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Optimisation of enzymatic synthesis of diacylglycerols in binary medium systems containing ionic liquids

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ABSTRACT

Two different approaches, namely the *n*-hexane/ionic liquid (IL) system and the binary IL system, were optimised as a reaction medium for selective production of diacylglycerols (DGs) by lipase-catalysed glycerolysis of triolein. ILs used are [BMIM]·[BF_4], [BMIM].[PF_6], [OMIM]·[PF_6], [TOMA]·[TFA], [TOMA]·[Tf_2N], and Ammoeng 120. The effect of mixing *n*-hexane with IL in different ratios and at different temperatures was found to depend on the polarity of the IL used. In general, the addition of *n*-hexane resulted in improvement of triacylglycerol (TG) conversion and DG yield. The enhancement of mass transfer due to the decrease of viscosity is a major reason. The binary IL systems were evaluated with the aim of increasing DG yield and TG conversion while decreasing monoacylglycerol (MG) content. The reaction conditions of glycerolysis in the binary IL system consisting of [TOMA]·[Tf_2N]/Ammoeng 102 were optimised by response surface methodology (RSM). Optimal conditions were recommended as temperature between 55–60 °C, glycerol/oil amount between 1.8–2 mmol/2 mmol, [TOMA]·[Tf_2N] concentration less than 10%, reaction time 24 h, and lipase amount 15 wt.% based on oil mass. Under these conditions, the glycerolysis reaction resulted in reasonably high DG yield (~70%) and TG conversion (~90%).

1. Introduction

Ionic liquids (ILs) are defined as organic salts which are liquid at ambient temperatures (Yang & Pan, 2005). Use of ILs in industry is not a new concept. They have been used in electrochemical and chemical applications and also for non-synthetic applications (Maase, 2008; Sheldon, 2001). Interest in these compounds as a medium has risen in recent years for a number of chemical as well as biocatalytic reactions as an alternative to organic solvents. The wide applications are mostly due to their unique properties, in which they are tunable including polarity, hydrophobicity, and solvent miscibility behaviour by modification of the cation and anion groups included (van Rantwijk & Sheldon, 2007). Besides, they are also known to have protective effects on enzymes or increase enzyme stabilities, to shift the reaction equilibrium to the desired side, and to be recoverable and recyclable (Guo & Xu, 2006). Several studies have showed that enzymes can not only tolerate ILs, but they are also stable in them and the activity is comparable to or even better than in organic solvents. Lipase-catalysed reactions in ILs are the most widely performed biocatalytic reactions (Bélafi-Bakó, Dörmõ, Ulbert, & Gubicsa, 2002; Guo & Xu, 2005; Ha, Lan, Lee, Hwang, & Koo, 2007; Irimescu & Kato, 2004; Itoh, Nishimura, Ouchi, & Hayase, 2003; Lau, van Rantwijk, Seddon, & Sheldon, 2000; Lozano, de Diego, Carrié, Vaultier, & Iborra, 2003; Mohile, Potdar, Harjani, Nara, & Salunkhe, 2004; Nara, Harjani, & Salunkhe, 2002; Persson & Bornscheuer, 2003; Schöfer, Kaftzik, Wasserscheid, & Kragl, 2001).

Diacylglyerols (DGs) are natural components of various edible oils, and are surface-active molecules widely used as emulsifiers in the food industry. The consumption of oils rich in DG, especially the 1,3-isomer, is proved to have positive effects on human health, namely the suppression of both postprandial serum triacylglycerol (TG) elevation and body fat accumulation (Yasukawa & Katsuragi, 2004). There have been a great number of studies focusing on enzymatic production of DG by esterification of glycerol with fatty acids or glycerolysis of oils (Guo & Sun, 2007; Kristensen, Xu, & Mu, 2005a, 2005b; Rosu, Yasui, Iwasaki, & Yamane, 1999; Watanabe, Sugiura, Sato, Yamada, & Nakanishi, 2005; Watanabe et al., 2003; Weber & Mukherjee, 2004). However, most of them were conducted in conventional solvents or solvent-free systems. Little work has been involved in the investigation of using ILs as a medium to perform similar reactions for the production of DG (Chen, Guo, Tan, & Xu, 2008; Guo, Chen, Murillo, Tan, & Xu, 2006; Guo & Xu, 2005, 2006). Very recently we investigated the lipase-catalysed glycerolysis of TG to produce DG in a variety of commercial ILs. The dependency of individual reaction profile on the molecular structure and property of corresponding ILs was observed. We also found that some

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ILs like [TOMA]·[Tf_2N] produced good yield of DG but low conversion of TG, while some ILs like Ammoeng 102 resulted in high TG conversion but selectivity towards MG over DG, the former of which is an undesirable by-product in this context (unpublished results). Thus, there exists a possibility to optimise the mixture solvents system containing ionic liquids in order to achieve both high TG conversion and good yields of DG (Guo, Kahveci, Özçelik, & Xu, 2009).

In this study, we started with the lipase-catalysed glycerolysis in the mixture of ILs with *n*-hexane. The focus was then placed on the evaluation on the reactions in binary IL systems, where response surface methodology (RSM) was also employed for the parameter optimisation of selected promising binary IL system including [TOMA]·[Tf_2N] and Ammoeng 102.

2. Materials and methods

2.1. Chemicals

Triolein with 90% purity was purchased from Dr. Frischer GmbH (Bremen, Germany) and glycerol of minimum 99% purity was from Sigma–Aldrich Co. (St. Louis, MO). Novozym 435 (*Candida antarctica* lipase B) was provided by Novozymes A/S (Bagsvaerd, Denmark). Ionic liquids including 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]·[BF_4]), 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]·[PF_6]), 1-methyl-3-octylimidazolium hexafluorophosphate ([OMIM]·[PF_6]), trioctylmethylammonium bis(trifluoromethylsulphonyl)imide ([TOMA]·[Tf_2N]), cocosalkyl pentaethoxi methyl ammonium methylsulfate (Ammoeng 100), tetraalkyl ammonium sulphate (Ammoeng 102), and quaternary ammonium sulphate (Ammoeng 120) were purchased from Solvent Innovation GmbH (Cologne, Germany), while methyltrioctylammonium trifluoroacetate ([TOMA]·[TFA]) was from Merck (Darmstadt, Germany). All solvents used were of chromatographic grade with the minimum purity of 96%.

2.2. Experimental procedure for glycerolysis in ILs

Two mmol of triolein and 1 mmol of glycerol were mixed with desired amount of solvent in a 25 mL jacketed reactor and premixed by magnetic agitation at 700 rpm. The reaction was initiated by the addition of the lipase (10 wt.% based on the oil mass) and conducted for 24 h. The reaction temperature was controlled by circulated water bath. Aliquots of 20 μL were taken periodically, and analysed by thin layer chromatography coupled with a flame ionisation detector (TLC-FID) (Iatroscan MK-6s, Bechenheim, Germany). The method is described elsewhere (Damstrup et al., 2005). All analyses were conducted in duplicates and the average was used for evaluation.

2.3. Investigation of n-hexane/IL systems

The *n*-hexane/IL systems were tested as a glycerolysis medium. The reactions were conducted with 2 mmol oil (1768 mg), 1 mmol glycerol (92 mg), 177 mg Novozym 435, 0.5 mL *n*-hexane, and 0.5 mL IL at 50 °C with 700 rpm stirring for 24 h. Furthermore, the effects of varying *n*-hexane/IL ratios and temperatures were investigated using 0.335:0.665 and 0.665:0.335 levels for *n*-hexane/IL (v/v, mL:mL) and 40 and 50 °C for temperature. The reaction progress was monitored similarly as abovementioned. Hexane was directly selected for its common use in food industry and its high solvability of oils and fats.

2.4. Investigation of binary IL systems

The reactions were performed with a mixture of two ILs in the ratio of 0.5:0.5, mL:mL at 60 °C. Tested binary systems, i.e. [TOMA]·[Tf_2N]/Ammoeng 100, [TOMA]·[Tf_2N]/Ammoeng 102, Ammoeng 120/Ammoeng 100, and Ammoeng 120/Ammoeng 102, were selected due to their ability to convert TG and/or produce DG to a high extent, which was observed in a preliminary study (unpublished results). The reaction progress was monitored similarly as abovementioned.

2.5. Optimisation of the reaction conditions for the selected binary IL system by RSM

According to the results of the previous step, [TOMA]·[Tf_2N]/Ammoeng 102 system was chosen for further optimisation by response surface methodology (RSM). A five-level and three-factor central composite rotatable design was used. The experimental settings were generated with the assistance of Minitab® Release 14 (Coventry, United Kingdom). Temperature, glycerol amount, and [TOMA]·[Tf_2N] concentration were selected as the factors of interest since reaction time and enzyme dosage were considered obvious and water content was not critical for Novozym 435. The ranges for the factors were set as follows based on the preliminary studies: Temperature, 45–75 °C; glycerol amount, 1.15–1.75 mmol; [TOMA]·[Tf_2N] concentration 25–75%. Two mmol oil, 15% (wt) lipase based on the oil mass, and 24 h reaction time were kept constant for all experiments. The reaction conditions for each experimental setting are shown in Table 1. Experiments were run randomly. The products were analysed by TLC-FID.

3. Results and discussion

3.1. Glycerolysis in n-hexane/IL systems

The relatively high viscosity of ILs is considered as a limiting factor for various applications. The addition of organic solvents as co-solvents to ILs can be an approach which aims for a dramatic reduction in the viscosity without changing the cations or anions in the system (Mantz & Trulove, 2008). As a common organic solvent for food industry and also friendly to enzyme activity, *n*-hexane was directly selected as a model system for initial evaluation. The acylglycerol compositions for products from the glycerolysis reactions in pure ILs and *n*-hexane/IL systems are given in Table 2. For all the ILs tested except [OMIM]·[PF_6], the addition of *n*-hexane resulted in improvements of both TG conversion and DG yield. However, the mixture of equal volume of *n*-hexane and [OMIM]·[PF_6] yielded similar results as in pure [OMIM]·[PF_6]. Poor mixing of the oil and solvent phases was observed when [OMIM]·[PF_6] was used.

The co-solvent addition approach was tested further at different *n*-hexane/IL ratios and temperatures (data not shown). The effect observed can be attributed to the change in medium polarity. For instance, DG yield and TG conversion both increased with increasing *n*-hexane/IL ratios in [OMIM]·[PF_6], likely due to the low polarity introduced to the system. This effect could have resulted in the improved solvation of the oil. Similarly, both DG yield and TG conversion decreased with increasing *n*-hexane/IL ratio in [BMIM]·[BF_4], probably due to the even more polar nature of the system compared to pure [BMIM]·[BF_4]. The effect of temperature was positive, though not dramatic, on the responses investigated (data not shown). With increasing temperature, both TG conversion and DG yield were improved, which was considered to be a result of the decrease in the viscosity of the system induced by increased temperatures. In general, the addition of *n*-hexane resulted in improvement of TG conversion and DG yield. The enhancement of mass transfer due to the decrease of viscosity was likely a major reason. The effect was more pronounced when hydrophilic ILs, i.e. [TOMA]·[TFA], [TOMA]·[Tf_2N], and Ammoeng

Table 1
Coded and decoded levels for RSM experiments, and responses obtained from the design for DG yield and TG conversion.

Exp. no.	Temperature (°C)		Glycerol amount (mmol)		[TOMA]·[Tf_2N] concentration (%)		DG yield (%)	TG conversion (%)
	Coded	Decoded	Coded	Decoded	Coded	Decoded		
1	−1	45	−1	1.15	−1	25	58.2	82.6
2	1	75	−1	1.15	−1	25	60.7	69.7
3	−1	45	1	1.75	−1	25	73.9	83.7
4	1	75	1	1.75	−1	25	60.6	80.1
5	−1	45	−1	1.15	1	75	59.8	64.9
6	1	75	−1	1.15	1	75	56.4	60.3
7	−1	45	1	1.75	1	75	59.5	65.3
8	1	75	1	1.75	1	75	61.2	66.3
9	−α	34.77	0	1.45	0	50	47.9	57.9[a]
10	+α	85.23	0	1.45	0	50	66.3	69.0
11	0	60	−α	0.94	0	50	59	64.7
12	0	60	+α	1.95	0	50	70.8	80.1
13	0	60	0	1.45	−α	7.95	71.9	85.9
14	0	60	0	1.45	+α	92.04	51.8	65.1
15	0	60	0	1.45	0	50	66.5	76.4
16	0	60	0	1.45	0	50	54.9	70.3
17	0	60	0	1.45	0	50	67.7	76.2

[a] The result was not included in the statistical analyses.

Table 2
Acylglycerol compositions obtained in pure IL and *n*-hexane/IL systems.[a]

Solvent		Acylglycerol composition (%)				
		TG	1,3-DG	1,2-DG	MG	FFA
[BMIM]·[BF_4]	IL	79.9	13.7	3.5	2.4	0.6
	n-Hexane/IL	77.7	16.4	3.6	2.0	0.2
[BMIM]·[PF_6]	IL	96.9	1.6	0.5	0.7	0.2
	n-Hexane/IL	85.2	10.0	2.7	1.7	0.3
[TOMA]·[TFA]	IL	64.9	8.8	14.4	11.0	0.9
	n-Hexane/IL	57.5	13.7	12.5	15.5	0.7
[OMIM]·[PF_6]	IL	94.8	2.9	0.9	0.9	0.3
	n-Hexane/IL	96.5	2.0	0.6	0.7	0.2
[TOMA]·[Tf_2N]	IL	66.8	24.2	7.9	2.5	1.0
	n-Hexane/IL	63.9	24.5	9.5	1.5	0.7
Ammoeng 120	IL	62.2	22.7	8.0	6.0	0.9
	n-Hexane/IL	45.7	33.2	10.3	9.7	1.1

[a] Reaction conditions: 2 mmol of oil (1768 mg), 1 mmol of glycerol (92 mg), 177 mg Novozym 435, 50 °C, 24 h, 700 rpm. Solvent used: 1 g IL for pure IL systems, 0.5 mL: 0.5 mL *n*-hexane/IL for mixed systems.

Table 3
Comparison of acylglycerol compositions obtained in pure ILs and binary IL systems.[a]

IL	Acylglycerol composition (%)				
	TG	1,3-DG	1,2-DG	MG	FFA
[TOMA]·[Tf_2N]	46.5	21.6	22.9	7.2	1.6
Ammoeng 100	13.6	29.2	15.9	31.7	9.5
Ammoeng 102	21.5	35.9	14.9	20.8	6.8
Ammoeng 120	46.1	30.8	11.7	9.1	2.2
[TOMA]·[Tf_2N]/Ammoeng 100	17.6	37.7	20.8	18.0	5.8
[TOMA]·[Tf_2N]/Ammoeng 102	43.0	36.9	14.2	3.2	2.6
Ammoeng 120/Ammoeng 100	59.9	16.0	7.3	11.6	5.2
Ammoeng 120/Ammoeng 102	43.9	30.2	11.0	12.5	2.2

[a] Reaction conditions: 2 mmol of oil (1768 mg), 1 mmol of glycerol (92 mg), 177 mg Novozym 435, 60 °C, 24 h, 700 rpm. Solvent used: 1 g IL for pure IL systems, 0.5 mL: 0.5 mL for binary IL systems.

120, were mixed with *n*-hexane, resulting in the higher solubility of oil in the system. Considering that the use of organic solvents will raise the concern of air pollution and process cost, this binary system was not given high attention for further optimisation, even though it indeed improved the performance significantly. Thus, we made our focus on the binary IL systems in the following sections. However, the conclusion of the binary systems containing organic solvents is still interesting and could provide clues for other potential systems.

3.2. Glycerolysis in binary IL systems

As shown in Table 2, none of the ILs, when used alone, resulted in a desired performance for all the responses considered such as DG yield, TG conversion, and MG concentration. To obtain a system which provides the best medium to obtain high DG yield and TG conversion with low MG concentration, the novel approach of binary IL systems was further evaluated in order to combine the selectivities of different ILs. Ammoeng 100 and Ammoeng 102 resulted in remarkably high TG conversion, and relatively high DG yield; however, selectivity towards MG over DG was unacceptably high as well (Guo et al., 2009). These two ILs were coupled with [TOMA]·[Tf_2N] and Ammoeng 120, the most effective ILs in the means of selective DG production with the drawback of relatively low TG conversion. The comparison of the acylglycerol compositions obtained by the binary IL systems can be seen in Table 3. [TOMA]·[Tf_2N]/Ammoeng 100 system resulted in high DG yield and TG conversion, while MG concentration was relatively lowered compared to the pure Ammoeng 100 system. Addition of Ammoeng 100 or Ammoeng 102 to Ammoeng 120 did not improve TG conversion; moreover, DG yield was decreased in Ammoeng 120/Ammoeng 100 systems compared to pure Ammoeng 120. This observation is believed to be resulted from the high specificity of Ammoeng 100 and Ammoeng 102 for MG production (Guo et al., 2006). Balancing the pros and cons of the evaluated binary IL systems, we decided to take the [TOMA]·[Tf_2N]/Ammoeng 102 system for further optimisation.

3.3. Optimisation of reaction conditions for [TOMA]·[Tf_2N]/Ammoeng 102 system by RSM

RSM was used to generate models to describe both DG yield and TG conversion (total of DG, MG and FFA produced) responses separately. Second order coefficients were generated by regression with backward elimination. Responses were fitted to the factors by multiple regressions. Responses obtained from the experimental design are given in Table 1. The accuracy of the models was evaluated by coefficient of determination (R^2) and absolute average deviation (*AAD*) and a test for lack of fit from ANOVA. It is suggested that R^2 and *AAD* are considered together for better control of the accuracy of the models (Baş & Boyacı, 2007).

AAD is calculated by the following equation:

$$AAD = \left\{ \frac{\left[\sum_{i=1}^{p} \left(\frac{|y_{i,exp} - y_{i,cal}|}{y_{i,exp}} \right) \right]}{p} \right\} \times 100$$

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively, and p is the number of experimental run. R^2 must be close to 1.0 and the *AAD* between the predicted and observed data must be as small as possible.

3.3.1. Modeling DG yield in [TOMA]·[Tf_2N]/Ammoeng 102 system

According to the statistical evaluation of the DG yield data (Table 1), R^2 and *AAD* values were found to be 0.7% and 5.4%, respectively. ANOVA (data not showed) presents that none of the investigated factors had a significant effect on DG yield, and the model had no significant lack of fit at a significance level of $P < 0.05$. The quadratic effect of glycerol amount was found to have the greatest effect on the response. As can be seen in the main effects plot (Fig. 1A), DG yield increased with increasing glycerol amount, the reason of which can be the improved availability of glycerol. In viscous and hydrophobic IL medium, it would be hard for the enzymes to interact with the highly hydrophilic glycerol. Temperature increase also had a positive effect on DG yield, especially when the temperature was increased from 34.7 to 45 °C. In the middle range, i.e. between 45 and 75 °C, the increase in the temperature did not influence the yield significantly. As [TOMA]·[Tf_2N] concentration in the medium decreased, in other words, Ammoeng 102 concentration increased, DG yield increased as well. This was an expected result, since Ammoeng 102 has high selectivity towards DG production, even at conditions favouring MG production (Guo et al., 2006).

The interaction between glycerol amount and [TOMA]·[Tf_2N] concentration was found to have the greatest effect on the DG yield. The contour plot generated for these two factors at the highest level of the third factor, temperature, can be seen in Fig. 1B. DG yield is predicted to be above 70% with glycerol higher than 1.5 mmol and [TOMA]·[Tf_2N] concentration lower than 50 when temperature is set at 75 °C. Response tends to get higher as glycerol amount increases and [TOMA]·[Tf_2N] concentration decreases simultaneously. At this point, one can question the reason for using a binary IL system instead of pure Ammoeng 102 IL, since the addition of [TOMA]·[Tf_2N] seems to have a negative effect on DG yield. However, it should be kept in mind that pure Ammoeng 102 resulted in unacceptably high MG concentration, which was the reason that this IL had not been chosen for further investigation. The binary system of [TOMA]·[Tf_2N]/Ammoeng 102, on the other hand, resulted in only 3% MG, which can be neglected (Table 3).

A chi-square (χ^2) test using four additional experimental sets chosen from the given ranges of reaction parameters was performed to examine the adequacy of the model established (data omitted), and it was observed that there were no significant differences between the observed and predicted values ($P < 0.05$).

3.3.2. Modeling TG conversion in [TOMA]·[Tf_2N]/Ammoeng 102 system

Similar statistical evaluations were performed on TG conversion data (Table 1) as well. R^2 and *AAD* values were 0.94% and 3.3%, respectively. ANOVA (data not showed) presented that the regression of the factors was significant, and the model had no significant lack of fit ($P < 0.05$). None of the factors was found to have a significant effect on TG conversion ($P < 0.05$). The main effects plot for TG conversion is given in Fig. 2A. In contrary to the model generated to describe DG yield, temperature had a slight effect on TG conversion. Considering the cost issues and enzyme stability, a temperature in the range of 45–60 °C can be chosen for the reaction. The effects of glycerol amount and [TOMA]·[Tf_2N] concentration were similar to the previous model; TG conversion increased with increasing glycerol and decreasing [TOMA]·[Tf_2N].

The interaction between glycerol amount and temperature was found to have the greatest effect on TG conversion. The contour plot generated for these two factors at the lowest level of the third factor, [TOMA]·[Tf_2N] concentration, can be seen in Fig. 2B. When [TOMA]·[Tf_2N] is set at the lowest level, which is 7.95%, TG conversion is expected to be higher than 80% for the whole range of glycerol amount used at 45–50 °C. Response tends to get higher as glycerol amount increases.

A χ^2 test was performed similarly (data omitted) which also indicated that there were no significant differences between the observed and predicted values ($P < 0.05$).

3.3.3. General discussion for [TOMA]·[Tf_2N]/Ammoeng 102 system

The reasons for using a binary IL system consisting of [TOMA]·[Tf_2N]/Ammoeng 102 for DG production can be summarised as (i) high DG yield, preferably with a high proportion of 1,3-DG isomer; (ii) high TG conversion; and (iii) low MG concentration. According to the results, these goals were generally achieved.

Reactions in the mentioned system resulted in 55–75% DG yield in the ranges used for investigated factors. This value is higher than

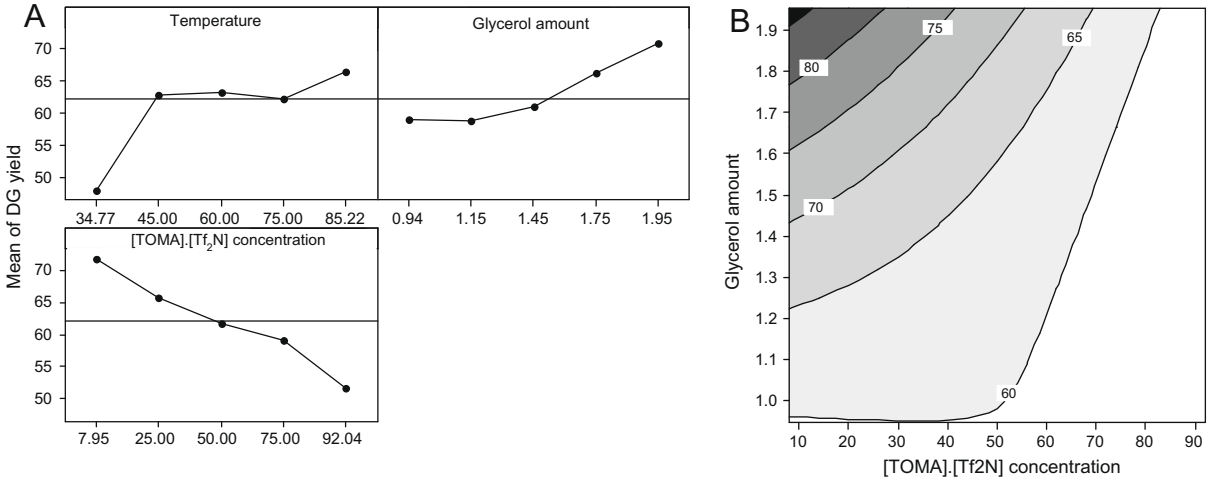

Fig. 1. (A) Main effects plot for the model describing DG yield. (B) Contour plot of interaction between glycerol amount and [TOMA]·[Tf_2N] concentration for the model describing DG yield.

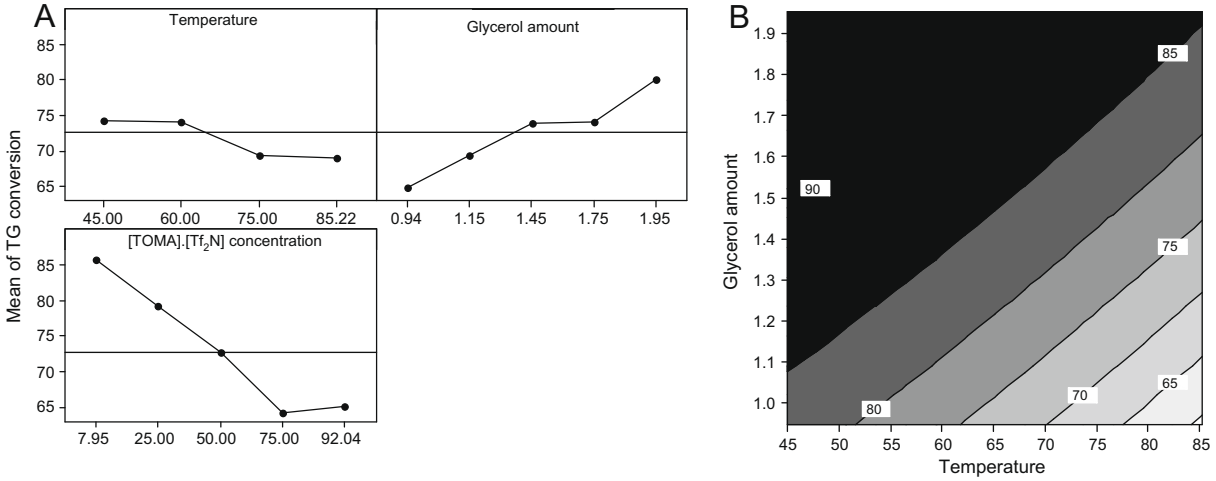

Fig. 2. (A) Main effects plot for the model describing TG conversion. (B) Contour plot of interaction between glycerol amount and temperature for the model describing TG conversion.

those obtained with the other ILs used for optimisation in the previous steps. Pure Ammoeng 102 had resulted in a higher DG yield; however, MG concentration in this IL was more than 20%, which was unacceptable. TG conversion of the binary system was also improved compared to the previously used ILs, again except Ammoeng 102, but as already mentioned, binary system was preferable considering all desired parameters. The yield is higher than that in solvent-free (Coteron, Martinez, & Aracil, 1998; Kristensen et al., 2005a, 2005b; Noureddini & Harmeier, 1998; Tuter, Babali, Kose, Dural, & Aksoy, 1999; Weber & Mukherjee, 2004) and in organic solvent glycerolysis reaction systems (Liao, Tsai, Chang, & Shieh, 2003). Considering the selective production of the 1,3-isomer, the developed reaction system should be a better solution.

As a conclusion for the optimisation, RSM was used to generate models for both DG yield and TG conversion responses in the [TOMA]·[Tf2 N]/Ammoeng 102 system. Both models can be considered well-fitting according to the R^2 and *AAD* values. Considering the optimum conditions chosen for the two models separately, it can be concluded that a temperature between 55 and 60 °C, glycerol amount between 1.8 and 2 mmol, and [TOMA]·[Tf_2N] concentration less than 10% can be chosen for high DG yield (∼70%) and TG conversion (∼90%).

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