Solvent Optimization for Efficient Enzymatic Monoacylglycerol Production Based on a Glycerolysis Reaction

M.L. Damstrup[a], T. Jensen[a], F.V. Sparsø[c], S.Z. Kiil[b], A.D. Jensen[b], and X. Xu[a,]*

[a]Food Biotechnology and Engineering, BioCentrum-DTU, DK-2800 Kgs. Lyngby, Denmark, [b]Department of Chemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, and [c]DANISCO A/S, DK-8220 Brabrand, Denmark

ABSTRACT: This study was aimed at screening solvent systems of varying polarities to identify suitable solvents for efficient and practical enzymatic glycerolysis. Several pure solvents and solvent mixtures were screened in a batch reaction system consisting of glycerol, sunflower oil, and Novozym® 435 lipase. Out of 13 solvents tested, *tert*-butanol and *tert*-pentanol were the only pure solvents suitable for a fast glycerolysis reaction with an acceptably high formation of MAG. In these systems, MAG contents of 68–82% were achieved within a few hours. Mixtures of *tert*-butanol/hexane, *tert*-pentanol/hexane, and *tert*-butanol/*tert*-pentanol in varying ratios also gave high MAG contents (58–78%). The tertiary alcohols *tert*-butanol and *tert*-pentanol, or mixtures of one of them with hexane, seemed to be the best choice among the solvents tested with respect to reaction efficiency, practical industrial applications, and steric hydroxyl group hindrance, which suppresses the ester formation with FA.

Paper no. J11089 in *JAOCS 82*, 559–564 (August 2005).

KEY WORDS: *Candida antarctica* lipase, glycerolysis, monoacylglycerols, organic solvents, sunflower oil, and tertiary alcohols.

The worldwide production of emulsifiers is estimated at approximately 200,000–250,000 metric tons per year (1,2). MAG or mixtures with DAG account for approximately 75% of the emulsifier production and have various applications in different fields (2). In the food industry, MAG are widely used in bakery products, margarines, dairy products, and confectionary because of their emulsifying, stabilizing, and conditioning properties. They are also important in cosmetic and pharmaceutical industries as drug carriers and for consistency improvements in creams and lotions. Owing to their lubricating and plasticizing properties, MAG are also used in textile and fiber processing, and in the production of plastics (3–7).

Commercial MAG are widely manufactured by the glycerolysis of fats or oils. The glycerolysis reaction is accelerated by the use of inorganic alkaline catalysts, such as NaOH or $Ca(OH)_2$, at high temperatures (220–260°C). The MAG content in the equilibrium mixture varies from 10 to 60%, depending on the glycerol-to-oil ratio in the reaction blend (2). Commercial synthesized glycerolysis processing usually provides a distribution between MAG, DAG, and TAG of 45–55, 38–45, and 8–12%, respectively (2). MAG are subsequently purified, typically by means of short-path distillation, to achieve a product of at least 90% purity (2,8–10).

This conventional process presents some disadvantages. The use of high temperature leads to the development of off-flavors and a dark color. It may also accelerate unwanted acyl migrations, in which FA residues migrate from one position of the glycerol backbone to another position [typically from the *sn*-2 position to the *sn*-1(3) positions] (2,4–8). Furthermore, the high-temperature chemical process is not suitable for the production of heat-sensitive MAG containing PUFA. Production of these heat-sensitive MAG is, however, of great commercial interest owing to their nutritional value, which could be applied in functional foods, pharmaceuticals, and the like.

Enzyme-catalyzed glycerolysis reactions are believed to be a potential alternative to the chemical process because of the much lower temperature required (below 80°C). The low temperature improves product quality and makes production of "new" heat-sensitive MAG feasible. Several investigations of low-temperature lipase-catalyzed glycerolysis have confirmed the potential of the enzyme-catalyzed processes, even though an industrial-scale process is still unavailable owing to the lack of efficiency (4–7,11–14). Long reaction times and/or low conversion of reactants, mostly attributable to the very poor miscibility of the hydrophilic glycerol and the lipophilic oil at low temperatures, make the reaction inefficient.

A suitable solvent system to improve the miscibility of substrates will result in a more homogeneous system and enhance the conversion of substrate, the reaction rate, and the product distribution in favor of MAG formation (7). Solvents such as *n*-hexane, *n*-heptane, dioxane, acetonitrile, acetone, isooctane, 2-methyl-2-propanol (*tert*-butanol), 2-methyl-2-butanol (*tert*-pentanol), or mixtures of some of them are useful in different lipase-catalyzed interesterification reactions. However, no careful solvent evaluation has been found in the literature concerning the enzymatic glycerolysis of vegetable oils (3,4,7,15–18).

This purpose of this study was to screen several pure and mixed solvents of varying polarities in order to evaluate solvents for an efficient enzymatic glycerolysis process with industrial applications. The reaction equilibrium should be achieved within a few hours; the reaction should favor the

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*To whom correspondence should be addressed at BioCentrum-DTU, Technical University of Denmark, Bldg. 221, DK-2800 Kgs. Lyngby, Denmark. E-mail: mali@biocentrum.dtu.dk

Address reprint requests to Xuebing Xu, BioCentrum-DTU, Technical University of Denmark, Bldg. 221, DK-2800 Lyngby, Denmark.

formation of MAG; and the MAG content achieved should be at least as high as the chemical process. Solvents with different octanol–water partition coefficients (P), which are used in the literature as a parameter to characterize solvent properties in relation to lipids and lipases, were chosen (19). In general, P (or, in its more common form of expression, log P) provides a measure of the lipophilic vs. hydrophilic nature of a compound. P describes the distribution of a compound in a two-phase system and is defined as the ratio of the equilibrium concentration of the compound in a 1-octanol-rich phase to the concentration in a water-rich phase (in which water and 1-octanol are in equilibrium). Log P tends to be largest for compounds with extended nonpolar structures and smallest or negative for compounds with highly polar groups (20).

MATERIALS AND METHODS

Materials. Aarhus United (Aarhus, Denmark) provided the sunflower oil; this oil was a TAG oil with 97.1% TAG, 2.5% DAG, and 0.4% MAG and a water content of less than 0.01%. The FA composition (% w/w) of the sunflower oil was: C14:0, 0.1; C16:0, 6.7; C16:1, 0.2; C17:0, 0.1; C18:0, 3.7; C18:1, 26.3; C18:2, 61.2; C18:3, 0.4; C20:0, 0.3; C20:1, 0.2; C22:0, 0.6; C24:0, 0.2. Glycerol was purchased from VWR International Ltd. (Albertslund, Denmark) (purity, 99.5% w/w). Novozymes A/S (Bagsvaerd, Denmark) supplied the lipase enzyme Novozym® 435. Novozym 435, which originates from *Candida antarctica* and is produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism, is subsequently adsorbed on a macroporous resin. All solvents used were of analytical grade with a purity of 98–99.9% and were provided by Sigma-Aldrich (Broendby, Denmark).

Enzymatic glycerolysis reaction. Enzymatic glycerolysis was performed in batch experiments. Reaction blends for solvent screening consisted of 10.0 g sunflower oil, 5.26 g glycerol, and 50 mL solvent. Reaction blends for the time courses of the reaction consisted of 10.0 g sunflower oil, 4.21 g glycerol, and 40 mL solvent. The mixtures were incubated in capped 80-mL flasks in a water bath with magnetic stirring at 50°C. The reaction was initiated by addition of 3.0 g lipase enzyme. After 150 min or the set time for solvent screening and the time course of the reaction, 2 mL of the reaction mixture was withdrawn and filtered through a syringe filled with cotton wool to remove the enzyme. Subsequently, samples were flushed with nitrogen to remove the solvent. All samples were stored at −20°C prior to analysis.

GC analysis. The distribution of compounds in the mixture was analyzed by a PerkinElmer Autosystem 9000 capillary gas chromatograph equipped with a FID and a wall-coated open tubular fused-silica column (CP Sil 8 CB; 12.5 m × 0.25 mm i.d. × 0.1 μm film thickness of 5% phenyl-methyl-silicone; Chrompack, Middelburg, The Netherlands). The sample (50 mg) was dissolved in 12 mL heptane/pyridine (2:1, vol/vol) containing heptadecane (0.5 mg/mL) as an internal standard. The 500-μL sample solution was transferred to a crimp-capped vial; 100 μL of *N*,*N*-bis-trimethylsilyl-trifluoroacetamide was added and reacted for 15 min at 60°C. The oven temperature was programmed from 70 to 240°C at a rate of 15°C/min, increased to 350°C at a rate of 10°C/min, and held for 12 min. A 1.0 μL sample was then injected by programmed split-splitless injection cold split injection (initial temperature: 50°C; heated to 385°C) in a helium carrier gas and detected by FID at 395°C. The compounds were identified by comparing their retention times with those of authentic internal standards from DANISCO A/S (Brabrand, Denmark). Results were calculated based on response factors for MAG, DAG, TAG, and FFA from the standard and expressed as weight percentages.

TLC-FID analysis. Lipid profiles (MAG, DAG, and TAG) were analyzed by an Iatroscan MK6 TLC-FID system (SES GmbH, Bechenheim, Germany). Samples (20 mg) were diluted in 1 mL chloroform/methanol (85:15 vol/vol). Then 1 μL of diluted sample was spotted onto silica-coated Chromarod® quartz rods by a semiautomatic sample spotter, Model SES 3202/IS-02. Samples were developed for 25 min in a DT-150 development tank with *n*-heptane/diethyl ether/acetic acid 35:35:1 (by vol) as the solvent for development. The rods were dried for 5 min at 120°C in a TK-8 chromarod dryer before being measured on the Iatroscan MK6. Data handling was performed on a PC equipped with SES I-Chromstar® 6.0 software. Standard curves based on straight lines between weight and area (FID response) were constructed for external standards of monoolein (MAG), diolein (DAG), and triolein (TAG) from Larodan Fine Chemicals (Malmö, Sweden). MAG contents were calculated based on the standard curves and expressed as weight percentages of the sum of MAG + DAG + TAG. Content values are reported as the means of duplicate determinations.

Water content. Water content was analyzed by volumetric Karl Fischer titration by a Model 701 KF Metrohm titrator (Titrino, Bie & Berntsen, Roedovre, Denmark). Approximately 2 g of sample was added directly to a cell filled with methanol as solvent. A one-component reagent, Hydranal®-Composite 5, with a titer value of 5.2234 mg/mL, was used for titration. The reagent contained all the reactants (i.e., iodine, sulfur dioxide, and imidazole), which were dissolved in diethyleneglycol monoethyl ether. Results were expressed as weight percentages. Water content values are reported as the means of duplicate determinations.

Analysis of equilibrium conditions by the UNIFAC method. The UNIFAC method enables liquid–liquid mixtures to be evaluated by checking the consistency and properties of the structural units in the mixture (subgroups/UNIFAC groups) from which the molecules are formed. The UNIFAC model described by Hansen *et al.* (21) was chosen to describe the excess Gibbs energy of a liquid–liquid mixture of solvent in glycerol and triolein. ICAs version 7.0 software (Computer Aided Process Engineering Center, Kgs. Lyngby, Denmark) was used to assist in calculating the upper critical solution temperature (UCST) and the m.p. and b.p. UCST expresses the lowest temperature required to obtain a miscible mixture under equilibrium conditions and was calculated to verify the miscibility of selected solvents in both glycerol and oil (triolein) under set conditions (5–70°C). The m.p. and b.p. were calculated for selected mixtures of solvents to estimate the displacement in properties compared with the pure compounds.

RESULTS AND DISCUSSION

Screening of pure solvents. Glycerolysis reactions were conducted in 13 different solvent systems with a range of log *P* values (Table 1). MAG formation in a solvent-free system was negligible after 150 min, as a result of the poor miscibility of glycerol and oil. Addition of a range of solvents such as alcohols and ketones undoubtedly enhanced the formation of MAG (Table 1). This is believed to be due to improvement in the miscibility of oil and glycerol, with resulting improvements in mass transfer. The tertiary alcohols improved MAG formation dramatically. With *tert*-butanol and *tert*-pentanol, it was possible to make a batch reaction system in which MAG contents as high as 83% (w/w) of lipids were achieved in a few hours.

Correlations between log *P* values and MAG contents after the glycerolysis reactions were done to investigate whether there was a pattern between the log *P* values of the solvents and the MAG formed (Fig. 1). The highest MAG contents were seen with solvents having log *P* values lower than one, and pronounced maximal yields were seen with log *P* values of 0.35 and 0.89 (*tert*-butanol and *tert*-pentanol, respectively) (Fig. 1). Hydrophilic solvents (such as isopropanol and ethanol, with log *P* values of 0.05 and –0.30) yielded higher MAG contents than hydrophobic solvents (such as *n*-heptane, *n*-hexane, and isooctane with log *P* values of 4.5, 4.0, and 5.15, respectively). However, the highest MAG content was obtained with *tert*-butanol and *tert*-pentanol as solvents. Reactions in 3-pentanone (log *P* value of 0.82) gave significantly lower MAG contents than in tertiary alcohols but higher contents than the most predominant hydrophilic and hydrophobic solvents. Thus, not only the polarity but also the functional groups played an important role in the solvent properties. The maximum MAG contents were obtained in solvents with log *P* values in the range of 0.3–1 and having a tertiary alcohol structure. The relatively low log *P* values of *tert*-butanol and *tert*-pentanol indicate both hydrophilic and hydrophobic characteristics, with predominantly hydrophilic characteristics. This is in accordance with the expectation that a solvent should have both water-like and octanol-like properties in order to be a suitable solvent for both oil and glycerol. Only *tert*-butanol and *tert*-pentanol were suitable solvents for an efficient industrial glycerolysis reaction with acceptably high contents of MAG.

The high content of MAG in the *tert*-butanol system agreed with Yang *et al.* (18). In that study, enzymatic production of MAG in *tert*-butanol was conducted at 40°C. They reached a MAG content of 60–70% (w/w) in the lipid phase in a stirred tank after a 2-h reaction. MAG contents up to 70% (w/w) of lipid phase [molar ratio of glycerol/oil (gly/oil) 4:1] were also reached in a packed bed reactor with a residence time of only 30–40 min (18).

Glycerolysis reactions in *tert*-butanol and *tert*-pentanol were further investigated by GC analysis. Typical time courses for MAG, DAG, and TAG in the two solvents systems are given in Figure 2. Equilibrium was reached after approximately 60 and 90 min in *tert*-pentanol and *tert*-butanol systems, respectively. The conversion of TAG was nearly complete at equilibrium conditions, and the equilibrium ratio of MAG/DAG was approximately 80:20 w/w%.

Alcohols can participate in lipase-catalyzed esterification reactions between FA and primary and secondary alcohols (23,24). Very little formation of unwanted FA esters took place (Table 2). The tertiary hydroxyl groups of the tertiary alcohols were sterically hindered toward the *C. antarctica* lipase and thus unable to form esters.

Unwanted FFA were formed during the reaction (Table 2). The formation of FFA indicates that water was present in the

TABLE 1
MAG Content After a Glycerolysis Reaction in Various Solvents and the Properties of the Solvents Screened

Solvent	MAG content[a]	Log *P* value[b]	m.p.[c] (°C)	b.p.[c] (°C)
Solvent free	0.0 ± 0.00			
Chloroform	0.0 ± 0.00	1.97	–63.41	61.17
n-Heptane	1.1 ± 0.02	4.50	–90.55	98.40
n-Hexane	1.4 ± 0.03	4.00	–95.35	68.73
Iso-octane	1.5 ± 0.17	5.15	–107.00	99.20
Acetonitrile	2.0 ± 0.07	–0.34	–43.82	81.65
Toluene	2.9 ± 0.20	2.73	–94.95	110.63
2-Butanone	5.4 ± 0.10	0.29	–86.64	79.59
Acetone	11.5 ± 0.73	–0.24	–94.70	56.05
Isopropanol	18.0 ± 0.31	0.05	–88.50	82.40
Ethanol	21.0 ± 0.18	–0.30	–114.14	78.29
3-Pentanone	29.4 ± 0.26	0.82	–39.00	101.00
tert-Pentanol	64.9 ± 1.12	0.89	–9.10	102.40
tert-Butanol	83.6 ± 0.14	0.35	25.69	82.40

[a] Weight percentages of lipids (MAG + DAG + TAG) ± SD. Reaction conditions: molar ratio glycerol/oil, 5:1; time, 150 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30% (w/w) of oil.
[b] Based on data from Reference 20. Log *P* value, octanol–water partition coefficients.
[c] Based on data from Reference 22.

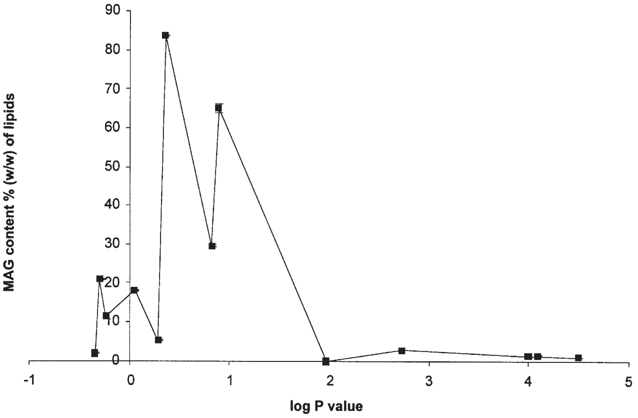

FIG. 1. Correlation between the log *P* value and MAG content achieved after the glycerolysis reaction. Reaction conditions: molar ratio glycerol/oil, 5:1; time, 150 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30% (w/w) of oil. Error bars represent SD based on double determinations. Log *P* value, octanol–water partition coefficients.

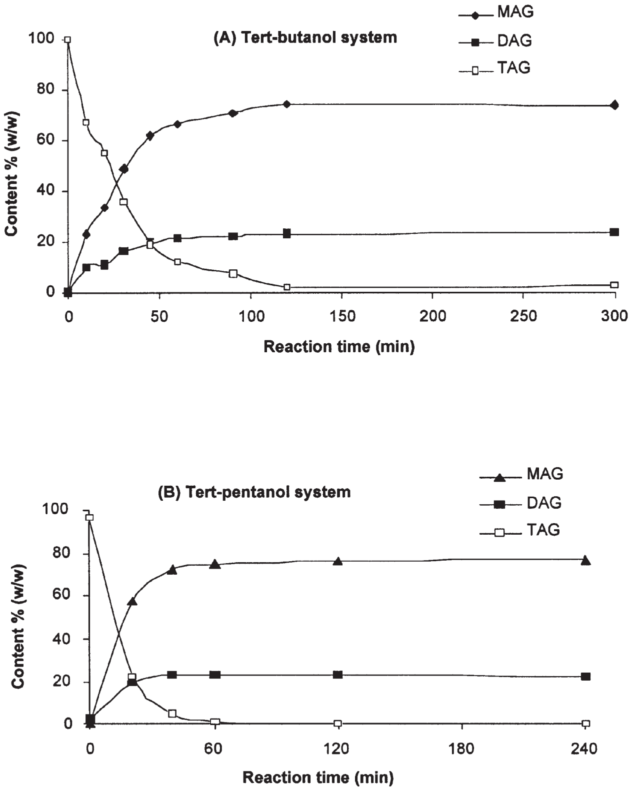

FIG. 2. Time course for the lipase-catalyzed glycerolysis reaction in two solvents: (A) *tert*-butanol, (B) *tert*-pentanol. Reaction conditions: Molar ratio glycerol/oil 4:1; temperature, 50°C; solvent, 40 mL/10 g oil; enzyme dosage, 30% (w/w) of oil.

reaction mixture. Since no water was added to the reaction mixture, the water originated from the reactants, i.e., solvent, enzyme, sunflower oil, and glycerol. The water contents of *tert*-butanol, *tert*-pentanol, Novozyme 435, sunflower oil, and glycerol were measured as 0.03, 0.09, 1.6, <0.01, and 0.06 wt%, respectively. These values were in accordance with the product descriptions from suppliers. Even these small amounts of water caused unwanted hydrolysis reactions. Identical substrates were used in both solvent systems, and the differences in water content found in the two solvents were negligible. Thus, no obvious explanation was found for the differences in FFA formation of 3.4 and 2.4% recorded for the *tert*-butanol and *tert*-pentanol systems.

In theory, a molar ratio (gly/oil) of 2 should result in optimal formation of MAG, but for several reasons this is not the case in enzyme-catalyzed glycerolysis. Higher molar ratios of gly/oil allow greater conversion of TAG to MAG. Therefore, in most reports of enzymatic glycerolysis, high ratios are used, and excessive quantities of unreacted glycerol are found at equilibrium (17). Reactions with a molar ratio (gly/oil) of 4 yielded approximately 60% MAG in an equilibrium mixture, corresponding to nearly 80% w/w MAG formation in the lipid phase (Table 2). This yield is undoubtedly very competitive with the yield of approximately 45–55 wt% for commercial chemical glycerolysis. The enzymatic glycerolysis process therefore seemed to be a very suitable alternative to the chemical process, not only in terms of the production of heat-sensitive compounds but also in terms of higher MAG contents. At equilibrium, 19% (w/w) unreacted/excess glycerol was obtained from the enzymatic glycerolysis with a molar ratio (gly/oil) of 4 (Table 2). Even with excess glycerol included in

TABLE 2
Composition of Reactant and Product Mixtures Before and After the Glycerolysis Reaction in *tert*-Pentanol and *tert*-Butanol Systems[a]

	tert-Pentanol system		*tert*-Butanol system	
Compound	Reactant mixture (wt%)	Product mixture (wt%)	Reactant mixture (wt%)	Product mixture (wt%)
FAE	0.1	1.9	0.0	0.6
FFA	0.3	2.7	0.8	4.2
MAG	0.3	59.1	0.3	57.3
DAG	1.9	17.2	1.9	17.8
TAG	74.0	0.4	73.7	0.5
Gly	23.4	18.7	23.3	19.6
Total	100	100	100	100

[a]Reaction conditions: molar ratio glycerol/oil 4:1; time, 240 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30% (w/w) of oil. FAE, FA ester; Gly, glycerol.

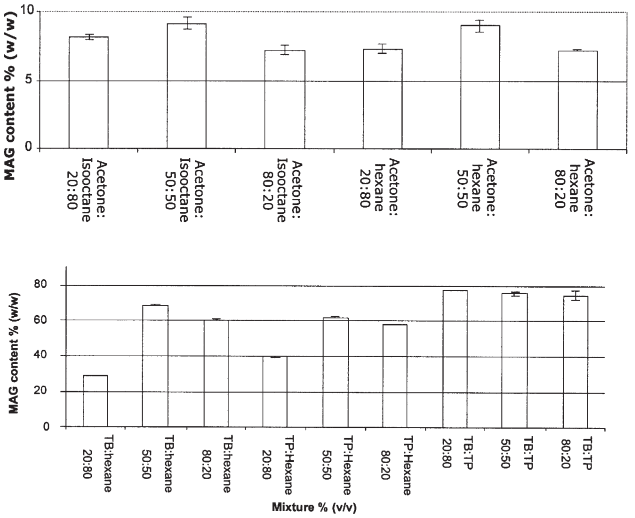

FIG. 3. MAG content [based on % (w/w) of MAG + DAG +TAG] for the lipase-catalyzed glycerolysis reaction in different solvent mixtures (% vol/vol). Reaction conditions: molar ratio glycerol/oil, 5:1; time, 150 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30 % (w/w) of oil. Error bars represent SD based on double determinations. TB, *tert*-butanol/2-methyl-2-propanol; TP, *tert*-pentanol/2-methyl-2-butanol.

the calculations, the high MAG obtained makes the process very attractive.

The UCST of *tert*-butanol and *tert*-pentanol were calculated by the UNIFAC group contribution method (21) to verify the miscibility of *tert*-butanol and *tert*-pentanol with both glycerol and oil (triolein) in the temperature range at which the experiments were conducted. Surprisingly, glycerol and triolein were estimated to be immiscible in a *tert*-butanol system at temperatures below 78.1°C. Glycerol and triolein were estimated to be immiscible in a *tert*-pentanol system at temperatures below 28.1°C. The calculations indicated that a two-phase system occurred during the reaction with *tert*-butanol, although a two-phase system was not observed during the reaction, probably owing to strong agitation. However, samples withdrawn from the *tert*-butanol system separated into two phases after only a few minutes' storage. The phase separation was expected to lower the MAG formation because of mass transfer limitations, but it did not seem to be the case for this system.

Evaluation of solvent mixture for enzymatic glycerolysis. Two of the obvious advantages of using solvents are improved reactant homogeneity (one-phase system) and reduced reaction time. Further aspects of the solvents should be considered to find one suitable for industrial applications. Unwanted side reactions with substrates or products, as well as the cost, energy required for removal from the product mixture, toxicity, and the like are other important aspects to be considered. *tert*-Pentanol is much more expensive than *tert*-butanol, especially if a purity of 99% (w/w) is needed. Even though a technical-grade *tert*-pentanol is available at a reasonable price, the presence of 1–3% impurities, such as secondary alcohols, causes unwanted side reactions. Using short-path distillation to purify a reaction mixture containing *tert*-butanol can be difficult. The temperature range between the m.p. and b.p. of *tert*-butanol is very narrow, allowing a risk of solvent crystallization during condensation. When crystallization occurs, it can be difficult to condense the solvent, which worsens the possibility of reuse.

These factors have a negative impact on industrial suitability. The glycerolysis reaction was thus carried out in several solvent mixtures (Fig. 3). Yields of 58–80% occurred in mixtures of *tert*-butanol/hexane and *tert*-pentanol/hexane (up to 50% vol/vol hexane) and *tert*-butanol/*tert*-pentanol in varying ratios (20:80, 50:50, and 80:20% vol/vol). It was not possible to obtain an efficient glycerolysis reaction without addition of *tert*-butanol or *tert*-pentanol. However, it was possible to replace up to 50% of the tertiary alcohols with hexane. The results agree with a previous study (19), in which mixtures of *n*-hexane/*tert*-butanol was used successfully as solvents in lipase-catalyzed MAG synthesis. In that study, Monteiro *et al.* (19) conducted the esterification reaction and achieved a homogeneous one-phase system of lauric acid and glycerol in the presence of *n*-hexane/*tert*-butanol 1:1 (vol/vol).

TABLE 3
Properties and Price Indexes of Selected Solvent Mixtures

					Price index[c]	
Solvent mix[a]	Molar ratio	Ratio (vol/vol)	b.p.[b] (°C)	m.p.[b] (°C)	Supplier 1[d]	Supplier 2[e]
TB/TP	100:0	100:0	82.35	24.85	100	100
TB/TP	82:18	80:20	86.85	6.85	142	124
TB/TP	54:46	50:50	91.85	−30.15	204	160
TB/TP	23:77	20:80	95.85	−39.15	267	196
TB/TP	0:100	0:100	101.95	−9.15	309	220
Hexane/TB	100:0	100:0	68.75	−95.15	340	272
Hexane/TB	15:85	20:80	70.85	8.85	148	134
Hexane/TB	42:58	50:50	64.85	−11.15	220	186

[a] TB, *tert*-butanol; TP, *tert*-pentanol.

[b] Based on UNIFAC calculations. The m.p. was calculated for nonideal solutions.

[c] Calculated as the relative price index between identical solvent amounts and purities. A price index of 100 was set for the cheapest solvent combination.

[d] Data for price index calculations were based on solvent amounts of 5 L and purities of 99% (obtained from www.sigmaaldrich.com).

[e] Calculated as the relative price index between solvent amounts of 5 metric tons with a purity of 99% for TB and hexane and a purity of 98% for TP obtained from DANISCO A/S (Brabrand, Denmark). For abbreviations see Table 2.

The m.p. and b.p. for mixtures of *tert*-butanol/*tert*-pentanol and *tert*-butanol/hexane were calculated by the UNIFAC method to see the effect on properties of the solvent mixtures compared with the pure solvents. Furthermore, the price index was compared for the different mixtures (Table 3). It is possible to change the m.p. and b.p. to obtain temperature ranges that can be operated easily in terms of engineering during distillation. From an economic point of view, *tert*-butanol was found to be most beneficial compared with *tert*-pentanol and hexane, but a "reasonable" price was also found by substituting 20% (vol/vol) of *tert*-butanol with *n*-hexane or *tert*-pentanol.

Enzymatic glycerolysis in certain solvent systems has potential as an industrial process for the production of MAG. The tertiary alcohols *tert*-butanol and *tert*-pentanol, or one of them mixed with *n*-hexane, provided good reaction efficiency, practical industrial applications, and steric hindrance to undesired esterification reactions with the FA. With enzymatic glycerolysis in a solvent system, it was feasible to produce a high content of MAG at lower temperatures than those of the current chemical glycerolysis processes.

ACKNOWLEDGMENTS

Financial support from DANISCO A/S (Brabrand, Denmark) is gratefully acknowledged. Advice from Jens Abildskov and technical assistance from Kaare Joergensen, Department of Chemical Engineering-DTU, are appreciated.

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[Received March 17, 2005; accepted June 20, 2005]