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Comparative qualitative assessment of DAG production from medium chain fatty acids mediated by enzymatic and chemical catalysts under individually optimized conditions

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ABSTRACT

The present study was aimed to assess the production attributes of synthesizing structured diacylglycerols from bioactive medium chain fatty acids in a stirred tank reactor by known methods of catalysis i.e. enzymatic and chemical on a comparative basis. Enzymatic esterification was performed using two commercially available immobilized lipases viz. *Rhizomucor meihei* and*Candida antarctica,* whereas, chemical was carried out using concentrated sulphuric acid (H_2SO_4) as acid catalyst. Two different medium chain fatty acids caprylic ($C_{8:0}$), capric ($C_{10:0}$) along with glycerol were used as the substrates for the esterification process. Reaction parameters, such as substrate molar ratio, time of reaction and acid values were standardized in the stirred tank reactor, keeping the catalyst (enzyme and chemical) concentration and reaction temperature constant. Chromatographic techniques were employed to monitor and separate the different isomers. Structured diacylglycerol yields of about $58.86 \pm 4.05\%$ and $53.77 \pm 3.74\%$ were obtained with $C_{8:0}$-RMIM and $C_{8:0}$-NS435 enzyme at 18th and 6^{th}hr respectively. About $55.41 \pm 3.95\%$, $51.89 \pm 2.60\%$ significant yields were also obtained in case of $C_{10:0}$-RMIM and NS435 enzymes at 18th and 6th h respectively. The study showed better eco-friendly qualitative yield of DAG production can be achieved with biological catalysts as compared to chemical catalysts.

1. Introduction

The nutritional advantages of diacylglycerols (DAGs) oils as nutraceuticals are now well established which includes suppression of post prandial serum triglyceride, high satiety value, lowering of blood glucose, prevention of postprandial hypertriglyceridemia and obesity (Ling and Jones, 1995; Murase et al. 2001, 2002; Watanabe et al., 1997; Taguchi et al., 2000).

According to the differences in chemical structure, there are various types of fatty acids that constitutes the triacylglycerol. Long-chain fatty acids (LCFAs) comprising 16 or more carbons constitute the major composition and medium-chain fatty acids (MCFAs) with 8–12 carbons constitute the minor composition of the naturally occurring triacylglycerols. Distinct differences in physical and chemical properties, *in vivo digestion/*absorption and biological adaptability exist between the LCFAs and MCFAs. Medium chain fatty acid (MCFA) containing triglycerides (MCTs) increase calorific importance of the food, enhances palatability, digestibility in addition to the higher absorption rate of nutrients when compared with long-chain fatty acid containing triglycerides (LCT). MCT cuts down the risk of vascular disease and helps in bodyweight maintenance (Taguchi et al., 2000; Sengupta and Ghosh, 2011).

Over the past few decades, extensive research work has already been undertaken to explore the nutritional benefits of MCFA-containing triacylglycerols (TAGs). Notably, although preparation of MCFA rich DAGs from natural TAG and LCFA rich DAG oils is an established phenomenon (Flickinger and Matsuo, 2003), reports on structured *de novo* DAG synthesis from MCFAs and LCFAs on glycerol backbone, have not been popularized.

Enzymes as well as chemical catalyzed reactions are widely practiced experimental methodologies for esterification processes. Chemical catalyzed esterification process is an age-old technique in the field of oleo chemistry. Extensive research work has been carried out with chemically derived esterified products in the field of lipids already. Chemically, DAG is commonly produced through a glycerolysis process which is conducted at high temperature of 220–260 °C in the presence of

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sodium hydroxide (NaOH), as catalyst. Every scientific research focuses on studying both the beneficial and detrimental effects of any process (both natural and synthetic). Same is also true for evaluating all the pros and cons of chemical synthesis for any product development, especially in the synthesis of DAGs from MCFAs. The high temperature required for chemical glycerolysis is unattractive from a production point of view especially for heat-sensitive polyunsaturated fatty acid (PUFA)-rich DAG (Sonntag, 1984). Progressive inventions have led to the concept of green chemistry and eventually, the idea of biocatalyst came into play. Enzymes, thus, gained importance worldwide. Enzymatically, DAG can be produced by direct esterification of glycerol and Fatty acids (FA), glycerolysis of triglycerides (TAG), partial hydrolysis of TAG, or a combination of partial hydrolysis and esterification (Cheong et al., 2007; Guo and Sun, 2007; Rosu et al., 1999; Weber and Mukherjee, 2004; Yang et al., 2004). Therefore, application of enzymes has attracted wide attention, due to mild reaction conditions, higher selectivity and low waste generation.

The present study provides a holistic and comparative account of production of DAGs from pure medium chain fatty acids (MCFAs) viz., caprylic acid ($C_{8:0}$) or capric acid ($C_{10:0}$) rich DAGs chemically and enzymatically to obtain the dual benefits of MCFAs and structured DAGs. Both Chemical and enzymatic catalysis leads to the formation of both the isomers 1, 3 and 1, 2/2, 3. As regards to the enzymatically catalyzed study, two commercially available immobilized lipases viz. *Rhizomucor meihei* (RMIM) and *Candida antarctica* (NS 435) – one being specific and the other non-specific respectively were employed.Whilst, for chemical catalysis reaction, concentrated sulphuric acid was involved as the chemical catalyst. The study highlights the production as well as cost aspects vis-à-vis downstream processing of the synthesis of DAGs from therapeutically potent MCFAs as a standard laboratory reference for pilot and higher scale studies.

2. Materials & methods

2.1. Chemicals

Two lipase enzymes *Rhizomucor meihei* (RMIM) and *Candida antarctica* (NS 435) were obtained from Novozyme India Ltd. These were used as biocatalysts. Concentrated sulphuric acid was used as the acid catalyst. MCFAs - caprylic acid ($C_{8:0}$) and capric acid ($C_{10:0}$) were purchased from Merck India Ltd, Mumbai, India and their purities were checked by gas chromatography (GC) at laboratory. Analytical grade glycerol (AR grade) used in this reaction was also obtained Merck India Ltd, Mumbai, India. All other chemicals and solvents were of analytical grade and procured from Merck India Ltd, Mumbai, India.

2.2. Production of MCFA containing structured diacylglycerol enzymatically

Glycerol and MCFAs were taken in different molar ratios of 1:1, 1: 2, 1:3, 1:4, 1:5 and 2:1 in presence of either RMIM or NS435 enzyme as catalysts. According to the previous work reported by Dhara and Singhal (2014), the enzyme percentage and reaction temperature during the reaction were kept at 10% (w/w) and 60 °C respectively (Dhara and Singhal, 2014). Stirring was provided by magnetic stirrer at 200 rpm and the reaction was carried out under vacuum. The molar ratio and reaction time were the two variable parameters those were optimized.

Samples were withdrawn intermittently from reaction mixture starting from 0 to 2, 4, 6, 16, 18, 20, 22, 24 h and analysed for the acid value and amount of DAG (% w/w) formed. Final product was filtered to remove enzymes and excess glycerol was removed initially by gravity separation and finally by water wash and then vacuum dried and stored (Wang et al., 2015, Yamada et al., 2001).

2.3. Chemical production of MCFA containing structured diacylglycerol

Glycerol and MCFAs were taken in different molar ratios of 1:0.5, 1:1, 1: 2, 1:3, and 2:1 in presence of concentrated sulphuric acid as chemical catalyst. The catalyst percentage and reaction temperature during the reaction were kept at 1.0% (w/w) and 180 °C respectively (Lucena et al., 2008; Jensen et al., 1979).

Stirring was provided by magnetic stirrer at 200 rpm and the reaction was carried out under vacuum. The molar ratio and reaction time were the two variable parameters those were optimized. Samples were withdrawn intermittently from reaction mixture starting from 0 to 3 h and analysed for the acid value and amount of DAG (% w/w) formed. Final product was filtered to remove the catalyst and excess glycerol was removed initially by gravity separation and finally by water wash and then vacuum dried and stored.

2.4. Purification of the product (DAG)

In order to purify and deodorize the structured-DAG oil from free acids, steam stripping with surface area (250–350 m^2/m^3) was employed for removing the free fatty acids. The counter-current interaction between the stripping steam and oil ensured high efficiency in removing free fatty acids over a short-contact time (Zehnder, 1995).

2.5. Determination of different parameters of DAG produced

2.5.1. Acid Value

Acid values of the samples were determined following the AOCS Official Method (Firestone, 2009).

2.5.2. Slip melting point

Slip melting point of the samples was determined by standard method of AOCS(Firestone, 2009).

2.5.3. Refractive index

Refractive index of the samples was determined by standard method of AOCS (Firestone, 2009).

2.5.4. Cloud point

Cloud point of the samples was determined following the AOCS Official Method (Firestone, 2009).

2.5.5. Viscosity

Viscosity of the samples was determined following the AOCS Brookfield Viscosity method (Firestone, 2009).

2.6. Thin layer chromatography (TLC)

The samples obtained at 2, 4, 6, 14, 16, 18, 20, 22, 24 h respectively during the entire reaction tenure was taken for thin-layer chromatographic analysis to find out the maximum conversion and the time for the maximum conversion for the three different enzymes (at a fixed enzyme concentration of 10% of the total weight of the substrates) and for the different molar ratios of the reactants at a constant temperature of 60 °C. Sample was fractioned on 20 × 20 cm plates spread with a 0.2 mm layer of Silica gel G. Sample of equal concentrations of different products were spotted on the TLC plate and the plate was applied uniformly along a line 1.5 cm from one edge of the plate and developed with solvent system at hexane-diethyl ether (80:20 by volume) and 1 mL glacial acetic acid with a continuous flow development of preparative TLC. After the complete run, the plate was removed from the solvent and allowed to evaporate the solvent completely. Then the plate was placed in the iodine chamber to visualize the separations. Then visual estimation of the maximum yield of DAG was observed and the time was noted along with the simultaneous quantitative yields of the respective samples (Firestone, 2009).

2.6.1. Quantification of the DAG isomers by high performance thin layer chromatography (HPTLC)

The estimation of DAG by HPTLC was done according to Macala et al. (Macala et al., 1983). Briefly, analyses were performed on TLC aluminium sheets 20 cm × 20 cm Silica Gel 60 F_{254} plates (Merck, Germany) with concentrating zone, 19 channels, and fluorescent indicator. Plates were developed to a distance of solvent front 65.0 mm, application position was 8.0 mm, vol 10 mL, solvent system used to develop plate was Di-ethyl-ether: hexane: 40:60 in a Camag HPTLC twin-trough chamber 10 × 10 cm, temperature is kept at 25 °C, lined with a saturation pad (Analtech, Newark, DE, USA) and the chamber was equilibrated with the mobile phase for 15 min before inserting the plate. Standards for sn-2, 3-DAG (99% pure, Sigma) and 1, 3-DAG (98% pure, Sigma) were applied on the plate, and the calibration curves were constructed by plotting the IOD (Integrated optical density) Vs the amount of the lipid (DAG samples) loaded. Approximately 30 mL mobile phase (15 mL in the trough containing the plate and 15 mL in the trough containing the pad) were used for each development, which required approximately 20 min. After development the plate was dried in air, in a fume hood, for 5 min, and sample and standard zones were quantified by linear scanning at 200 nm by use of a Camag TLC Scanner 3″ (Scanner 3–130214" S/N 130214) with a D_2 source, (5 × 0.45 mm, micro) and a scanning speed of 20 mm/s, data resolution 100 μm/step. The WINCATS-3 software controlling the densitometer produced a calibration plot by linear regression relating standard zone weights to their scan areas and the experimental weight of DAG samples were automatically interpolated from the calibration curve.

2.7. Statistical analysis

All the analysis was done in triplicates (n = 3). All the data are represented as means with their standard errors (Mean ± SEM). Statistical comparisons between groups were performed using one-way ANOVA. Level of significance $p < 0.05$ was considered by Tukey test (using Origin Pro 8 software).

3. Results & discussion

Using the two distinct lipases (RMIM and NS 435) the yield and characteristics of the esterified products were studied. At constant temperature of 60 °C, enzymatic esterification reaction of 1:1, 1:2, 1:3, 1:4, 1:5 and 2:1 M ratios of glycerol and $C_{8:0}$ and $C_{10:0}$ were performed in presence of RMIM and NS435 enzymes for 0, 2, 4, 6, 14, 16, 18, 20, 22, 24 h respectively. Acid value is considered to be a primary tool for the observation procedure of successful DAG production. The acid values at 0, 2, 4, 6, 16, 18, 20, 22, 24 h for 1:1, 1:2, 1:3, 1:4, 1:5 and 2:1 M ratios of glycerol and $C_{8:0}$were determined for two different enzymes as catalysts i. E RMIM and NS 435 respectively at 60 °C.Incase of RMIM, 1:1, 1:2 and 1:5 M ratios of glycerol and $C_{8:0}$gave no significant results as the acid values did not drop with time noticeably (Fig. 1A).The line graphs plotted in Fig 1A& Fig 2Ashowed higher acid values for 1:1, 1:2 1:5 M ratios at 60 °C irrespective of the type of lipase used (RMIM and NS435). When the inverse ratio of 2:1 pattern was introduced, acid values did not decrease with time, further with poor DAG yields, which is very clear in the supplemented graphically represented figures (Fig. 1C and D respectively). No more inverse ratio patterns were employed as increased concentration of glycerol than the particular MCFA, $C_{8:0}$, resulted in retarding the effective DAG yield. Similar findings on application of random lipase, NS435 as biocatalyst, could be found that are evident in Fig. 2C and Fig. 2D. All other conditions remaining constant,1: 4 ratios portrayed a different outlook. Figs. 1A and 2A also depicted remarkable results for 1:3 M ratios.

When Glycerol and $C_{8:0}$were reacted in presence of immobilized RMIM enzyme (10% w/w) at 1:3 ratio at 60 °C for the time interval of 0–24 h, Fig. 1A showed that, with time there was decrease in acid values initially. Significant fall in the acid value was observed at the 18th hr. But after the 18thhr, acid values began to increase progressively again. Therefore, from Fig. 1A, it was revealed that at the 18thhr for 1:3 M ratios, significant ($p < 0.05$) drop of acid value was obtained when compared to the other molar ratios in case of RMIM enzyme and at all the hours. For the other molar ratios at different time scale at 60 °C, acid

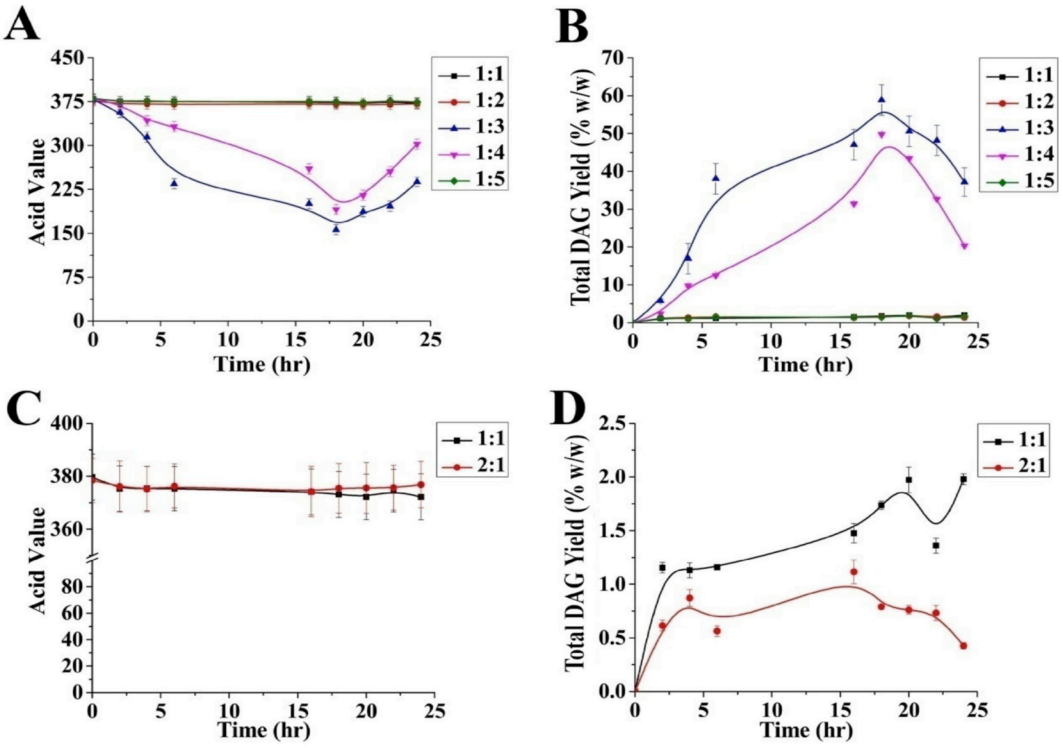

Fig. 1. A, 1B and 1C and 1D Comparison of the acid values and structured DAG yields of all the products of different molar ratios (1:1, 1:2, 1:3, 1:4, 1:5, 2:1) of glycerol and $C_{8:0}$using RMIM as biocatalyst (10% w/w) at different time intervals (0–24 h) at 60 °C (Values are Mean ± SEM,$p < 0.05$, hr = hour, n = 3).

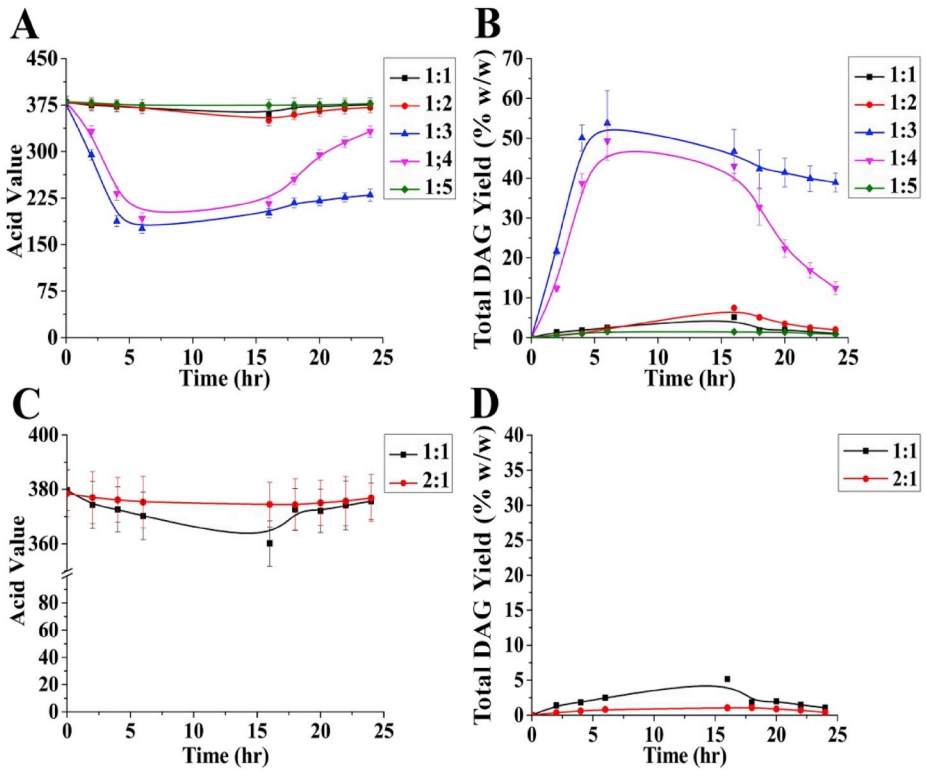

Fig. 2. A, 2B, 2C and 2D Comparison of the acid values and structured DAG of all the products of different molar ratios (1:1, 1:2, 1:3, 1:4, 1:5, 2:1) of glycerol and $C_{8:0}$ using NS 435 enzyme as biocatalyst (10% w/w) at different time intervals (0–24 h) at 60 °C (Values are Mean ± SEM,$p < 0.05$, hr = hour, n = 3).

values were found to gradually diminish but didnot fall as low as when compared to the 18thhr time for 1:3 M ratios. Furthermore, formation of structured DAG was confirmed by TLC analysis. Quantitative analysis (Fig. 1B) was also performed for all the esterified products of all the said molar ratios of glycerol and $C_{8:0}$at 0, 2, 4, 6, 16, 18, 20, 22 and 24 hrtime interval at 60 °C at 10% (w/w) RMIM lipase respectively to ascertain the actual maximum DAG yield.

When immobilized NS 435 was applied instead of RMIM enzyme, keeping the other reaction parameters same as in the previous case, the acid value at the 6th hr was found to be the least for 1:3 M ratios, as observed in Fig. 2A. Prominent DAG spot was exhibited by TLC, followed by quantitative yield analysis.Fig. 2B depicted the maximum structured DAG yield percentage at the 6thhrsignificantly ($p < 0.05$). In case of other molar ratios (1:1, 1:2, 1:5) at 0, 2, 4, 6, 16, 18, 20, 22 and 24 h time scale, the acid values dropped to a certain extent but was not found to be as low as for 1:3 M ratios at the 6th hour time interval.From Fig. 2C and D, it is evident that, when 2:1 ratio (glycerol and $C_{8:0}$) was carried out using random NS435 lipase at 10% w/w at 60 °C, significantly high acid values along with very negligible structured DAG yields. Greater concentration of glycerol might be a possible reason behind the slower esterification reaction procedure, with least DAG formation. 1:4 M ratio earned good quantitative DAG yields but was significantly low compared to that of 1:3 M ratio (Fig. 2B).

Therefore, from Fig. 1A, B, C D, the optimal conditions for the production of maximum DAG was maintained at 18thhr reaction time at 1:3 M ratios of glycerol and MCFA, $C_{8:0}$in presence of the 1, 3 specific-immobilized RMIM at 60 °C.Thus, the standardized reaction parameter for maximum structured DAG yield in case of $C_{8:0}$, the enzyme being NS 435, was found at 6thhr with the reactant molar ratios of 1:3 and at 60 °C reaction temperature (Fig. 2 A, B, C and D).

It could be concluded from these above set of results obtained from Fig. 1 A, B, C, D and Fig. 2 A, B, C and D,that, both RMIM and NS 435 enzymes played a significant role in the reaction system of glycerol and $C_{8:0}$ for the productive yields of structured DAG to a greater extent. The same reaction parameters were set for $C_{10:0}$as MCFA.

1:1, 1:2, 1:3, 1:4, 1:5 and 2:1 M ratios of glycerol and MCFA, $C_{10:0}$were taken into consideration for the experimental enzymatic esterification. 0–24 h time interval was selected for the entire reaction process. RMIM and NS435 enzymes were engaged separately at 10% (w/w) at 60 °C. 1:1, 1:2 and 1:5 M ratios provided no fruitful results. Acid value drop with time progressively indicated a chance of fair DAG yield followed by TLC analysis for its final confirmation. The decrease in acid value was not significant for 1:1, 1:2 and 1:5 M ratios with time, as exhibited in Fig. 3A. Quantitative DAG yield for these molar ratios remained very less after proper TLC (Fig. 3B) The pattern of graph for 1:3 and 1: 4 M ratios using both 1, 3 specific RMIM and random NS435 lipases exhibited a progressive decrease in acid value till the 18thhr and 6thhr respectively followed by progressive increase again. Results for 1:3 were more prominent. Least acid values were obtained at 18th and 6thhr for 1: 3 M ratios with both the lipases, respectively as indicated in Figs. 3A and 4A. Furthermore, quantitative TLC study revealed maximum DAG yields at those two-time intervals significantly. DAG yields for the other molar ratios were also depicted in Fig. s. 3B and 4B for both the lipases. 2:1 M ratio was also considered but gave no significant output as evident from Fig. 3C and D and Fig. 4C and D.

From Fig. 3A, the standard reaction parameter was found to be at the 18th hour for 1:3 M ratios of glycerol and $C_{10:0}$as MCFA at 60 °C when RMIM enzyme was applied. When the acid values of all the molar ratios (1:1, 1:2, 1:3 and 1:4) at 0–24 h time interval was plotted using the statistical software (Fig. 3A), significant ($p < 0.05$) drop of acid value was observed at the 18thhr for the 1:3 M ratios. Qualitative TLC was performed along with the quantitative analysis of this hour for visual estimation and production of structured DAG.

When the same thing was repeated with the enzyme being NS 435, the significant least acid value ($p < 0.05$) was found at the 6thhr for 1:3 M ratios. Comparison among the obtained acid values of the other molar ratios (1:1, 1:2, 1:4) from 0 to 24 h time interval in a line graph using the same statistical software (Fig. 4A), lead to the selection of the

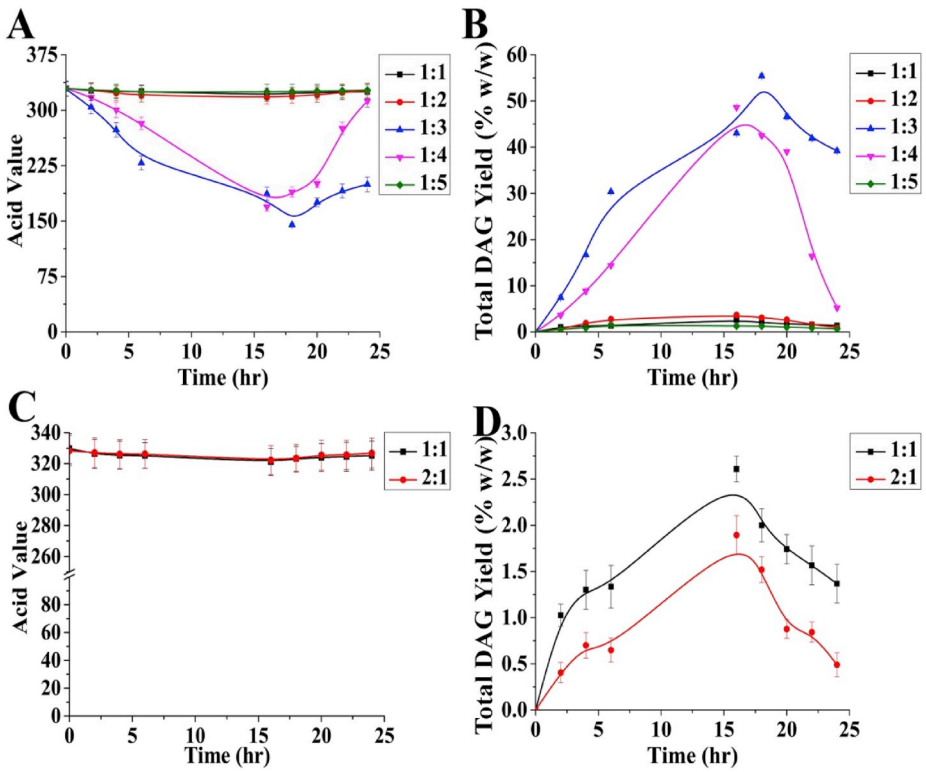

Fig. 3. A, 3B, 3C and 23D Comparison of the acid values and structured DAG of all the products of different molar ratios (1:1, 1:2, 1:3, 1:4, 1:5, 2:1) of glycerol and $C_{10:0}$RMIM as biocatalyst (10% w/w) at different time intervals (0–24 h) at 60 °C (Values are Mean ± SEM,$p < 0.05$, hr = hour, n = 3).

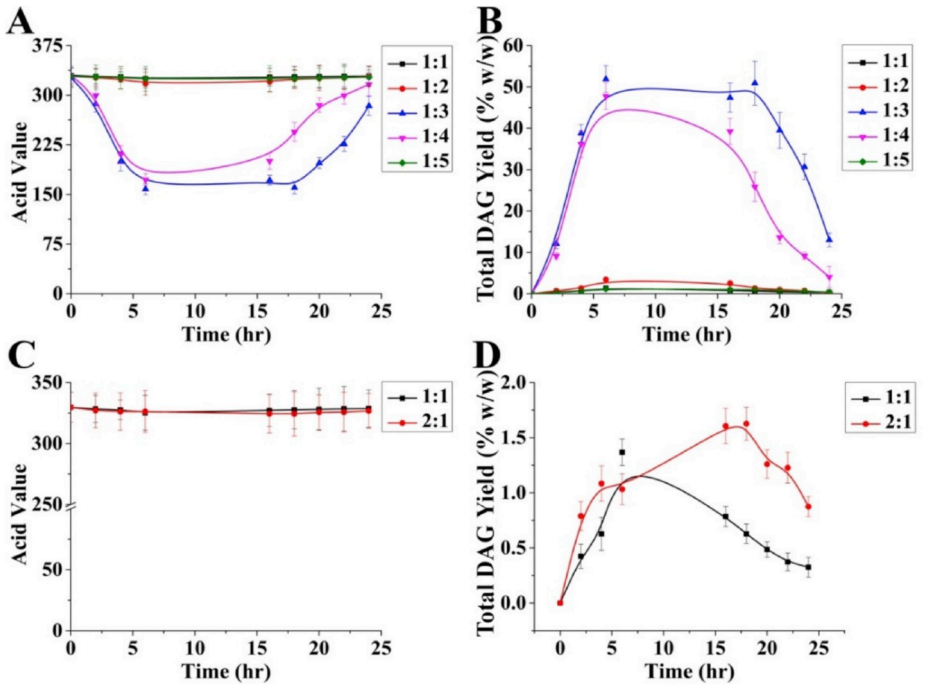

Fig. 4. A, 4B, 4C and 4D Comparison of the acid values and structured DAG of all the products of different molar ratios (1:1, 1:2, 1:3, 1:4, 1:5, 2:1) of glycerol and $C_{10:0}$ NS435as biocatalyst (10% w/w) at different time intervals (0–24 h) at 60 °C (Values are Mean ± SEM,$p < 0.05$, hr = hour, n = 3).

6^{th}hr of 1:3 M ratios at 60 °C as the optimized reaction parameter.

All the optimized products were made to undergo HPTLC for isomer separation. Table2represents the presence of the two different isomers 1, 3 and 2, 3 from the same product obtained at the 18th and 6^{th}hr of 1:3 M ratios of glycerol and $C_{8:0}$and glycerol and $C_{10:0}$in presence of RMIM and NS 435 enzyme (10% w/w) at 60 °C respectively, using HPTLC methodologies. Moreover, HPTLC confirmed very little acyl migration in case of RMIM lipases which could be ignored.

Using HPTLC measures, two different results came into play. There was a maximum yield of 1, 3 DAG only in case of RMIM enzyme,

irrespective of the fatty acid type ($C_{8:0}$ and $C_{10:0}$). There was very negligible acyl migration, hence it could be ignored. But the introduction of NS 435 enzyme in the esterification reaction gave a different direction in case of both the fatty acids.

Table 1 shows the determined percentage of lipid content of the resultant products. RMIM catalyzed reaction provided the best results for the synthesis of 1, 3-dicaprylin and 1, 3-dicaprin. Maximum conversion of 58.86 ± 4.05% and 55.41 ± 3.95% of 1, 3 DAG from two MCFAs; $C_{8:0}$ and $C_{10:0}$ using RMIM lipase was obtained from the reaction mixture (Tables 1 and 2). 2, 3 (1, 2) DAG isomers of $C_{8:0}$ and $C_{10:0}$ were also formed under the same reaction parameters but in lesser amounts irrespective of the type of lipases used (RMIM and NS435). Immobilized lipase at 10% w/w (RMIM and NS435) at an optimized temperature of 60 °C accelerated the fatty acid conversion, as well as the triacylglycerol formation owing to rapid acyl group migration. Formation of triacylglycerols was less compared to that of DAG part in both the enzymatic esterification processes of $C_{8:0}$ and $C_{10:0}$. Less amounts of Monoacylglycerol (MAG) and Free Fatty acid (FFA) were also found to be present within the respective reaction mixtures as has been shown in Table 1.

One-wayANOVA using statistical software (Origin Pro 8) was performed to compare the best yields of structured DAGs from $C_{8:0}$and $C_{10:0}$using RMIM and NS435 respectively. Structured 1, 3 DAG synthesized enzymatically (NS435) using 1:3 M ratio of Glycerol and MCFA, $C_{8:0}$ at 6thhr at 60 °C was significantly lower ($p < 0.05$) than that of the same produced using RMIM at 18th hr. Graphical representation of such significance is depicted in Fig. 5A. Similar scenario is evident in the pictorial representation of the comparative graph (Fig. 5B) for the best DAG yield.Structured 1, 3 DAG synthesized enzymatically at 1:3 M ratio of Glycerol and $C_{10:0}$at 6thhr at 60 °C using NS435was significantly lower ($p < 0.05$) than the yields at 18thhr using RMIM respectively.

From the appended set of figures (Fig. 5), it can be said that, in both cases of MCFAs, i. e, $C_{8:0}$and $C_{10:0}$, significant ($p < 0.05$) production of structured DAG was observed when RMIM and NS435 enzymes were employed as intervening catalysts albeit under same experimental parameters the RMIM lipases provided higher efficacy in the structured DAG production as compared to NS435. The fact that RMIM gave better results when reacted with MCFAs could be due to its site specificity attribute as compared to NS435, a random lipase. Further, it was also conspicuous that the yield of DAG production from $C_{8:0}$ was much higher than $C_{10:0}$.The higher viscosity of $C_{10:0}$could have resulted in lower yield in DAG incorporation than $C_{8:0}$ during enzymatic conversion (Macala et al., 1983). Also, due to the smaller size of the $C_{8:0}$molecule it can be assumed that the acyl migration in case of DAG was more favored for the $C_{8:0}$molecule than the $C_{10:0}$molecule, in case of NS435 enzyme.

Interestingly, esterification reaction with acid catalyst for both the MCFAs in separate reaction sets led to successful incorporation as structured DAGs. Concentrated sulphuric acid (H_2SO_4) at 1.0% w/w was used as the choice of chemical catalyst. During optimizing reaction parameters, it was found that 1:1 M ratio of glyceroland MCFAs; for both $C_{8:0}$ and $C_{10:0}$ resulted in maximum yield although optimum time of reaction for the maximum yield differed at 1 and 2 h respectively (Figs. 6 and 7). The reaction period, prior to optimization was set for 6 h. However, during this period, experimental products got charred and separation and isolation of the desired product was a major issue. Consequently, the reaction tenure was set to 3 h for the easy collection and purification of the products. When 1:0.5 and 2:1 M ratio was attempted, there was no successful DAG yield, as confirmed by qualitative TLC.

When Glycerol and $C_{8:0}$ were reacted in presence of concentrated H_2SO_4 as catalyst (1.0% w/w) at 1:1, 1:2, 1:3 M ratios at 180 °C for the time interval of 0–3 h, there was decrease in acid values initially with time as is evident in Fig. 6A. Significant fall in the acid values was observed at the 1st hr. But acid values began to increase progressively again. Therefore, from Fig. 6A, it was revealed that at the first hour for

Table 2
Determination of the percentage of yield of the two different isomers 1, 3 and 2, 3/(1, 2) of DAGs from $C_{8:0}$ and $C_{10:0}$ with the enzymes RMIM & NS 435 respectively.

Type of MCFA	Type of Enzyme with reaction time in hour (h)	Composition [Mean ± SEM]	
		Type of isomers of DAG	
		1, 3	2, 3/(1, 2)
$C_{8:0}$	RMIM (18 h)	91.79 ± 0.42*	8.21 ± 0.01
	NS435(6 h)	84.86 ± 4.69	15.14 ± 2.69
$C_{10:0}$	RMIM(18 h)	90.11 ± 0.014**	9.89 ± 0.02
	NS435(6 h)	86.69 ± 0.33	13.31 ± 0.27

Values are represented as Mean ± SEM, n = 3.
*Significant at $p < 0.05$ of $C_{8:0}$ 1, 3 DAG yield percentage (%) using RMIM vs. NS435.
**Significant at $p < 0.05$ of $C_{10;0}$ 1, 3 DAG yield percentage (%) using RMIM vs. NS435.

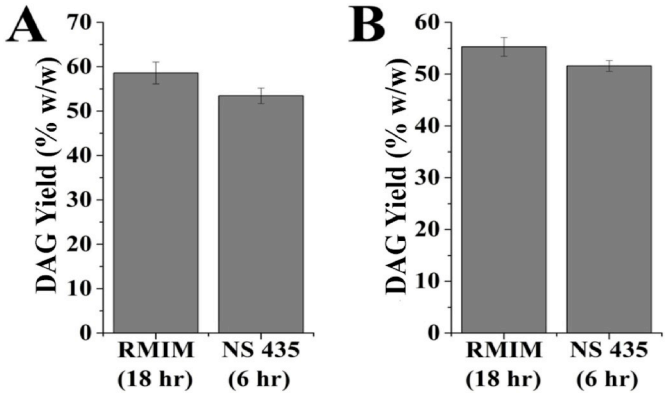

Fig. 5. A and 5B Comparison of the yield (%) of the structured DAGs (1, 3) from glycerol-$C_{8:0}$ and glycerol-$C_{10:0}$combinations of reactants, at 1:3 M ratios using RMIM and NS 435 enzymes separately, with the reaction time at the 18th hour and the 6th hour respectively at 60 °C. (Values are Mean ± SEM, $p < 0.05$, n = 3).

Table 1
Percentage of lipid constituents within the esterified products of glycerol and the MCFAs; caprylic ($C_{8:0}$) and capric acid ($C_{10:0}$) at 1:3 M ratio at 10% enzyme concentration (w/w) of immobilized RMIM and random immobilized NS435 lipases respectively at 60 °C.

Type of MCFA	Type of Enzyme with reaction time in hour (h)	Composition of lipid (%) [Mean ± SEM]			
		MAG	DAG	FFA	TAG
$C_{8:0}$	RMIM (18 h)	4.62 ± 0.11	**58.86 ± 4.05***	4.16 ± 0.01	34.36 ± 0.09
	NS435 (6 h)	8.93 ± 0.02	**53.77 ± 3.74**	7.85 ± 0.01	29.45 ± 0.02
$C_{10:0}$	RMIM (18 h)	6.12 ± 0.06	**55.41 ± 3.95****	11.15 ± 0.04	27.32 ± 0.02
	NS435 (6 h)	8.95 ± 0.05	**51.89 ± 2.60**	13.89 ± 0.02	25.27 ± 0.07

Values are represented as Mean ± SEM, $n = 3$.
*Significant at $p < 0.05$ of $C_{8:0}$-DAG yield percentage (%) using RMIM vs. NS435.
**Significant at $p < 0.05$ of $C_{10:0}$-DAG yield percentage (%) using RMIM vs. NS435.

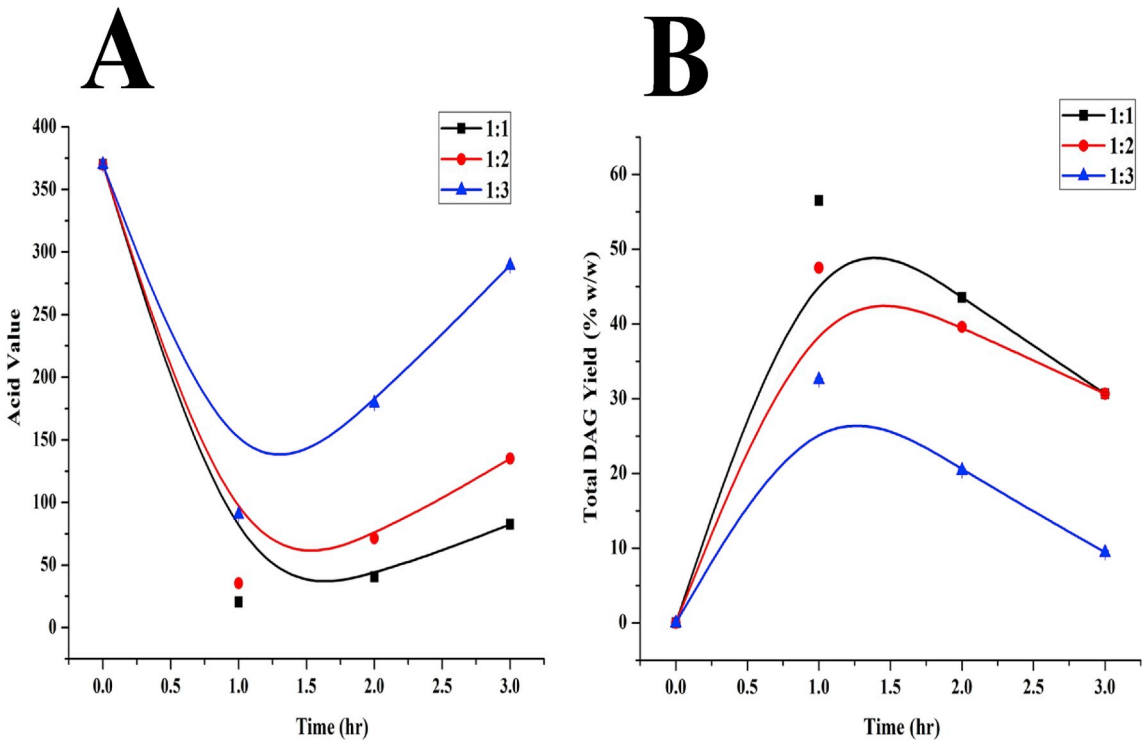

Fig. 6. A and 6B Comparison of the acid values and structured DAG yields of all the products of different molar ratios (1:1, 1:2, 1:3) of glycerol and $C_{8:0}$ using concentrated sulphuric acid (1.0% w/w) at different time intervals (0–3 h) at 180 °C chemically (Values are Mean ± SEM,$p < 0.05$, hr = hour, n = 3).

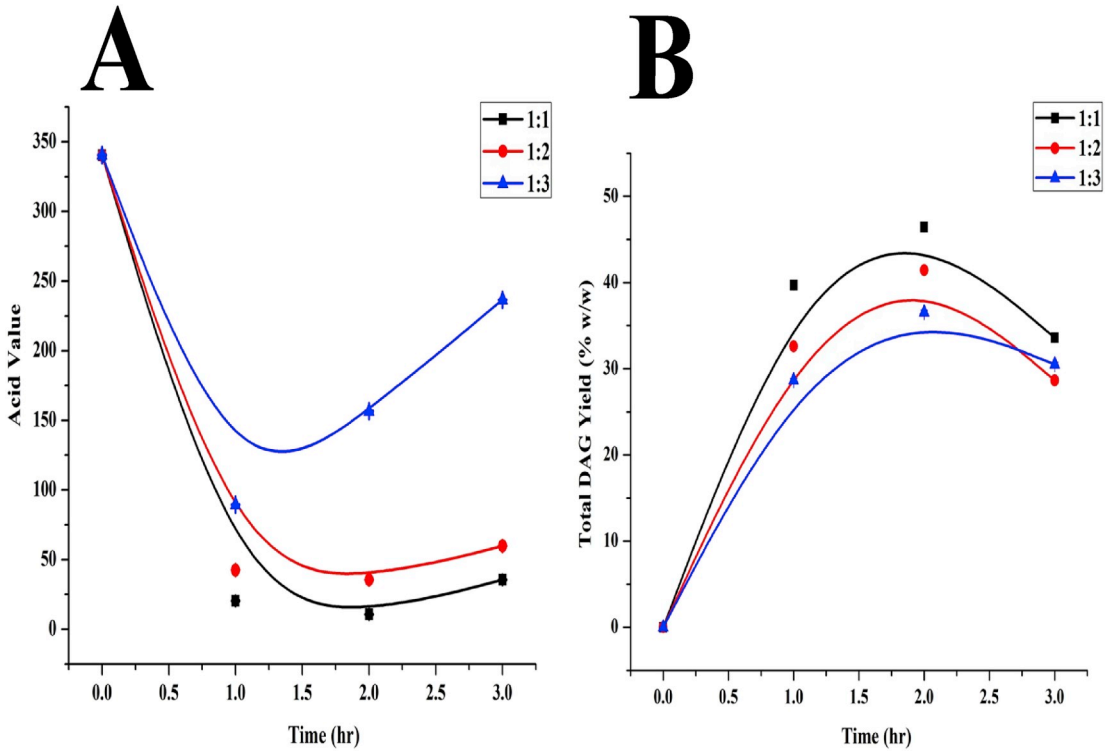

Fig. 7. A and 7B Comparison of the acid values and structured DAG yields of all the products of different molar ratios (1:1, 1:2, 1:3) of glycerol and $C_{10:0}$ using concentrated sulphuric acid (1.0% w/w) at different time intervals (0–3 h) at 180 °C chemically (Values are Mean ± SEM, $p < 0.05$, hr = hour, n = 3).

1:1 M ratio, significant ($p < 0.05$) drop of acid value was obtained when compared to the other molar ratios and at all the hours. Subsequently, the structured DAG yields of all the molar ratios (1:1, 1:2, and 1:3) during different time intervals from 0 to 3 h were determined by TLC analysis, followed by column chromatography (Fig. 6B). Chemical esterification of glycerol and $C_{8:0}$ at 1:1 M ratio for 1 h using concentrated sulphuric acid at 1.0% w/w at 180 °C gave significant ($p < 0.05$) DAG yields (Fig. 6B).

When 1:0.5 and 2:1 M ratios of glycerol and $C_{10:0}$ were taken into consideration for 0–3 h at 180 °C using 1.0% chemical catalyst, there was no successful DAG yield, as was determined by qualitative TLC. Consequently, 1:1, 1:2, and 1:3 M ratios were also executed under similar experimental conditions. Acid value drop is considered as a benchmark for subsequent structured DAG formation. Fig. 7A exhibits the pattern of acid values that was found for the time period of 0–3 h for all the said molar ratios (1:1, 1:2, and 1:3).1:1 M ratio showed most significant ($p < 0.05$) drop in acid values initially with least being at the 2nd hour. 1: 2 and 1:3 also showed similar patterns (Fig. 7A). TLC, following column chromatographic analysis revealed the significant DAG yields for all the molar ratios from 0 to 3 h. Structured DAG yield was found to be significantly ($p < 0.05$) higher in case of 1:1 M ratio of glycerol and MCFA- $C_{10:0}$ at the 2ndhr, other experimental conditions remaining the same (Fig. 7B). 1:1 M ratio of glycerol and $C_{10:0}$ at the 2nd hour using concentrated sulphuric acid as the chemical catalyst at 1% w/w at 180 °C was considered as the optimized reaction parameter. The different lipid constituents present within the chemically esterified product of the two MCFAs was depicted in Table 3. HPTLC analysis also has revealed the significant ($p < 0.05$) yield of 1, 3 isomer of structured DAG from 1:1 M ratio of glycerol and MCFA- $C_{8:0}$ chemically using 1.0 w/w concentrated sulphuric acid as acid catalyst at 180 °C than 2, 3 (1, 2) forms. Similar observations were determined in case of $C_{10:0}$ at the 2nd hr chemically. 1, 3 isomers were significantly higher than the 2, 3 (1, 2) ones, as noted from Table 4.

The study revealed that initial esterification leads to formation and increase in MAG concentration that acts as natural substrates for immobilized non-specific lipases such as RMIM and NS 435 (Ortiz et al., 2019). Consequently, as has been asserted by other investigators (Von der Haar et al., 2015) that MAG concentration in reaction mixture of enzymatic esterification reaction has significant impact. It was expected that due to the non-specific activity of NS-435 catalyst and chemical catalysts, much of the formed DAGs will proceed to form triacylglycerols (TAGs) which will result in higher amount of TAG in the mixture than the RMIM catalyzed reactions. But in the present study it was observed that amount of TAG present in the RMIM catalyzed reaction was higher than the random catalysis. This may be due to longer reaction time in case of RMIM which led to acyl migration and in turn higher amount of TAG was formed (Ortiz et al., 2019; Von der Haar et al., 2015). Similarly higher amount of FFA was reacted due to prolonged reaction time to produce MAG, DAG and TAG.

After estimation of the structured DAG yields from both the reactions, enzymatically and chemically, using TLC and HPTLC techniques, physical characterization was performed with the best yields. Viscosity, Refractive Index, Slip Melting Point and Cloud Point being some of the landmark physical characterization parameters were performed for the final set of four 1, 3 DAGs produced following standardized AOCS Official methods (Firestone, 2009).

Cloud point is referred to as the cold temperature operability of oils, where, at a certain temperature the oil possesses a cloudy physical appearance following the onset of freezing. $C_{8:0}$ is such a MCFA, which does not turn to freeze even if the temperature goes down to the freezing

Table 3
Amount (%w/w) of lipid constituents produced during the esterified products of glycerol and the MCFAs; caprylic ($C_{8:0}$) and capric acid ($C_{10:0}$) at 1:1 M ratio at 1.0% concentration (w/w) of chemical catalyst at 1 and 2 h at 180 °C repectively

Fatty acids	Lipid components (Mean ± SEM) in %w/w			
$C_{8:0}$ (1hr)	MAG	DAG	TAG	FFA
	31.45 ± 0.28	**56.52 ± 0.28***	7.55 ± 0.23	4.49 ± 0.14
$C_{10:0}$ (2hr)	19.48 ± 0.20	**46.38 ± 0.24****	22.56 ± 0.22	11.42 ± 0.23

Values are represented as Mean ± SEM, $n = 3$.
*Significant at $p < 0.05$ of $C_{8:0}$-DAG yield percentage (%) using chemical catalyst.
**Significant at $p < 0.05$ of $C_{10:0}$-DAG yield percentage (%) using chemical catalyst.

Table 4
Yield (% w/w) of the two different isomers 1, 3 and 2, 3/(1, 2) of DAGs from $C_{8:0}$ and $C_{10:0}$ with the chemical catalyst.

Type of MCFAs	Composition of the isomers of DAGs [Mean ± SEM] in % w/w	
	1, 3	2, 3/(1, 2)
$C_{8:0}$	86.69 ± 1.36*	13.31 ± 0.93
$C_{10:0}$	84.86 ± 4.23**	15.14 ± 2.09

Values are represented as Mean ± SEM, n = 3.
*Significant at $p < 0.05$ of $C_{8:0}$ 1, 3 DAG yield percentage (%) using chemical catalyst.
**Significant at $p < 0.05$ of $C_{10;0}$ 1, 3 DAG yield percentage (%) using chemical catalyst.

temperature. Hence, addition of $C_{8:0}$enhances the cold temperature operability of the structured DAG (1, 3). RMIM added an extra benefit to this condition of the reaction process as is evident from Table 2 data. The cloud point of the pure $C_{10:0}$was quite higher (15.32 ± 0.10 °C) in comparison to that of $C_{8:0}$. This was a disadvantage in case of $C_{10:0}$. But, application of RMIM as the immobilized lipase enzyme might enhance the cold temperature property of 1, 3 DAG from $C_{10:0}$. Presence of glycerol in the reaction system might act as a chemical agent which enhances the cold temperature property of $C_{10:0}$rich structured 1, 3 DAG. It was observed from Table 5, that the cloud point of the $C_{10:0}$rich 1, 3 DAG using NS435 was significantly ($p < 0.05$) higher than the 1, 3 DAG produced using RMIM. Therefore, the performance of RMIM again revealed greater influence on the production and physical property of the structured DAGs (1, 3).

Minor changes in refractive index of both the MCFA DAGs were noted in Table 5 in comparison to the pure MCFAs. This could have occurred due to the reaction of MCFAs with glycerol leading to formation of 1, 3 DAGs. The carbon chain length of the MCFAs ($C_{8:0}$and $C_{10:0}$) might contribute to one of the possible reasons of difference in the refractive index data. Better refractive index was observed in both cases of $C_{8:0}$and $C_{10:0}$rich 1, 3 DAG using RMIM. Eventually, 1, 3 specific RMIM earned a better proficiency during the production of 1, 3 DAGs in both cases of MCFAs; $C_{8:0}$and $C_{10:0}$than the random immobilized NS435 lipases.

It can be seen from Table 5, that due to higher slip melting point values, there was less significant ($p < 0.05$) incorporation of C:8 when NS435 was the biocatalyst in the reaction system whereas, RMIM usage with $C_{8:0}$gave higher significance ($p < 0.05$) with less slip melting values. Similar incidents were observed for $C_{10:0}$. After thorough observation from the result pattern obtained in Table 2, it might be stated that RMIM played a vital role as a biocatalyst in the incorporation of the MCFAs; $C_{8:0}$and $C_{10:0}$in the production of 1, 3 DAGs in comparison to NS435 by enhancing the entire reaction process. This might be a probable cause behind the significant ($p < 0.05$) yields of 1, 3 DAGs of both $C_{8:0}$and $C_{10:0}$.

In chemical catalysis substrates are reacted directly with each other whereas in enzyme catalysis reaction first enzyme-substrate complex is formed and the reaction between two substrates is occurred in that particular site and finally the enzyme leaves the product. Therefore, enzymatic glycerolysis takes longer time to reach the equilibrium compared to chemical catalysis reaction. Random nature of chemical glycerolysis produces equal proportions of different isomers after the reaction. However, 1, 3 DAG is thermodynamically stable than 1, 2 (2, 3) DAG due to steric effect. Therefore, 1, 2 (2, 3) DAG will migrate to form 1, 3 DAG in every case especially where the reaction time span is longer. From the complete set of results, it was evident that, enzymatic esterification required 6–18 h to produce maximum yield whereas the chemical esterification process completed such conversion within 1–2 h for both the MCFAs. A significantly less reaction time was required chemically in case of $C_{8:0}$ to produce the same amount of DAG than that of the $C_{10:0}$, possibly due to the fatty acid chain length.

Table 5
Physical characterization of caprylic acid, capric acid, and their DAGs produced at 1:3 M ratio of glycerol and particular medium chain fatty acid; $C_{8:0}$/and $C_{10:0}$ at 60 °C temperature at 10% (w/w) immobilized enzyme concentrations (RMIM and NS435) at 18 h and 6 h respectively.

Properties	Values					
	Type of medium chain fatty acid (MCFA)					
	Caprylic Acid ($C_{8:0}$)			Capric Acid ($C_{10:0}$)		
	Pure	DAG		Pure	DAG	
		Reaction time			Reaction time	
		18 h	6 h		18 h	6 h
		RMIM	NS435		RMIM	NS435
Viscosity (Cp) at 21.6 °C	111.70 ± 0.42	64.80 ± 0.27*	103.80 ± 0.76	175 ± 0.35	75.30 ± 0.35$^{\#}$	110.06 ± 0.34
Refractive Index at 40 °C	1.42 ± 0.01	1.46 ± 0.01**	1.44 ± 0.01	1.46 ± .003	1.45 ± 0.01$^{\#\#}$	1.46 ± 0.003
Slip Melting (°C)	16.5 ± 0.12	14.98 ± 0.20***	15.55 ± 0.21	30.49 ± .28	25.49 ± 0.12$^{\#\#\#}$	28.17 ± 0.48
Cloud Point (°C)	8.52 ± 0.04	8.33 ± 0.04****	9.3 ± 0.04	15.32 ± 0.10	9.49 ± 0.10$^{\#\#\#\#}$	10.34 ± 0.07

Values are represented as Mean ± SEM, n = 3.
*Significant at $p < 0.05$ of $C_{8:0}$; Viscosity of DAG produced by using RMIM vs. NS435.
**Significant at $p < 0.05$ of $C_{8:0}$; Refractive Index of DAG produced using RMIM vs. NS435.
***Significant at $p < 0.05$ of $C_{8:0}$; Slip Melting of DAG produced using RMIM vs. NS435.
****Significant at $p < 0.05$ of $C_{8:0}$; Cloud Point of DAG produced using RMIM vs. NS435.
$^{\#}$Significant at $p < 0.05$ of $C_{10:0}$; Viscosity of DAG produced by using RMIM vs. NS435.
$^{\#\#}$Significant at $p < 0.05$ of $C_{10:0}$; Refractive Index of DAG produced using RMIM vs. NS435.
$^{\#\#\#}$Significant at $p < 0.05$ of $C_{10:0}$; Slip Melting of DAG produced using RMIM vs. NS435.
$^{\#\#\#\#}$Significant at $p < 0.05$ of $C_{10:0}$; Cloud Point of DAG produced using RMIM vs. NS435.

4. Conclusion

Major findings of the present research work included a small-scale production of the different isomers of structured DAGs as result of both chemical and enzyme catalyzed esterification reactions separately. Chemically synthesized structured DAGs from MCFAs $C_{8:0}$and $C_{10:0}$ was found to be noticeably less quantitatively when compared to that of the enzymatic ones. The entire reaction set up was kept solvent free for both the processes. MCFAs $C_{8:0}$and $C_{10:0}$both gave better DAG yields when subjected to lipozyme RMIM in comparison to NS435. Therefore, RMIM played a pivotal role as a biocatalyst not only in case of MCTs and LCFAs, but also for MCFAs. Although the chemical process required less reaction time in the maximum DAG conversion, due its high reaction temperature is not considered eco friendly process by the environmental scientists. Enzyme catalysis involving more reaction time but with mild reaction temperature, selectivity and low waste generation have satisfied the criteria of green chemistry. Therefore, it could be concluded by saying that, the production of pure structured DAG [1, 3 and/or 2, 3 (1, 2) isomers] from MCFAs, using eco-friendly biocatalysts is a novel approach in the field of structured lipids and oil industry. This resulted in a new concept of designer lipids which in future will aid in the biological sphere for the prevention of various kinds of patho-physiological conditions. These products, if prepared in large scale in industries might provide a different dimension to the future technologists and biologists.

Declaration of competing interest

Authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101422.

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