3.8±0.1^{b}	6.0±0.0^{c}	11.8±0.0^{ab}	17.1±0.1^{c}	9.6±0.1^{bcd}
SP 1:0.5	-16.6±0.3^{g}	2.7±1.4^{d}	-0.6± 0.2^{def}	-12.7±0.7^{c}	14.4±0.7^{a}	15.2±1.6^{a}	22.0±2.0^{b}	20.5±0.2^{b}

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different (α = 0.05) within each enzyme system.

SB: Soybean oil, SP: SB mix with palmitic acid at 24-hour reaction with three different blends ratios (1:0.1, 1:0.25, and 1:0.5 v/v), IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus,* ND: Not detected.

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Table 6. Comparison of peak temperature (Tp) of soybean oil after lipase-catalyzed interesterification before and after removing fatty acids.

Sample	Before removing fatty acids Tp (°C)	After removing fatty acids Tp (°C)
IMT2		
SB	-10.4±0.0	-10.4±0.0
SP 1:0.1	29.9±0.7	4.1±0.4
SP 1:0.25	33.3±0.4	37.1±0.7
SP 1:0.5	40.6±0.0	40.3±0.3
ILGS		
SB	-10.4±0.0	-10.4±0.0
SP 1:0.1	20.7±0.5	13.7±0.3
SP 1:0.25	38.5±0.1	22.4±2.3
SP 1:0.5	49.7±0.1	26.1±1.0
ILAF		
SB	-10.4±0.0	-10.4±0.0
SP 1:0.1	12.7±0.6	13.3±0.3
SP 1:0.25	30.7±2.4	17.1±0.1
SP 1:0.5	44.3±0.0	22.0±2.0

Values are presented as mean±SD. Values with different superscripts within the same column are significantly different ($\alpha = 0.05$) within each enzyme system.
SB: Soybean oil, SP: SB mix with palmitic acid at 24-hour reaction with three different blends ratios (1:0.1, 1:0.25, and 1:0.5 v/v), IMT2: commercial immobilized lipase, ILGS: immobilized lipase from *Geobacillus stearothermophilus,* ILAF: immobilized lipase from *Anoxybacillus flavithermus.*

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