Lipase-catalyzed synthesis of partial acylglycerols of acetoacetate*

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Immobilized *Rhizomucor miehei* lipase (Lipozyme RMIM) was employed in the synthesis of partial acylglycerols of acetoacetate (C₄H₆O₃, 3-oxobutanoic acid). Both 1(3)-sn-monoacetoacetyl glycerol (MAcG) and 1,3-sn-diacetoacetyl glycerol (DAcG) resulting from esterification of glycerol with ethylacetoacetate were isolated and identified by ¹H and ¹³C NMR. An HPLC method for the separation and quantitation of these species was developed. The effects on product yield of coordinate variations in the amounts of enzyme, water, ethyl acetoacetate, and the ratio of immobilizing silica to glycerol were explored. This allowed creation of predictive equations relating these variables to product yield. Reaction conditions were thereby identified and validated under which maximum yields of MAcG and/or DAcG or total ester were predicted. The production of both MAcG and DAcG was markedly sensitive to water, with optimal yields obtained within a narrow range of added water contents. Substantial excesses of ethylacetoacetate ameliorated the inhibitory effect of water. MAcG was effectively produced with the lowest amount of RMIM investigated. It was necessary to use larger amounts of lipase to achieve high yields of DAcG, and even then actual yields were only 30% of theoretical maximum. In the absence of silica only MAcG was produced.

Practical applications: Aceotacetyl esters of glycerol are potential prochiral building blocks for further chemical synthesis and exhibit the chemical reactivity of both the acetoacetyl moiety and of glycerol. They are thus of potential use in, for example, the production of biopolymers exhibiting any of a variety of features and functionalities. The use of enzymatic catalysis rather than chemical synthesis for their production offers advantages of reduced degradation and contamination due to the gentler reaction conditions characteristic of enzymatic catalysis, as well as a potential reduction in the costs to procure and dispose of catalyst.

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1 Introduction

Growing appreciation of the finite supply and uncertain economics of crude oil has stimulated efforts to develop bio-based replacements for petroleum products. In addition, the growth of the biodiesel industry has produced substantial quantities of coproduct glycerol. These facts have triggered research to develop routes for the production of new materials from glycerol [1].

One family of potentially useful glycerol-based compounds is that wherein the esterifying acid contains additional, internal, chemical functionality. Among such acids is acetoacetate (C₄H₆O₃, 3-oxobutanoic acid). Not only is its keto group a prochiral center and thus a potentially useful chemical synthon, but the high nucleophilicity of the carbon lying between its carbonyl groups suggests the potential for further facile chemical reactions by acylglycerols containing it. For instance, it is conceivable that the di- and triacetoacetyl esters of glycerol could participate in further reactions, generating cross-linked bio-based polymers of various sizes, reactivities, chemical and physical properties, *Disclaimer: Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.*
applications, and biodegradabilities. Also, a partial acylglycerol of acetoacetate could be further esterified at its free hydroxyl group(s) to bring other functionalities into the molecule.

Traditional conditions for ester synthesis can involve destructively high temperatures and/or require expensive catalysts. The former can jeopardize the use of labile and reactive species such as acetoacetate as reagents unless protected, and the latter can render a reaction scheme too expensive for application except to synthesize high value compounds such as pharmaceuticals. Thus, using solketal (acetone ketal of glycerol), diketene (anhydride of acetoacetate), \( t \)-butylacetoacetate, and toluene as solvent, the synthesis of mono- and triacetoacetyl glycerols and their use in the production of hydroxybutyrylglycerols has been described [2]. Some steps in this synthesis involved reflux at 120 °C for 14 h.

Enzymes are attractive potential alternative catalysts in such situations since they are active under mild reaction conditions, which reduces energy inputs and the degradation of reactants. In addition they often employ relatively simple, affordable substrates; display positional and stereo selectivities that can be difficult to achieve with conventional chemical catalysis; and do not catalyze undesirable side reactions. There has been substantial progress in the development of lipases, enzymes that form and hydrolyze carboxylic acid esters, and related structures, as applied catalysts [3, 4]. Thus, the use of enzymatic catalysis in the acetoacetyl glycerol synthesis cited above [2] would be expected to reduce reaction temperatures, eliminate the need for derivatization of the starting materials, and probably eliminate the use of toluene as solvent. By employing a positionally selective lipase as catalyst it also could provide a route to the previously unreported 1,3-diacylglycerol. Cross-linked polymers derived from this DAG should display physical properties different from those of polymers produced from the tri-substituted acylglycerol. Also, by esterification of other functionalities to the available hydroxyl groups of 1(3)-monoaacetoacetyl glycerol (MAcG) and 1,3-diacoacetyl glycerol (DAcG), products with multiple types of chemical reactivity could be synthesized. In the present work we describe the use of enzymatic catalysis for the first reported production of the mono- and diacetoacetyl esters of glycerol.

Enzymatic catalysis has been previously employed for the production of the acetoacetyl esters of a number of primary alcohols, for use as prochiral synthons [5, 6]. To our knowledge, however, this is the first report of the use of enzymatic catalysis for the synthesis of the acetoacetyl esters of a polyol such as glycerol (Fig. 1).

2 Materials and methods

2.1 Materials

Lipozyme RMIM (Rhizomucor miehei lipase immobilized on ion exchange resin), a generous gift from Novozymes North America, Franklinton, North Carolina, was dried at 55 °C under vacuum for 24 h and stored at RT over anhydrous calcium sulfate. Drying reduced the water content of the preparation from 4.3 wt%, as received, to 0.68 wt% as determined by Karl Fischer titration. Ethylacetoacetic acid (EAA) (’’98% min’’), purchased from EMD (Gibbstown, NJ), had a water content by Karl Fischer titration of 0.08 wt%. It was stored over 3 Å molecular sieves previously activated by drying for 6 h at 350 °C. Anhydrous glycerol (’’ultra’’ grade), purchased from Fluka (Steinheim, Germany), had a water content of 0.14 wt% by Karl Fischer titration. Silica gel (230–400 mesh), from Fisher Chemicals (Fair Lawn, NJ), was dried at 110 °C for 24 h and stored over anhydrous calcium sulfate. TLC plates (HPTLC-GHL normal phase, 150 μm, inorganic binder) were obtained from Analtech (Newark, DE).

Silica-immobilized glycerol was prepared by adding a desired amount of silica to a preweighed amount of glycerol in a screw capped vial. The materials were mixed with a spatula to a uniform flowable consistency and the vial was sealed with a rubber-lined cap and stored over anhydrous calcium sulfate at RT.

2.2 Reactions

Reactions contained EAA, water, enzyme, and 3 mmol of glycerol immobilized on silica, and were conducted in 11 cm × 2.5 cm OD (’’11 dram’’) flat bottom glass vials capped with rubber-lined screw top lids. Reagents were allocated to vials of an experimental group before enzyme,
which was added last. Reactions were conducted on a Glas-Col Laboratory Rotator (Terre Haute, IN) rotary mixer at 20 rpm and 55°C. This mixer rotated the vials through a complete circle in the vertical dimension, which achieved optimal mixing of reaction components without loss of enzyme to the tube walls. Reaction time was 18 h.

Following reaction, the vials were allowed to cool to RT. Ethyl acetate sufficient to bring the total liquid volume to 25 mL was added. (Reactions requiring more than 25 mL of liquid substrate were diluted to 30 mL, with subsequent correction for this difference in final volume.) The vials were shaken vigorously to ensure that any product adsorbed to the silica would be released into the solvent phase. Preliminary experiments had shown this protocol to achieve full release of the products. Samples of liquid phase were filtered through polytetrafluoroethylene (PTFE, 0.45 μm, Alltech, Deerfield, IL). Filtrates were stored in screw capped vials at 4°C until assayed for product.

2.3 Purification of reaction products

For structural identification and use as chromatography standards, MAcG and DAcG were separated and isolated by preparative silica gel column chromatography. Several of the reactions described above, less aliquots retained for quantitation, were pooled and their ethyl acetate and co-product ethanol removed under vacuum. A sample (1–2 g) of the remaining material was applied to a silica column (30 cm × 3.2 cm diam.) in hexane. The column was then sequentially washed, using nitrogen pressure to achieve a flow rate of 12 mL/min, with 250 mL hexane, 240 mL 1:1 v/v hexane/ethyl acetate, and 1250 mL ethyl acetate. Fractions (8.5 mL) were collected, and aliquots subjected to TLC using ethyl acetate as developing solvent. Following chromatography, MAcG and DAcG were detected on the air dried plates by dipping them in sulfuric acid in methanol (10 v/v%) and char-ring on a hot plate. MAG and DAG were cleanly separated from one another by this method (Rf of MAcG: 0.54; Rf of DAcG: 0.74). Material for characterization was obtained by pooling appropriate fractions and removing solvent under vacuum.

2.4 NMR Characterization of reaction products

1H and 13C NMR spectra were recorded on a Varian Inova 9.4 Tesla spectrometer (Palo Alto, CA) operating at a frequency of 399.93 and 100.56 MHz, respectively, using a 5 mm direct-detect broadband probe at 25°C. The samples were dissolved in deuterochloroform (Sigma, St. Louis, MO). Chemical shifts are reported as ppm from a tetramethylsilane internal standard.

2.5 HPLC

The MAcG and DAcG were separated and quantitated using a Lichrosorb Si60-5 silica column (10 cm, Varian, Walnut Creek, CA) on a Hewlett-Packard (Valley Forge, PA) model 1050 HPLC chromatograph equipped with an Alltech Varex MKIII evaporative light scattering detector (nitrogen flowrate: 1.77 SLPM, drift tube temperature: 33°C). The analytes were cleanly baseline separated from one another using linear solvent gradients of ethyl acetate, isopropanol, and hexane (Table 1) at a flow rate of 0.5 mL/min. Retention times were approximately 18 min for DAcG and 23 min for MAcG. Detector response was calibrated by chromatography of known amounts of purified MAcG and DAcG. Calibrations were performed in duplicate. R2 values of linear fits to the means of the resulting data exceeded 0.99. SDs of replicate determinations were no greater than 3% of the mean of the determinations.

2.6 Optimization of reaction conditions

Using a constant glycerol content of 3 mmol (0.27 g) throughout, Central Composite Response Surface design methods [7] were employed to investigate the effects and interactions of the ratio of silica to glycerol, and amounts of added water, immobilized enzyme, and EAA in the production of MAcG, DAcG, and total esterified glycerol in 18 h reactions conducted at 55°C. The ranges of these variables and the combinations in which they were tested, as specified by the experimental design, are listed in Tables 2a and 2b.

3 Results and discussion

3.1 Structural identification of MAcG and DAcG by NMR

Peaks from both tautomers of EAA were seen in the spectra of both the mono- and the diacetoacetyl acylglycerols.

3.1.1 Characterization of monoacetoacetyl glycerol

1H and 13C NMR spectra of MAcG (Fig. 2) contained peaks assignable to both the 1-and the 2-substituted glycerol isomers. All peaks due to the 2-isomer were minor relative to the 1-isomer. This is consistent with the known high
selectivity of the \textit{R. miehei} lipase in Lipozyme for the esterification of primary versus secondary alcohol moieties.

The $^1$HNMR spectrum (Fig. 2a) contained peaks consistent with a 1-acyl glycerol in the region 4.26–3.60 ppm. Peaks assignable to the keto tautomer of acetoacetic acid resolved at 3.55 ppm (alpha hydrogens) and at 2.29 ppm (terminal hydrogens).

The presence of the 2-position isomer was indicated on the $^1$HNMR spectrum by a multiplet at 5.04 ppm unique to an acyl substitution on the secondary carbon of glycerol. Other peaks overlapped with those assigned to the 1-position isomer. Peaks assignable to the enol tautomer of MAcG resolved at 11.88 ppm (the acidic hydrogen peak) and 5.04 ppm (the vinylic hydrogen). The terminal CH$_3$ signal shifted to 1.98 ppm. Peaks assignable to the 2-position enol tautomer were not seen.

The expected integration for 1-MAcG is as follows (shifts in ppm): 4.26–4.24 (2H), 3.96 (1H), 3.88–3.60 (2H), 3.55 (2H), 2.29 (3H). Observed integrations unique to the 1-position isomer were consistent with expected values. Overlap between the isomers led to an integrated value greater than 2H in the region 3.88–3.60 and greater than 3H at 2.29.

The $^{13}$C NMR spectrum (Fig. 2b) showed three distinctive peaks assignable to the glycerol carbons of the 1-position isomer at 69.9, 66.1, and 63.2 ppm. The ketone and ester carbons of acetoacetic acid resolved at 201.4 and 167.2 ppm, respectively, while its alpha carbon resolved at 50.0 ppm and its terminal carbon at 30.3 ppm.

\begin{table}[h]
\centering
\caption{Variables and the amounts of each studied, in the statistically designed optimization of the Lipozyme RMIM-catalyzed production of mono- and diacetoacetylglycerols}
\begin{tabular}{lcc}
\hline
Variable & Amounts studied \\
\hline
Ethyl acetoacetate (mL) & 2.55, 8.29, 14.02, 19.76, 25.49 \\
Water (\textmu L) & 50, 175, 300, 425, 550 \\
Lipozyme (g) & 0.05, 0.175, 0.300, 0.425, 0.550 \\
Mass ratio: silica/glycerol & 0.5, 1.0, 2.0, 3.0, 4.0 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Combinations of reagents and product yields, in the statistically designed optimization of the Lipozyme RMIM-catalyzed synthesis of mono- and diacetoacetylglycerols}
\begin{tabular}{llllllll}
\hline
Reaction & Water (\textmu L) & Silica (g) & Ethylacetoacetate (mL) & Lipozyme (g) & Monomer (mmol) & Dimer (mmol) & Total (mmol) \\
\hline
1 & 425 & 3.0 & 19.76 & 0.175 & 2.12 & 0.71 & 2.83 \\
2 & 300 & 2.0 & 14.02 & 0.300 & 2.06 & 0.87 & 2.92 \\
3 & 425 & 3.0 & 8.29 & 0.425 & 1.71 & 0.46 & 2.17 \\
4 & 425 & 1.0 & 19.76 & 0.425 & 2.09 & 0.89 & 2.98 \\
5 & 425 & 1.0 & 8.29 & 0.175 & 1.56 & 0.39 & 1.95 \\
6 & 175 & 3.0 & 8.29 & 0.175 & 1.91 & 0.74 & 2.65 \\
7 & 175 & 1.0 & 8.29 & 0.425 & 1.78 & 0.79 & 2.57 \\
8 & 175 & 3.0 & 19.76 & 0.425 & 1.63 & 1.22 & 2.86 \\
9 & 175 & 1.0 & 19.76 & 0.175 & 2.12 & 1.04 & 3.16 \\
10 & 300 & 2.0 & 14.02 & 0.300 & 1.98 & 0.85 & 2.83 \\
11 & 175 & 3.0 & 19.76 & 0.175 & 1.92 & 0.96 & 2.89 \\
12 & 175 & 1.0 & 8.29 & 0.175 & 2.07 & 0.71 & 2.78 \\
13 & 175 & 1.0 & 19.76 & 0.425 & 1.79 & 1.24 & 3.03 \\
14 & 300 & 2.0 & 14.02 & 0.300 & 1.76 & 0.77 & 2.53 \\
15 & 175 & 3.0 & 8.29 & 0.175 & 2.01 & 0.66 & 2.67 \\
16 & 425 & 1.0 & 19.76 & 0.425 & 1.66 & 0.44 & 2.10 \\
17 & 425 & 3.0 & 8.29 & 0.175 & 1.53 & 0.72 & 2.25 \\
18 & 425 & 1.0 & 19.76 & 0.425 & 1.70 & 0.81 & 2.50 \\
19 & 425 & 3.0 & 8.29 & 0.425 & 1.76 & 0.78 & 2.53 \\
20 & 300 & 2.0 & 14.02 & 0.300 & 2.02 & 0.91 & 2.93 \\
21 & 300 & 2.0 & 14.02 & 0.300 & 1.74 & 0.39 & 2.13 \\
22 & 50 & 2.0 & 14.02 & 0.300 & 1.54 & 0.45 & 1.99 \\
23 & 300 & 2.0 & 2.55 & 0.300 & 1.64 & 0.68 & 2.32 \\
24 & 300 & 4.0 & 14.02 & 0.300 & 1.53 & 0.68 & 2.21 \\
25 & 300 & 0.5 & 14.02 & 0.300 & 1.64 & 0.68 & 2.32 \\
26 & 550 & 2.0 & 14.02 & 0.300 & 1.54 & 0.45 & 1.99 \\
27 & 300 & 2.0 & 25.49 & 0.300 & 1.61 & 0.94 & 2.56 \\
28 & 300 & 2.0 & 14.02 & 0.050 & 1.70 & 0.43 & 2.13 \\
29 & 300 & 2.0 & 14.02 & 0.300 & 1.55 & 0.70 & 2.24 \\
30 & 300 & 2.0 & 14.02 & 0.300 & 1.53 & 0.70 & 2.23 \\
\hline
\end{tabular}
\end{table}
The $^{13}$C NMR spectrum also showed minor peaks characteristic of the 2-position isomer at 76.3, 61.8, 50.3, and 29.7 ppm, belonging to the glycerol carbons, and the alpha and terminal carbons of acetoacetate.

### 3.1.2 Characterization of diacetoacetyl glycerol

The $^1$H and $^{13}$C NMR (Fig. 3) spectra of DAcG contained peaks assignable to both the 1,3- and 1,2-diacetoacetyl isomers. All peaks due to the 1,2-isomer were minor relative to the 1,3-isomer, consistent with the known positional selectivity of the enzyme catalyst employed.

The $^1$H NMR spectrum (Fig. 3a) contained peaks expected for a 1,3-acyl glycerol in the region 4.37–4.19. Peaks assignable to the keto tautomer of acetoacetic acid resolved at 3.54 ppm (alpha hydrogens) and at 2.29 ppm (terminal hydrogens). The peaks integrated as expected on the basis of these assignments.

The presence of the 1,2-isomer was indicated on the $^1$H NMR spectrum by a multiplet at 5.19 ppm unique to an acyl substitution on the secondary carbon of glycerol. Peaks assignable to the keto tautomer of acetoacetic acid resolved at 3.51 ppm (alpha hydrogens) and at 2.27 ppm (terminal hydrogens).
Peaks assignable to the enol tautomer of 1,3-DAcG resolved at 11.88 ppm (acidic hydrogen) and 5.04 ppm (vinyllic). The terminal hydrogens appeared at 1.98 ppm.

The $^{13}$C NMR spectrum (Fig. 3b) showed two distinct peaks assignable to the glycerol carbons of the 1,3-isomer at 67.5 and 65.6 ppm. The ketone and ester carbonyl carbons of acetoacetate resolved at 201.0 and 166.9 ppm, respectively. The alpha carbons of the acetoacetyl residues resolved at 49.9 ppm. Their terminal carbons resolved at 30.3 ppm.

The $^{13}$C NMR spectrum also showed peaks characteristic of the 1,2-isomer at 73.1, 65.8, and 62.9 ppm, belonging to the carbons on glycerol, and at 50.0 ppm for the alpha carbons on the acetoacetyl chains and 29.7 ppm for their terminal carbons.

Thus, NMR analysis (a) indicated the presence of MAcG and DAcG, (b) verified that the primary locations of esterification of the glycerol were the 1 and 3 positions, (c) indicated the presence of both the keto- and enol-isomers of acetoacetate in the acylglycerol products, and (d) established the presence of minor amounts of 2-substituted glycerol esters in the preparation. The latter was probably produced by acyl migration from the 1 and 3 positions, which is known to occur at low rates.
3.2 Impacts of reaction components on product yields

Statistical experimental design techniques were adopted to identify the effects of the amounts of EAA, enzyme, and water, and of the ratio of immobilizing silica to glycerol, on the production of MACG and DACG. The product yields obtained in these studies are listed in Table 2b. Linear second order least square regression equations correlating product yield with reaction compositions were constructed using the PROC RSREG function of SAS/STAT software [8]. Preliminary experiments also indicated that the yield of acetoacetylglycerol was not increased by the presence of an organic solvent (hexane), reaction temperatures above approximately 55°C, or the use of inorganic salts (LiCl, K₂CO₃, KI, KCl; water activities of saturated solutions: 0.11, 0.43, 0.64, 0.80, respectively), to control the water activity of the system. Therefore these were not further examined. No product was made in the absence of enzyme.

Glycerol was immobilized on silica before addition to the reaction since this was shown to increase the degree of esterification. Prior reports have shown this approach to effectively provide glycerol to enzymatic reactions in organic solvents [9, 10]. The ratio of silica to glycerol in this immobilization was one of the variables examined in the statistically designed optimization of reaction conditions.

3.3 Reaction conditions for predicted optimum production of monoacetoacetylglycerol

Response surface experimental methodology indicated that the effects of variations in reaction parameters on the production of MACG could be expressed as:

\[
\begin{align*}
\text{MACG (mmol)} &= 1.74 - 6.66 \times 10^{-2}W - 1.05 \times 10^{-1}SG \\
&+ 2.88 \times 10^{-1}EAA - 5.89 \times 10^{-2}E + 4.39 \times 10^{-2}W^2 \\
&+ 8.31 \times 10^{-2}(W)(SG) + 3.55 \times 10^{-1}(W)(EAA) \\
&+ 3.40 \times 10^{-1}(W)(E) + 5.04 \times 10^{-2}SG^2 \\
&- 2.62 \times 10^{-2}(SG)(EAA) - 7.44 \times 10^{-2}(SG)(E) \\
&- 4.31 \times 10^{-1}EAA^2 - 1.75 \times 10^{-1}(EAA)(E) \\
&+ 2.54 \times 10^{-1}E^2 \quad (R^2 = 0.868)
\end{align*}
\]

where E is the enzyme (g), SG the mass ratio of silica to glycerol, W the water (μL), and EAA is the ethyl acetoacetate (mL).

The \(R^2\) value of 0.868 for the MACG model is significantly \((p < 0.05)\) nonzero. It indicates that the model explains 86.8% of the total variability in the experimental MACG responses, which is an acceptable fit of the model to the raw data obtained.

The coefficients in Eq. (1) were calculated by expressing the values of the settings of the variables as coded terms ranging from −1 for the lowest amount examined to +1 for the highest amount (Table 2a). This approach more clearly illustrates the relative impacts of the variables studied since it eliminates the effects of differences in the breadth of the ranges of variables examined and of differences in the magnitudes of the units used to express amounts of each (e.g., water is expressed in microliters while amounts of enzyme are expressed in grams). The sizes of the coefficients in Eq. (1) indicate that increases in the amount of ethylacetoacetate had the largest positive effect on MACG yield, with both the linear and quadratic terms for EAA being highly significant \((p < 0.01)\). This is illustrated in Fig. 4, with reduced amounts of MACG product at the lower and higher EAA levels, and maximum synthesis at approximately 18–20 mL EAA. This represents a minimum molar excess of EAA over glycerol of 47-fold. This suggests that EAA plays a solvent-like role in the reaction, perhaps preventing enzyme inhibition by diluting excess water or the ethanol coproduct of the reaction.

It is also possible that enzymatic hydrolysis of EAA, yielding acetoacetic acid and ethanol, is the basis for the requirement of high levels of this substrate. In fact it has recently been shown that such generation of acetoacetic acid, which can subsequently decompose to acetone and carbon dioxide, is a feasible route for the removal of water generated during enzyme-catalyzed esterification reactions, an approach termed “chemical drying” [11]. However, it can be calculated that application of all the water in the reactions conducted here, both that added at the outset of the reactions and that generated by glycerol esterification, to the hydrolysis of EAA would degrade less than 1 mL of this substrate. Therefore, hydrolysis is not a significant contributor to the observed requirement for substantial amounts of EAA.

The sizes of the cross product terms in Eq. (1) indicate that in terms of interactions between reaction variables, those between water and EAA and water and enzyme caused the greatest increases in yield. The amount of catalyst had little effect on the yield of monosubstituted glycerol under most conditions examined. Thus, a fivefold increase in enzyme gave no better than a predicted 10–20% increase in MACG (Fig. 4a and b). The lowest levels of enzyme examined gave a predicted 2/3 maximum theoretical product yield under appropriate conditions (Fig. 4a), and only small increases were obtained under any conditions with the use of additional catalyst (Fig. 4b). Thus, only small amounts of catalyst are needed for fairly effective production of MAG in this reaction. Higher amounts reduced yield under some conditions (Fig. 4a and b).
Preliminary experiments had shown that the yield of DAG was very poor in reactions containing 40 μL or less of water. Therefore, the minimum water level examined in these experiments was 50 μL. At all but the higher enzyme levels examined, MAcG yield was greatest at the lowest water levels examined. Higher amounts of water generally inhibited reaction, although EAA at elevated levels counteracted this inhibition (Fig. 4a and b).

MAG production was not substantially affected by the amount of silica used to immobilize the glycerol substrate, with the lowest silica/glycerol ratio examined (0.5) giving MAcG yields comparable to those at higher ratios (Fig. 4b and c). In the complete absence of silica no DAG was produced (data not shown). This suggests a route for the production of high purity MAcG if desired.

In this system, containing 3 mmol of glycerol, maximum MAcG yield was predicted to occur in reactions generally consisting of 0.05–0.1 g enzyme, 8–18 mL EAA, 50–100 μL water, and a silica/glycerol mass ratio of 0.5–1.0 (Fig. 2a). Predicted MAcG yields were nearly 90% of maximum theoretical, with the remainder of the glycerol predicted to be converted to DAcG.

Figure 4. Predicted yields of monoacetoacetyl glycerol as a function of added water and EAA at selected settings of catalyst and mass ratio of silica to glycerol. (a) Enzyme: 0.1 g; silica/glycerol mass ratio: 0.5, (b) enzyme: 0.5 g; silica/glycerol mass ratio: 0.5, and (c) enzyme: 0.5 g; silica/glycerol mass ratio: 4.0.
3.4 Reaction conditions for optimum production of diacetoacetylglycerol

Table 2b lists the amounts of product obtained in reactions designed to identify the relationship between esterification conditions and glyceride ester yield.

The equation relating the yield of DAcG to reaction variables, again expressed with coefficients as defined for Eq. (1) was:

\[
\text{DAcG (mmol)} = 7.71 \times 10^{-3} - 3.44 \times 10^{-3} W \\
- 2.19 \times 10^{-2} SG + 3.28 \times 10^{-1} EAA + 1.55 \times 10^{-1} E \\
+ 8.93 \times 10^{-2} W^2 + 3.06 \times 10^{-2} (W)(SG) \\
- 3.00 \times 10^{-2} (W)(EAA) - 2.00 \times 10^{-2} (W)(E) \\
- 4.32 \times 10^{-2} SG^2 - 2.63 \times 10^{-2} (SG)(EAA) \\
- 3.50 \times 10^{-2} (SG)(E) - 7.57 \times 10^{-2} EAA^2 \\
+ 1.55 \times 10^{-1} (EAA)(E) - 7.07 \times 10^{-2} E^2 (R^2 = 0.975)
\] (2)

The \(R^2\) value of 0.975 for the DAcG model is highly significantly \((p < 0.01)\) nonzero. This indicates a good fit between the model and the experimental data, with the model explaining 97.5% of the total variability in the experimental responses. Water, enzyme, and EAA were the most significant variables in the model, with their linear terms being highly significant \((p < 0.01)\).

The variable space examined here was identified by preliminary investigations as yielding the greatest production of DAG. Nonetheless, predicted DAcG production rarely exceeded 1.5 mmol or 50% of maximum theoretical glycerol conversion.

As with MAG, predicted DAcG yield increased as the amount of EAA in the reaction increased (Fig. 5a). As opposed to MAcG, however, there was no reduction in DAcG yield at the highest levels of EAA addition examined. Levels of EAA higher than shown here were not explored since reaction volumes were judged to be excessive under such conditions.

The production of DAcG was very water sensitive, with the highest predicted yields attained at the lowest water level examined (50 µL per reaction; Fig. 5a–c). Regardless of the amounts of other reaction components present, predicted DAcG yields were reduced above this amount. Preliminary experiments demonstrated that below about 40 µL of water DAcG synthesis was very poor. Thus, the “window” of water concentrations for effective DAG production was quite small.

The production of DAcG increased as the amount of catalyst increased. However, the effect was small and nonlinear, with the 11-fold range in enzyme amounts examined here giving only an approximately 60% change in predicted DAcG yield (Fig. 5a and b).

As in the production of MAG, the reaction was not markedly sensitive to the silica/glycerol ratio. Maximum DAcG yield was predicted at the lowest ratio and was not substantially affected by an increase in silica (Fig. 5b and c).

Equation (2) predicted maximum DAcG yields in reactions of 3 mmol glycerol with 0.4–0.6 g lipase, 25–28 mL EAA, 50 µL water and a silica/glycerol mass ratio of 0.5–2.0. Under these conditions the predicted yields of DAcG were 56–63% of maximum theoretical, with the balance of the reactant glycerol being present as MAcG.

3.5 Reaction conditions for the optimum production of total acetoacylglycerols

The predicted yield of total acylglycerol, i.e., the sum of MAcG and DAcG, as a function of reaction variables, and with normalized coefficients as in Eq. (1 and 2), could be expressed as:

\[
\text{Total Acetoacetylglyceride (mmol)} = 2.51 - 4.14 \times 10^{-1} W \\
- 1.25 \times 10^{-1} SG + 6.19 \times 10^{-1} EAA + 9.49 \times 10^{-2} E \\
+ 1.38 \times 10^{-1} W^2 + 1.07 \times 10^{-1} (W)(SG) \\
- 3.18 \times 10^{-1} (W)(EAA) + 3.18 \times 10^{-1} (W)(E) \\
+ 1.08 \times 10^{-2} SG^2 - 5.03 \times 10^{-2} (SG)(EAA) \\
- 1.12 \times 10^{-1} (SG)(E) - 4.98 \times 10^{-2} EAA^2 \\
- 2.28 \times 10^{-2} (EAA)(E) + 1.88 \times 10^{-1} E^2 (R^2 = 0.921)
\] (3)

The \(R^2\) value of 0.921 for the total model is highly significantly \((p < 0.01)\) nonzero. It indicates that the model fits the experimental data well, explaining 92.1% of the total variability in the experimental responses for total acetoacetylglyceride.

Water and EAA were the most significant variables in the model, with their linear and quadratic (EAA only) terms being highly significant \((p < 0.01)\). The EAA effect reflects its impact on the production of both MAG and DAGs, and thus total product yield was most affected by the amount of EAA in the reaction. This is indicated by the large EAA coefficient in Eq. (3) as well as by plots of yield versus reaction composition (Fig. 6). Yields were substantial at low levels of EAA, with esterification of all available glycerol predicted in reactions containing as little as approximately 6 mL of EAA under some conditions (Fig. 6a).

Total acylglycerol yield was relatively insensitive to the amount of enzyme present, with maximum theoretical yield predicted for even the lowest amount of catalyst examined under some reaction conditions (Fig. 6a). However, the enhancement of DAcG synthesis at elevated enzyme levels (Fig. 5a and b), the effect of increased enzyme would be to enrich the product for DAG, reducing MAG levels proportionately.

As noted above for MAcG and DAcG, the sum yield of these species was sensitive to the water content of the reaction, with maximum ester production at the smallest amount examined.
The yield of total substituted glycerol was not substantially impacted by the ratio of silica to glycerol (Fig. 6a–c). Maximum theoretical ester yield was predicted at the lowest silica/glycerol ratio examined (Fig. 6a).

Maximum theoretical combined ester yields were predicted by a number of combinations of the variables studied here. Among the most economical in terms of reagent usage was enzyme: 0.05–0.1 g; EAA: 6 mL; water: 50 mL; silica/glycerol: 0.5 (Fig. 6a).

It is possible that greater DAcG yields could be achieved by synthesizing MAcG under its optimum conditions, isolating the product, and then identifying and applying optimal conditions for its conversion to DAcG. This approach was not explored here.

3.6 Validation of the predictions of reaction conditions for optimal product yield

To validate the predictions of the RSM studies, reactions were conducted at 55°C using the conditions predicted to yield maximum mono- or disubstituted glycerol. Multiple independent reaction tubes were run at each condition, and product yields at selected times were determined by assaying the contents of an entire tube. The reaction conditions were: (a) for MAcG: glycerol: 3 mmol; enzyme: 0.1 g, ethylacetoacetate: 8.3 mL, silica/glycerol mass ratio: 0.5; water: 50 μL; (b) for DAcG: glycerol: 3 mmol; enzyme: 0.45 g, ethylacetoacetate: 27.5 mL, silica/glycerol: 0.5, water: 50 μL. The levels of both mono- and disubstituted glycerol were not substantially impacted by the ratio of silica to glycerol (Fig. 6a–c). Maximum theoretical ester yield was predicted at the lowest silica/glycerol ratio examined (Fig. 6a).

Maximum theoretical combined ester yields were predicted by a number of combinations of the variables studied here. Among the most economical in terms of reagent usage was enzyme: 0.05–0.1 g; EAA: 6 mL; water: 50 mL; silica/glycerol: 0.5 (Fig. 6a).

It is possible that greater DAcG yields could be achieved by synthesizing MAcG under its optimum conditions, isolating the product, and then identifying and applying optimal conditions for its conversion to DAcG. This approach was not explored here.

Figure 5. Predicted yields of diacetoacetyl glycerol as a function of added water and EAA at selected settings of catalyst and mass ratio of silica to glycerol. (a) Enzyme: 0.1 g; silica/glycerol mass ratio: 0.5, (b) enzyme: 0.5 g; silica/glycerol mass ratio: 0.5, and (c) enzyme: 0.5 g; silica/glycerol mass ratio: 4.0.
acylglycerols in all reactions were determined. Total yield was determined by summing these data. Results are shown in Fig. 7.

Under the conditions tested for monoglyceride production, the statistical model predicted a yield of 2.5 mmol (83% maximum theoretical conversion). MAcG production was steady throughout the incubation and had not reached a plateau by the 24 h sampling. Longer reaction times were deemed unwieldy. A yield of 2.1 mmol (70% max. theoretical) was observed at 24 h (Fig. 7a). This is acceptably close to the predicted yield, and validates the fit of the model to reality. DAcG production occurred at a slower pace, reaching a maximum of 0.4–0.5 mmol (13–17% maximum theoretical yield) under these reaction conditions. Since MAcG is probably a precursor to DAcG, the production of the latter reduces the ultimate achievable levels of the former. Overall production of substituted glycerols reached 2.5 mmol (Fig. 7a), representing esterification of 83% of the available glycerol.

Higher levels of DAcG were achieved under reaction conditions predicted to be optimal for this species (Fig. 7b). Nonetheless, yields reached only 0.9 mmol (30% maximum theoretical yield), and required 18 h of reaction. This is low compared to a predicted conversion

Figure 6. Predicted yields of total glycerol esters as a function of added water and EAA at selected settings of catalyst and mass ratio of silica to glycerol. (a) Enzyme: 0.1 g; silica/glycerol mass ratio: 0.5, (b) enzyme: 0.5 g; silica/glycerol mass ratio: 0.5, and (c) enzyme: 0.5 g; silica/glycerol mass ratio: 4.0.
of approximately 55% based on Eq. (2). It is unclear why the reaction failed to achieve the predicted yield. The reaction conditions did correspond to the edge of the variable space examined, similar to Fig. 5b at the highest EAA and lowest water values. The predictive value of a statistical model is known to be weakest at the edges of a response surface plot, due to the low number of data points collected in these regions, which might explain this anomaly.

The derived response surface models also predicted maximum theoretical yield of MAcG + DAcG under the reaction conditions for optimal DAcG synthesis. This was achieved in tests of the validity of the predicted optimal conditions, with full conversion of available glycerol being achieved by 18 h of reaction (Fig. 7b). Thus, validation experiments generally affirmed the validity of the derived predictive equation.

4 Conclusions

The enzyme-catalyzed synthesis of the mono- and diacetyloxyglycerols of glycerol has been achieved and confirmed.

Reaction conditions predicted to achieve the complete conversion of added glycerol to a mixture of the mono- and diacetyglycerols have been identified.

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