Biotechnology Reports xxx (2018) e00246

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Synthesis of designer triglycerides by enzymatic acidolysis

Monali R. Kavadia, Manish G. Yadav, Annamma A. Odaneth*, Arvind M. Lali

DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology Matunga, Mumbai, 400019, India

ARTICLE INFO

Article history:
Received 22 November 2017
Received in revised form 27 February 2018
Accepted 6 March 2018
Available online xxx

Keywords:
Acidolysis
Fully hydrogenated soybean oil
Caprylic acid
Immobilized lipase

ABSTRACT

Enzymatic acidolysis process was developed for modification of fully hydrogenated soybean oil (FHSO) by incorporation of caprylic acid, a medium chain fatty acid. Immobilized sn-1,3 specific lipase PyLip was used to modify FHSO to produce a new fat with improved physico-chemical and functional properties. PyLip mediated acidolysis resulted in 88% reduction of substrate triglycerides and 45.16% incorporation of caprylic acid in FHSO at molar ratio of 1:3 of FHSO and caprylic acid in 60 min reaction time. HPLC analysis revealed formation of mono-substituted and di-substituted TAGs post enzymatic acidolysis. Physical properties of synthesized lipid were studied using DSC and XRD and considerable change was observed in the final product compared to the starting material. The present study reports a faster acidolysis process in the presence of solvent enhancing the modification of FHSO with caprylic acid and having no side products formation (monoglycerides and diglycerides) making the entire process highly efficient and commercially attractive.

1. Introduction

Designer triacylglycerols (TAGs) are TAGs that have been modified by incorporation of new fatty acids, restructured to alter the fatty acid position or composition of natural oils and fats to obtain desired physico-chemical, nutritional, and functional properties. Such designer lipids are expected to be an efficient source of medium chain and essential fatty acids [1]. Acidolysis involves reaction between a fatty acid and oil/fat and the fatty acid and TAG source can be selected based on the desirable final properties and application of product. Using acidolysis strategy, physico-chemical properties of oils and fats can be altered to suit specific applications and can provide a useful means for production of new class of lipids. Few designer lipids are being manufactured commercially having novel combination of fatty acids by acidolysis route. Caprenin, a structured lipid produced by Procter & Gamble Company consists of caprylic acid: capric acid:behenic acid and resembles cocoa butter in physical properties but has only half of its calories. Benefat developed by Nabisco Foods is another commercially produced structured lipid that contains a mixture of short chain and long chain fatty acids. Other products available commercially include Captex, Neobee, Intralipid which are also composed of a mixture of long chain and medium chain fatty acids in different combinations. However, all of these products are manufactured industrially by chemical based processes. Chemical methods are catalyzed by alkali metals under high temperatures and anhydrous conditions. Due to harsh reaction conditions, several unwanted products are formed that require extensive post-processing steps. On the other hand, attempts, have been made by the food industry to employ enzymatic acidolysis for production of designer lipids and very few products are being manufactured commercially through this route such as Betapol produced by Loders Croklaan. The synthesis methodology for these products basically involves use of single step process where either a fatty acid or fatty acid ester is used as acyl donor to bring about modification of substrate TAGs by 1, 3-regiospecific lipase. In this process of synthesis, different types of acyl donors such as ethyl stearate, stearic acid, palmitic acid, caproic acid have been reported to modify various types of substrate oils of vegetable and animal origin such as palm oil midfraction, safflower oil, linseed oil [2,3].

sn-1,3 specific lipases are effective catalysts for carrying out acidolysis reaction over classical chemical catalysts because of their ability to perform regiospecific alteration on glycerol backbone of TAG producing specific restructured end products [4,5]. Although lipase catalyzed acidolysis has significant benefits, the industrial application of the technology has been slow because of several constraints such as longer reaction times (6 h to 72 h), use of commercial expensive biocatalysts, low conversion/fatty acid incorporation, use of high molar ratio of substrates, low yield due to hydrolysis and formation of secondary products (MAGs and

* Corresponding author.
E-mail addresses: monali.kavadia@gmail.com (M.R. Kavadia), manishy222@gmail.com (M.G. Yadav), a.annamma@ictmumbai.edu.in (A.A. Odaneth), am.lali@ictmumbai.edu.in (A.M. Lali).

https://doi.org/10.1016/j.btre.2018.e00246

DAGs), extensive downstream separation of by-products [6–14]. For widespread industrial use, the process needs to be technically and economically feasible. In the present study, an efficient enzymatic acidolysis process was developed to overcome the shortcomings reported in literature. In the present study, acidolysis reaction was carried out between fully hydrogenated soybean oil (FHSO) and caprylic acid as starting materials using indigenously immobilized sn-1,3 specific lipase preparation PyLip. FHSO a low-cost product with limited applications was chosen as starting material for acidolysis reaction. FHSO is a hard fat produced by hydrogenation of soybean oil composed of stearic acid and palmitic acid which is used as frying oil, ingredient for margarine, shortenings, as confectionary fat, and in stabilizer applications [15]. Soybean oil has a high content of monounsaturated and polyunsaturated fatty acids whereas FHSO consists of entirely saturated fatty acids namely stearic and palmitic acid of which stearic forms the major component. As a result, FHSO has a better oxidative stability as compared to soybean oil and hence is a good candidate for value addition with medium chain fatty acids for obtaining steeper melting profiles coupled with good oxidative stability. Clinical studies have shown that the effect of stearic acid in spite of being saturated fatty acid is similar to that of oleic acid on plasma lipoprotein concentrations and has a neutral effect on cholesterol and TAG levels in the body. This is in contrast to the hypercholesterolemic effect of other long-chain saturated fatty acids and hence stearic acid has no cardiovascular risk which makes FHSO a good candidate for value addition by incorporation of medium chain fatty acid (MCFA) to produce metabolically useful TAGs [16,17]. Enzymatic acidolysis of FHSO by caprylic acid in the present study led to synthesis of designer lipids containing both medium chain fatty acid (MCFA) and long chain fatty acid (LCFA) in the same TAG molecule. Effect of various key reaction parameters such as molar ratio of FHSO and caprylic acid, enzyme load, substrate concentration, reaction time and reaction temperature on acidolysis efficiency was investigated. The final product recovered after acidolysis exhibited formation of new triglycerides and significant changes in the physical properties were observed by differential scanning calorimetry and X-ray diffraction. The developed single step acidolysis process yields 88% conversion of the substrate TAG, shortened reaction time, high incorporation of caprylic acid (45%), use of low cost indigenously immobilized lipase and no by-production formation (MAGs and DAGs) greatly minimizing downstream processing steps. The reaction scheme for lipase catalyzed acidolysis reaction is shown in Scheme 1.

2. Materials and methods

2.1. Chemicals and lipases

All chemicals and solvents used were of analytical or chromatographic grade and purchased from SD Fine Chemicals (Mumbai, India). Soluble *Thermomyces lanuginosus* lipase [TLL] was purchased from DK Enzymes & Chemicals (India). Indion PA 500 resin was purchased from Ion Exchange, (India). TLL was indigenously immobilized at DBT-ICT Centre for Energy Biosciences, Mumbai, India. Reference standards of tristearin, distearin, caprylic acid and TAGs of varying equivalent carbon number (ECN) ($>99.0\%$) were purchased from Sigma-Aldrich (USA). Fully hydrogenated soybean oil was provided by General Mills, USA. Caprylic acid was purchased from SD Fine Chemicals (Mumbai, India). All chemicals and solvents were used without any modification/purification.

2.2. Experimental methodology

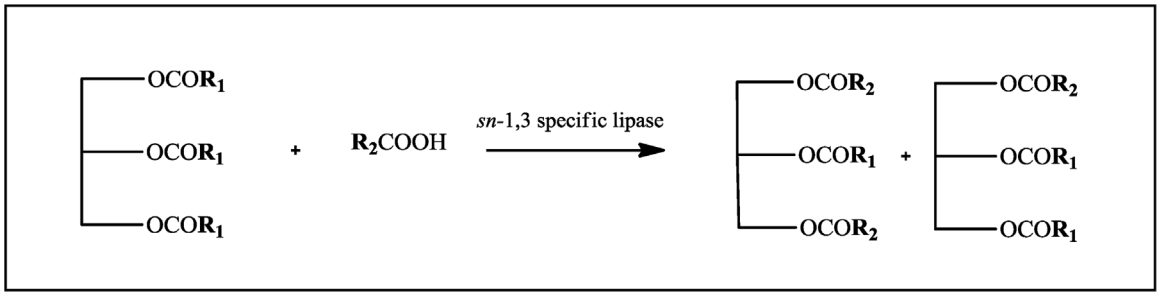
2.2.1. Lipase-catalyzed acidolysis reaction

Acidolysis reaction between fully hydrogenated soybean oil (FHSO) and caprylic acid was set up in 100 mL conical flask with stoppered cap, thermostated to the desired operating temperature and reaction blend was mixed using orbital shaker (Thermo Scientific MaxQ 4000 benchtop orbital shaker). Appropriate amount of PyLip enzyme was added to the reaction mixture to start acidolysis reaction. Samples were withdrawn from reaction mixture at pre specified time intervals, solvent was evaporated and 10 mg/mL sample was prepared in HPLC grade chloroform. Sample were analyzed using method developed on reversed-phase high performance liquid chromatography (RP-HPLC) equipped with evaporative light scattering detector (ELSD) as explained in Section 2.3.

2.3. Analysis of reaction mixture for determination of tristearin reduction by HPLC

Reaction products were analyzed by reversed-phase high performance liquid chromatography (HPLC) in an Agilent 1200 Chromatography System (CA, USA) equipped with a quaternary solvent delivery module, and an Agilent evaporative light scattering detector (ELSD). Hexane was evaporated from the reaction mixture and 10 mg/mL of sample was prepared in HPLC grade chloroform and 10 μL sample was automatically injected on Agilent Zorbax XDB ODS 5 μM, 4.6 × 150 mm column. Separation was obtained by gradient elution using acetonitrile and dichloromethane as solvent system. The flow rate was maintained at 1 mL/min and total run time was kept at 30 min. Calibration curve was plotted for reference standard of tristearin and equation was calculated which was used for determination of percent reduction of tristearin. Triglycerides of varying equivalent carbon number (ECN) were used to identify new TAGs formed post acidolysis reaction. Percentage conversion was determined on the basis of reduction of tristearin peak in the final reaction mixture using standard calibration curve of tristearin as follows:

$$\%conversion = \frac{mgtristearininfinalreactionmixture}{mgtristearininstartingreactionmixture} \times 100 \qquad (1)$$



Scheme 1. Reaction scheme showing lipase catalyzed acidolysis reaction.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

2.4. Analysis of percent incorporation of caprylic acid post acidolysis by HPLC

HPLC method developed in Section 2.3 for determination of tristearin reduction prevented estimation of caprylic acid incorporation because of very low scattering of small fatty acids on ELSD. Hence caprylic acid incorporation was determined using a different method developed on HPLC with UV detector at 205 nm. Caprylic acid was extracted from starting and final reaction mixture using methanol. Sample was treated with methanol three times and methanol fraction was pooled and centrifuged. Upper layer was separated and solvent was removed by distillation. The obtained sample was dissolved in HPLC grade *tert*-butyl alcohol and 10 μL sample was automatically injected on Agilent Zorbax XDB ODS 5 μM, 4.6 × 150 mm column. Separation was obtained by gradient elution using acetonitrile and water as solvent system. The flow rate was maintained at 1 mL/min and total run time was kept at 20 min. Calibration curve was plotted for reference standard of caprylic acid and equation was calculated which was used for determination of percent incorporation of caprylic acid. Percentage incorporation was determined on the basis of reduction of caprylic acid peak in the final reaction mixture using standard calibration curve of caprylic acid as follows:

$$\%incorporation = \frac{mgcaprylicacidinstartingreactionmixture - mgcaprylicacidinfinalreactionmixture}{mgcaprylicacidinstartingreactionmixture} \times 100 \quad (2)$$

2.5. Purification of triglycerides

Separation and recovery of triglycerides (TAGs) post acidolysis was done by titration of the reaction mixture using 0.5 N aq. NaOH. This base treatment led to neutralization of residual free fatty acid (both unreacted caprylic acid and released stearic acid due to acidolysis) and its conversion into sodium salts of fatty acid. TAGs and sodium salts of fatty acids were separated by liquid-liquid extraction using hexane and methanol-glycerin mixture. The hexane layer was collected and evaporated to obtain purified TAGs free from any fatty acids. The presence of pure TAG in the extract was checked by HPLC acylglycerol analysis using the method described in Section 2.3. The obtained sample was then subjected to estimation of saponification value, DSC analysis and XRD analysis (Fig. 1).

2.6. Saponification value estimation

Saponification value of purified sample was determined according to AOCS method Cd 3–25. In summary, 20 mL of 1N alcoholic KOH and 25 mL of solvent mixture (2:1 ratio of methanol and isopropanol) was added to 2 g of purified reaction mixture and this mixture was subject to reflux for 1 h to carry out saponification. The obtained sample solution was then titrated using 0.5 N HCL to obtain the saponification value.

2.7. DSC analysis

Differential scanning calorimetry (DSC) 2010 (Mettler Toledo, STARe software) was used to obtain the melting thermograms of the starting material and final reaction mixture. An empty aluminum pan was used as a reference, and each sample was accurately weighed (5 ± 0.1 mg) for DSC analysis. The sample was heated to 80 °C and held for 10 min. Thereafter, the temperature was decreased at 5 °C/min to −40 °C. After holding for 10 min at −40 °C, the melting curve was obtained by heating to 80 °C at 5 °C/min.

2.8. X-ray diffraction

Melted fat samples were placed on rectangular plastic molds, and tempered at 24 °C for 16 h. Polymorphic forms of the samples were determined by XRD-6100 X-ray diffractometer with a fine copper X ray tube, operating at 40 kV and 35 mA. The short spacing was observed in the 2θ range of 10 to 40°, and scan rate was set at 2°/min. The $\beta\not{c}$ form was identified with 2 strong spacing at approximately 3.8 and 4.2°A whereas β form was identified with strong spacing at approximately 4.6°A [18].

2.9. Statistical analysis

All tests were performed in duplicate. The effect of different reaction parameters such as molar ratio of substrates, reaction

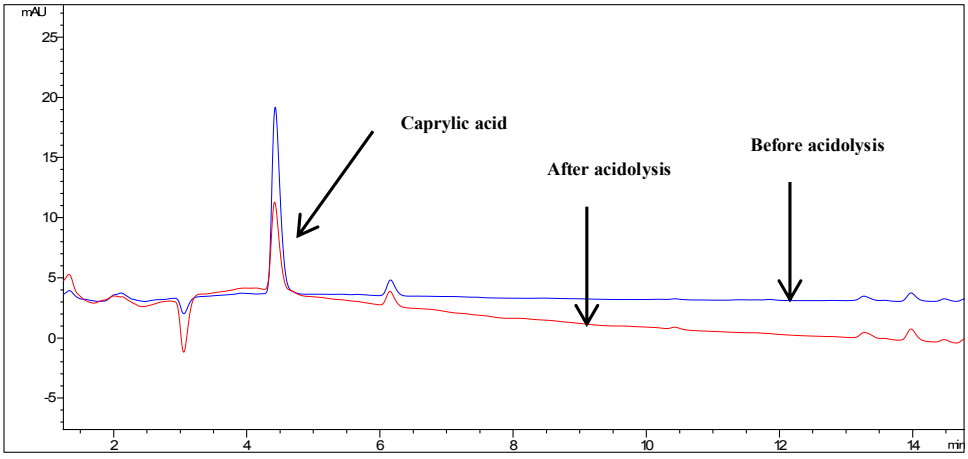


Fig. 1. Overlay HPLC chromatogram showing caprylic acid before and after acidolysis reaction of FHSO. Extraction and separation was performed as described in Section 2.4.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

time, reaction temperature, enzyme load and substrate concentration on caprylic acid incorporation and tristearin reduction and their interaction were analyzed using ANOVA (two-way) statistical analysis using Graphpad Prism (Version 7.0, USA). The results were given as means ± standard deviation (SD). For all statistical comparisons, the level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Selection of lipase for acidolysis reaction

Soluble *Thermomyces lanuginosus* lipase [TLL] is a sn-1, 3 specific lipase that targets the terminal positions of triglycerides and is a highly effective catalyst to carry out position specific replacement of fatty acids in triglycerides [19]. In the present study, TLL was immobilized on polystyrene divinyl-benzene beads of Indion PA 500 resin by physical adsorption using method adapted from Yadav et al. [20,21]. Indion PA 500 is a polystyrene divinyl-benzene support which is highly hydrophobic in nature and is a cheaper alternative to hydrophobic supports available commercially. The immobilized *Thermomyces lanuginosus* enzyme preparation was named as PyLip and used as biocatalyst for acidolysis reaction. The hydrolytic and esterification activities were measured using tributyrin hydrolytic assay and butyl oleate assay as shown in Table 1. Before carrying out acidolysis reaction between FHSO and caprylic acid, properties of indigenously immobilized PyLip lipase and commercial Lipozyme TL IM were determined. Table 1 gives details of properties of Lipozym TL IM and indigenously immobilized PyLip lipase. From the data obtained, it was found that PyLip lipase is competitive with merchandized Lipozyme TL IM and was used for synthesis of modified fat by acidolysis of FHSO and caprylic acid.

3.2. Preliminary experimental data

Fully hydrogenated soybean oil used in the present study consisted of two types of triglycerides which were identified as tristearin (ECN = 54) and a TAG (ECN = between 48 and 54) determined by HPLC analysis of reference standards of a range of TAGs with varying ECN values. Percent distribution of the two TAGs was found to be 85% tristearin and 15% other TAG as per HPLC analysis described in Section 2.3. Also the fatty acid composition of fully hydrogenated soybean oil was determined by GC–MS analysis and found to be consisting of 90% stearic acid and 10% palmitic acid. Based on HPLC and GC–MS analysis, it was concluded that the product TAG has a mixed composition consisting of both stearic acid and palmitic acid. In a preliminary experiment, reaction was set up using 1:2 molar ratio of FHSO and caprylic acid at 10% w/v substrate concentration in hexane using 20% w/w PyLip lipase at 60 °C. Reaction was carried out for 24 h and reaction mixture was analyzed using method described in Section 2.3. Reduction in tristearin content and incorporation of caprylic acid was taken as the basis for determination of degree of acidolysis. HPLC chromatograms showed appearance of two new peaks of triglycerides post acidolysis reaction of FHSO with caprylic acid by PyLip lipase. Taking into account the results obtained in preliminary experiment, detailed study of various reaction parameters influencing acidolysis reaction was carried out.

Table 1
Comparative details of commercial Lipozyme TL IM and indigenously immobilized PyLip lipase [19].

	Lipozyme TL IM	PyLip
Tributyrin hydrolytic activity (U)	554	593
Butyl oleate esterification activity (U)	159	186
Protein load (mg/mL of resin)	–	60
Surface area (m^2/g)	–	500
Water content (%)	1–2%	0.5–1%
Immobilization matrix	Granulated silica gel	Indion PA 500-poly styrene divinyl benzene

3.3. Effect of molar ratio of substrates

In the present study, reduction in tristearin content was taken as the basis for determination of degree of acidolysis. Effect of molar ratio of FHSO and caprylic acid was studied to obtain the most optimum substrate molar ratio that will give maximum degree of acidolysis. Optimum molar ratio of substrates is required to obtain maximum reduction of substrate TAGs and formation of new TAGs enriched with medium chain fatty acid. Molar ratio of FHSO to caprylic acid was varied from 1:1 to 1:4. The amount of PyLip lipase was kept at 20% w/w of total substrates and reaction was carried out for 24 h. As shown in Fig. 2, when molar ratio was increased from 1:1 to 1:3 reduction in tristearin increased from 27.56% to 88.76% respectively whereas incorporation of caprylic acid increased from 12.31% to 44.89% respectively. No significant increase in tristearin reduction or caprylic acid incorporation was observed for 1:3.5 and 1:4 molar ratios. Appearance of two new peaks was observed post acidolysis reactions having retention time of 8.1 min and 9.9 min respectively (shown in Fig. 3). The first peak (retention time: 8.1 min) was identified as tristearin substituted at two positions by caprylic acid and the second peak (retention time: 9.9 min) was identified as tristearin substituted at one position by caprylic acid. Thus, both di-substituted (containing two caprylic acid and one stearic acid) and mono-substituted triglycerides (containing one caprylic acid and two stearic acid) in the increasing order of their retention time were formed as a result of acidolysis reactions which were identified by HPLC analysis of reference TAGs standards of varying ECN values. In the present study, 44.89% incorporation of fatty acid was obtained with 88.76% tristearin reduction using molar ratio of 1:3 by indigenously immobilized PyLip lipase. Also, no diglyceride formation was observed in the present study. The support matrix used for immobilization was of hydrophobic nature whereas for commercial TL preparation – Lipozyme *TLIM*, silica based matrices which are primarily hydrophilic in nature are employed. The difference in the support matrix could be one of the reasons for no/low formation of by-product such as diglycerides.

The two-way ANOVA analysis (Table 2) indicates that the degree of acidolysis (incorporation of caprylic acid and tristearin reduction) at different substrate molar ratios (1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, and 1:4) and the interaction seem to be statistically significant because *F* values for substrate molar ratio, degree of acidolysis, and their interaction are high compared to the *F* table values ($F_{6,14}$, $F_{1,14}$ and $F_{6,14}$ values are 2.85, 4.60, and 2.85, respectively at α=0.05).

3.4. Effect of reaction time

For an enzymatic process to be efficient on an industrial scale, it is desirable to have shortened reaction time and high conversion. Optimum interaction between substrate and enzyme is needed to obtain good conversion rate [21]. During time course reaction study, rapid reduction in tristearin was observed such that reduction of tristearin reached up to 65.44% in the first 30 min of reaction. After 1 h reaction time; 88.66% reduction in tristearin was observed. Reaction was monitored up to 8 h and no significant reduction in tristearin occurred beyond 1 h of reaction time in the

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

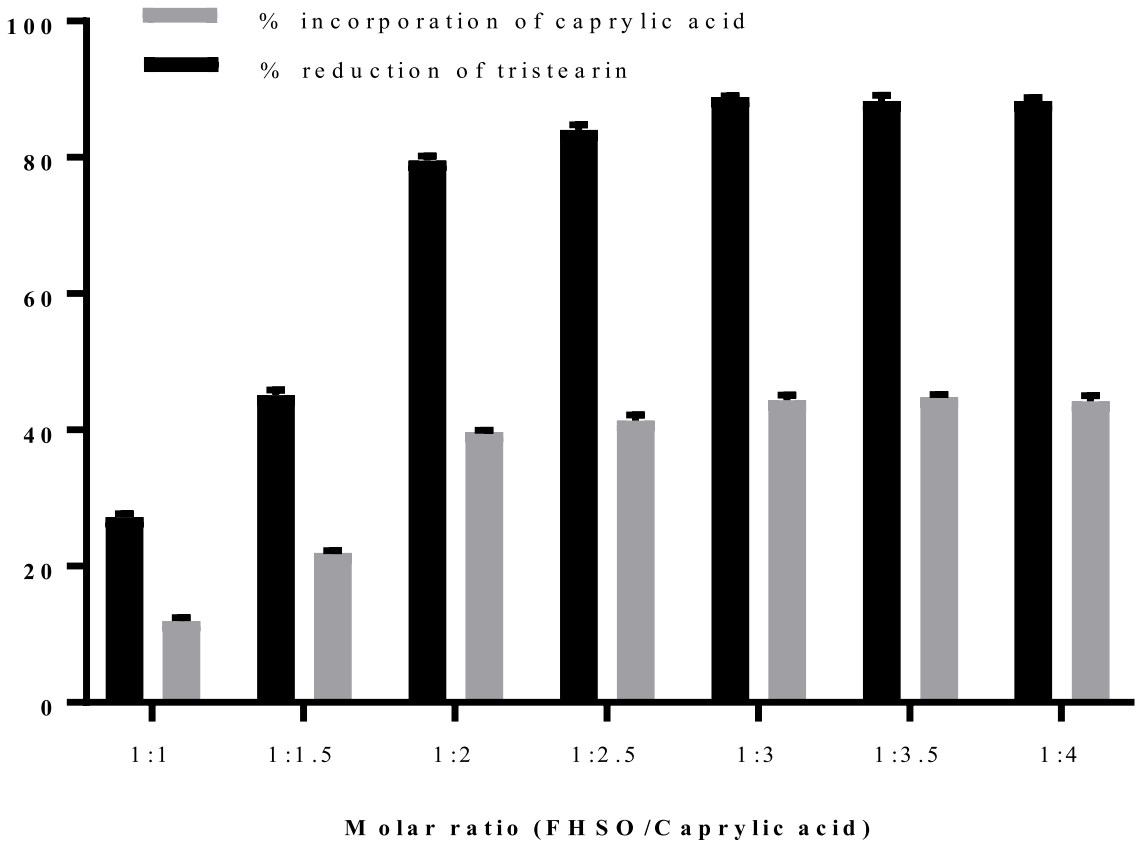


Fig. 2. Effect of molar ratio of FHSO and caprylic acid. Reaction conditions: 20% w/w PyLip lipase, 10% substrate concentration, temperature: 60 °C. Vertical bars represent standard deviation.

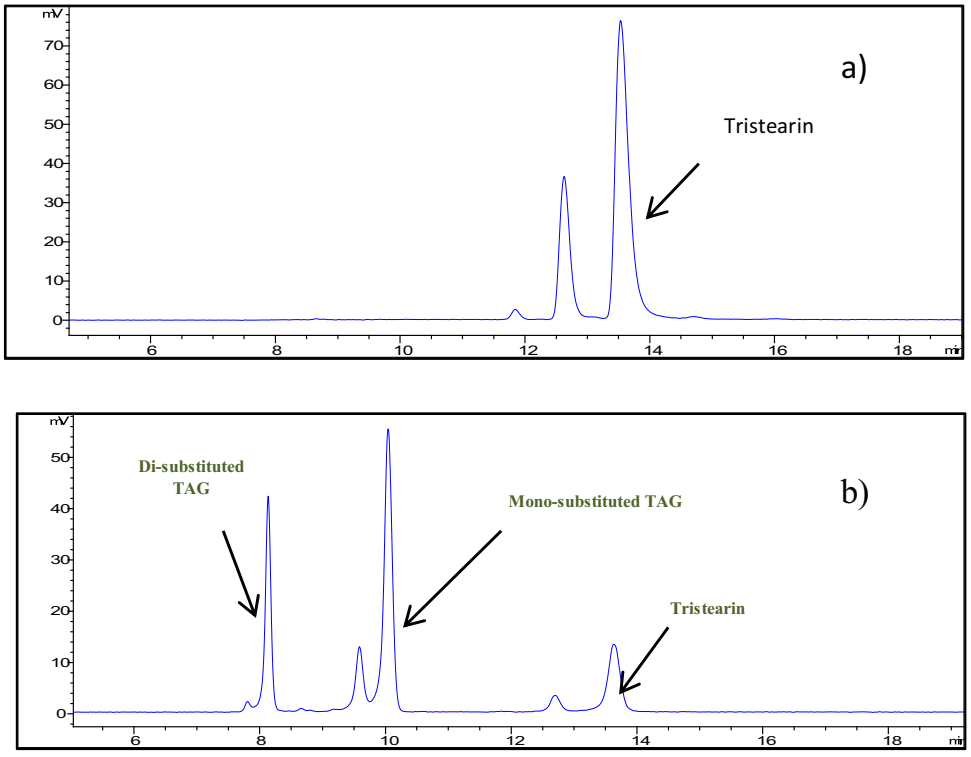


Fig. 3. a) HPLC chromatogram showing triglycerides of FHSO. b) HPLC chromatogram showing triglycerides post acidolysis reaction. Separation was performed as described in Section 2.3.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

Table 2
Anova table for experimental results of degree of acidolysis at different molar ratio of substrates.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	851.2	6	141.9	F (6, 14) = 355.4	P < 0.0001
Molar ratio	8700	6	1450	F (6, 14) = 3632	P < 0.0001
Degree of acidolysis	9137	1	9137	F (1, 14) = 22887	P < 0.0001
Residual	5.589	14	0.3992		

Df degrees of freedom; *SS* sum of squares; *MS* mean square.

Table 3
Anova table for experimental results of degree of acidolysis at different reaction time.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	576.1	5	115.2	F (5, 12) = 432.9	P < 0.0001
Reaction time	5925	5	1185	F (5, 12) = 4453	P < 0.0001
Degree of acidolysis	8433	1	8433	F (1, 12) = 31686	P < 0.0001
Residual	3.194	12	0.2661		

Df degrees of freedom; *SS* sum of squares; *MS* mean square.

present reaction system (Fig. 4). Reaction time ranging from 6 h to 72 h have been reported for acidolysis of different oils and fats such as mustard oil, canola oil as well as reference triglycerides standards such as tripalmitin and triolein using varying chain length fatty acids such as capric acid, caprylic acid, lauric acid, stearic acid to produce designer lipids with unique fatty acid composition for which various commercial immobilized lipases have been examined for their acidolysis ability such as Novozym 435, Lipozym RM IM, and Lipozym TL IM [5,9,12,22,23]. In the present study, rapid acidolysis was observed which could be attributed to effect of PyLip resulting in 88.66% reduction of tristearin in only 1 h reaction time.

The two-way ANOVA analysis (Table 3) indicates that the degree of acidolysis (incorporation of caprylic acid and tristearin reduction) at different reaction time (0.25, 0.5, 1, 3, 5, and 8 h) and the interaction seem to be statistically significant because F values for reaction time, degree of acidolysis, and the interaction are high compared to the F table values ($F_{5,12}$, $F_{1,12}$ and $F_{5,12}$ values are 3.11, 4.75, and 3.11 respectively at $\alpha = 0.05$).

3.5. Effect of enzyme load

Low amount of immobilized enzyme is needed for any enzymatic process to be economically feasible at an industrial scale [24]. In the present study, effect of enzyme load was studied for acidolysis reaction between FHSO with caprylic acid to determine the optimum enzyme concentration to achieve maximum triglyceride reduction and caprylic acid incorporation and is depicted in Fig. 5. Acidolysis of FHSO with caprylic acid was performed using 1–25% w/w PyLip lipase as the biocatalyst. In this study, substrate molar ratio was kept at 1:3 ratio of FHSO to caprylic acid. The optimum enzyme load was found to be 15% w/w of substrates at which reduction of tristearin reached a maximum of 88.5%. Extent of reduction of tristearin increased from 34.81% to 88.5% with an increase in enzyme load from 1% to 15%. Literature reports also show that increase in enzyme load leads to increased incorporation of fatty acid into substrate TAGs [11,12,25]. Increasing enzyme load beyond 15% did not lead to any significant increase in tristearin reduction indicating that for the present reaction system, 15% w/w of PyLip lipase is the optimum enzyme load to effect maximum reduction of tristearin.

The two-way ANOVA analysis (Table 4) indicates that the degree of acidolysis (incorporation of caprylic acid and tristearin reduction) at different enzyme load (1, 5, 10, 15, 20, and 25% w/w of substrates) and the interaction seem to be statistically significant because F values for enzyme load, degree of acidolysis, and the interaction are high compared to the F table values ($F_{5,12}$, $F_{1,12}$ and $F_{5,12}$ values are 3.11, 4.75, and 3.11 respectively at α=0.05).

3.6. Effect of reaction temperature

Effect of reaction temperature on the acidolysis of fully hydrogenated soybean oil with caprylic acid was investigated. Temperature is a crucial parameter that has influence on reaction efficiency of lipase by affecting its catalytic activity. Higher temperature leads to an increase in reaction velocity; however every lipase has an optimum temperature range beyond which reduction in its activity is observed. In the present study, acidolysis reaction was set up at 40 °C, 50 °C and 60 °C (Fig. 6). At 40 °C, there was 68.79% tristearin reduction and 35.09% incorporation of

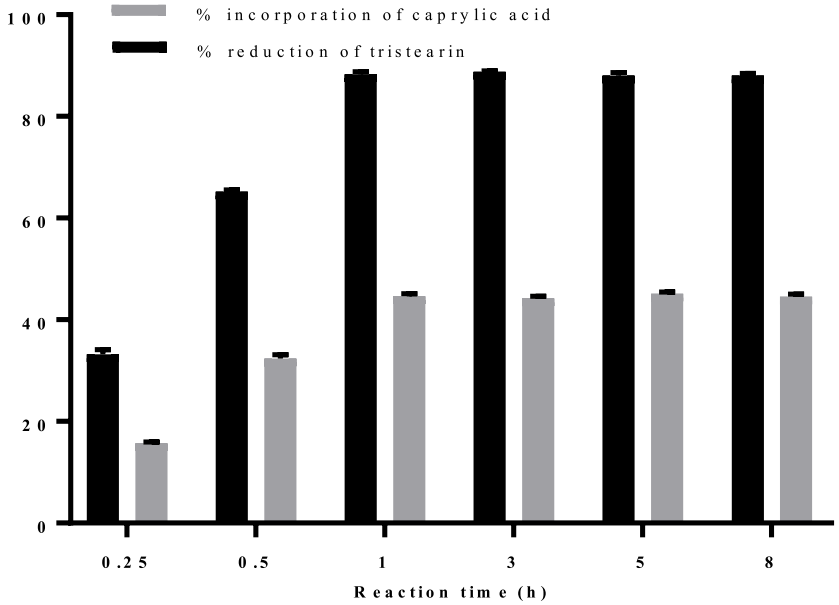


Fig. 4. Effect of reaction time on acidolysis reaction between FHSO and caprylic acid. Reaction conditions: 1:3 molar ratio of FHSO/caprylic acid, 10% w/v substrate concentration, 20% w/w PyLip lipase, 60 °C. Vertical bars represent standard deviation.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

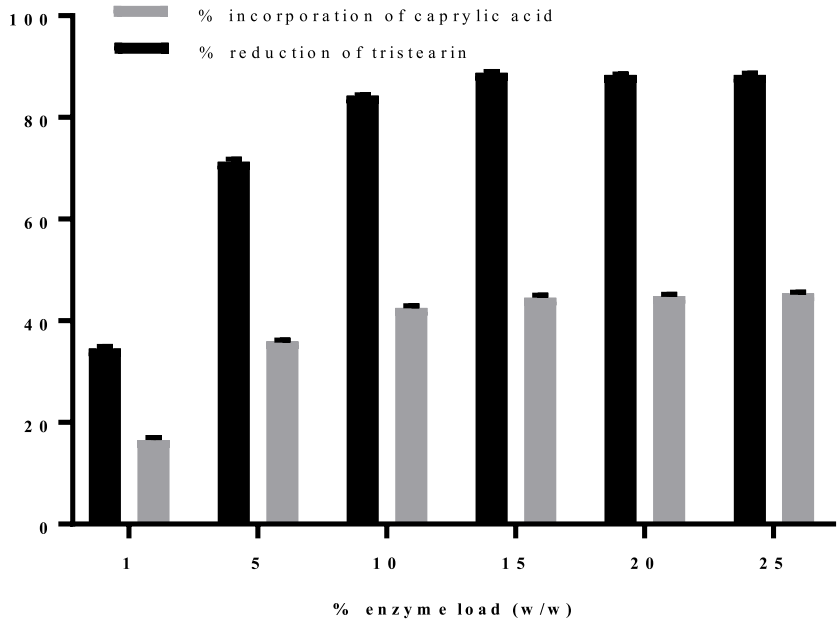


Fig. 5. Effect of enzyme load on acidolysis of FHSO with caprylic acid. Reaction conditions: 1:3 molar ratio of FHSO/caprylic acid, 10% w/v substrate concentration, 60 °C, 1 h reaction time. Vertical bars represent standard deviation.

Table 4
Anova table for experimental results of degree of acidolysis at varying enzyme load.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	514.9	5	103	F (5, 12) = 828.2	P < 0.0001
Enzyme load	5308	5	1062	F (5, 12) = 8537	P < 0.0001
Degree of acidolysis	8487	1	8487	F (1, 12) = 68251	P < 0.0001
Residual	1.492	12	0.1244		

Df degrees of freedom; *SS* sum of squares; *MS* mean square.

caprylic acid. At 50 °C, there was significant increase in tristearin reduction and was found to be 82.35% with 42.11% incorporation of caprylic acid. At 60 °C, there was almost 6% increase in tristearin reduction and 44.99% incorporation of caprylic acid. In the present reaction system, reduction in tristearin increased as the temperature increased with a concomitant increase in caprylic acid incorporation.

The two-way ANOVA analysis (Table 5) indicates that the degree of acidolysis (incorporation of caprylic acid and tristearin reduction) at different reaction temperature (40, 50, and 60 °C) and the interaction seem to be statistically significant because F values

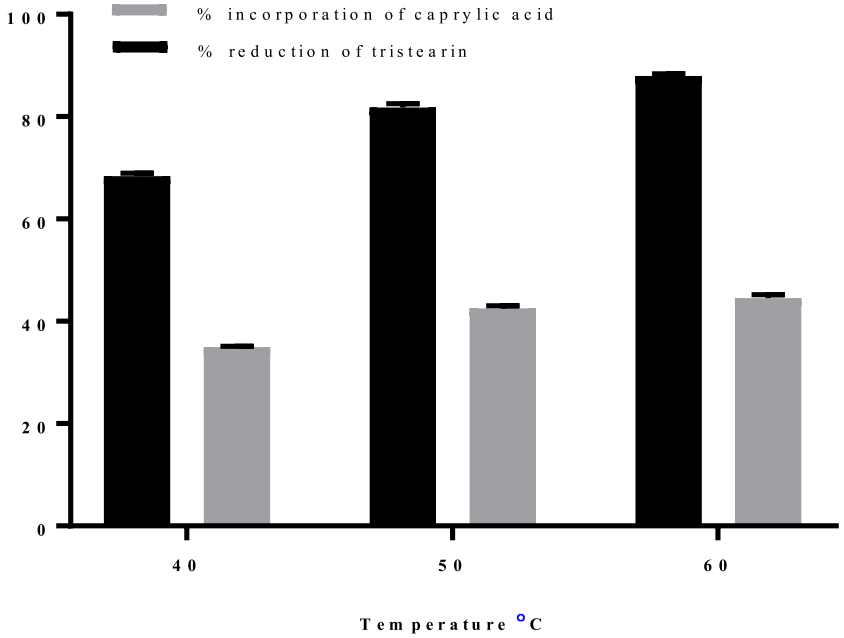


Fig. 6. Effect of reaction temperature on acidolysis of FHSO with caprylic acid. Reaction conditions: 1:3 molar ratio of FHSO/caprylic acid, 10% w/v substrate concentration, 1 h reaction time, 15% w/w PyLip lipase. Vertical bars represent standard deviation.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

Table 5
Anova table for experimental results of degree of acidolysis at different reaction temperature.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	49.95	2	24.98	F (2, 6) = 81.32	P < 0.0001
Reaction temperature	453	2	226.5	F (2, 6) = 737.5	P < 0.0001
Degree of acidolysis	4496	1	4496	F (1, 6) = 14638	P < 0.0001
Residual	1.843	6	0.3071		

Df degrees of freedom; *SS* sum of squares; *MS* mean square.

Table 6
Anova table for experimental results of degree of acidolysis at different substrate concentration.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	200.6	7	28.66	F (7, 16) = 108.8	P < 0.0001
Substrate concentration	2076	7	296.5	F (7, 16) = 1126	P < 0.0001
Degree of acidolysis	12310	1	12310	F (1, 16) = 46736	P < 0.0001
Residual	4.214	16	0.2634		

Df degrees of freedom; *SS* sum of squares; *MS* mean square.

for enzyme load, degree of acidolysis, and the interaction are high compared to the F table values ($F_{2,6}$, $F_{1,6}$ and $F_{2,6}$ values are 5.14, 5.99, and 5.14 respectively at $\alpha = 0.05$).

3.7. Effect of substrate concentration

Introduction of non-polar solvent like hexane into reaction system is considered to have positive impact on enzyme catalysis because non-polar solvents lead to better solubility of lipidic substrates favoring interaction of substrates without altering enzyme structure/conformation. Polar solvents on the other hand; are not useful for structured lipid synthesis because they provide little solvation of substrates. Polar solvents penetrate hydrophilic layer of enzyme stripping water from enzyme which hampers enzyme catalysis. Klibanov et al. [26,27] and Gupta et al. [28] have discussed in detail the advantages of using organic solvents in enzyme catalysis. Zhao et al. [25] has reported that solvents with logP values >3 (hexane logP value = 3.5) were most suitable media for enzymatic acidolysis increasing the solubility of non-polar substrates and shifting the reaction equilibrium toward synthesis rather than hydrolysis. As shown in Fig. 7, solvent-free acidolysis resulted in 59.55% reduction in tristearin with 30.14% incorporation of caprylic acid. For substrate concentrations; 5% to 40% w/v; almost same reduction in tristearin (~88%) was obtained. A decline in tristearin conversion was observed upon increasing substrate concentration beyond 40% w/v. At 60% w/v substrate concentration, tristearin reduction was 70.23% and incorporation of caprylic acid was reduced to 36.24%. A decrease in degree of acidolysis beyond 40% substrate concentration could be attributed to increase in the viscosity of reaction medium preventing adequate contact between substrates and between substrates and enzyme. Also, presence of solvent was found to increase degree of acidolysis as compared to solvent-free conditions. This could be attributed to the fact that solvents like hexane lower viscosity of reaction medium enabling free access of substrates to enzyme active site [29]. In the present study, maximum reduction in tristearin was obtained when 5% to 40% w/v substrate concentration was used.

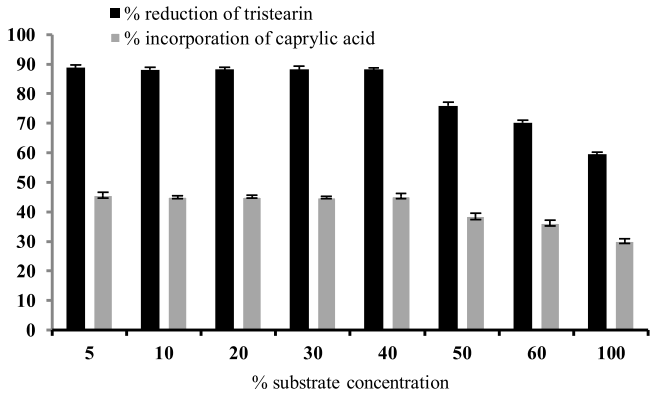


Fig. 7. Effect of substrate concentration on acidolysis of FHSO with caprylic acid. Reaction conditions: 1: 3 molar ratio of FHSO/caprylic acid, 15% w/w PyLip lipase, 60 °C. Vertical bars represent standard deviation.

The two-way ANOVA analysis (Table 6) indicates that the degree of acidolysis (incorporation of caprylic acid and tristearin reduction) at different substrate concentration (0, 5, 10, 20, 30, 40, 50, and 60% w/v) and the interaction seem to be statistically significant because F values for substrate concentration, degree of acidolysis, and the interaction are high compared to the F table values ($F_{7,16}$, $F_{1,16}$ and $F_{7,16}$ values are 2.66, 4.49, and 2.66 respectively at $\alpha = 0.05$).

3.8. Comparative study of indigenously immobilized Thermomyces lanuginosus lipase and Lipozyme TL IM

Using optimized reaction conditions such as 1:3 molar ratio of FHSO and caprylic acid, 15% w/w enzyme load, 40% w/v substrate concentration, acidolysis reaction was set up using commercial sn-1,3 specific lipase Lipozyme TL IM which has been reported in various studies on acidolysis [12,25,30] to compare its efficiency with PyLip lipase. Fig. 8 shows tristearin conversion and caprylic acid incorporation for the two enzymes used in the present study. It was observed that indigenously immobilized *Thermomyces lanuginosus* soluble lipase PyLip resulted in significantly higher tristearin reduction and caprylic acid incorporation than commercial lipase Lipozyme TL IM. This can be attributed to the difference in adsorbent support characteristics of commercial TL IM and PyLip lipase. The matrix used for immobilization of Lipozyme TL IM is a granulated silica gel carrier and is less hydrophobic than polystyrene divinyl benzene used as support in the present study for production of PyLip. The reaction mixture consisted of hexane as solvent which is highly non-polar in nature (log P > 3) and hence the interaction of substrates in hexane with enzyme PyLip might be better than with TL IM due to greater hydrophobicity of the surface of PyLip than TL IM which led to increased acidolysis when PyLip was used. This study therefore indicates the feasibility of using cheaper alternatives to commercial lipase Lipozyme TL IM for designer lipid synthesis.

3.9. Reusability studies in batch mode

Operational stability of immobilized lipase, PyLip, was studied and analyzed for synthesis of modified fats by the acidolysis route in batch mode. After each reaction run (60 min), PyLip was filtered and washed with hexane to remove any residual substrate. The recovered enzyme was then used for next batch of reaction and the reusability of enzyme preparations was thus evaluated for 10 cycles. As can be seen from Fig. 9, PyLip resulted in consistent conversion of tristearin and caprylic acid incorporation for synthesis of modified fat for 10 consecutive cycles. From this study, it can be concluded that the prepared PyLip lipase has good operational stability for synthesis of designer lipids.

3.10. DSC analysis

Differential scanning calorimetry (DSC) was used to obtain the melting thermograms of starting material and final synthesized sample as described in Section 2.7. DSC is a useful technique to study melting characteristics and to analyze the transition of

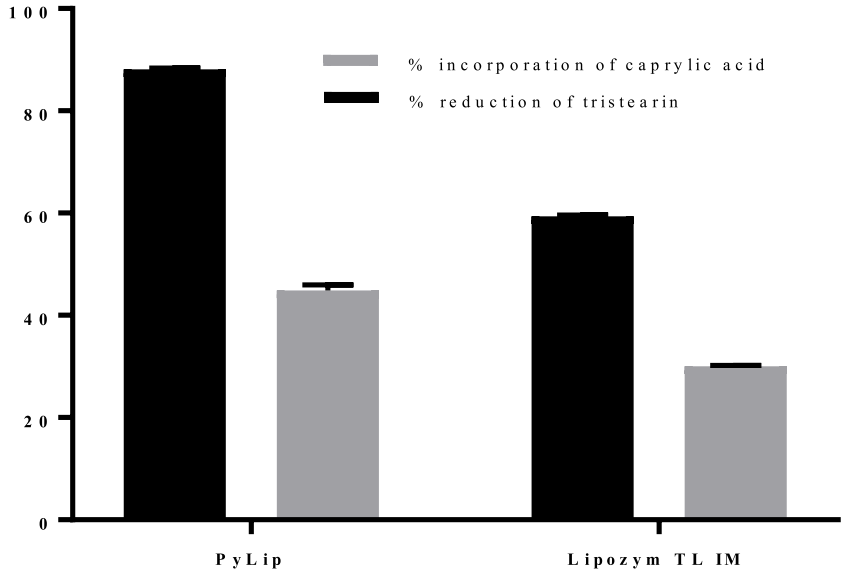


Fig. 8. Comparative acidolysis study between Lipozym TL IM and PyLip lipase. Reaction mixture consisted of 1: 3 molar ratio of FHSO/caprylic acid at 40% w/v substrate concentration; 15% w/w enzyme load, operating temperature was 60 °C. Vertical bars represent standard deviation.

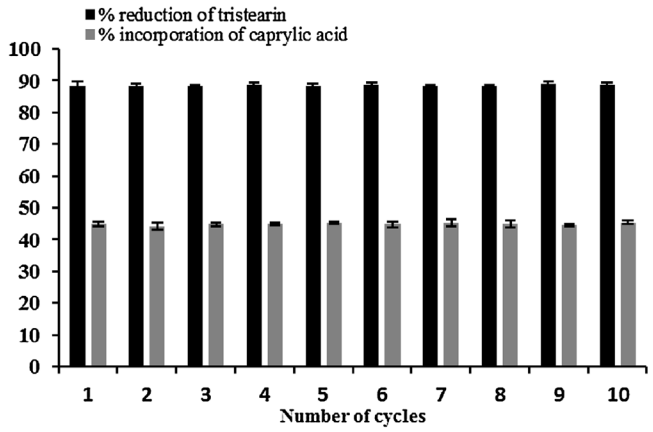


Fig. 9. Batch operational study for synthesis of modified fat using PyLip lipase. Reaction mixture consisted of 1: 3 molar ratio of FHSO/caprylic acid at 40% w/v substrate concentration; 15% w/w enzyme load, operating temperature was 60 °C. Vertical bars represent standard deviation.

crystal forms during melting of fats. DSC melting thermograms of FHSO and acidolyzed product are presented in Fig. 10. The melting thermogram obtained from FHSO showed 2 distinct separated peaks at 53 °C and 62 °C, belonging to TAG species composed of long chain saturated fatty acids. A similar result was also reported by [18]. Acidolysed product showed three distinct broad peaks at 23 °C, 37 °C and 55 °C which can be attributed to formation of unique triglycerides post reaction. In the reaction product, melting peaks were observed at lower temperatures than starting material due to the formation of new triglycerides consisting of both long chain fatty acid (stearic acid) and medium chain fatty acid (caprylic acid) which have lower melting temperature range than triglycerides containing only long chain fatty acids. Thus significant change in physical properties of acidolysed sample was observed due to altered triglyceride composition and fatty acid composition.

3.11. XRD study

The polymorphism of acidolyzed product and FHSO were measured by X-ray diffraction spectroscopy. The three representative polymorphic forms of fat crystals are α, β, and β′ which are an important criteria for functional properties of lipids used in

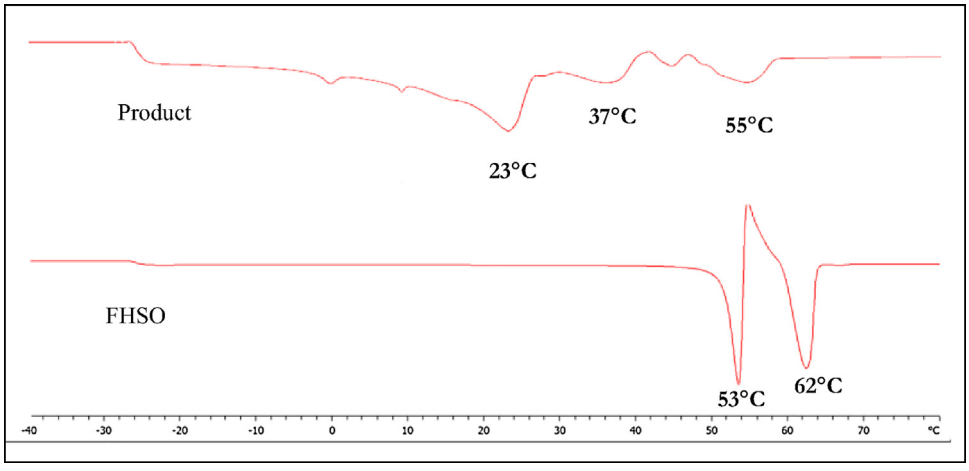


Fig. 10. DSC melting thermogram of FHSO and acidolysed product.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246

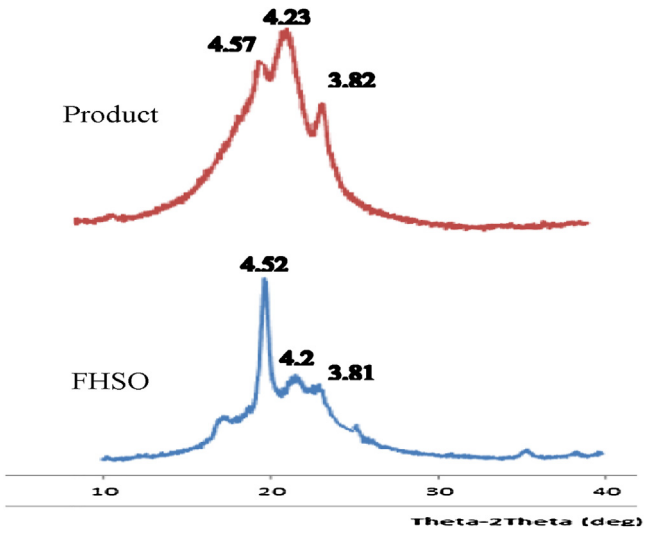


Fig. 11. X-ray diffraction profiles of FHSO and acidolysed product.

bakery and confectionary industry [31]. The starting material FHSO showed strong intensities of short spacing at 4.52°A (β), relatively weaker intensities at 4.2°A (β') and 3.81°A (β') whereas acidolyzed product showed weaker intensity of short spacing at 4.57 (β)°A, and relatively stronger intensities at 4.23 (β')°A, and 3.82 (β')°A. After acidolysis of FHSO with caprylic acid by PyLip lipase, the intensity of short spacing for β form was reduced and β' form was dominant (Fig. 11). That could be due to the substitution of long chain fatty acid (stearic acid) with medium chain fatty acid (caprylic acid) on TAG backbone after acidolysis reaction. β' form indicates presence of small-to-moderate crystal size that allow for smooth texture and is generally desirable for application in food products.

4. Conclusion

In the present study, enzymatic acidolysis process for production of modified fats was developed using indigenously immobilized lipase PyLip. Lipase-catalyzed acidolysis reaction resulted in substantial replacement of long chain fatty acid residues in FHSO with caprylic acid residues to produce new triglycerides which significantly altered the physico-chemical properties of the newly formed product which were assessed using DSC analysis and XRD study. The present study reports efficient green enzymatic single step process for carrying out acidolysis reaction for bulk production of designer fats catalyzed by stable low-cost indigenously immobilized lipase PyLip in shortened reaction time, and no by-product formation making the overall process clean and environment friendly. FHSO enriched by caprylic acid offers a new lipid with better metabolic capabilities due to its improved functionality and physical properties.The present study has potential to be a useful reference for development of industrial process for enzymatic production of modified fats for use in functional food formulations.

Declaration

The authors have declared no conflicts of interest.

Acknowledgement

The authors are grateful to the financial support provided by Department of Biotechnology under the Ministry of Science and Technology, Govt of India.

References

[1] J. Gokce, N.S. Yesilcubuk, G. Ustun, Enzymatic production of low-calorie structured lipid from Echium seed oil and lauric acid: optimisation by Response Surface Methodology, Int. J. Food Sci. Technol. (2013) 1–7.
[2] X. Xu, Production of specific-structured triacylglycerols by lipase catalyzed reactions: a review, Eur. J. Lipid Sci. Technol. (2000) 287–303.
[3] M.L. Ferreira, G.M. Tonetto, Enzymatic Synthesis of Structured Triglycerides: From Laboratory to Industry, Springer Briefs in Molecular Science, 2017 ISBN 978-3-319-51574-8.
[4] A.B.A. Mizan, M.K. Ayob, A.G. Ma, M.M. Yusof, I. Izzreen, Optimization of the synthesis of structured lipids using medium chain fatty acids through acidolysis, Am.-Eurasian J. Agric. Environ. Sci. 16 (2016) 698–706.
[5] P. Villeneuve, Lipases in lipophilization reactions, Biotechnol. Adv. 25 (2007) 515–536.
[6] S.P.J.N. Senanayake, F. Shahidi, Acidolysis of seal blubber oil with lauric acid, J. Food Lipids 14 (2007) 78–96.
[7] A. Sengupta, S. Roy, S. Mukherjee, M. Ghosh, Production of medium chain fatty acid rich mustard oil using packed bed bioreactor, J. Oleo. Sci. 64 (2015) 153–159.
[8] C.C. Akoh, C.O. Moussata, Lipase-catalyzed modification of borage oil: incorporation of capric and eicosapentaenoic acids to form structured lipids, JAOCS 75 (1998) 697–701.
[9] B.H. Jennings, C.C. Akoh, Lipase-catalyzed modification of rice bran oil to incorporate capric acid, J. Agric. Food Chem. 48 (2000) 4439–4443.
[10] F. Hamam, F. Shahidi, Synthesis of structured lipids via acidolysis of docosahexaenoic acid single cell oil (DHASCO) with capric acid, J. Agric. Food Chem. 52 (2004) 2900–2906.
[11] Y. Wang, L. Xia, X. Xu, L. Xie, Z. Duan, Food and Bioproducts Processing Lipase-catalyzed acidolysis of canola oil with caprylic acid to produce medium-, long- and medium-chain-type structured lipids, Food Bioprod. Process. 90 (2012) 707–712.
[12] L.B. Fomuso, C.C. Akoh, Enzymatic modification of high-laurate canola to produce margarine fat, J. Agric. Food Chem. 49 (2001) 4482–4487.
[13] D. Zhou, X. Xu, H. Mu, C.E. Hoy, J. Adler-Nissen, Synthesis of structured triacylglycerols containing caproic acid by lipase-catalyzed acidolysis: optimization by response surface methodology, J. Agric. Food Chem. 49 (2001) 5771–5777.
[14] D. Savaghebi, M. Safari, K. Rezaei, P. Ashtari, J. Farmani, Structured lipids produced through lipase-catalyzed acidolysis of canola oil, J. Agric. Sci. Technol. 14 (2012) 1297–1310.
[15] E.G. Hammond, L.A. Johnson, C. Su, T. Wang, P.J. White, Soybean oil, in: F. Shahidi (Ed.), Bailey's Industrial Oil and Fats Products, sixth edition, Wiley-Interscience, 2005, pp. 577–653.
[16] A.P.B. Ribeiro, R. Grimaldi, L.A. Gioielli, L.A.G. Gonçalves, Zero trans fats from soybean oil and fully hydrogenated soybean oil: physico-chemical properties and food applications, Food Res. Int. 42 (2009) 401–410.
[17] R. Rao, B.R. Lokesh, TG containing stearic acid synthesized from coconut oil, exhibit lipidemic effects in rats similar to those of cocoa butter, Lipids 38 (2003) 913–918.
[18] P. Adhikari, J.A. Shin, J.H. Lee, J.N. Hu, K.T. Hwang, K.T. Lee, Enzymatic production of trans-free hard fat stock from fractionated rice bran oil, fully hydrogenated soybean oil, and conjugated linoleic acid, J. Food Sci. 74 (2009) 87–96.
[19] A.A. Odaneth, R.N. Vadgama, A.D. Bhat, A.M. Lali, Tuning lipase reaction for production of fatty acids from oil, Appl. Biochem. Biotechnol. 180 (2016) 504–515.
[20] M.G. Yadav, M.R. Kavadia, R.N. Vadgama, A.A. Odaneth, A.M. Lali, Green enzymatic production of glyceryl monoundecylenate using immobilized *Candida antarctica* lipase B, Prep. Biochem. Biotechnol. 47 (2017) 1050–1058.
[21] Production of 6-O-L-Ascorbyl Palmitate by Immobilized *Candida antarctica* Lipase B, Appl Biochem Biotechnol. (2017) Online ISSN 1559-0291.
[22] W. Tsuzuki, Acidolysis between triolein and short-chain fatty acid by lipase in organic solvents, Biosci. Biotechnol. Biochem. 69 (2005) 1256–1261.
[23] N. Sahin, C.C. Akoh, A. Karaali, Lipase-Catalyzed acidolysis of tripalmitin with hazelnut oil fatty acids and stearic acid to produce human milk fat substitutes, J. Agric. Food Chem. 53 (2005) 5779–5783.
[24] R.N. Vadgama, A.A. Odaneth, A.M. Lali, Green synthesis of isopropyl myristate in novel single phase medium Part I: Batch optimization studies, Biotechnol. Rep 8 (2015) 133–137.
[25] H. Zhao, Z. Lu, X. Bie, F. Lu, Z. Liu, Lipase catalyzed acidolysis of lard with capric acid in organic solvent, J. Food Eng. 78 (2007) 41–46.
[26] A.M. Klibanov, Enzymes that work in organic solvents, Chem. Tech. 16 (1986) 354–359.
[27] A.M. Klibanov, Improving enzymes by using them in organic solvents, Nature 409 (2001) 241–246.
[28] M.N. Gupta, Enzyme function in organic solvents, Eur. J. Biochem./FEBS 203 (1992) 25–32.
[29] M.E. Carrin, G.H. Crapiste, Enzymatic acidolysis of sunflower oil with a palmitic −stearic acid mixture, J. Food Eng. 84 (2008) 243–249.
[30] I. Kim, H. Kim, K. Lee, S. Chung, S. Ko, Lipase-Catalyzed acidolysis of perilla oil with caprylic acid to produce structured lipids, JAOCS 79 (2002) 363–367.
[31] T. Jeyarani, S.Y. Reddy, Effect of enzymatic interesterification on physicochemical properties of mahua oil and kokum fat blend, Food Chem. 123 (2010) 249–253.

Please cite this article in press as: M.R. Kavadia, et al., Synthesis of designer triglycerides by enzymatic acidolysis, Biotechnol. Rep. (2018), https://doi.org/10.1016/j.btre.2018.e00246