İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PRODUCTION OF DIGLYCERIDES BY LIPASE-CATALYZED GLYCEROLYSIS IN IONIC LIQUIDS

M.Sc. Thesis by
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Programme: Food Engineering

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İYONİK SIVI ORTAMINDA, ENZİMATİK GLİSEROLİZ REAKSİYONU KULLANILARAK DİGLİSERİT ÜRETİMİ

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PREFACE

The present project was conducted and defended in Food Production Engineering Group, at Technical University of Denmark in the period of October 2007 – March 2008 while I was an exchange student at the mentioned university by the Erasmus Exchange Programme. The project was a 50 ECTS points exam project and will also be presented in Istanbul Technical University to fulfill the degree of Master of Science in the Food Engineering Department.

I would like to thank my supervisor at ITU, Beraat Özçelik, who I have been working with both as a student and a research assistant since 4 years. She has always encouraged me to express my ideas, to take responsibilities and to improve myself as an academic staff candidate. I also would like to thank my colleagues as a research assistant for being so friendly and helpful to me since I became a part of the department.

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Finally, I would like to dedicate my thesis to my mother Hesna, my father Mustafa, and my sister Esra. I have always felt their love and support, which made me hold on during the 8-months period away from home.

June 2008 **Derya KAHVECİ**

CONTENTS

LIST of ABBREVIATIONS

DG	: Diacylglycerol
IL	: Ionic liquid
MG	: Monoacylglycerol
TG	: Triacyglycerol
[3-MBP][$N(CN)_2$]	: 1-Butyl-3-methylpyridinium dicyanamide
[BMIM].[BF_4]	: 1-Butyl-3-methylimidazolium tetrafluoroborate
[BMIM].[PF_6]	: 1-Butyl-3-methylimidazolium hexafluorophosphate
[BMIM].[CF_3SO_3]	: 1-Butyl-3-methylimidazolium trifluoromethanesulfonate
[DMIM].[DMP]	: 1,3-Dimethylimidazolium dimethylphosphate
[EMIM].[MDEG.SO_4]	: 1-Ethyl-3-methylimidazolium2 (2methoxyethoxy)ethylsulfate
[EMIM].[OctSO_4]	: 1-Ethyl-3-methylimidazolium n-octylsulfate
[MeBuPyO].[$N(CN)_2$]	: 1-Butyl-3-methylpyrrolidinium dicyanamide
[MeEtPy].[$C_4F_9SO_3$]	: 1-Ethyl-3-methylpyridinium perfluorobutanesulfonate
[MeOcPy].[BF_4]	: 3-Methyl-1-octylpyridinium tetrafluoroborate
[MTOA].[TFA]	: Methyltrioctylammonium trifluoroacetate
[OMIM].[BF_4]	: 1-Methyl-3-octylimidazolium tetrafluoroborate
[OMIM].[PF_6]	: 1-Methyl-3-octylimidazolium hexafluorophosphate
[TOMA].[Tf_2N]	: Trioctylmethylammonium bis(trifluoromethylsulfonyl)imide

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PRODUCTION OF DIGLYCERIDES BY LIPASE-CATALYZED GLYCEROLYSIS IN LONIC LIQUIDS

SUMMARY

Nineteen different ionic liquids (ILs) were tested as a reaction medium for lipase-catalyzed glycerolysis of triolein aiming to establish an efficient protocol for diacylglycerols (DGs) production. In comparison with conventional organic solvents, i.e. *tert*-butanol and *tert*-pentanol, 6 types of the screened ILs showed better product selectivity towards DGs and thus were chosen for further investigation. The factors of interest in this study are the ratio between the substrates and IL (substrates/IL), temperature (T), ratio between oil and glycerol (oil/glycerol), and the type of lipase. It was seen that the effects of the individual factors on the reaction performance in terms of TG conversion and product selectivity (DG/MG) differ from case to case due to greatly diversified properties and structures of ILs. Therefore, to generalize some observations are quite difficult. On the other hand, some efftects can be concluded, e.g. within a certain range, enhancement of temperature generally leads to increased DG yield and TG conversion.

To tune the property of the medium for a better result, two different binary-mixing systems, namely, *n*-hexane/IL system and binary IL system were selected for further examination. The results showed that the introduction of *n*-hexane into the ILs evaluated does not result in significant improvement in either conversion or product selectivity, and the effects strongly depend on the polarity of the IL employed. Primary evaluation of binary IL mixing system showed that mixture of the IL with better DG selectivity and the IL with higher TG conversion could yield promising results. Thus, a representative binary IL system consisting of [TOMA].[Tf_2N]/AMMOENG 102 was chosen for optimization by response surface methodology. Considering the optimum levels on desired responses, it is concluded that a temperature between 55-60^oC, glycerol mole between 1.8-2 mmol, and [TOMA].[Tf_2N] concentration less than 10%, 24 h reaction time with 2 mmol oil and lipase in the amount of 15 wt % based on oil mass can be recommended for enzymatic glycerolysis of oils and fats, which resulted in over 70% DG yield and around 90% TG conversion.

In summary, this study described a first attempt to produce DGs by enzymatic glycerolysis of oils employing neoteric green solvents (ILs) as reaction media. The results indicated that better product selectivity and higher conversion employing ILs as media, over those by most efficient conventional solvents, could be obtained through selection of proper ILs or/and tuning property by mixing different ILs. These experiences are believed to be useful for molecular design of task-specific ILs for enzymatic production of DG, with practically industrial potentials.

İYONİK SIVI ORTAMINDA, ENZİMATİK GLİSEROLİZ REAKSİYONU KULLANILARAK DİGLİSERİT ÜRETİMİ

ÖZET

On dokuz farklı iyonik sıvı (İS), verimli bir digliserit (DG) üretim yöntemi belirlenmesi amacıyla, trioleinin lipaz katalizörlüğünde gliserolizi reaksiyonunda reaksiyon ortamı olarak test edilmiştir. Geleneksel olarak kullanılan organik çözgenlerle (*tert*-butanol ve *tert*-pentanol) karşılaştırılan İS'lerden 6 tanesi, diğerlerinden daha yüksek ürün seçiciliği göstermiş ve ileri araştırmalar için seçilmiştir. Çalışmada etkisi incelenen faktörler, substratlar ile İS arasındaki oran, sıcaklık, substratların bibirlerine oranı, ve lipaz türüdür. Trigliserit (TG) dönüşümü ve digliseritin monogliserite oranı (DG/MG) bakımından incelendiğinde, faktörlerin reaksiyon üzerindeki etkilerinin, İS'lerin birbirlerinden oldukça farklı özelliklerine ve yapılarına bağlı olarak, her bir İS için farklı olduğu gözlenmiştir. Bu nedenle, sonuçlar hakkında genelleme yapmak oldukça zordur. Diğer yandan, bazı etkiler üzerinde genel sonuçlara varılabilinir; örneğin belli bir aralık dahilinde, sıcaklığın arttırılması ile TG dönüşümü ve DG verimi artmaktadır.

Ortam özelliklerini, daha iyi verim alabilmek amacıyla değiştirmek için, hekzan/İS ve İS/İS olmak üzere iki farklı karışım sistemi de denenmiştir. Sonuçlara bakıldığında, hekzan ilavesinin incelenen parametrelere belirgin bir etkisi olmadığı, ve etkini İS polaritesine bağlı olarak değiştiği görülmüştür. İkili İS siteminde nispeten daha yüksek DG verimi sağlayan İS ile daha yüksek TG dönüşümü sağlayan İS'nin karışımının umut verici gelişmelerle sonuçlandığı görülmüştür. Temsil edici olarak, [TOMA].[Tf_2N]/AMMOENG 102 içeren ikili İS sistemi seçilmiş ve tepki yüzey yöntemi kullanılarak optimize edilmiştir. Elde edilmesi amaçlanan optimum değerler göz önüne alındığında, 55-60^oC sıcaklık aralığı, 1.8-2 mmol gliserol miktarı, ve 10%'dan daha düşük [TOMA].[Tf_2N] konsantrasyonu, 24 saat reaksiyon süresi, 2 mmol triolein ve yağın ağırlıkça 15 %'i kadar lipaz kullanılarak gerçekleştirilen gliseroliz reaksiyonu ile 70%'in üzerinde DG verimi ve 90% civarında TG dönüşümü elde etmek mümkün olmaktadır.

Özetle bu çalışma, İS ortamında yağların enzimatik gliserolizi yöntemiyle digliserit üretilmesi konusunda yapılan ilk çalışma olma özelliğini taşımaktadır. Bu amaçla kullanılan organik çözgenler yerine uygun İS ya da ikili İS sistemleri seçilerek daha yüksek ürün seçiciliği elde edilebileceği görülmüştür. Elde edilen bulguların, DG üretilmesi için ve endüstriyel uygulamalarda kullanılacak özel amaçlı İS'lerin moleküler düzeyde tasarlanmasında faydalı olacağı düşünülmektedir.

1. INTRODUCTION

It is well known that diets high in fat are risk factors for obesity and coronary heart diseases. Changes in dietary habits and exercise are recommended for preventing obesity and metabolic syndrome, i.e. the complication of multiple risk factors to which obesity and overweight lead, such as adverse effects on blood pressure, cholesterol, triglycerides, and insulin resistance (Mokdad *et al.*, 2003). In 1980s and 90s, fat substitutes involving the use of starch, cellulose, protein, pectin, and dextrose were developed to replace fat in food products. These substitutes, however, were not always applicable for use due to taste, smell, texture, physical chemistry, and safety issues. Therefore, the use of natural oils which have positive effects on lipid metabolism is preferred over the use of fat substitutes (Yasukawa and Katsuragi, 2004).

Diacylgycerols (DGs) are shown to have beneficial effects compared to conventional triacylglycerol (TG) oil. Two main health benefits of DG are: (a) the suppression of postprandial serum TG elevation, (b) the suppression of body fat accumulation (Yasukawa and Katsuragi, 2004). There have been a great number of studies focusing on the production of DG oils in recent years. DG oil has become commercially available as a cooking oil in March 1999 in Japan under the brand name of Econa® Cooking Oil (Kao Corporation, Tokyo, Japan) and is approved as FOSHU (Food for Specified Health Use) by the Japanese Ministry of Health, Labor and Welfare pertaining to post-meal blood lipids and body weight. In the United States, same product is sold under the name of Enova® Oil since December 2003. The Food and Drug Administration (FDA) accepted the GRAS (Generally Recognized as Safe) notification for Enova Oil (Yasukawa and Katsuragi, 2004).

Enzymatic reactions were interestingly shown to be more effective in anhydrous media than the aqueous one, which is the natural environment for enzymes. Thus,

biocatalysis in organic media has been a major area of interest over the last two environmental issues, and to safety issues as well for food production in particular. To overcome these drawbacks, ionic liquids (ILs) were suggested to replace organic solvents as a media for enzyme-catalyzed reactions. ILs are substances that are composed of ions and are liquid at or close to room temperature. Their unique properties, such as near-zero vapor pressure, high chemical and thermal stability, and being able to have desired physicochemical properties by modifying the ionic structure, made these materials popular as a media for chemical and biochemical reactions in the last decade (van Rantwijk and Sheldon, 2007). From the point of decades. However, the use of organic solvents was undesirable in most cases due to biocatalytic uses, ILs, so called 'green solvents', are also known to have protective effects on enzymes or increase their stabilities, to shift the reaction equilibrium to the desired side, and to be recoverable and recyclable (Guo and Xu, 2006).

There exists no study on the development of a suitable IL system to produce DG with a high selectivity in the literature. The present project aimed to fill this gap. The effects of different reaction parameters were also investigated to understand the reaction mechanism better.

2. DIACYLGLYCEROLS

2.1 Introduction

Diacylglycerols (DGs) are natural components of various edible oils, and are surface-active molecules widely used as emulsifiers in the food industry. Commercial preparations are mixtures of monoacylglycerols (MGs) and DGs generated during chemical glycerolysis of triacylglycerols (TGs). Novel uses of DGs include modifiers of TG crystal structure in fat-containing foods, and intermediates in the chemical synthesis of drug conjugates, phospholipids, and glycolipids. For many of these reactions, pure substrates are required. Therefore, lipase-catalyzed production of DGs is preferred due to the unique regioselectivity of lipases (McNeill, 1998).

There are two isomers of DG, named as 1,3-DG and 1,2(2,3)-DG according to the positions of fatty acids on the glycerol backbone (Figure 2.1).

Figure 2.1: Structure of DG isomers. R: Fatty acid.

Generally, the concentration of DG in edible oils is below 5%; however, there are some exceptions, as shown in Table 2.1 (Yasukawa and Katsuragi, 2004). The ratio of 1,3-DG to 1,2(2,3)-DG in naturally occurring edible oils is reported to be 7:3 approximately. The isomerization of DG is likely to be caused by a steric effect due to the conversion to the more thermodynamically stable linear form of 1,3-DG (Nakajima *et al.*, 2004).

Table 2.1: Acylglycerol contents of various edible oils (g/100 g).

	TG	Total DG	1,2-DG	1,3-DG	MG	Others
Soybean	97.9	1.0	ND	ND	0.0	1.1
Palm	93.1	5.8	ND	ND	<DL	1.1
Cottonseed	87.0	9.5	ND	ND	0.2	3.3
Corn	95.8	2.8	1.5	2.9	<DL	1.4
Safflower	96.0	2.1	1.2	2.7	<DL	1.9
Olive	93.3	5.5	ND	ND	0.2	2.3
Rapeseed	96.8	0.8	ND	ND	0.1	2.3
Canola	97.1	2.9	1.0	1.9	<DL	ND
Sesame	95.2	4.1	1.2	2.9	0.8	ND
Rice	92.4	7.6	2.4	5.2	<DL	ND
Grapeseed	94.2	5.8	2.1	3.7	<DL	ND

TG: triacylglycerol; DG: diacylglycerol; MG: monoacylglycerol; ND: not determined; <DL: below detectable limit

2.2 Digestion, Absorption and Metabolic Effects of Diacylglycerols

The main reason for DG oil to gain a particular interest recently is that consumption of oil rich in DG, especially the 1,3- isomer, is proved to have positive effects on human health. Before detailed explanations of these effects, the mechanism underlying them, which is the difference between digestion and absorption of conventional TG oil and DG rich oil, should be cleared.

TG oil is generally hydrolyzed by pancreatic lipase to free fatty acids (FFAs) and 2-MG in the small intestine lumen. These hydrolysis products form micelles with bile salts and are absorbed by intestinal epithelial cells. Following the absorption, TG is resynthesized via 2-MG pathway by the substrates of 2-MG and two fatty acids. Newly formed TG is incorporated into chylomicrons, released into the lymphatic

system, and poured into the bloodstream (Gotoh and Shimasaki, 2004; Ikeda and Yanagita, 2004). Chylomicron-TG is hydrolyzed by lipoprotein lipase to remnants and FFA. Chylomicron remnants are further taken up by liver (Cristophe, 2004).

In the case of DG oil, digestion and absorption of two isomers are different from each other. 1,2-DG is hydrolyzed to 2-MG and FFA, and follows the same steps as TG. 1,3-DG, on the other hand, is hydrolyzed by pancreatic lipase to 1-MG and FFA. 1-MG is not a suitable substrate for TG synthesis via 2-MG pathway in intestinal cells. Alternatively, a much smaller amount of TG is synthesized via the glycerol phosphate pathway, which involves the utilization of glycerol-3-phosphate as the starting point of TG synthesis (Flickinger, 2004). As a result, amount of TG released to the bloodstream through the lymphatic system is reduced after 1,3-DG consumption (Murata *et al.*, 1994). Besides, increased release of FFA into portal circulation enhances β-oxidation in liver, which further contributes to a reduction in body fat accumulation (Watanabe *et al.*, 1997).

2.3 Health Benefits of Diacylglycerols

Since diets high in lipid are potential risks for coronary heart diseases and obesity, most mortal diseases of the century, the health benefits of DG are of great importance.

DG has two main health benefits: (a) the suppression of postprandial serum TG elevation, (b) the suppression of body fat accumulation (Yasukawa and Katsuragi, 2004). It was shown that energy value of DG oil was nearly 98% of TG oil with the similar fatty acid composition. Besides, bioavailability (Taguchi *et al.*, 2001) and absorption rates (Murase *et al.*, 2001) of these two oils were not different from each other. Thus, health benefits of DG oil are considered to be the results of the different metabolic fate after absorption by intestinal epithelial cells, as described above. It should be noted that, in most of the studies summarized below, DG was mainly composed of oleic and linoleic acids. Thus, there observed some different effects with the DG samples including long chain fatty acids as well.

2.3.1 Effects of diacylglycerols on postprandial lipid profile

DG oil was shown to activate lipid catabolism in small intestine by a study conducted on rats which have diet-dependent obesity (Murase *et al.*, 2002). Synthesis of enzymes involved in β-oxidation was elevated by DG consumption, independent from the fatty acid composition of DG. Murata *et al.* (1997) furthermore proved by an animal study that ingestion of DG resulted in significant decreases in the activities of enzymes involved in fatty acid synthesis, in the serum TG concentration, and in the liver TG content. In addition to that, in a study on rats, it was found that increases in serum chylomicron-TG and very low density lipoprotein (VLDL)-TG were also suppressed by DG loading compared to TG (Nakagiri *et al.*, 2002). The most recent study on the subject (Kim *et al.*, 2007) supported the previous results by lower plasma TG, FFA, and phospholipid concentrations and hepatic TG level, increased β-oxidation, and decreased fatty acid synthesis enzyme activity in DG-fed rats compared to TG-fed ones.

A DG-rich oil containing conjugated linoleic acid (CLA) and capric acid was proposed to combine the positive effects of DG with those of the mentioned fatty acids (Kim *et al.*, 2006). On the other hand, a recent study comparing the effects of docosahexaenoic acid (DHA) on hyperlipidemia when it was consumed in the form of DG, TG, or concentrate revealed that the form of the lipid caused no significant differences between the effects of the oils (Tamai *et al.*, 2007). The form of CLA was also found to have no effect on plasma or liver cholesterol or plasma lipoprotein cholesterol concentrations (Porsgaard *et al.*, 2006). Similarly, simultaneous intake of indigestible dextrin, a type of dietary fiber that modulates intestinal functions and decreases serum cholesterol and TG concentrations, and DG did not result in a synergistic effect on cholesterol-fed rats. The authors commented that simultaneous intake of food components with similar physiologic functions did not necessarily produce additive or synergistic physiologic benefits (Nagato and Saito, 2006).

Investigation of effects of DG oil on serum lipids of healthy men showed that serum TG, serum phospholipid (Taguchi *et al.*, 2000; Tada *et al.*, 2001) and VLDL-TG levels were significantly decreased, whereas high density lipoprotein (HDL) level was slightly increased compared to TG oil consumption (Taguchi *et al.*, 2000). A typical meal containing DG oil was able to reduce postprandial TG, chylomicron TG,

and remnant-like particle cholesterol levels of healthy people (Tomonobu *et al.*, 2006).

Tada *et al.* (2005) investigated the effects of DG on postprandial lipids in diabetics. DG loading significantly suppressed increases in postprandial serum TG and lipids in remnant-like particles as compared with TG. However, there were no differences between the changes in serum levels of insulin, free fatty acids, and ketone bodies during either DG or TG loading. The authors suggested that substituting DG for TG might be beneficial to moderately controlled diabetic patients due to its effect in reducing postprandial hyperlipidemia. A single dose ingestion study showed that DG oil vs. TG oil reduced postprandial lipidemia especially in subjects with insulin resistance (Takase *et al.*, 2005).

Yamamoto *et al.* (2005) investigated postprandial and long-term effects of dietary DG on serum triacylglycerol TG levels in a 34-year old man homozygous for complete lipoprotein lipase (LPL) deletion. Lack of this metabolism results in severe hyperchylomicronemia and hypertriglyceridemia, often accompanied by severe hypercholesterolemia. Since medium-chain fatty acid TG (MCT) is absorbed without being reesterified to TG, MCT is currently the only cooking oil available for patients with LPL deletion. Long-term use of MCT, however, might cause an insufficient intake of essential fatty acids, which are indispensable for growth. Mentioned study showed that postprandial serum TG levels after DG oil ingestion were lower than those after TG oil ingestion and similar to those after MCT oil ingestion. Moreover, substitution of 12.0 g/d DG (equivalent to 15 g DG oil) for TG oil had the same effect as reducing TG oil consumption for controlling the serum TG levels in the LPL-depleted patient with hypertriglyceridemia. In conclusion, the results of the study demonstrated that DG oil might be replaced by MCT oil as cooking oil for those with LPL deletion.

Besides positive effects on lipid metabolism, the altered digestion and absorption of DG oil may have influence on the absorption of fat-soluble vitamins as well. In a human study focusing on this point, levels of fat-soluble vitamins, namely vitamin A, vitamin E, 25-hydroxyvitamin D, and 1-α,25-dihydroxyvitamin D, were not affected by DG consumption (Watanabe *et al.*, 2001a).

2.3.2 Effects of diacylglycerols on obesity

Obesity is a metabolic disorder which results from the lack of equilibrium between energy uptake and expenditure, and is a major risk factor for a number of life-related diseases, such as diabetes, hypertension, hyperlipidemia, and atherosclerosis (Hase and Itakura, 2004). DG oil has been proved to have preventive effects against obesity.

Rats fed with either DG or TG oil as 10% of their diet for a month were compared. The percentage of body fat was significantly lower in the DG-fed group (Watanabe *et al.*, 1997). Another study conducted on C57BL/6J mice, a model of diet-induced obesity and diabetes, revealed that DG consumption as a part of a high-fat and high-sucrose diet suppresses the accumulation of body fat, especially in the liver, and prevents hyperinsulinemia (Sugimoto *et al.*, 2003).

Mice prone to obesity and diabetes had lower body weight, body fat, insulin and leptin levels when they were fed with DG oil for 5 months compared to TG-fed ones (Murase *et al.*, 2001).

DG consumption was followed by an increase in energy expenditure in rats, either with long-term ingestion (Watanabe *et al.*, 2001b), or with a single dose (Watanabe *et al.*, 1997), which can result in the suppression of diet-induced obesity.

In a human study (Kamphuis *et al.*, 2003), although energy expenditure was not affected by DG oil consumption, fat oxidation was increased and the decreases in hunger, appetite, estimated food intake, and desire to eat were significant compared to TG oil. On the other hand, a more recent human study (Saito *et al.*, 2006) resulted in higher postprandial energy expenditure after DG oil consumption compared to that of TG oil. Moreover, serum insulin level 0.5 h after loading with DG oil was significantly lower than that after loading with the TG oil, which suggested that there was a smaller stimulus in the direction of fat storage after loading with DG oil.

The effects of long-term consumption of DG and TG oil-enriched Western-type diets on glucose and lipid metabolism in mice lacking brown adipose tissue (BATless mice) were investigated (Saito *et al.*, 2007). Such mice are models of high-fat diet-induced insulin resistance and obesity. Although BATless mice became obese on both DG and TG diets, the ones in the DG group gained less weight and had less body fat accumulation. The results of glucose tolerance tests conducted after 5 weeks

were not different. However, after 10 weeks, impaired glucose tolerance developed in the TG group but was prevented in the DG one.

Umeda *et al.* (2006) replaced a portion of the fat in dog food with either DG or TG, and fed overweight beagle dogs for a 6-week period. Even though the food compositions other than fat type were identical, dogs fed the DG diet showed a statistically significant reduction in body weight. In addition, the DG group also showed a reduction in body fat content, serum TG and total cholesterol concentrations.

Since it was shown that the beneficial effects of DG oil were independent from the fatty acid compositions (Murase *et al.*, 2002), combining these effects with those of polyunsaturated fatty acids was an interesting approach. In this concept, α-linolenic acid-rich DG (ALA-DG) was used in studies conducted on C57BL/6J mice. It was observed that, after a month, the group fed with ALA-DG had significantly lower body weight gain and total visceral fat than the group fed with a high fat diet (Hun *et al.*, 1999). When the study was reproduced at a longer period (20 weeks), in addition to the previous results, it was also noted that serum insulin and leptin concentrations were significantly lower in ALA-DG group, which can be considered as a preventive effect against diabetes (Hase *et al.*, 2001).

Human studies on the antiobesity effects of DG oil have also been promising. In a 4-months study conducted on healthy but near-obese men, decreases in body weight, body mass index, total fat area, visceral fat area, and hepatic fat content were significantly higher in the group consuming DG oil-rich foods, compared with the TG oil one (Nagao *et al.*, 2000). When DG oil was incorporated into a reduced-energy diet of men and women for a 6-months period, it was seen that, although TG- and DG-fed groups both lost weight, it was more pronounced on the DG-fed group (Maki *et al.*, 2002).

Although all these results can be seen strong enough to prove antiobesity effects of DG oil, it should be kept in mind that all the studies summarized previously were done under strict diet conditions. To benefit mentioned effects of DG oil, its consumption under *ad libitum*, i. e. free-feeding, conditions should result in the same way. A number of studies were conducted accordingly. Obese and overweight men and women replaced their regular cooking oil with either DG or TG oil without any additional dietary restrictions for a 1 year period. Although not as significant as the

previous studies, DG oil consumption still resulted in reduced body weight compared to TG oil. The mild effect was explained to be due to the relatively small amount of DG oil intake and to the free-living environment (Koyama *et al.*, 2003). In a similar study with the same conditions (Kawashima *et al.*, 2008), body weight decreased significantly in the DG group compared to the TG group, which was attributed to the substitution of usual home cooking oil with DG, because total energy intake and physical activity did not differ between groups. Participants with higher initial body mass index or greater percentage of total fat intake as DG exhibited greater reduction in body weight. In a monadic study for 1 year (Yasukawa and Yasunaga, 2001; Katsuragi *et al.*, 1999), body weight and body fat decreased significantly with DG oil consumption. Although serum TG levels of subjects with normal initial serum TG levels did not change, this parameter significantly decreased at hypertriglyceridemic subjects. In addition, HDL cholesterol significantly increased, whereas low density lipoprotein (LDL) cholesterol significantly decreased. Obese and/or hypertriglyceridimia subjects taking part in a monadic study for 2 years had significantly decreased body weight, body fat and body mass index, and significantly increased HDL cholesterol (Ostuki *et al.*, 2003). To investigate the effects of DG oil in children as well, a study was conducted on obese and/or hypertriglyceridimia children in which they consumed DG oil *ad libitum* for 5 months (Matsuyama *et al.*, 2000; Matsuyama, 2001). Although the level of obesity generally increased over the study period, there were significant decreases at total fat area, serum leptin and serum TG levels, and a significant increase in HDL cholesterol level, without any adverse effects on growth and development of the children.

2.3.3 Effects of diacylglycerols on life-style related diseases

Since DG oil has a number of beneficial effects on lipid profile, which is considered as a potential risk factor for some life-style related diseases, consumption of such oil can also be beneficial for those diseases. An important example is type II diabetes, the type that accounts most of the diabetes cases worldwide. Obesity and overweight result in an increased risk of type 2 diabetes, which further can lead to hypertriglyceridemia (Yamamoto and Asakawa, 2004). The potential of DG to suppress postprandial TG was investigated on Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Mori *et al.*, 2005). This type of rats has some characteristics of human type II diabetes and exhibit severe hypertriglyceridemia with decreased plasma

lipoprotein lipase activity. Oral DG loading reduced the serum TG increase. DG also markedly reduced the serum FFA concentration increase due to the fat load. After 22 weeks of feeding, dietary DG reduced serum TG levels in the non-fasting state. Moreover, an oral glucose tolerance test revealed enhanced glucose disposal in the DG-fed rats compared with the TG-fed rats. Dietary DG was proven to extenuate arterial thrombus formation compared to TG oil in diet-sensitive congenital apolipoprotein E and LDL receptor double deficient mice (Ijiri *et al.*, 2006). The effects of α-linolenic acid-rich DG on lipid accumulation and the expression of genes involved in lipid metabolism in the liver were investigated on Zucker rats (Murase *et al.*, 2005). These animals, which are reported to be a good model of non-insulin-dependent diabetes, are characterised by hyperphagia, excessive body weight gain, and lipid deposition in the liver and adipose tissue. It was shown that such DG significantly inhibited hepatic TG accumulation and might be useful in the prevention of fatty liver formation. In a recent study conducted on diabetic apoE-deficient mice, the effects of long-term feeding of 1,3-DG oil on the dyslipidemia and atherosclerosis were investigated (Fujii *et al.*, 2007). ApoE-deficient mice are models of hypercholesterolemia, and they are highly susceptible do develop atherosclerotic lesions when they are fed with a Western-type diet. Additionally, diabetic apoE-deficient mice are highly vulnerable to dyslipidemia and atherosclerosis even without supplemental fat and cholesterol. The authors concluded that 1,3-DG oil reduced atherosclerosis in diabetic apoE-deficient mice. This was reported to be the first study to show that dietary DG-rich oil improves not only dyslipidemia but also atherosclerosis associated with diabetes.

Cho *et al.* (2006) proposed a new functional oil composed of DG (40%), MG (15%), and TG (45%) which had blood cholesterol-lowering effect and inhibited atherogenic enzymes on C57BL/6 mice.

Yamamoto *et al.* (2001) performed a study on type II diabetic patients to determine the effect of long-term consumption of DG oil on fasting TG levels. Subjects who replaced their ordinary cooking oil with DG oil for 3 months had significantly lower serum TG levels than those consuming TG oil. To evaluate the effect of DG on postprandial hyperlipidemia in insulin resistance and glucose intolerance, a study was conducted on subjects with a normal glucose tolerance (NGT) and subjects with impaired glucose tolerance (IGT) (Ai *et al.*, 2007). In the IGT subjects, after the DG

and TG load, the serum concentrations of TG, remnant lipoprotein-TG, and remnant lipoprotein-cholesterol increased throughout the 4-h study. The responses of these variables above baseline after the DG load were significantly smaller than those after the TG load. In contrast, in the NGT subjects, changes in these parameters were much smaller than those observed for IGT subjects. DG was more effective in insulin resistant and hyperinsulinemic participants regardless of glucose intolerance, and may be beneficial in reducing the extent of CAD risk in such individuals.

Type II diabetic patients generally develop small dense LDL (sLDL), a subclass of LDL, which is reported to be associated with a relative increase in serum TG levels, reduced levels of HDL, and an increased risk of coronary artery disease compared with subjects with larger LDL (Feingold *et al.*, 1992). The effects of DG oil on sLDL were investigated on type II diabetic patients for 3 months (Yamamoto *et al.*, 2003), and it was seen that TG levels in the LDL fraction and sLDL level were significantly decreased, whereas HLD cholesterol significantly increased in DG oil consuming patients, compared to TG oil consuming ones. The long-term intake of DG oil prevented the progression of renal function in the subjects of type II diabetes with nephropathy, delaying the onset of dialysis (Yamamoto *et al.*, 2006).

Phytosterols (PS) are known to reduce serum total cholesterol and LDL cholesterol levels. The limited use of PS, which are barely soluble in oil or water, was overcame when it was found that PS could be dissolved in DG at high levels (Meguro *et al.*, 2001), and several studies were conducted to see if DG oil could be used to enhance beneficial effects of PS. PS in combination with DG (PS/DG) feeding to cholesterol-fed rabbits for 14 weeks resulted in significantly lower serum total cholesterol level than those fed with PS in combination with TG (PS/TG). Atherosclerosis formation was inhibited in PS/DG group (Meguro *et al.*, 2003). In a human study with the same concept (Meguro *et al.*, 2001), PS/DG consumption reduced total and LDL cholesterol levels significantly compared to PS/TG. Serum TG level was also decreased in PS/TG group. *Ad libitum* studies supported these results as well (Saito *et al.*, 2001; Takeshita *et al.*, 2001). PS/DG was recently shown to reduce total cholesterol, LDL cholesterol and apolipoprotein B concentrations of postmenopausal women with mild to moderate hypercholesterolemia (Takeshita *et al.*, 2007). In summary, PS/DG can be considered as a useful ingredient to prevent hypercholesterolemia and initiate atherosclerosis.

2.4 Production of Diacylglycerols

As mentioned before, DG and MG are used as emulsifiers and stabilizers in the food industry, and in pharmaceutical and cosmetic applications as well. Mixtures of MG and DG are generally synthesized from TG and glycerol by a chemical glycerolysis reaction at temperatures above 200^{o}C using alkaline catalysts. The selectivity of the reaction can usually not be modified and depends on the molar ratio of glycerol to TG used in the reaction. DG fraction in the resulting product mixture has physicochemical characteristics that are intermediate between those of TG and MG; thus, the separation process is difficult for the purpose of making a DG-rich oil product (Watanabe *et al.*, 2004).

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are a group of ubiquitous enzymes, which can catalyse not only hydrolysis of fat and oils, but also various reverse reactions, such as esterification, acidolysis, interesterification, etc. Lipase-catalyzed enzymatic reactions have a number of advantages over chemical ones for DG production, such as mild reaction conditions, high selectivity, and low energy costs.

The main enzymatic reactions used to produce DG are esterification (Figure 2.2) and glycerolysis (Figure 2.3), which are the reaction between glycerol and FFAs or glycerol and TGs, respectively. Glycerolysis reaction, in which DG can be produced both by removal of a fatty acid from a TG molecule and by acylation of a MG formed during the reaction, can be chosen over esterification, since it is a simple process where oil can be used directly without the need to liberate FFA prior to the reaction (Kristensen *et al.*, 2005a). Some selected studies are summarized below.

Early studies involved enzymatic reactions in organic media. The use of organic solvents improves the solubility of substrates and results in more efficient contact between them and enzyme. Li and Ward (1993) used esterification reaction between glycerol and omega-3 polyunsaturated fatty acids in various organic solvents and suggested isooctane, hexane, heptane, and pentane to be useful for DG synthesis with the 1,3-specific *Rhizomucor miehei* lipase.

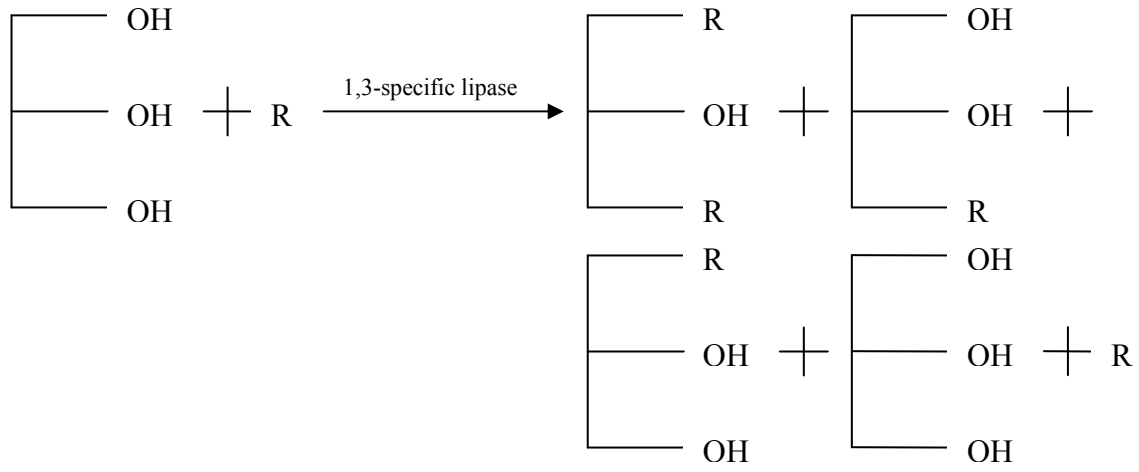

Figure 2.2: Esterification reaction between glycerol and a fatty acid (R) catalyzed by a 1,3-specific lipase.

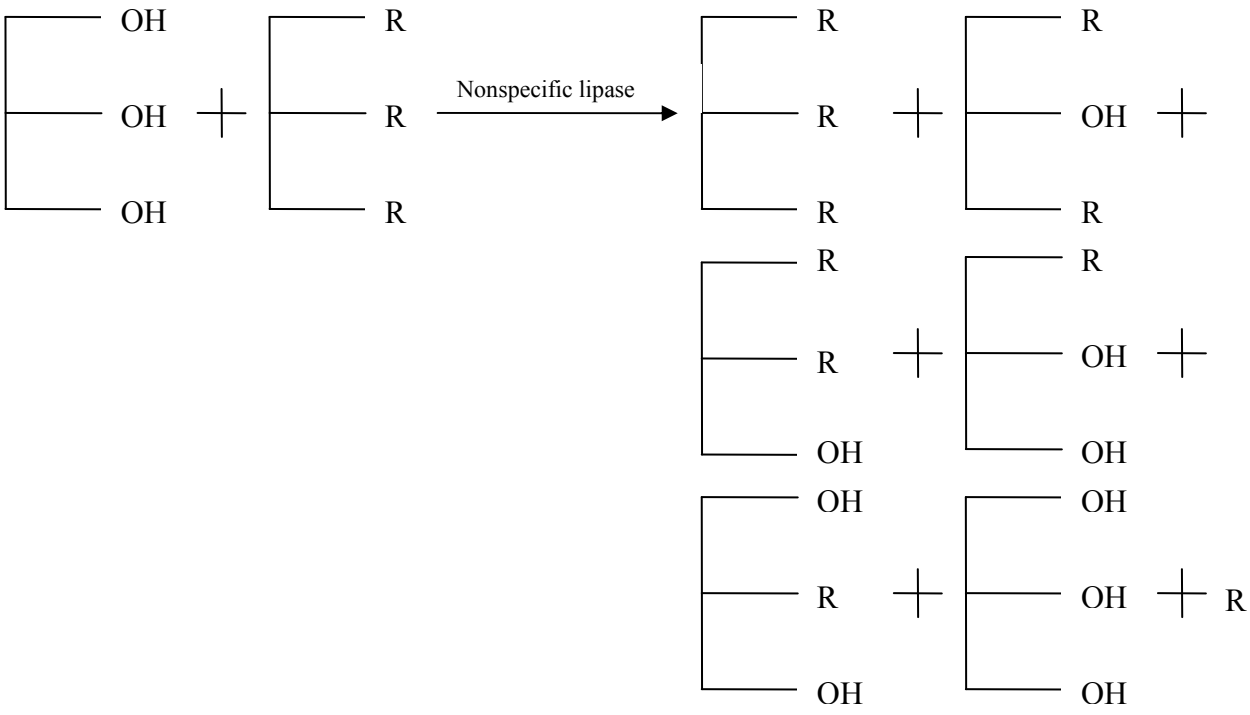

Figure 2.3: Glycerolysis reaction between glycerol and TG catalyzed by a nonspecific lipase (R: Fatty acid).

DG was produced by enzyme-catalyzed alcoholysis of TG by various lipases in organic media (Fureby *et al.*, 1997). Alcohol concentration and chain length of the alcohol had significant effects on yield. The lipase from *Penicillium roquefortii* was

shown to produce a high content of 1,2-DG. DG yield was 75%, 95% of which was 1,2-DG.

Since glycerol is immiscible in organic solvents, a different approach was suggested which involves the adsorbing of glycerol onto a solid support (Berger *et al.*, 1992). Investigation of different solid supports showed that inorganic supports resulted in a higher reaction rate than others. The esterification reaction was observed even with the ratio of 1:10, support to glycerol, but optimum ratio was 1:1. The solid support was also shown to be suitable for reuse after consecutive applications. The reactions were carried out in *n*-hexane, diethyl ether, or *tert*-butylmethyl ether.

In recent years, use of organic solvents for such reactions was not preferred. Although organic solvent usage can result in shifting the equilibrium towards 1,3-DG product, an esterification reaction in an organic solvent is not suitable for the food industry, since the recovery of the solvent is costly and time-consuming, and not preferred for food-grade productions as well. Watanabe *et al.* (2003) used direct esterification of glycerol with FFA to produce DG in a solvent-free system with a 1,3-specific lipase from *Rhizomucor miehei*. It was found that the rate of esterification was increased with an increase in either temperature or the enzyme used, but the 1,3-DG yield did not change. In addition, the temperature dependency of the acyl migration rate from 1,3- to 1,2-DG was stronger than that of the 1,3-DG synthesis rate; so 1,3-DG purity (i.e. the ratio between 1,3- and 1,2-DG) was higher in lower temperatures. 1,3-DG yield was 81% after 4 hours with the substrate molar ratio of 2:1 (FFA:glycerol), 5 wt % of enzyme on the basis of total substrates, under 3 mm Hg vacuum, at 50°C. However, it was later commented that the stirred-tank reactor used in the study might not be suitable for long term and industrial-scale production because the immobilized enzyme resins cannot be loaded into the reactor at a high density and they would be susceptible to breaking by the stirring shear force (Watanabe *et al.*, 2005). Alternatively, a packed bed bioreactor was generally considered suitable for long term continuous operations, but the problem with this sytem was that the water content along the axial direction of the column would be perturbed due to the formation of water during the esterification. The removal of water produced during reaction resulted in the shift of equilibrium towards esterification. The authors suggested circulation of the substrates between a packed

bed column and a water removal vessel to overcome these disadvantages. A 1,3-DG yield of 70% was achieved.

Esterification of glycerol with CLA catalyzed by various lipases in a solvent-free system was performed (Guo and Sun, 2007). To produce 1,3-DG, Novozym 435 (nonspecific *Candida antarctica* lipase B immobilized on macroporous acrylic resin) was preferred over Lipozyme TL IM (1,3-specific *Thermomyces lanuginosa* lipase immobilized on granulated silica) and Lipozyme RM IM (1,3-specific *Rhizomucor miehei* lipase immobilized on macroporous anion-exchange resin).

DG was produced by solid-phase lipase-catalyzed glycerolysis of hydrogenated beef tallow (Yamane *et al.*, 1994). The temperature control was important since the substrate was solidified stepwise. DG yield was approximately 90%, 95% of which was 1,3-DG.

Esterification of glycerol with various FFAs in a solvent-free system was performed (Rosu *et al.*, 1999). A 1,3-spesific *Rhizomucor miehei* lipase was used as the catalyst. Water was removed to shift the equilibrium towards DG. 1,3-DG yield was around 85% after 8 hours of reaction with the substrate mole ratio of 2:1 (FFA:glycerol) and 4 wt % of enzyme on the basis of total substrates, under 3 mm Hg vacuum, at 25°C. The authors tested removal of water produced from the reaction medium and using excess of FFA to shift the equilibrium towards DG. Water removal greatly improved conversion rate. Optimum susbtrate molar ratio was found to be 4:1 (FFA:glycerol). It was also reported that the increase in reaction temperature also increased TG production from the 1,2-DG substrate, which was an undesired effect.

Kristensen *et al.* (2005a) performed a solvent-free glycerolysis reaction catalyzed by Novozym 435 with the substrates rapeseed and sunflower oils and glycerol. They optimized the reaction conditions with respect to the total DG yield and concluded that the parameter which influenced the DG yield the most was reaction temperature. Water addition was the only factor with a negative effect on the DG yield. Optimal conditions were no water addition, low content of glycerol (molar ratio of 2:1, oil:glycerol), temperature of 60-65°C, 4-5 h reaction time, and 5 wt % lipases on the basis of oil mass, which resulted in 60% DG yield. To shift the acylglycerol equilibrium, MG addition and lowering the temperature approaches were tried, both of which did not lead to higher DG yield.

Weber and Mukherjee (2004) compared the DG yield of various methods, i.e. interesterification between TG and MG with or without vacuum, esterification between FFA and MG with vacuum, esterification between FFA and glycerol with vacuum, and glycerolysis of TG with vacuum, in a solvent-free medium. The lowest DG yield (40-45%) was obtained by the esterification between FFA and glycerol, whereas the highest one (75%) obtained from the interesterification between TG and MG under vacuum.

Several commercially available lipases were screened for their ability to synthesize DG in a glycerolysis reaction in a solvent-free system (Kristensen *et al.*, 2005b). It is generally accepted that lipase-catalyzed reaction occurs at the oil/water interface. When an immobilized enzyme is used, the reaction takes place not in the glycerol phase but in the oil phase. The authors concluded that the reason for the esterification not to take place when the enzymes immobilized on hydrophilic supports first came into contact with glycerol was the formation of glycerol layer around the enzyme particles. Novozyme 435 was found to result in the highest DG yield (62%). Glycerol was either added at the beginning of the reaction (batch reaction), or stepwise during the reaction (fed-batch reaction), and it was seen that although the latter one reached equilibrium faster than the former, DG yield was not different between them. Another observation was that nonspecific lipases yielded more 1,3-DG in a shorter time compared to 1,3-specific lipases. This effect was expected since nonspecific lipases can produce 1,3-DG both by removing an acyl moiety from TG and acylation of 1 and 3 positions of glycerol, whereas 1,3-specific lipases produce 1,2(2,3)-DG by the former mechanism.

Acyl migration, in which 1,3-DG is converted to 1,2-DG or 1-MG to 2-MG, is an important issue in esterification reactions, especially when it is aimed to produce DG high in 1,3-DG isomer. The effects of various factors, such as water content, temperature, enzyme load, and reaction time, on acyl migration were investigated (Xu *et al.*, 1998; Xu *et al.*, 1999) and all mentioned factors were found to have effects on acyl migration.

Hydrolysis of TG was also shown to be a useful way to produce a mixture of DG and MG (Pluo *et al.*, 1996). The rate of the reaction was dependent on the regioselectivity of the different lipases used and the hydrophilicity of supports on which enzymes were immobilized. Cheong *et al.* (2007) recently optimized reaction parameters, such

as water content, enzyme load, temperature, and reaction time, for partial hydrolysis using Lipozyme RM IM lipase in a solvent-free system to produce a DG-enriched palm olein.

2.5 Properties and Use of Diacylglycerols in Food Products and Consumer Evaluation of Such Products

The physicochemical properties of DG oil are almost the same as those of TG. On the other hand, since DG has a hydroxyl group in the molecule, the properties related to hydrogen bonding are different compared to those of TG. These differences greatly influence application of DG oil to food products by affecting the retention of flavor components, hydrolysis during heating, selection of emulsifier, and solubilization of functional substances (Nakajima *et al.*, 2004).

Autoxidation stability and thermal oxidation stability were not different between DG and TG oil (Nakatsugawa *et al.*, 2001; Nishide *et al.*, 2004). Deep-frying tests under severe conditions showed that DG oil was more susceptible to hydrolysis than TG oil (Nishide *et al.*, 2004). DG dissolves more moisture than TG because of the presence of a hydroxyl group, which results in hydrolysis (Nakajima *et al.*, 2004). On the other hand, the difference between hydrolysis indicators in DG and TG oils was not significant under in-home cooking conditions (Nishide *et al.*, 2004). DG oil produced from sunflower oil was oxidatively less stable as compared to TG sunflower oil, but they had similar sensory quality. DG butter blends produced from sunflower and rapeseed oils did not have different stabilities than their control TG blends. However, they had significantly less salty and buttery flavours, which was thought to be a result of much smaller water droplet size causing a delayed sensory perception in the mouth (Kristensen *et al.*, 2006).

DG oil was shown to be useful to replace TG either partially or completely for production of oil-in-water foods, such as mayonnaise and salad dressing (Kawai, 2004); water-in-oil foods, such as margarine, spreads, and butter cream (Masui, 2004); baked goods, nutritional beverages and bars, sauces, and gravies (Sikorski, 2004).

Sensory evaluations were conducted for several fried foods, a stir-fry, and a sauté prepared using DG and TG oils (Ogawa *et al.*, 2001a). No significant differences

were observed between two groups immediately after cooking. However, 30 minutes after cooking, evaluations of potato chips cooked with DG oil were significantly lower than those with TG oil, which was considered as a result of high moisture absorption capacity of DG oil. A deep-frying study showed that sensory evaluations of foods fried with TG oil were better than those with DG oil (Fukuda *et al.*, 2002). Home-made confectionaries prepared with either DG or TG oil showed no significant sensory differences, with the exception of chiffon cake. The antifoaming effect of DG caused by adsorption to the interface between air and water (Nakajima *et al.*, 2004) might have influenced the homogeneity of the chiffon cake (Ogawa *et al.*, 2001b). When ordinary cooking oil was replaced with DG oil for 3 weeks, participants reported that DG oil was superior to the ordinary one. Dishes prepared with DG oil were noted to be not oily and to have a light taste. Some subjects also reported the absence of a heavy stomach feeling (Masui *et al.*, 2001). The last effect was considered to be a result of the shorter gastric emptying time of DG oil compared to TG oil (Yasunaga *et al.*, 2000).

2.6 Safety Issues of Diacylglycerols

Several toxicological and clinical studies were conducted to assess the safety of DG oil. No toxic effects were observed on acute animal studies (Borzellaca *et al.*, 2004). Repeated-dose animal studies also resulted in no treatment-related, dose-dependent toxicological effects (Soni *et al.*, 2001; Borzellaca *et al.*, 2004; Chengelis *et al.* 2003).

In addition to the toxicological studies in animals, DG oil was also tested for possible mutagenicity in *in vitro* bacterial systems, cultured mammalian cells, and *in vivo* mammalian systems. DG oil was shown to have no mutagenic potential (Borzellaca *et al.*, 2004). DG oil did not cause a higher risk of carcinogenity (Chengelis *et al.*, 2006) and did not exert modifying potential on tumor development in the colon or any other organ (Ichihara *et al.*, 2008) compared to TG oil on rats.

Clinical studies were also conducted to examine human tolerance to DG oil. No adverse effects were reported and DG oil was well tolerated by the subjects in single-dose (Taguchi *et al.*, 2000), repeated-dose (Nagao *et al.*, 2000; Watanabe *et al.*, 2001a; Yasunaga *et al.*, 2004), and *ad libitum* (Katsuragi *et al.*, 1999) studies with healthy volunteers, as well as subjects with hyperlipidemia (Matsuyama *et al.*, 2000;

Teramato *et al.*, 2000), overweight (Maki *et al.*, 2002), and diabetes (Yamamoto *et al.*, 2001). As a conclusion, DG oil can be considered safe for food use in a manner similar to other edible oils.

3. IONIC LIQUIDS

3.1 Introduction

Ionic liquids (ILs) are organic salts, and are liquid at ambient temperatures (Yang and Pan, 2005). Interest in these compounds as a medium for several reactions, including biocatalysis, as an alternative to organic solvents has risen in the recent years, due to their unique properties, such as near-zero vapor pressure, high chemical and thermal stability, ability of tuning a number of properties like polarity, hydrophobicity, and solvent miscibility behavior by modification of the cation and the anion included (van Rantwijk and Sheldon, 2007). Besides, ILs, so called 'green solvents', are also known to have protective effects on enzymes or increase their stabilities, to shift the reaction equilibrium to the desired side, and to be recoverable and recyclable (Guo and Xu, 2006). Several studies showed that enzymes not only tolerate ILs, but they are also stable and the activity is comparable or even better than in organic solvents.

3.2 Properties of Ionic Liquids

Ionic liquids are organic salts with low melting points (<100^{o}C) which remain liquid in a wide temperature range (<300^{o}C) (Yang and Pan, 2005). Before discussing the physical and chemical properties of ILs, it must be remembered that they can be dramatically altered by the presence of impurities. Purification of the ILs is essential not only to avoid possible interactions between reactants and impurities, but also because they can change the nature of these solvents. The main contaminants are halide anions and organic bases, arising from unreacted starting material and water (Chiappe and Pieraccini, 2005). Some early results with biocatalysis in ionic liquids proved to be difficult to reproduce, probably due to the presence of such impurities (van Rantwijk and Sheldon, 2007).

In order to be liquid at room temperature, the cation of the IL should preferably be unsymmetrical. The melting point is also influenced by the nature of the anion (Sheldon, 2001). Some researches (Bonhôte *et al.*, 1996; Hagiwara and Ito, 2000) indicated that there appears to be no overall correlation between the composition of an IL and its melting point, based on non-systematic changes in cation and anion types. The melting points decrease with incorporation of larger, more asymmetrical cations (Ngo *et al.*, 2000). Further, an increase in the branching on the alkyl chain increases the melting point (Chiappe and Pieraccini, 2005).

Most of the ILs exhibit high thermal stability. The decomposition temperatures reported are generally >400^{o}C, with minimal vapor pressure below their decomposition temperatures. The onset of thermal decomposition is furthermore similar for the different cations but appears to decrease as the anion hydrophilicity increases (Chiappe and Pieraccini, 2005). On the other hand, it was shown that high decomposition temperatures do not imply long-term thermal stability below these temperatures. After 10 hours, even at temperatures as low as 200^{o}C, 1-alkyl-3-methylimidazolium hexafluorophosphate and 1-decyl-3-methylimidazolium triflate showed appreciable mass losses (Kosmulski *et al.*, 2004).

ILs have higher viscosity than typical organic solvents, which is considered as one of the largest barriers to the application of ILs. A high viscosity may produce a reduction in the rate of many organic reactions and a reduction in the diffusion rate of the redox species (Chiappe and Pieraccini, 2005). Normally, viscosity increases with an increase in alkyl chains on the cation and in the size of the anion. It should also be noted that polarity decreases as well in these conditions (Yang and Pan, 2005). Viscosity of ILs is highly dependent on temperature and show a great variety because of the impurities included. If needed, the addition of cosolvents to ILs can be an alternative which results in dramatic reductions in the viscosity without changing the cations or anions in the system (Mantz and Trulove, 2008).

In general, ILs have higher density than water. The molar mass of the anion significantly affects the overall density of ILs (Chiappe and Pieraccini, 2005).

ILs are generally considered as highly polar but weakly coordinating solvents. They are shown to have polarities similar to those of short-chain alcohols. The polarity varies depending on the nature of the IL's moieties. For example, the solubility of

water in an IL can be varied from completely miscibile to almost immiscible by changing the anion from Cl^- to $[PF_6]^-$ (Cocalia *et al.*, 2008). In general, it can be stated that for 1-alkyl-3- methylimidazolium [C_nMIM] ILs, the polarity is determined by the anion when the 1-alkyl groups are short (C_n<6) and by the cation when the 1-alkyl groups are long (C_n>6) (Cantone *et al.*, 2007). In a similar way, lipophilicity of ILs can be modified by the degree of cation substitution (Cocalia *et al.*, 2008). Due to their tunable properties, many compounds, i. e. polar or nonpolar organic, inorganic, and polymeric, are soluble in ILs (van Rantwijk and Sheldon, 2007). ILs are generally immiscible with many organic solvents. The immiscibility of ILs with either water or organic solvents has made them attractive to be used to form biphasic systems (Yang and Pan, 2005). The solubility of gases, such as H_2, CO, and O_2, is also high in ILs, which makes them attractive solvents for several reactions like catalytic hydrogenation, carbonylation, hydroformylation, and aerobic oxidation (Sheldon, 2001).

ILs are widely used in catalysis reactions carried out under multiphase homogeneous conditions, that are believed to occur at the interface between the IL and the overlying organic phase. Therefore, these reactions should be dependent on the access of the catalyst to the surface and on the transfer of the material across the interface, so it can be said that the rate of these processes depends on surface tension. In general, liquid/air surface tension values for ILs are higher than those for conventional solvents, although not as high as for water, and differ in a wide range. Surface tension values vary with temperature and both the surface excess entropy and energy are affected by the alkyl chain length, decreasing with increasing length. For a fixed cation, in general, the compound with the larger anion has the higher surface tension (Chiappe and Pieraccini, 2005).

As the interest in ILs increases, their being ‘green’ is questioned in more details. Near-zero vapor pressure of ILs results in no contribution to air pollution, but it should be kept on mind that most ILs are water soluble and thus, can enter the environment via this path (Wagner and Hilgers, 2008).

Involatility had been assumed to be a property common to all ILs that do not undergo thermal decomposition. Earle *et al.* (2006) recently showed that certain IL structures known for their very high thermal stability, in particular bis(trifluoromethyl)sulphonylamide salts, can be evaporated and recondensed under

relatively mild conditions. For example, the distillation of 1-hexyl-3-methylimidazolium bis(trifluoromethyl)-sulphonylamide was reported to occur at 170°C under a pressure of 0.07 millibar. There was no direct proof for the presence of ions in the gas phase of the distillation processes, but the study showed that there were several instances of IL evaporation and recondensation, and that non-volatility is not a property which can be assumed for all thermally stable ILs. Vapour pressure is just another physical-chemical property that depends on an IL's cation-anion combination, and will in the future have to be checked experimentally for each new liquid. It was, however, clearly stated that the vapour pressure of ILs remains negligible at near ambient conditions, and many ILs show no signs of distillation below the temperature of their thermal decomposition; so these developments cannot be said to bring into question the view that ILs are 'green solvents'.

For an IL to be considered as a green solvent, its life cycle analysis has to be taken into account. Kralish *et al.* (2005) examined the cumulative energy demand, a measure of energetic impact, environmental and economic impact of ILs. The cumulative energy demand takes the supply of raw materials, recycling and disposal of chemicals, the energies required for heating, stirring, pumping and providing cooling water into account. The choice of cation, anion and reaction solvents were found to have a large effect on the cumulative energy demand, toxicity and economics. To improve the sustainability of ILs, using biorenewable raw materials was suggested. Imidazolium ILs were synthesized from fructose (Handy *et al.*, 2003) and shown to be able to replace the conventional imidazolium-based ILs for some applications (Handy and Okello, 2003).

Safety aspects of ILs still need to be investigated. Since ILs can consist of so many different types of cations and anions, toxicity studies cannot be generalized and have to be done case by case. Some recent data on the issue is summarized in Table 3.1. As can be seen, ILs can be toxic as well as harmless and biodegradable (Maase, 2008). 1,3-dialkylimidazolium cation was shown to be an important determinant of IL toxicity. Toxicity increased as alkyl chain length increased (Ranke *et al.*, 2004).

To produce non-toxic ILs, a new approach which involves the use of anions based on non-nutritive sweeteners of known toxicity, such as saccharinate and acesulfamate, was suggested (Carter *et al.*, 2004; Pernak *et al.*, 2005). ILs were also produced by

anions of known toxicity and biodegradability using 20 natural amino acids combined with the $[EMIM]^+$ cation (Fukumoto *et al.*, 2005). Commercial suppliers of ILs are also preparing several kinds based on ions of known toxicity. Sachem produce Terrasail™ ILs based on the docusate(dioctylsulfosuccinate) anion. Solvent Innovation GmbH produces ECOENG ™ and AMMOENG ™ IL families, which are based on alkylsulfate and alkoxysulfate anions and ethylene glycol based ammonium cations, respectively. These ions have already been studied with regards to toxicity and are already utilized in other industries (Gordon and Muldoon, 2008).

Table 3.1: Toxicity of some IL examples.

	BMIM.Cl[1]	EMIM.$EtOSO_3$[2]	MTEOA.MeOS O_3[3]
Acute oral toxicity	toxic	not harmful	not harmful
Skin irritation	irritant	non-irritant	non-irritant
Eye irritation	irritant	non-irritant	non-irritant
Sensitization	non-sensitizing	non-sensitizing	non-sensitizing
Mutagenicity	non-mutagenic	non-mutagenic	non-mutagenic
Biodegradability	not readily degradable	not readily degradable	not readily degradable
Toxicity to daphnia	acutely toxic	acutely not harmful	acutely not harmful
Toxicity to fish	acutely not harmful	-	acutely not harmful

[1]: 1-Butyl-3-methylimidazlium chloride
[2]: 1-Ethyl-3-methylimidazolium ethlysulfate
[3]: Tris-(2-hydroxyethyl=-methylammonium methylsufate

The biodegradability of commonly used ILs such as 1-butyl-3-methylimidazolium tetrafluoroborate and 1-butyl-3-methylimidazolium hexafluorophosphate were found to be negligible; hence, these should be expected to persist in the environment for long periods (Gathergood *et al.*, 2004). Efforts to produce biodegradable ILs by using ester or amide derivatives of bromoacetic acid (Gatherhood *et al.*, 2004; Garcia *et al.* 2005) showed that ones with an ester group on the cation showed the greatest

biodegradation, while the octyl sulfate anion was the most easily degraded anion among those used.

3.3 Applications of Ionic Liquids

Use of ILs in the industry is not a new concept. They have been used in electrochemical and chemical researches. Some important applications of ILs in chemical processes are acid scavenging, extractive distillation, chlorination with nucleophilic HCl, cleavage of ethers, dimerization and oligomerization of olefins, hydrosilylation, and fluorination. ILs can also be used for some electrochemical applications, such as electroplating of chromium and electropolishing, as antistatic additives for cleaning fluids, as compatibilizers for pigment pastes, and for storage of gases (Maase, 2008). Present uses as well as potential uses in the future are summarized in Figure 3.1 (Plechkova and Seddon, 2008).

ILs are reported to be useful in synthetic applications and catalysis, as well as non-synthetic ones. Hydrogenation, hydroformylation, carbonylation, olefin dimerisation, oligomerisation, Heck reactions, catalytic oxidations, Lewis and Brønsted acid catalysis are some examples for synthetic applications in ILs reported so far (Sheldon, 2001). It has become clear that frequently used and well-established homogeneous catalysts need to be modified in order to work effectively in ILs. For instance, it was shown that activation of a pre-catalyst can be thermodynamically hindered in an IL, resulting in no catalysis at all if reaction conditions are not adapted properly (Daguenet and Dyson, 2004). In addition to catalyst activation, sufficient retention of the catalyst in the IL is important in order to provide good recycling. Fixing homogeneous catalysts in IL phases is to attach charged groups to the ligands bound to the metal catalyst centre has been used for this purpose in imidazolium ILs (Dyson and Geldbach, 2007).

ILs have further proven to be excellent solvents to both immobilize and stabilize nanoparticle catalysts. Controlled preparation of transition metal nanoparticles in ILs by reduction of the metal complex with molecular hydrogen in the absence of stabilizers was reported (Dupont *et al.*, 2002).

As mentioned above, yields and selectivities can vary according to the batch of IL used due to the impurities such as halides, and to lesser extent organic compounds of

low volatility. To overcome this problem, synthesis of highly pure ILs can be an approach. In addition, designing ILs, so-called task specific ILs (*i.e.*, ILs containing functional groups) that exhibit specific properties to enhance catalytic activity has been studied. For example, nitrile functionalized ILs were developed to improve catalyst retention in reactions employing simple palladium salts as catalyst precursors. Such ILs have been utilized in Suzuki and Stille coupling reactions and they were shown to exhibit excellent recycling properties compared to other ILs (Zhao *et al.*, 2004a; Chiappe *et al.*, 2006). Task specific ILs may also act as supports

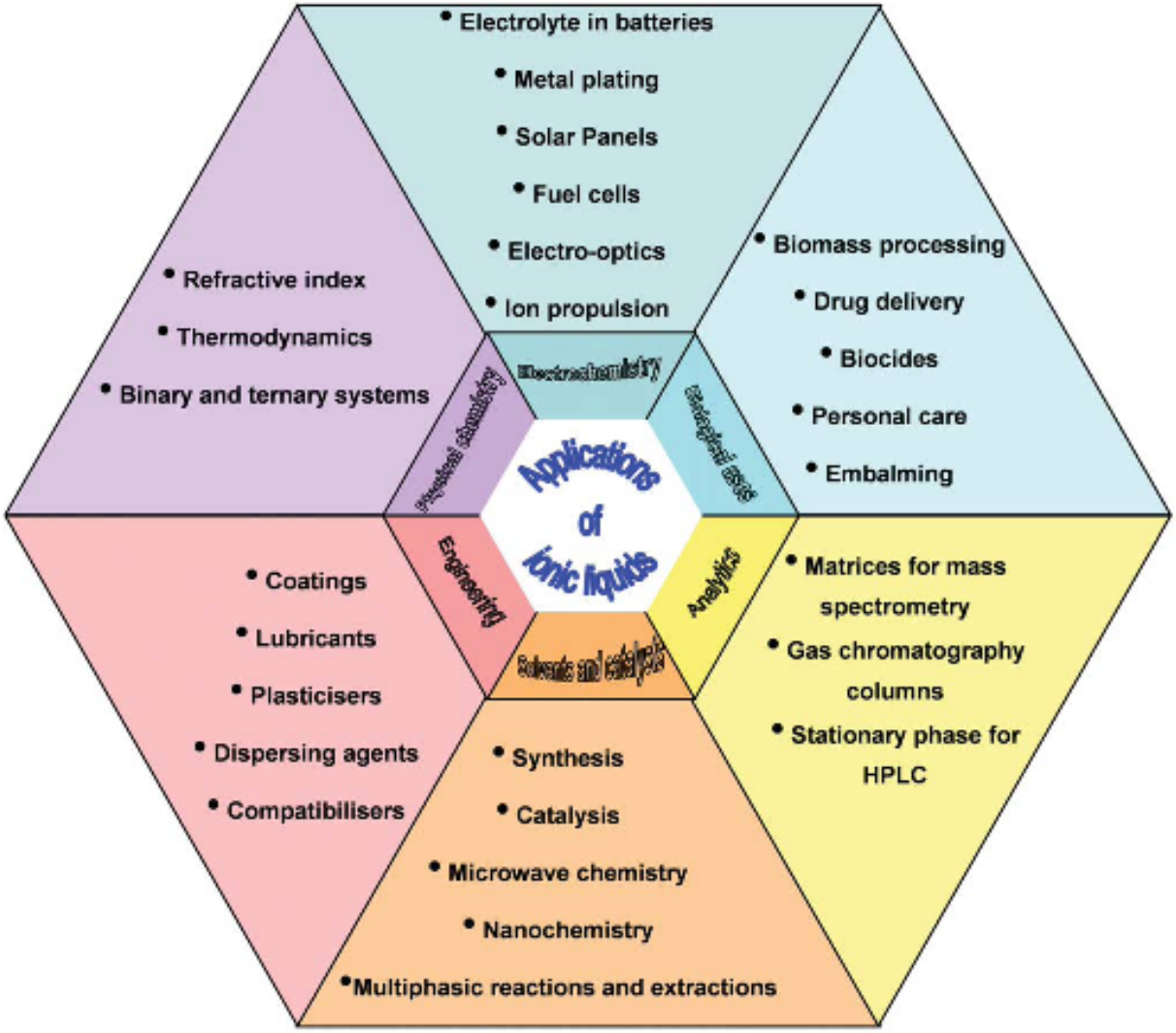

Figure 3.1: Fields of applications for ILs.

for the synthesis of certain molecules, thereby facilitating purification and isolation. Advantages of using a task specific IL relative to other supports results from their lower cost, high loading capacity and facile analysis of the intermediates by

spectroscopic methods (Dyson and Geldbach, 2007). One potential problem with task specific ILs is that their viscosities tend to be higher than their non-functionalized analogues, which has recently overcomed by the founding that the viscosity of these ILs can be reduced by using asymmetric functionalized anions in combination with the functionalized cation (Zhao *et al.*, 2004b).

As ILs are still expensive compared to conventional solvents, efficient recycling of them is an important issue, especially in large-scale applications. Recycling protocols generally make use of the low solubility of the ILs in organic solvents such as toluene. This allows products and residual organics to be extracted using an organic solvent, while salt by-products present in water-immiscible ILs can be washed out with water. Water-miscible ILs, on the other hand, are more difficult to recycle as inorganic by-products cannot be easily removed. Furthermore, if the reaction products are sufficiently volatile, they can be distilled directly from the IL, since ILs are inherently non-volatile (Wagner and Hilgers, 2008). Membrane techniques were also shown to be suitable to recycle ILs (Wagner and Hilgers, 2008).

3.4 Ionic Liquids and Enzymes

Since ILs are composed of anions and cations, it could be useful to summarize the ion effects on enzymes before discussing enzymatic reactions in such media in details. The strongly hydrated ions that increase the structure of water are called kosmotropes, so-called ‘structure-makers’, and those weakly hydrated ions that decrease the structure of water are known as chaotropes, ‘structure-breaker’. Kosmotropes are usually small and highly charged, while chaotropes are large and low-charged. All multivalent ions are highly hydrated, therefore, are kosmotropic. Many studies concluded that kosmotropic anions and chaotropic cations stabilize enzymes, while chaotropic anions and kosmotropic cations destabilize them. Multivalent anions are better enzyme stabilizers or activators due to their kosmotropic nature. Generally cations have less influence on the enzyme activity and stability than anions do; however, it is not always the case. When the enzyme surface is negative charged, the cations may have considerable interaction with the enzyme. In addition, when a cation is the co-factor to form ion-enzyme complexes, the cation may be essential for the enzymatic activity (Zhao, 2005). At high concentrations, it was found that both kosmotropic and chaotropic anions inhibited the enzyme by

different mechanisms. Kosmotropic anions decrease the apparent Michaelis constant and increase the activation barrier, while chaotropic ones have the opposite effect. The active site flexibility is important in the function of the enzyme. The kosmotropic anions induce high rigidity, while chaotropic anions induce high flexibility. Both situations lowered the enzyme activity (Žoldák *et al.*, 2004).

The high hydrophobicity, i.e. large log *P*, of ILs could be beneficial to the enzyme activity. The more hydrophobic of an IL with less negative log *P* value, the less water is required for maintaining optimal enzyme activity and enantioselectivity. When the IL is hydrophilic, more water presence is needed because high ionic strength of hydrophilic ILs tend to remove the essential water from the enzyme causing the deactivation (Zhao, 2005). It was shown that higher enzyme activity might be observed in ILs with larger cations, such as $[OMIM]^+$, than those with smaller ones, such as $[BMIM]^+$. The longer hydrophobic alkyl chain in the cation has less tendency to take away the essential water from the enzyme (Yang and Pan, 2005).

The main reason for ILs being considered as a biocatalysis medium was the desire to improve the low turnover rate of enzymes in organic media (van Rantwijk *et. al*, 2003). The first publication describing the preparative use of an enzymatic reaction in ILs involved the synthesis of the dipeptide Z-aspartame catalyzed by protease thermolysin (Erbeldinger *et al.*, 2000). The reaction rates were comparable to those in organic solvents such as ethyl acetate. Additionally, the enzyme stability was increased in the IL. Since then, various enzymes are reported to be active in many ILs, and their activities are comparable with or even higher than those obtained in organic solvents. A list of examples of enzyme applications in ILs is given in Table 3.2 (Yang and Pan, 2005).

The majority of enzymes reported to be active in ILs so far are lipases. Lau *et al.* (2000) published the second study demonstrating the potential use of enzymes in ILs, which was the first one on lipases. They compared the activity of *Candida antarctica* lipase in ILs such as $[BMIM].[PF_6]$ and $[BMIM].[BF_4]$ with that in organic solvents. Alcoholysis, ammoniolysis and perhydrolysis reactions were investigated, and it was found that the reaction rates were similar for all of them. Since then, several studies were conducted in ILs using lipase-catalyzed reactions, such as direct esterifications (Bélafi-Bakó *et al.*, 2002) and transesterifications (Nara *et al.*, 2002; Lozano *et al.*,

2003), enantioselective transesterifications, (Schöfer *et al.*, 2001; Itoh *et al.*, 2003; Persson and Bornscheuer, 2003), enantioselective hydrolyses (Mohile *et al.*, 2004), enantioselective acylation of amines (Irimescu and Kato, 2004), ammoniolysis (Lau *et al.*, 2000), and direct glycerolysis of oils (Guo and Xu, 2005). Lipase-catalyzed biodiesel production from soybean oil in ILs was also published recently (Ha *et al.*, 2007).

One of the particular features of ILs is their ability to act as solvents for both hydrophobic and hydrophilic compounds, such as carbohydrates. The lipase-catalyzed esterification of carbohydrates in ILs was shown to be more advantageous as compared to that occurring in organic solvents because of the enhanced regioselectivity (Park and Kazlauskas, 2001). The regioselective acylation of glucose in [MOEMIM].[BF_4] resulted in 99% yield and 93% selectivity, both of which were higher than those obtained with organic solvent use.

Several research groups have reported that lipases showed higher enantioselectivity when used for kinetic resolution of chiral alcohols (Kim *et al.*, 2001; Schöfer *et al.*, 2001; Eckstein *et al.*, 2002; Kiebasiski *et al.*, 2002) and esterification (Ulbert *et al.*, 2004) in ILs.

Since enzyme stability is usually much higher in organic media, especially at a low water activity, than in aqueous solution, it is expected that ILs should have the same effect on stabilizing enzymes. Indeed, the stability of α-chymotrypsin was studied by incubation in four different ILs and the half-life of the enzyme was significantly enhanced compared to that in 1-propanol. The increase in the enzyme's half-life seemed to be in agreement with the increase in hydrophobicity of the ILs. It is reasonable that a hydrophobic reaction medium would allow the preservation of the essential water molecules surrounding the protein structure, thereby reducing the protein-ion direct contact and enhancing the enzyme stability toward denaturation (Lozano *et al.*, 2001a). At elevated temperatures, *Candida rugosa* lipase in ILs kept its activity and enantioselectivity much better than in traditional organic solvents (Fráter *et al.*, 2003).

Table 3.2: Examples of using enzymes in ionic liquids.

Biocatalyst	Reaction	Ionic liquid	Reference
Lipase	Transesterification	[BMIM].[PF_6]	Kaar *et al.*, 2003
	Alcoholysis, ammoniolysis, perhydrolysis	[BMIM].[PF_6], [BMIM].[BF_4]	Lau *et al.*, 2000
	Kinetic resolution of chiral alcohols	[BMIM].[Tf_2N]	Schöfer *et al.*, 2001
	Resolution of amino acid ester	[EPy].[BF_4], [EMIM].[BF_4]	Zhao *et al.*, 2003
	Kinetic resolution of *P*-chiral hydroxymethanephosphinates	[BMIM].[PF_6]	Kiebasiski *et al.*, 2002
	Esterification of carbohydrates	[MOEMIM].[BF_4]	Park and Kazlauskas, 2001
	Synthesis of polyesters	[BMIM].[PF_6]	Kaar *et al.*, 2003
Alcohol dehydrogenase	Enantioselective reduction of 2-octanone	[BMIM].[Tf_2N]	Eckstein *et al.*, 2004
Thermolysin	Synthesis of *Z*-aspartame	[BMIM].[PF_6]	Erbeldinger *et al.*, 2000
α-Chymotrypsin	Transesterification	[EMIM].[Tf_2N], [MTOA].[Tf_2N]	Lozano *et al.*, 2001a

Table 3.2: Examples of using enzymes in ionic liquids (continued).

Biocatalyst	Reaction	Ionic liquid	Reference
Esterase	Transesterification	[BMIM].[PF_6]	Persson and Bornscheuer, 2003
Subtilisin	Resolution of amino acid ester	[EPy].[TFA]-H_2O (15:85, v/v)	Zhao and Malhotra, 2002
β-Galactosidase	Synthesis of *N*-acetyllactosamine	[MMIM].[$MeSO_4$]-H_2O (25:75, v/v)	Kaftzik *et al.*, 2002
Peroxidase	Oxidation of guaiacol	[BMIM].[PF_6]	Laszlo and Compton, 2002
Laccase	Oxidation of anthracene	[BMIM].[PF_6]	Hinckley *et al.*, 2002
Formate dehydrogenase	Regeneration of NADH	[MMIM].[$MeSO_4$]-H_2O (25:75, v/v)	Kaftzik *et al.*, 2002
Baker's yeast	Enantioselective reduction of ketones	[BMIM].[PF_6]-H_2O (10:1)	Howarth *et al.*, 2001
Lactobacillus kefir cells	Asymmetric reduction of 4-chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol	[BMIM].[PF_6], [BMIM].[Tf_2N], [OMA].[Tf_2N]	Pfruender *et al.*, 2004

The stabilization effect of ILs on enzymes can be further improved by incubating the enzyme in the presence of the substrate. The reuse of free *Candida antarctica* lipase B in [BMIM].[PF_6] in continuous operation cycles showed a half-life of 2300 times greater than that observed when the enzyme was incubated in the absence of substrate (Lozano *et al.*, 2001b). The authors concluded that the association of the substrate to the enzyme may cause a conformational change that activates the enzyme, and this active conformation is well-retained during the presence of the substrate.

Supercritical CO_2 (sc-CO_2) has been widely described as an excellent solvent for hydrophobic compounds, and is considered as clean technology for a range of industrial extractive processes, which is also used to extract organic compounds dissolved in the IL phase (Blanchard *et al.*, 1999). sc-CO_2 might exert adverse effect on biocatalysts, due to pH changes caused by the carbonic acid that forms during the reaction, or due to conformational changes observed during pressurisation or depressurisation. In order to overcome these limitations, a new concept for continuous biphasic biocatalysis, where a homogeneous enzyme solution is dissolved in ILs (working phase) and substrates and products reside largely in a supercritical phase (extractive phase), has been proposed (Lozano *et al.*, 2002). An aqueous solution of native *Candida antarctica* lipase B was dissolved in [EMIM].[Tf_2N] or [BMIM].[Tf_2N] and the synthesis of butyl butyrate was studied in a continuous packed bed reactor system. The reactor was coupled with a high-pressure sc-CO_2 extraction apparatus. The low water content of the system (4%, v/v) resulted in high selectivity and good conversions. The ILs exerted a protective effect against enzyme deactivation by temperature and sc-CO_2 which was proved by the ability of usage of the enzyme for ten reaction cycles. A few other studies using a similar process for acylation of 1-octanol with vinyl acetate (Reetz *et al.*, 2002), kinetic resolution of chiral secondary alcohols (Reetz *et al.*, 2003), and enantioselective synthesis of glycidyl esters (Lozano *et al.*, 2004) are published.

4. MATERIALS and METHODS

4.1 Materials

Glycerolysis reactions were performed between the substrates of glycerol and triolein. 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, lot no: LC200207), Lipozyme RM IM (*Rhizomucor miehei*, lot no: LUX00205), and Lipozyme TL IM (*Thermomyces lanuginosa*, lot no: LA300205) were provided by Novozymes A/S (Bagsvaerd, Denmark). All ILs were purchased from Solvent Innovation GmbH (Cologne, Germany), except methyltrioctylammonium trifluoroacetate which was from Merck (Darmstadt, Germany). All solvents used were of chromatographic grade with the minimum purity of 96%.

4.2 Methods

The project can be divided into four different stages, which are (1) the screening of different ILs with respect to the DG yield observed and comparison of the results with conventional organic solvents, (2) the investigation of the effects of different reaction parameters on the yield in selected ILs, (3) the use of *n*-hexane/IL and binary IL systems, and (4) the optimization of the reaction conditions for the selected binary IL system.

4.2.1 Screening of different IL types

As a first step, 19 different ILs (Appendix A) were tested concerning their DG production abilities and the results were compared to those obtained in *tert*-butanol and *tert*-pentanol systems. Glycerolysis of high oleic acid sunflower oil catalyzed by Novozym 435 was performed in 25 mL jacketed reactors. Typically, 1 mmol of oil and 0.5 mmol of glycerol were mixed with 1 g of IL. The substrates and IL are

mixed by magnetic agitation at 700 rpm, thereafter the reaction was initiated by the addition of the lipase (10 wt % based on the oil mass). The reaction was thermostated at 60^{o}C by circulated water bath. Aliquots of 20 μL were withdrawn at 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h, dissolved in 0.8 mL of chloroform/methanol mixture (2:1, v/v), and analyzed by thin layer chromatography (TLC) coupled with a flame ionization detector (FID; Iatroscan MK-6s, Bechenheim, Germany). 1 μL of diluted sample was spotted onto silica-coated Chromarod® quartz rods by a semiautomatic sampe spotter, Model SES 3202/IS-02. Samples were developed in a DT-150 development tank with the developing system of *n*-hexane, diethyl ether, and acetic acid (45:25:1, v/v/v). The rods were dried for 2 minutes at 120^{o}C in a TK-8 chromarod dryer prior to analysis. Data handling was performed on a computer equipped with SES I-Chromstar® 6.0 software. The area percentages of TG, DG (1,3- and 1,2- isomers separately), MG, and FFA were used for the calculation of product yields. An exception in the procedure was [TOMA].[Tf_2N], which gave an ion peak at the same time interval with MG. To seperate MG precisely, reaction product was mixed with aqueous solution of ammonium acetate (10%, w/w) to exchange anions between that and the IL, following sample extraction by chloroform, prior to the TLC-FID analysis.

Comparative reactions in *tert*-butanol and *tert*-pentanol were conducted in 25 mL jacketed reactors with 4 mmol oil, 2 mmol glycerol and lipase in the amount of 10 wt % based on the oil mass, at 60^{o}C, with 200 rpm stirring, for 24 h. The reaction progress was monitored similarly.

4.2.2 Investigation of the effects of different reaction parameters on the yield in selected ILs

According to the results of the previous step, 6 out of 19 ILs were selected for the rest of the project: [BMIM].[BF_4], [BMIM]. [PF_6], [OMIM].[PF_6], [MTOA].[TFA], [TOMA].[Tf_2N], AMMOENG™ 120. The parameters, the effects of which were investigated, are ratio between the substrates and IL (substrates/IL), temperature (T), and ratio between oil and glycerol (oil/glycerol), respectively. All reactions were performed in 25 mL jacketed reactors with 1 g of IL and lipase in the amount of 10 wt % based on the oil mass, for 24 h, with 700 rpm stirring. After evaluating the overall results, 3 ILs, namely [MTOA].[TFA], [TOMA].[Tf_2N], and AMMOENG

120, were selected for further examination at optimized conditions with different types of lipases. The varying reaction conditions are summarized in Table 4.1. The reaction progress was monitored by periodical sampling and TLC-FID analysis.

Table 4.1: Reaction conditions for parameter investigation experiments.

Parameter	Substrates, oil/glycerol, mmol:mmol	Lipase	T, ^{o}C
Substrates/IL	0.5:0.25	Novozym 435	60
	2:1	Novozym 435	60
Temperature	2:1	Novozym 435	50
	2:1	Novozym 435	70
Oil/glycerol	2:1.25	Novozym 435	60
	2:1.5	Novozym 435	60
Lipase type*	2:1.5	Lipozyme RM IM	60
	2:1.5	Lipozyme TL IM	60

*: Reactions were performed only with [MTOA].[TFA], [TOMA].[Tf_2N], and AMMOENG 120.

4.2.3 Use of *n*-hexane/IL systems

Solvent addition is a useful approach to decrease the viscosity of ILs, which may cause mass transfer problems and result in low conversion in biocatalytic reactions. To investigate this approach, *n*-hexane/IL systems with 6 different ILs, i.e. [BMIM].[BF_4], [BMIM]. [PF_6], [OMIM].[PF_6], [MTOA].[TFA], [TOMA].[Tf_2N], AMMOENG 120, were tested as a glycerolysis medium. The reactions were performed in 25 mL jacketed reactors with 2 mmol oil, 1 mmol glycerol and lipase in the amount of 10 wt % based on the oil mass, with 600 rpm stirring, for 24 h. The varying reaction conditions are summarized in Table 4.2. The reaction progress was monitored similarly.

Table 4.2: Reaction conditions for *n*-hexane/IL experiments.

n-hexane/IL, mL:mL	T, ^{o}C
0.5:0.5	40
0.5:0.5	50
0.5:0.5	60*
0.665:0.335	50
0.335:0.665	50

*: Reactions were performed in screw capped tubes in order to prevent n-hexane loss by evaporation.

4.2.4 Use of binary IL systems

The first step of the study, which was the screening of the IL types, showed that none of the ILs resulted in desired levels for all the parameters considered such as DG yield (i.e. total DG/total components in the final mixture, %), TG conversion (i.e. total components in the final mixture – TG, %), MG concentration (i.e. MG/total components in the final mixture, %). To obtain a system which provides the best medium to obtain high DG yield and TG conversion with low MG concentration, the use of binary IL systems was tested. The reactions were performed in 25 mL jacketed reactors with 2 mmol oil, 1 mmol glycerol, lipase in the amount of 10 wt % based on the oil mass, and a mixture of two ILs in the ratio of 0.5:0.5, mL:mL, at 60^{o}C, with 600 rpm stirring, for 24 h. Tested binary systems were [TOMA].[Tf_2N]/AMMOENG 100, [TOMA].[Tf_2N]/AMMOENG 102, AMMOENG 120/AMMOENG 100, and AMMOENG 120/AMMOENG 102. The reaction progress was monitored similarly.

4.2.5 Optimization of the reaction conditions for the selected binary IL system with response surface methodology

According to the results of the previous step, [TOMA].[Tf_2N]/AMMOENG 102 binary IL system was chosen for further optimization. A five-level central composite rotatable design was used for the RSM studies, and the experimental settings

determined by Minitab Release 14 were generated with three factors. Temperature, glycerol dosage, and [TOMA].[Tf_2N] percentage were the factors investigated. The ranges for the factors were determined as follows: Temperature, 45-75 ^oC; glycerol dosage, 1.15-1.75 mmol; [TOMA].[Tf_2N] percentage 25-75. 2 mmol oil, lipase in the amount of 15 wt % based on the oil mass, and 24 h reaction time were constant for all experiments. The reaction conditions for each experimental setting are shown in Table 4.3. Experiments were run randomly.

RSM was used to generate models for both DG yield and TG conversion responses. Second order coefficients were generated by regression with backward elimination. Responses were fitted to the factors by multiple regressions. 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. R^2 alone is not a measure of the model's accuracy; it is a measure of the amount of the reduction in the variability of response obtained by using the repressor variables in the model. However, a large value of R^2 does not necessarily imply that the regression model is a good one. Adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significant or not. Thus, evaluation of R^2 and AAD values together is said to be better to check the accuracy of the model. R^2 must be close to 1.0 and the AAD between the predicted and observed data must be as small as possible (Baş and Boyacı, 2007).

AAD is calculated by the following equation:

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

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively, and p is the number of experimental run.

Table 4.3: Coded and decoded levels for RSM experiments.

	Temperature		Glycerol mol		[TOMA].[Tf_2N] percent	
Exp. no	Coded	Decoded	Coded	Decoded	Coded	Decoded
1	-1	45	-1	1.15	-1	25
2	1	75	-1	1.15	-1	25
3	-1	45	1	1.75	-1	25
4	1	75	1	1.75	-1	25
5	-1	45	-1	1.15	1	75
6	1	75	-1	1.15	1	75
7	-1	45	1	1.75	1	75
8	1	75	1	1.75	1	75
9	-α	34.7731	0	1.45	0	50
10	+α	85.2269	0	1.45	0	50
11	0	60	-α	0.94546	0	50
12	0	60	+α	1.95454	0	50
13	0	60	0	1.45	-α	7.9552
14	0	60	0	1.45	+α	92.0448
15	0	60	0	1.45	0	50
16	0	60	0	1.45	0	50
17	0	60	0	1.45	0	50

5. RESULTS and DISCUSSION

5.1 Screening of Different IL Types

As a first step, 19 different ILs (Appendix A) were tested due to their DG production abilities and the results were compared to those obtained in *tert*-butanol and *tert*-pentanol systems. Time courses of the glycerolysis reactions performed in *tert*-butanol and *tert*-pentanol are shown in Figure 5.1 and 5.2, respectively.

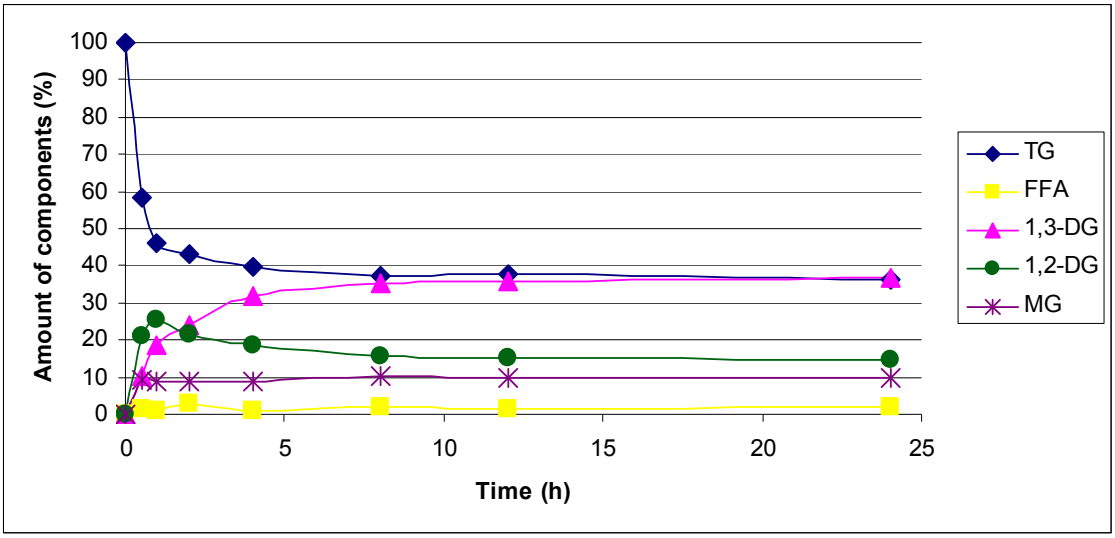

Figure 5.1: Time course of the glycerolysis reaction in *tert*-butanol. Reaction conditions: Enzyme dosage 10 wt % of oil mass, oil:glycerol ratio 4:2 (mmol:mmol), 5 ml solvent, temperature 60^{o}C, stirring 200 rpm.

Glycerolysis system with an immobilized lipase as catalyst is a three-phase system: a hydrophobic oil phase, a hydrophilic glycerol phase, and a solid enzyme phase. Since the more hydrophilic characteristics of the enzyme, glycerol often binds to the enzyme particles so that the excess of the oil molecules to the enzyme is difficult. Therefore, a solvent medium is an important solution to improve the homogeneity of the system. The number of the solvents which can be used for this purpose is very limited due to safety issues for food production processes and also the effects of

solvents on enzymatic activity. A few alcohols more than five carbons can be considered since they contain a polar -OH group and a nonpolar carbon chain, which

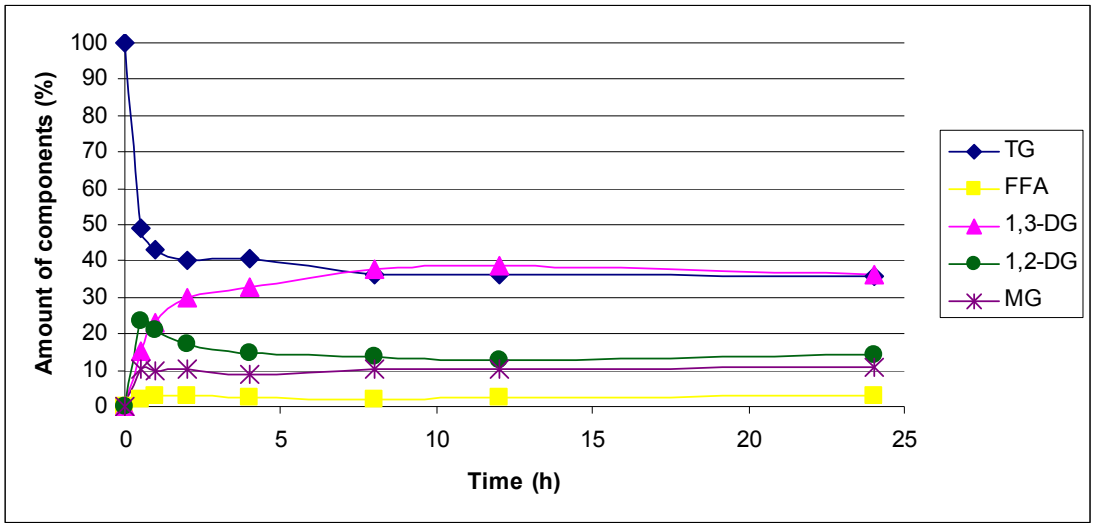

Figure 5.2: Time course of the glycerolysis reaction in *tert*-pentanol. Reaction conditions: See Figure 5.1.

gives a possibility to hold oil and glycerol in one system. The use of tertiary alcohols is considered to be appropriate since the tertiary structure will have strong steric hindrance for the enzyme activity (Yang *et al.*, 2005a). Enzymatic glycerolysis in the organic solvents *tert*-butanol or *tert*-pentanol has been investigated and found to be very efficient for partial acylglycerol production (Liao *et al.*, 2003; Damstrup *et al.*, 2005; Yang *et al.*, 2005a; Damstrup *et al.*, 2006a). *tert*-butanol only differs from *tert*-pentanol by one more carbon atom placed in the alcohol chain, which slightly affects the polarity (Yang *et al.*, 2005b). Since the polarity is the defining factor for the solvent selection in biocatalysis reactions, the yields of the reactions in *tert*-butanol and *tert*-pentanol are expected to be similar.

As can be seen in the figures, glycerolysis in both *tert*-butanol and *tert*-pentanol solvents reached to equilibrium at 8 h and yielded in 50% DG yield, 36% of which was 1,3-DG isomer. Kristensen *et al.* (2005b) performed a solvent-free glycerolysis reaction catalyzed by Novozym 435 which resulted in 62% DG yield, 40% of which was 1,3-DG. When a 1,3-spesific lipase was used, DG yield of the glycerolysis reaction was in the range of 30-45%, depending on the oil type used (Tüter *et al.*, 1999). Chemical glycerolysis of butterfat yielded in 40-45% DG (Yang *et al.*, 2004). Compared to these results, the yield of the reaction can be considered sufficient; however, unconverted TG and by-product MG concentrations are still undesirably

high, which are 36.5% and 10% for *tert*-butanol, whereas 36% and 10.5% for *tert*-pentanol, respectively.

Solvent selection for biocatalytic reactions has been evaluated on solvent polarity. It was shown that as the polarity of the solvent increases, the hydrophilicity also increases, which results in the dehydration of the enzyme because of the stripping off the bound water by the solvent (Gorman and Dordick, 1992). On the other hand, studies showed that solvent polarity cannot be a universal measure for enzyme activity, and that polarity of the substrates and products should also be considered. It is accepted that optimum enzyme activity can be expected when the difference between the polarity of the solvent and the product is minimum, which results in scavenging of the product by the solvent immediately after production, therefore avoiding possible product inhibition and shifting the equilibrium to the desired direction (Laane *et al.*, 1987). *tert*-butanol and *tert*-pentanol are both polar solvents, with log *P* values of 0.6 and 1.4, respectively (Janssen *et al.*, 1993). This phenomenon can be an explanation of the high MG production, which is a polar compound itself. Indeed, there are several studies published aimed at MG production in *tert*-butanol and *tert*-pentanol medium (Rendón *et al.*, 2001; Liao *et al.*, 2003; Damstrup *et al.*, 2005; Yang *et al.*, 2005a; Yang *et al.*, 2005b; Damstrup *et al.*, 2006a). It was also shown that not only the polarity, but also the functional groups of the solvent played an important role in the solvent properties. Damstrup *et al.* (2005) studied enzymatic glycerolysis reaction in different solvents and concluded that the maximum MG contents were obtained in solvents with log *P* values in the range of 0.3-1 and having a tertiary alcohol structure. Such structure enables the alcohol's participation in the esterification reaction (Janssen *et al.*, 1993).

The results from the screening of 19 different ILs were evaluated and compared with those obtained from organic solvents according to not only DG yield, but also TG conversion and MG production. Time courses of the glycerolysis reactions performed in all ILs can be seen in Appendix B. Although none of them resulted in higher total or 1,3-DG than compared conventional organic solvents, there were some promising ILs for further investigation. The results obtained from the selected ILs and a few others which gave interesting, yet not useful for the aim of the project, are summarized below.

Glycerolysis performed in 1-butyl-3-methylimidazolium tetrafluoroborate, i.e. [BMIM].[BF_4] (Figure 5.3), yielded in 30% DG yield, 23% of which was 1,3-DG. The reaction, which seems to have an induction period, leading to almost no reactivity for the first 2 hours, reached equilibrium around 24 h. 1,3-DG ratio in the total DG fraction was the highest in 36 h which was 30%, then was reduced to 23% at 48 h, which may be a consequence of the inhibition of enzyme access to the substrates due to the product accumulation. MG yield was very low, 2.5% at most which can be neglected.

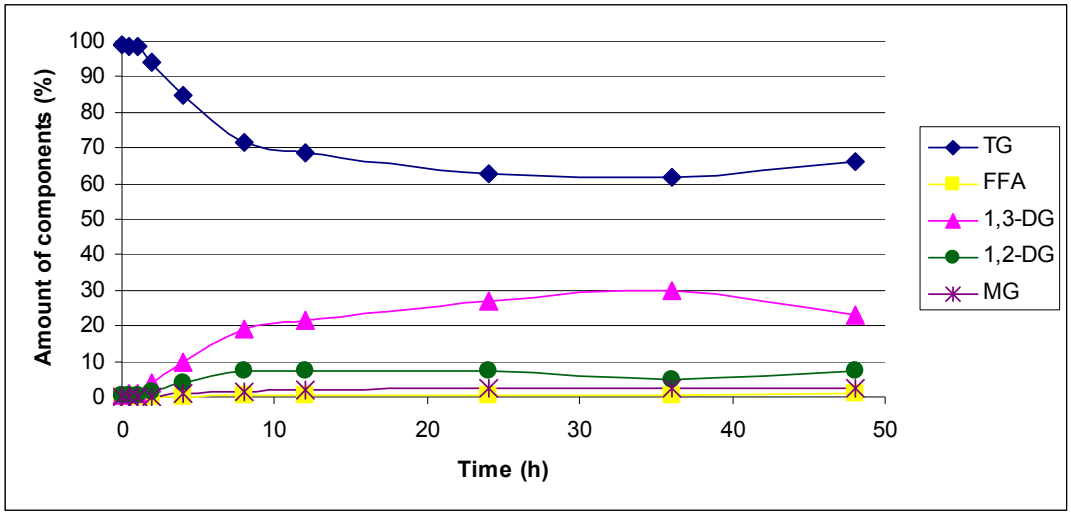

Figure 5.3: Time course of the glycerolysis reaction in [BMIM].[BF_4]. Reaction conditions for Figures 5.3-10: Enzyme dosage 10 wt % of oil mass, oil:glycerol ratio 1:0.5 (mmol:mmol), 1 g IL, temperature 60°C, stirring 700 rpm.

[BMIM].[BF_4] is one of the most studied ILs for biocatalysis applications. It has been reported that [BMIM].[BF_4] is suitable for alcoholysis, ammoniolysis, and perhydrolysis reactions catalyzed by Novozym 435 (Lau *et al.*, 2000), transesterification reaction catalyzed by free as well as immobilized *Pseudomonas cepacia* lipases (Nara *et al.*, 2002) or free *Candida antarctica* lipase B (de los Ríos *et al.*, 2007). However, the activity of lipases were shown to be much lower in this IL compared to organic solvents and other ILs based on the same cation (Ulbert *et al.*, 2004; de los Ríos *et al.*, 2007), which was said to be a consequence of the hydrophilic nature of the $[BF_4]^-$ anion, resulting in the stripping off the essential water layer surrounding the enzyme and thus, inactivating the enzyme. It was even reported that no glycerolysis reaction took place in [BMIM].[BF_4] medium due to the

low solubility of oil in the IL phase, again as a result of the hydrophilic behavior (Guo and Xu, 2005). The contrary results can be stemmed from the different conditions used in the previous studies. For instance, de los Ríos *et al.* (2007) performed the transesterification reaction at 30°C. Temperature is known to have a great influence on the viscosity of ILs and the ability of them to dissolve oil. In addition, [BMIM].[BF_4] is shown to have an induction period for glycerolysis reaction, which is also greatly dependent on temperature: Longer induction periods are reported with lower temperatures (Chen *et al.*, 2008). Use of low temperature, as low as the half of the temperature used in the present study, could have resulted in the poor solubility of the oil leading to low reactivity in [BMIM].[BF_4] medium reported previously. Longer induction period could also be the reason for the IL considered unsuitable for lipase-catalyzed glycerolysis. In addition, using higher dosage of glycerol (oil/glycerol, 1:5, mmol/mmol) could be one of the reasons that the results in this work differ from the previous publication (Guo and Xu, 2005), because high glycerol applied will reduce the solubility of oil in ILs.

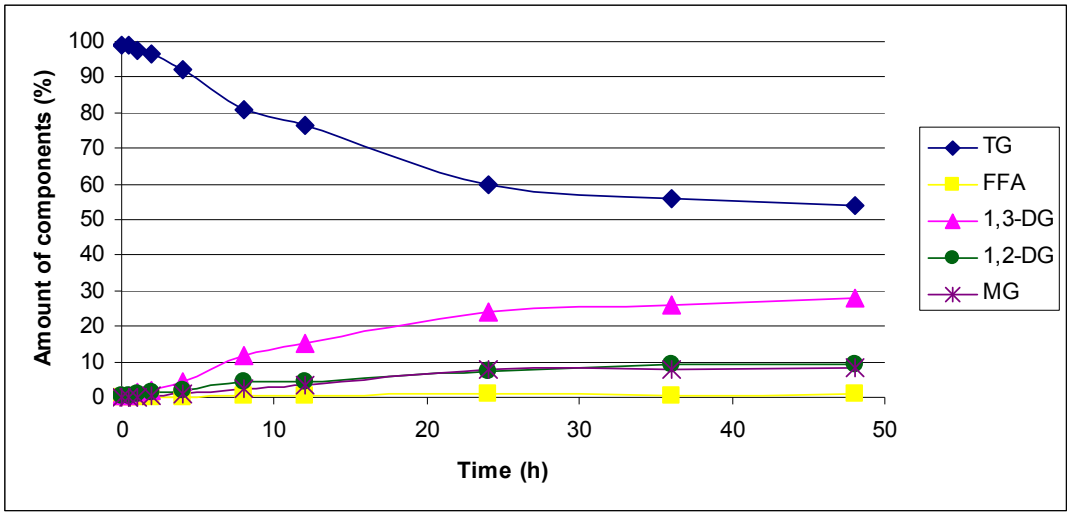

Figure 5.4: Time course of the glycerolysis reaction in [BMIM].[PF_6].

Another imidazolium type IL, 1-butyl-3-methylimidazolium hexafluorophosphate, i.e. [BMIM].[PF_6], was also selected for the rest of the project. Time course of the glycerolysis reaction performed in [BMIM].[PF_6] is given in Figure 5.4. The reaction yielded in 37% DG at the end of 48 h period. Equilibrium was reached at 24 h. 1,3-DG ratio was 24% at that point and it slightly increased at 36 h and 48 h to 26% and 28%, respectively. Considering cost issues, 24 h of reaction can be enough for this

type of IL. MG yield was around 8% after 24 h of reaction and increased slightly until the end of the reaction.

Similarly to [BMIM].[BF_4], [BMIM].[PF_6] has also been widely investigated due to its suitability for lipase-catalyzed reactions. Enantioselectivity of immobilized *Candida antarctica* lipase B for the transesterification reaction was higher in [BMIM].[PF_6] compared to [EMIM].[BF_4], due to the hydrophobic nature of the former IL (Kim *et al.*, 2001). Interestingly, [BMIM].[PF_6] is a polar, yet hydrophobic solvent, which was said to be the reason that free *Candida antarctica* lipase B had improved activity in [BMIM].[PF_6] compared to [BMIM].[BF_4], an IL with a lower polarity (Lozano *et al.*, 2001b). Synthetic activity of free *Candida antarctica* lipase B in several ILs was explained due to two properties, namely hydrophobicity of the cation and nucleophilicity of the anion. $[PF_6]^-$ anion has low nucleophilicity, which prevents the conformation of enzyme by interacting with the positively charged sites in the enzyme's structure (de los Ríos *et al.*, 2007). Kaar *et al.* (2003) reported that Novozym 435 was inactivated in [BMIM].[PF_6] medium, which should be a result of the impurities included in the IL, since it was synthesized in the laboratory scale instead of purchasing the pure form. Indeed, the purity of ILs with $[PF_6]^-$ anion were shown to have a great impact on enzyme activity due to their tendency to hydrolytic decomposition (Hernández-Fernández *et al.*, 2007). Even the commercial IL products with claimed purity of >99% maintained improved enzyme activity when they were purified further. Relatively high MG yield obtained in [BMIM].[PF_6] can also be attributed to the polar nature of the IL.

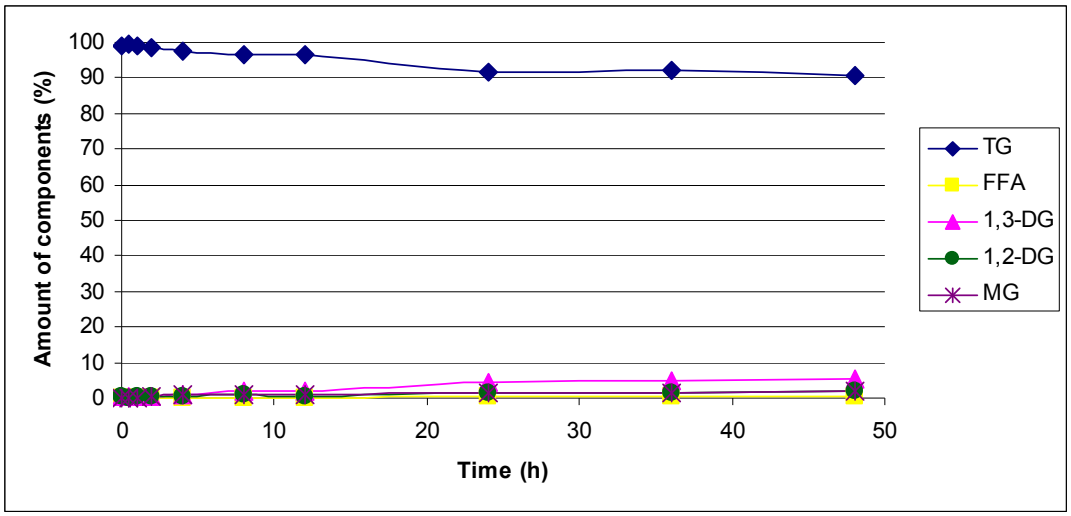

Figure 5.5: Time course of the glycerolysis reaction in [OMIM].[PF_6].

The last imidazolium type IL selected was 1-methyl-3-octylimidazolium hexafluorophosphate, i.e. [OMIM].[PF_6]. As can be seen in Figure 5.5, a very little reaction occurred. All the products reached their highest level at 48 h, with the 1,3-DG being the dominant one, but still the amount of them were very low. The reason for this IL being investigated further was that, according to the parallel studies performed in our working group, it was seen that improved results can be obtained by optimizing the reaction conditions for [OMIM].[PF_6]. Previous studies using [OMIM].[PF_6] as a medium had contrary results. Highly polar nature of [OMIM].[PF_6] as a result of long alkyl group on the cation and relatively lower nucleophilicity of the anion, both of which lead to hydrophobicity, was claimed to be the reason for this type of IL to stabilize free *Candida antarctica* lipase B and to increase the half-life of the enzyme up to 48 hours. Accordingly, synthetic activity was also found to be higher than that observed from ILs with same anion but shorter alkyl group on the cation (de los Ríos *et al.*, 2007). However, Chen *et al.* (2008) recently reported that solubility of oil in [OMIM].[PF_6] was very low, resulting in poor glycerolysis reaction. In fact, the solubility of oil in [OMIM].[PF_6] is higher than that in [BMIM].[PF_6]; however, as mentioned above, the application of higher glycerol dosage in the cited study could have resulted in poor solubility of oil in the IL. The contrary results can also be attributed to the purity issue of the ILs used. Hernández-Fernández *et al.* (2007) reported that the synthetic activity of free *Candida antarctica* lipase B in [OMIM].[PF_6] increased from 0 U/mg protein to 116.1 U/mg protein when the IL, which was claimed to have >99% purity, was further purified.

In a similar way, methyltrioctylammonium trifluoroacetate, i.e. [MTOA].[TFA], was also selected in spite of the poor results (Figure 5.6), based on the data which claims that glycerolysis reaction catalyzed by Novozym 435 in this IL resulted in good TG conversion (Chen *et al.*, 2008).

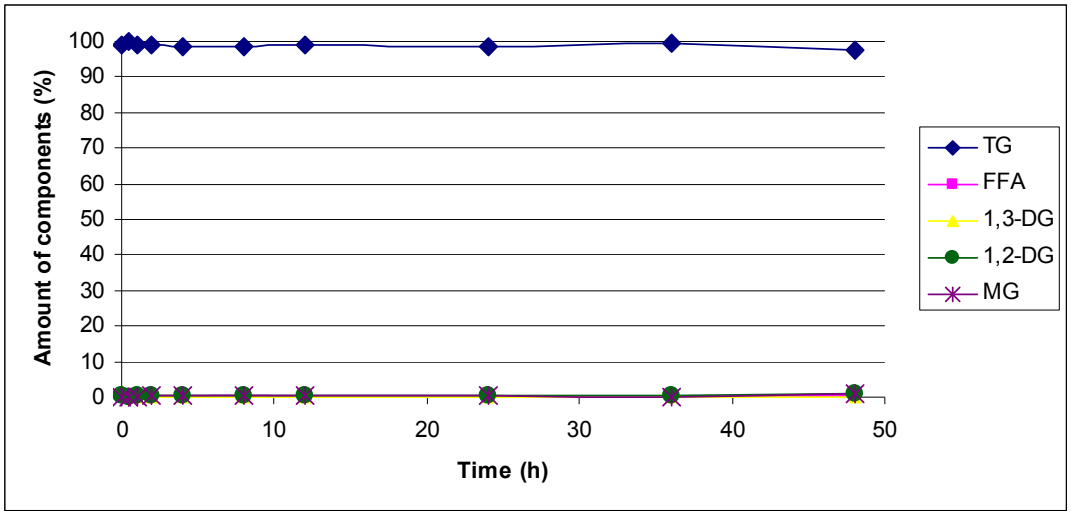

Figure 5.6: Time course of the glycerolysis reaction in [MTOA].[TFA].

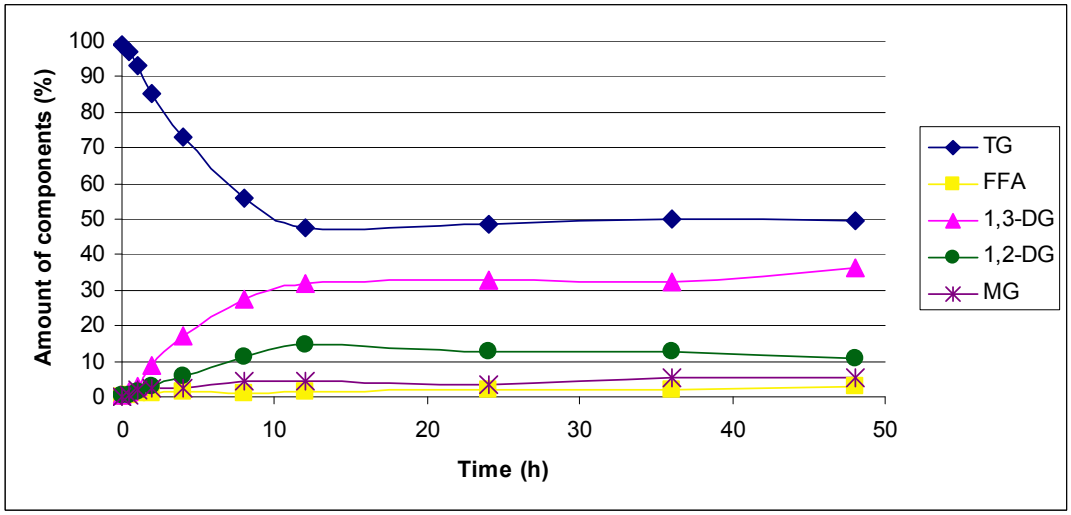

Figure 5.7: Time course of the glycerolysis reaction in [TOMA].[Tf_2N].

Another IL based on the same cation with [MTOA].[TFA], trioctylmethylammonium bis(trifluoromethylsulfonyl)imide, i.e. [TOMA].[Tf_2N] (Figure 5.7), gave interestingly different results from the previous one. Reaction started from the first half an hour and reached equilibrium around 12 h. TG conversion and DG yield were both high. At the end of the 48 h of reaction period, 50% of TG was converted into partial acylglycerols and FFAs. At the equilibrium point (12 h) DG yield was 46%, 32% of which was 1,3-DG. MG concentration was 4% at 12 h, which increased to 5% during the rest of the reaction period. The great difference between the yields obtained in [MTOA].[TFA] and in [TOMA].[Tf_2N] can be considered as a support to the assumption that enzyme activity in ILs is anion dependent (Kaar *et al.*, 2003). $[Tf_2N]^-$ anion is known to be hydrophobic, which makes the IL including this anion

water-immiscible and thus, suitable candidates for lipase-catalyzed reactions. de Diego *et al.* (2005) stated that water-immiscible ILs including $[Tf_2N]^-$ anion form a strong ionic matrix which retains enzyme molecules in an adequate microenvironment, resulting in a supramolecular net able to keep the protein conformation active. These ILs, according to the authors, should be regarded as being an immobilization support, rather than a reaction medium. In addition, the solubility of oil in IL was shown to increase with increasing hydrophobicity (Chen *et al.*, 2008), which can be considered as a reason for the high TG conversion in [TOMA].[Tf_2N].

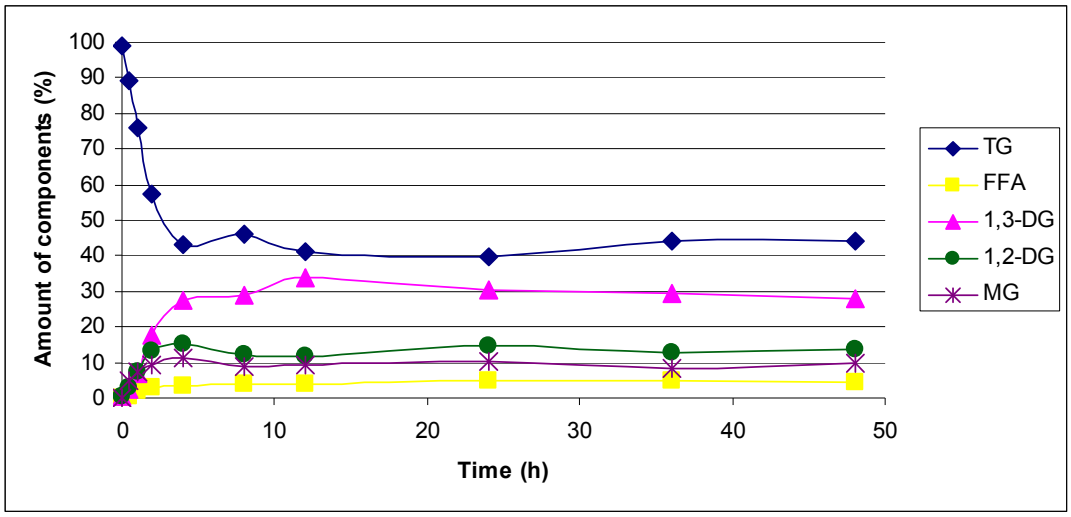

Figure 5.8: Time course of the glycerolysis reaction in AMMOENG 120.

The last IL used for the rest of the study, AMMOENG 120 (Figure 5.8), was the only selected one from the AMMOENG™ series. Glycerolysis reaction had a high rate; in the first half an hour, 11% of the TG was converted into partial acylglycerols and FFAs. The reaction equilibrium was reached around 12 h, at which point DG yield was 45% (34% 1,3-DG) and MG concentration was 9%. The results are parallel to the previous studies. Guo *et al.* (2006) reported that AMMOENG 120 yielded in high DG, even to such an extent that this IL could be considered as 'a DG-specific solvent' (Chen *et al.*, 2008). The oil solubility was high, resulting in good TG conversion (Chen *et al.*, 2008).

Although not used for the following experiments, AMMOENG 100 and AMMOENG 102 also gave interesting results, which can be seen in Figures 5.9 and 5.10, respectively, that needs to be discussed here.

The glycerolysis reaction in AMMOENG 100 reached the equilibrium around 8 h-12 h. Almost all of TG (97%) was converted into partial acylglycerols and FFAs; however, the dominant product was MG with the ratio of 57% at the end of 48 h period, which was the reason that the IL was not used further in this study. Similar results were reported previously, and explained by the ability of the moiety of ployethoxyl alcohol in the IL molecule to form hydrogen bonds with the hydroxyl group in the glycerol moiety of MG, which results in unreactivity of hydroxyl group of MG for reverse esterification (Guo and Xu, 2005). The authors concluded that the action of AMMOENG 100 in the glycerolysis reaction system was not only just as a solvent but also as a "trap" for MG molecules, which shifted the reaction towards the synthesis of MG (Guo and Xu, 2005; Chen *et al.*, 2008).

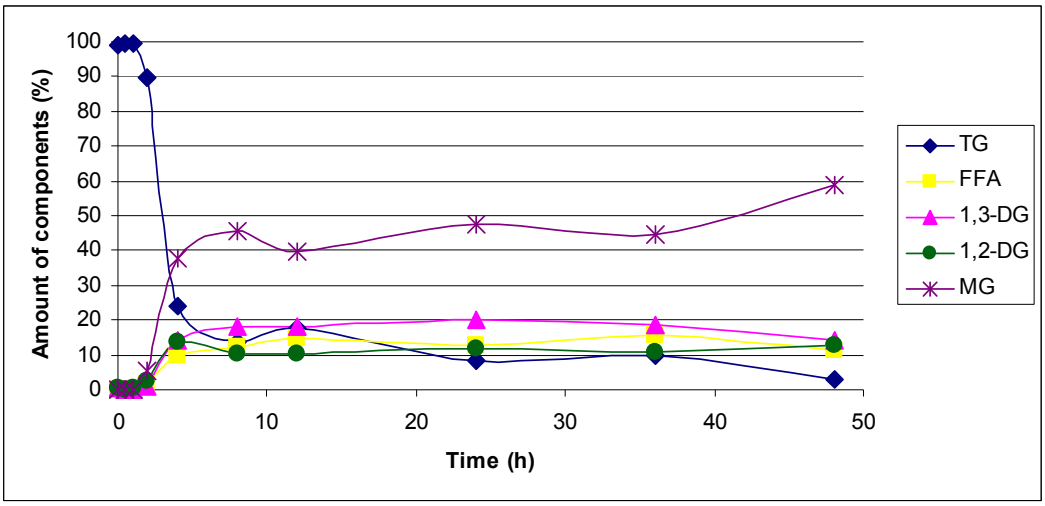

Figure 5.9: Time course of the glycerolysis reaction in AMMOENG 100.

Similarly, the TG conversion was promising in the glycerolysis reaction performed in AMMOENG 102 as well. The reaction rate was dramatically increased between 2 h and 4 h; conversion ratio of TG was 19% at 2 h, and it was increased to 80% at 4 h. The dominant product was MG but the difference between MG and DG concentrations was not very high, which were 39% and 34% at the end of the reaction, respectively. The molecular structures of AMMOENG 100 and AMMOENG 102 were reported to be similar, which leads to similar glycerolysis yields (Guo *et al.*, 2006). Even at the 1:1 ratio of glycerol to TG, at which the stoichiometric ratio should theoretically yield equimolar amounts of DG and MG, the

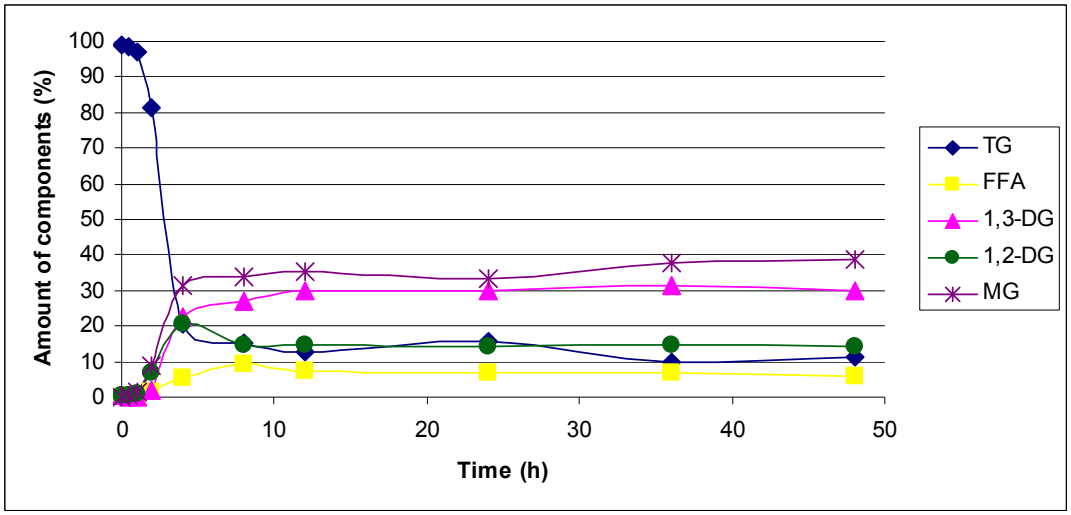

Figure 5.10: Time course of the glycerolysis reaction in AMMOENG 102.

glycerolysis reaction in AMMOENG 102 yielded 80% MG and MG/DG ratio was 9. The authors concluded that there was a strong urge in the AMMOENG 102 system to push the reaction towards the formation of MG. It was recently stated that the different induction periods of AMMOENG 100, AMMOENG 102 and AMMOENG 120 were results of different oil solubility, with AMMOENG 120 having the greatest solvation power, and thus, the shortest induction period (Chen *et al.*, 2008), which also was observed in the present study.

5.2 Effects of Different Reaction Parameters on the Yield in Selected ILs

According to the results of the previous step, 6 ILs out of 19 were selected for the rest of the project: [BMIM].[BF_4], [BMIM]. [PF_6], [OMIM].[PF_6], [MTOA].[TFA], [TOMA].[Tf_2N], AMMOENG 120. The parameters, the effects of which were investigated, are ratio between the substrates and IL (substrates/IL), temperature (T), and ratio between oil and glycerol (oil/glycerol), respectively. After evaluating the overall results, 3 ILs, i.e. [MTOA].[TFA], [TOMA].[Tf_2N], and AMMOENG 120, were further investigated at optimized conditions with different types of lipases.

5.2.1 Effect of substrates/IL ratio

The IL medium can help to improve the system homogeneity and stability as well as shifting the equilibrium towards the desired product. On the other hand, the medium will decrease the concentration of substrates, which might possibly lead to decreasing the reaction rates due to dilution effect. In addition, since the viscosity of

the ILs are relatively high (Chiappe and Pieraccini, 2005), the low ratio of the substrates to the IL would result in a limited access of the enzyme to the substrates.

The effect of the substrates/IL ratio was investigated at different oil and glycerol amounts with the constant ratio and a set amount of IL. Time courses of the glycerolysis reactions performed to investigate the effect of substrates/IL ratio, and the comparison of the three different levels used can be seen in Appendixes C1-3. The mentioned effect was pronounced the most in the glycerolysis reaction performed in [MTOA].[TFA] (Figure 5.11). The reaction yielded in almost no conversion of TG when the ratio used was 0.5:0.25, oil/glycerol, mmol/mmol, whereas this value increased to 18% when the ratio was 2:1, oil/glycerol, mmol/mmol. The other ILs also showed decreases or increases depending on the substrates/IL ratio, but none of the differences were significant enough. Since both DG yield and TG conversion generally tended to increase with the increase in substrates/IL ratio, this parameter was set at 2:1, oil/glycerol, mmol/mmol, for the following steps of the project.

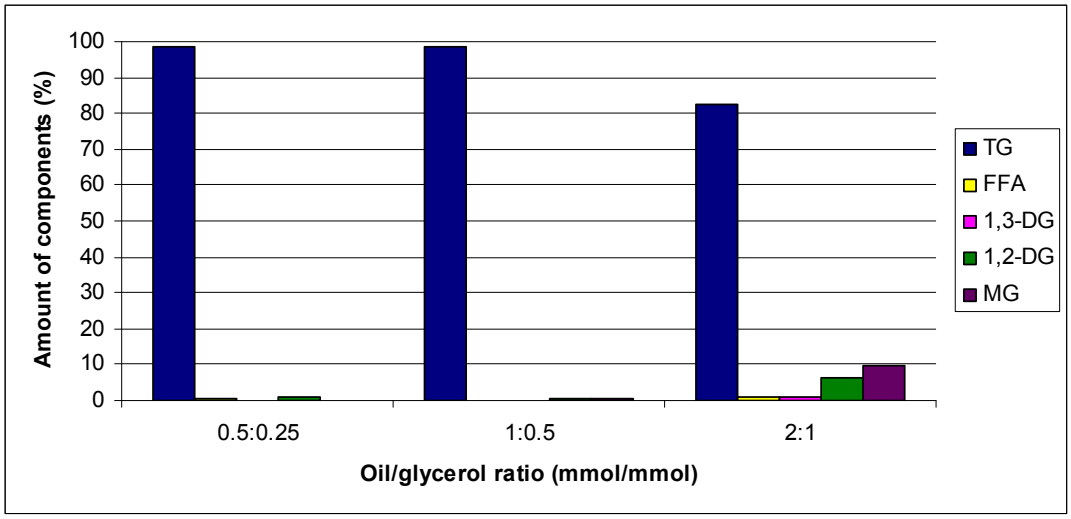

Figure 5.11: Comparison of the results at different substrates/IL ratios in [MTOA].[TFA].

Reaction rates were tended to be higher in the middle level used, which was 1:0.5, oil/glycerol, mmol/mmol. The low concentration of the substrates in the system resulted in lower reaction rate, as stated above. On the other hand, higher amount of substrates could have caused a bulk mass through which enzyme particles could not diffuse efficiently, resulting in a lower reaction rate. Katsoura *et al.* (2007) studied the effects of reaction parameters on the lipase-catalyzed selective acylation of

polyhydroxylated natural compounds in ILs. They reported that an increase of the concentration of substrates up to a specific value led to an increase in the conversion, while a further increase of the concentration had a negative effect on the conversion yield, which was similar to the results obtained in the present study. The authors concluded that the inhibitory effect of higher substrate concentration might be attributed to a possible increase of the viscosity and/or a change in the polarity of the reaction medium so as to influence the active conformation of the enzyme and lead to mass transfer limitations and substrate partition effects.

5.2.2 Effect of temperature

Enzymatic reactions are highly dependent on reaction temperature. An increase above the limits can cause denaturation of the enzyme and thus, result in no catalytic activity, whereas too low temperature is also not desirable since every enzyme needs a defined temperature to be activated. Kristensen *et al.* (2005a) reported that temperature was the most effective factor on DG yield in a lipase-catalyzed glycerolysis reaction. For biocatalytic reactions taking place in IL medium in particular, temperature has additional effects on reaction yields and rates. Increasing temperature was shown to decrease viscosity of ILs, and to increase the oil solubility in these media, simultaneously, both of which lead to improved enzymatic activity (Chen *et al.*, 2008).

The effect of temperature on the glycerolysis reactions in selected ILs were investigated at three different values, which are 50^{o}C, 60^{o}C, and 70^{o}C. Time courses of the glycerolysis reactions performed to investigate the effect of temperature, and the comparison of the three different levels used can be seen in Appendixes D1-3. All of the ILs tested showed improved DG yields and TG conversions when the temperature was increased, except [TOMA].[Tf_2N] which had the highest TG conversion value at 60^{o}C. On the other hand, the increase in the temperature caused DG yield to decrease while MG yield increased; hence, 70^{o}C should be considered to give the best results for this IL as well. In the cases of [MTOA].[TFA] (Figure 5.12) and [OMIM].[PF_6] (Figure 5.13), both the reaction yields and rates showed significant improvements with the increasing temperature, which suggests that there could be an induction period for the glycerolysis reaction in these ILs. The increase in both yield and rate of lipase-catalyzed reactions with increased temperature has already been reported by several authors in solvent-free medium (Rosu *et al.*, 1999;

Watanabe *et al.*, 2003; Kristensen *et al.*, 2005a; Watanabe *et al.*, 2005), in organic solvents (Martinez *et al.*, 2005) and in ILs (Gubicza *et al.*, 2003; Guo and Xu, 2006; Katsoura *et al.*, 2007; Chen *et al.*, 2008; Fehér *et al.*, 2008). It was stated the temperature must be kept in a narrow range, since further increase can result in deactivation of the enzyme (Noureddini and Harmeier, 1998).

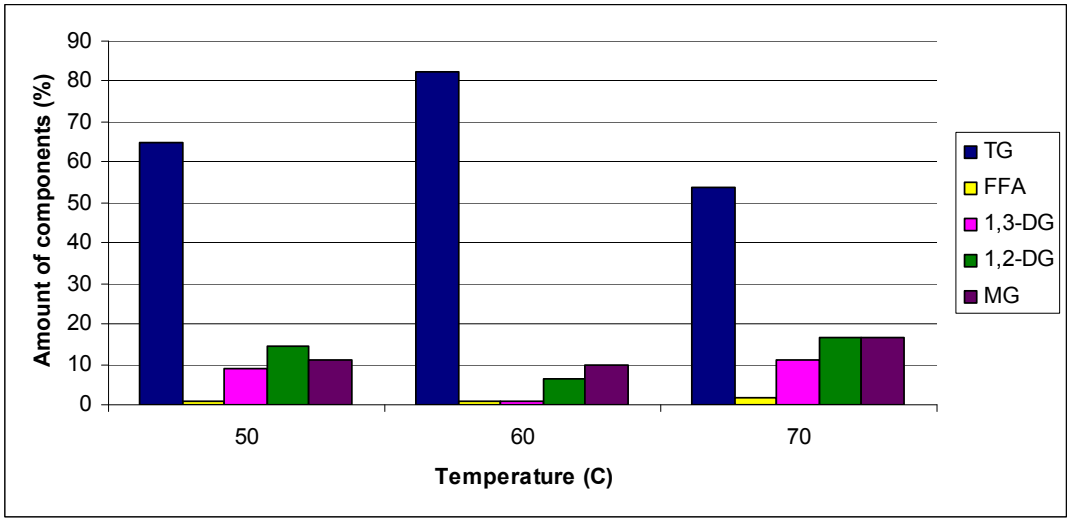

Figure 5.12: Comparison of the results at different temperatures in [MTOA].[TFA].

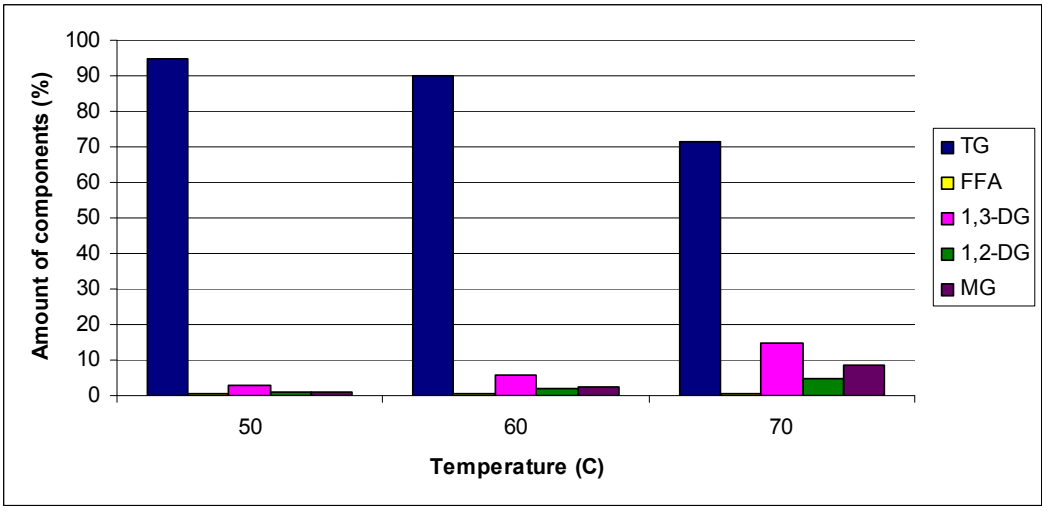

Figure 5.13: Comparison of the results at different temperatures in [OMIM].[PF_6].

5.2.3 Effect of oil/glycerol ratio

The molar stoichiometry of the glycerolysis reaction to obtain DG is as follows:

$$2\ [TG] + [glycerol] \rightarrow 3\ [DG]$$

Low glycerol content is preferable, since glycerol increases the viscosity and the polarity of the medium, both of which would not be favorable for enzymatic reaction (Damstrup *et al.*, 2006b). An excess amount of glycerol was shown to have no improving effect on DG yield based on the equation above (Kristensen *et al.*, 2005a). The effect of the oil/glycerol ratio on the glycerolysis reaction was investigated at three different values. Time courses of the glycerolysis reactions performed to investigate the effect of oil/glycerol ratio, and the comparison of the three different levels used can be seen in Appendixes E1-3. All of the ILs tested showed improved DG yields and TG conversions when the oil/glycerol ratio was decreased, except [BMIM].[PF_6] in which both DG yield and TG conversion values were slightly higher in the oil/glycerol molar ratio of 2:1.25 compared to that in 2:1.5. The most dramatic effect was observed in [MTOA].[TFA] (Figure 5.14). DG yield value was increased from 7% to 43% while TG conversion value was also increased from 18% to 74% when the oil/glycerol ratio was increased from 2:1, mmol/mmol to 2:1.5, mmol/mmol. The reason for the yield increasing in the IL medium with decreasing oil/glycerol ratio can be the improved availability of glycerol, since in viscous and hydrophilic IL medium, it could be harder for the enzymes to interact with hydrophilic glycerol. Ferreira-Dias *et al.* (2001) also stated that decreasing oil/glycerol ratio had a little effect on DG yield in a Novozyme 435-catalyzed glycerolysis reaction.

In IL medium, which generally includes 1-2% water, hydrolysis becomes more remarkable as a competitive reaction to glycerolysis when glycerol is reduced (Chen *et al.*, 2008). As a result, FFA concentration increases, which also was observed in the present study.

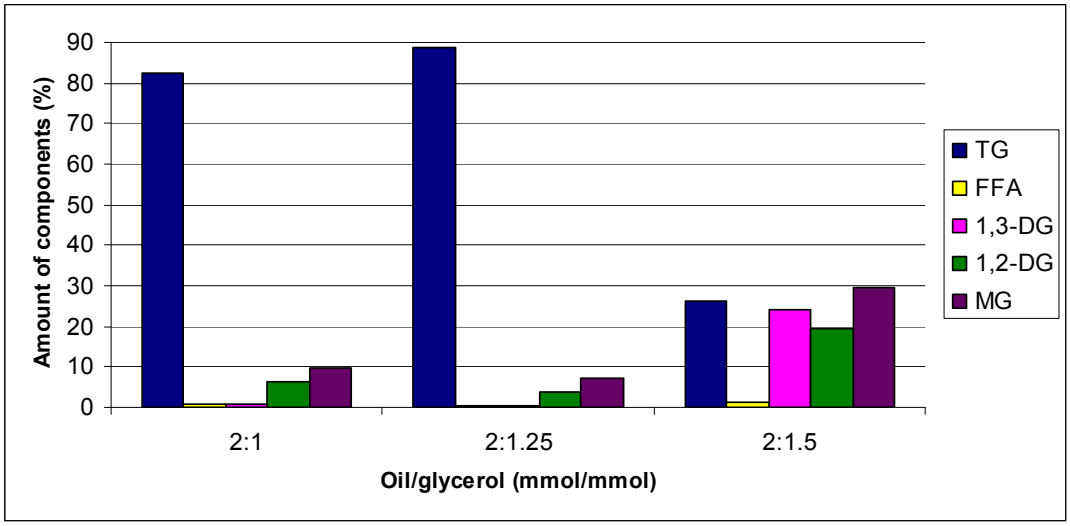

Figure 5.14: Comparison of the results at different oil/glycerol ratios in [MTOA].[TFA].

5.2.4 Screening of different lipases at optimized conditions

Commercially available lipases were screened previously to test their their abilities for DG production (Kristensen *et al.*, 2005b) and it was shown that Novozym 435 was the most effective lipase for this purpose. On the other hand, since ILs have many unpredictable properties due to their varied molecular structures, the behavior of the lipases could also be different in these media.

According to the evaluation of the optimization studies, three different reaction systems were selected to screen different types of lipases. Time courses of the glycerolysis reactions performed to screen different lipases, and the comparison of the three different lipase types used can be seen in Appendixes F1-3. Lipozyme RM IM gave the highest DG yields for [TOMA].[Tf_2N] and AMMOENG 120; however, FFA and MG concentrations were also high, both of which are undesired by-products of DG production process. Novozym 435, on the other hand, resulted in a significantly lower MG and FFA concentrations in the price of a slightly lower DG yield, especially for [TOMA].[Tf_2N]. The highest DG yield was observed by the Lipozyme TL IM-catalyzed reaction in [MTOA].[TFA], but all three lipases gave unacceptably high MG and FFA concentrations in this IL.

Similar conversion performances of Novozym 435 and Lipozyme RM IM were previously reported in a tetraammonnium-based IL (Guo *et al.*, 2006).

Kristensen *et al.* (2005b) discussed the DG production potential of different lipase types due to their carrier material. They concluded that the hyrophobicity of the carrier had a great effect on the enzyme activity. Both Lipozyme RM IM and Lipozyme TL IM have hydrophilic carriers, namely macroporous anion-exchange resin (i.e. Duolite) and granulated silica, respectively. When they come to contact with a hydrophilic substrate, glycerol in this case, the substrate forms a layer on the immobilized lipase particles and thus, prevents the contact of them with the hydrophobic substrate, which is oil in this reaction. This hypothesis was further proved when the reaction was performed with glycerol absorbed on silica gel, resulting in high TG conversions and DG yields for both lipases. Novozym 435, on the other hand, has a hydrophobic carrier, macroporous acrylic resin, which enables is to disperse in the reaction medium and to contact with both of the substrates. A similar study was conducted in IL medium recently (Lozano *et al.*, 2007). It was found that both free silica and modified silica with ionizable (or polar) groups provided the worst catalytic parameters for immobilized *Candida* antarctica lipase B derivatives, while the use of silica modified with hydrophobic alkyl chains resulted in good catalytic parameters for immobilized derivatives, dependent on the length of side chain.

Despite the similar carriers, Lipozyme TL IM had somewhat worse activity than Lipozyme RM IM in the present study, as reported previously (Yang *et al.*, 2005b).

5.3 Use of *n*-hexane/IL Systems

The relatively high viscosity of ILs is considered as a limited factor for various applications. It was reported that, the addition of cosolvents to ILs can be an alternative which results in dramatic reductions in the viscosity (Mantz and Trulove, 2008). Therefore, glycerolysis reactions were performed in *n*-hexane/IL system with different mixing ratio at varied temperatures. Time courses of the glycerolysis reactions performed in *n*-hexane/IL system, and the comparison of the system with pure ILs can be seen in Appendixes G1 and G2, respectively.

Time courses of the glycerolysis reactions performed to investigate the effect of temperature on the *n*-hexane/IL system, and the comparison of the three different levels used can be seen in Appendixes H1-3. DG yields and TG conversions were not effected by temperature in [TOMA].[Tf_2N] and AMMOENG 120, whereas almost no

reaction occurred in the other ILs when the temperature was 40^{o}C, probably due to the low viscosity of the substrates and IL both and poor mixing of the *n*-hexane and IL phases.

Time courses of the glycerolysis reactions performed to investigate the effect of *n*-hexane/IL ratio on the *n*-hexane/IL system, and the comparison of the three different levels used can be seen in Appendixes I1-3. The trend obtained when the effect of *n*-hexane/IL ratio on the glycerolysis reaction can be attributed to the change in medium polarity. For instance, DG yield and TG conversion both increased with increasing *n*-hexane/IL ratio in [OMIM].[PF_6], due to the low polarity introduced to the system. This effect could have improved the solvation of oil more efficiently. Similarly, DG yield and TG conversion values 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]. Biphasic media including an organic solvent and an IL is not intensively investigated. It was recently reported (Lozano *et al.*, 2007) that a loss in synthetic activity was observed in *n*-hexane/[TOMA].[Tf_2N] media, as compared to that obtained in *n*-hexane. This type of IL is insouble in *n*-hexane. Thus, the results were explained by the biotransformation occuring within a liquid/liquid biphasic system, which involves an increase in mass-transfer limitations in the reaction media and results in decreased enzymatic activity. Same observations were made in the present study as well.

5.4 Use of Binary IL Systems

The first step of the study, which was the screening of the IL types, showed that none of the ILs resulted in desired way for all the parameters 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 use of binary IL systems was tested. AMMOENG 100 and AMMOENG 102 were promising from the point of high TG conversion, and in addition, they resulted in relatively high DG yields, but MG concentration was too high as well. These two ILs were coupled with [TOMA].[Tf_2N] and AMMOENG 120, the most effective ILs with the drawback of relatively low TG conversion. Time courses of the glycerolysis reactions performed in binary IL systems, and the comparison of the yields can be seen in Appendixes J1-3. [TOMA].[Tf_2N]/AMMOENG 100 system resulted in high

DG yield and TG conversion, and MG concentration was lowered compared to pure AMMOENG 100 system, but still undesirably high. 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 system compared to pure AMMOENG 120. Although TG conversion was not as high as expected, consideration of DG yield and MG concentration leaded to the choice of [TOMA].[Tf_2N]/AMMOENG 102 system for further optimization.

5.5 Optimization of the Reaction Conditions for [TOMA].[Tf_2N]/AMMOENG 102 System with Response Surface Methodology

Response surface methodology (RSM) is a design of experiment which collects statistical and mathematical techniques useful for developing, improving, and optimizing processes. The main reason for RSM to be preferred recently in many studies is that, in addition to defining the effects of independent variables on the response of interest, it also enables to see the effects of the interactions of the variables (Baş and Boyacı, 2007). This property is of importance especially for biocatalytic reactions, in which the single and combined effects of variables can be differing in a wide range.

5.5.1 Model generated for DG yield

Responses obtained from the experimental design are given in Table 5.1. R^2 and AAD values are 0.7 and 5.42% for DG yield, respectively. ANOVA (Appendix K) showed that none of the factors were significant for DG yield, and the model had no significant lack of fit at a significance level of $P<0.05$. The regression coefficients and *P* values for DG yield are given in Appendix K. The quadratic effect of glycerol mole was found to be the greatest effect. As can be seen in the main effects plot (Figure 5.15), DG yield increases with increasing glycerol mole. The reason for the yield increasing in the IL medium with decreasing oil/glycerol ratio can be, as previously discussed in Section 2.3., the improved availability of glycerol, since in viscous and hydrophobic IL medium, it could be harder for the enzymes to interact with hydrophilic glycerol. Temperature increase also had a positive effect on DG yield, especially when the temperature increased from 34.7^{o}C to 45^{o}C. In the middle of the range, i.e. between 45^{o}C and 75^{o}C, the increase in the temperature did not

influence the yield significantly. As [TOMA].[Tf_2N] percent in the medium decreases, in other words, AMMOENG 102 percent increases, DG yield increases as well. This effect was expected, since AMMOENG 102 has high selectivity towards DG production, even at conditions favoring MG production (Guo *et al.*, 2006). The observed values vs. predicted values plot, residual plots, contour plots, and surface plots for DG yield are given in Appendix L.

Table 5.1: Responses for DG yield.

Exp. no	Temperature	Glycerol mole	TT percent	DG yield, %
1	45	1.15	25	58.2
2	75	1.15	25	60.7
3	45	1.75	25	**73.9**
4	75	1.75	25	60.6
5	45	1.15	75	59.8
6	75	1.15	75	56.4
7	45	1.75	75	59.5
8	75	1.75	75	61.2
9	34.77	1.45	50	47.9
10	85.22	1.45	50	66.3
11	60	0.94	50	59
12	60	1.95	50	**70.8**
13	60	1.45	7.95	**71.9**
14	60	1.45	92.05	51.8
15	60	1.45	50	66.5
16	60	1.45	50	54.9
17	60	1.45	50	67.7

TT percent: [TOMA].[Tf_2N] percent.

The interaction between glycerol mole and [TOMA].[Tf_2N] percent was found to have the greatest effect on DG yield. The contour plot generated for these two factors at the highest level of the third factor, temperature, can be seen in Figure 5.16. DG yield is above 70% with glycerol mole higher than 1.5 mmol and [TOMA].[Tf_2N] percent lower than 50 when temperature was set at 75^{o}C. Response tends to get higher as glycerol mole increases and [TOMA].[Tf_2N] percent 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 yield, 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% of MG, which can be neglected (Appendix J3).

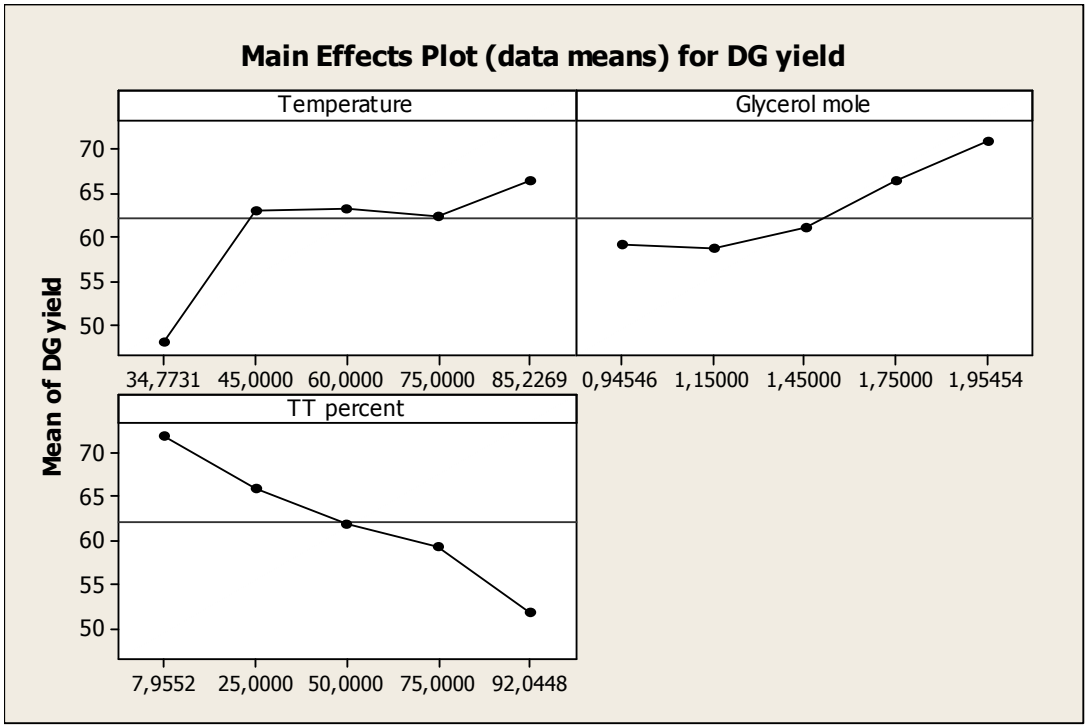

Figure 5.15: Main effects plot for DG yield.

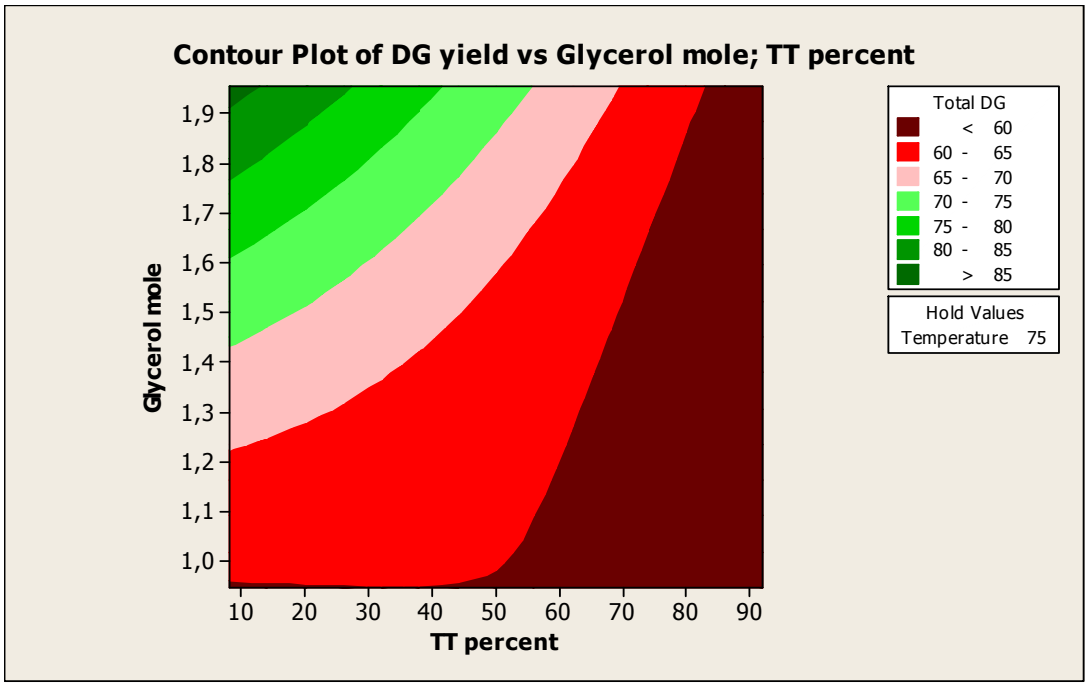

Figure 5.16: Contour plot of interaction between glycerol mole and [TOMA].[Tf_2N] percent.

A chi-square test using four additional experimental sets chosen from the given ranges of reaction parameters was performed to examine the adequacy of the model established. The chi-square test for DG yield indicated that there were no significant (P<0.05) differences between the observed and predicted values since the chi-square value (7.62) was smaller than the cutoff point (7.82) at $\alpha = 0.05$ and DF = 3. The detailed calculations of the AAD value and the chi-square test of the model generated for DG yield are given in Appendix M.

Using the response optimizer function of the software, following conditions were found to be the optimum ones: 69°C temperature, 1.95 mole glycerol, and 7.95% [TOMA].[Tf_2N]. Predicted response at these conditions was 87% DG yield; however, the reaction resulted in 72% DG yield.

5.5.2 Model generated for TG conversion

Responses obtained from the experimental design are given in Table 5.2. Regression analysis revealed that the experiment no. 9 was an outlier, thus, was not included in the statistical analyses. R^2 and AAD values are 0.94 and 3.3% for TG conversion, respectively. ANOVA (Appendix N) showed that the regression of the factors was significant for TG yield, and the model had no significant lack of fit at a significance

level of $P<0.05$. The regression coefficients and P values for TG conversion are given in Appendix N. None of the factors had a significant effect on TG conversion at a significance level of $P<0.05$. The main effects plot for TG conversion is given in Figure 5.17. In contrary to the model generated for DG yield, temperature had a slight effect on TG conversion. Considering the cost issues and enzyme stability, a

Table 5.2: Responses for TG conversion.

Exp. no	Temperature	Glycerol mole	TT percent	TG conversion, %
1	45	1.15	25	82.6
2	75	1.15	25	69.7
3	45	1.75	25	83.7
4	75	1.75	25	80.1
5	45	1.15	75	64.9
6	75	1.15	75	60.3
7	45	1.75	75	65.3
8	75	1.75	75	66.3
9*	34.77	1.45	50	57.9
10	85.22	1.45	50	69.0
11	60	0.94	50	64.7
12	60	1.95	50	80.1
13	60	1.45	7.95	85.9
14	60	1.45	92.05	65.1
15	60	1.45	50	76.4
16	60	1.45	50	70.3
17	60	1.45	50	76.2

*: The result was not included in the statistical analyses.
TT percent: [TOMA].[Tf_2N] percent.

temperature in the range of 45-60°C can be chosen for the reaction. The effects of glycerol mole and [TOMA].[Tf_2N] percent were similar to the previous model; TG conversion increased with increasing glycerol and decreasing [TOMA].[Tf_2N]. The observed values vs. predicted values plot, residual plots, contour plots, and response surface plots for DG yield are given in Appendix O.

The interaction between glycerol mole 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] percent, can be seen in Figure 5.18. When [TOMA].[Tf_2N] was set at the lowest level, which is 7.95%, TG conversion was above 80% for the whole range of glycerol mole at 45-50°C. Response tends to get higher as glycerol mole increases.

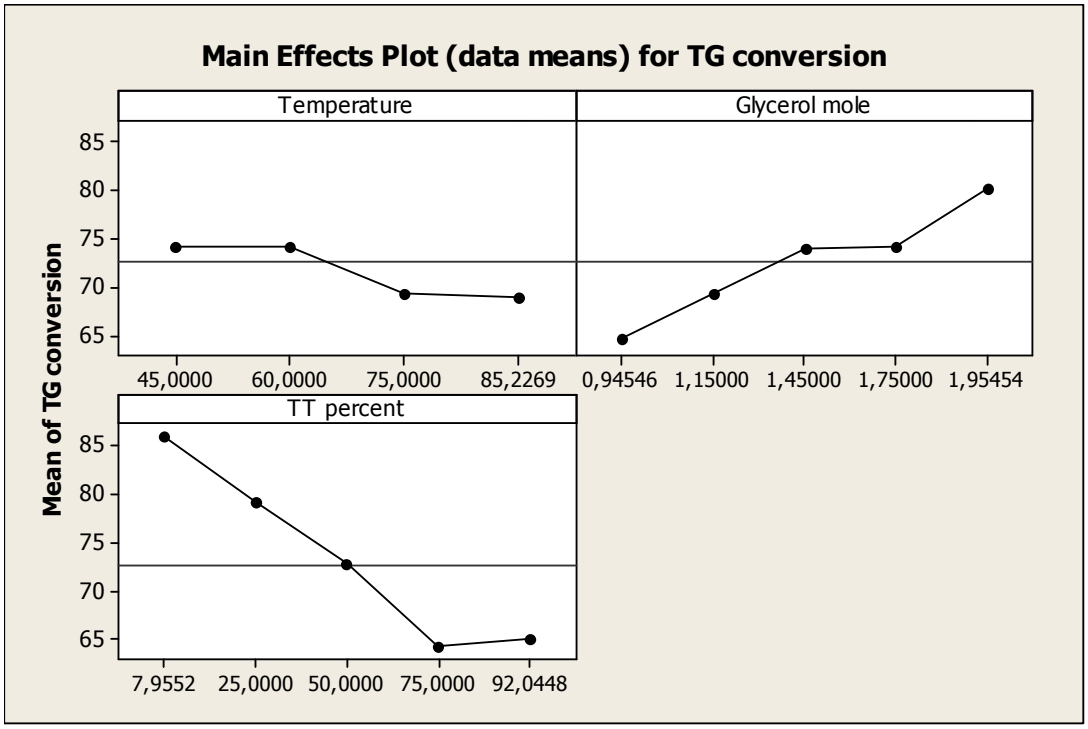

Figure 5.17: Main effects plot for TG conversion.

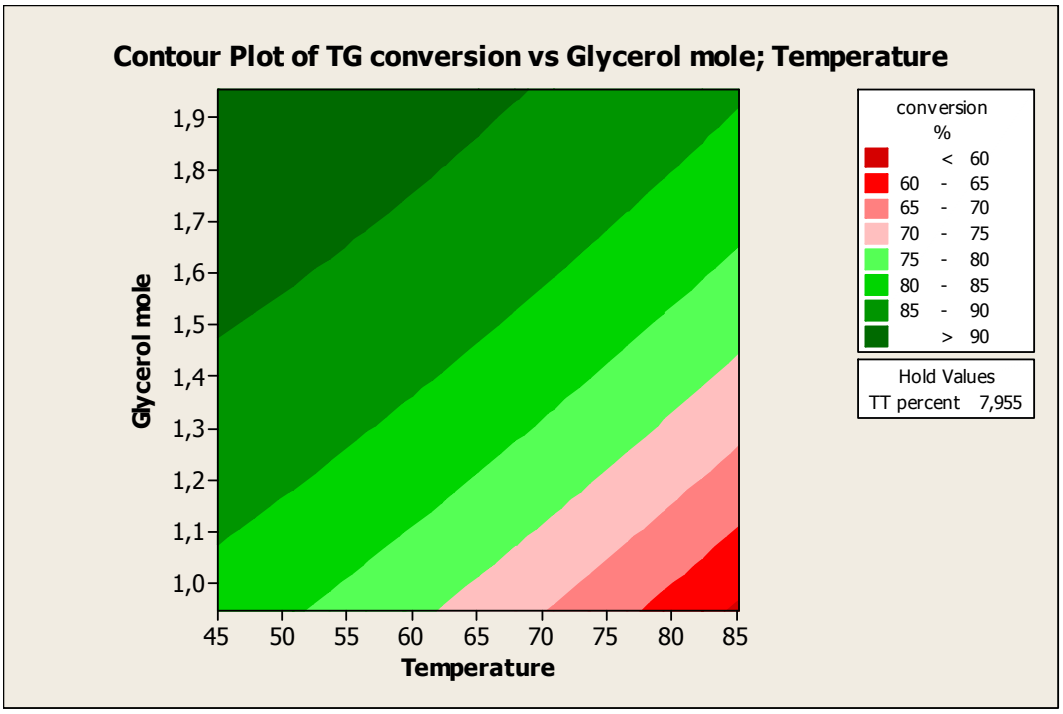

Figure 5.18: Contour plot of interaction between glycerol mole and temperature.

A chi-square test using four additional experimental sets chosen from the given ranges of reaction parameters was performed to examine the adequacy of the model established. The chi-square test for TG conversion indicated that there were no significant (P<0.05) differences between the observed and predicted values since the chi-square value (4.67) was smaller than the cutoff point (7.82) at $\alpha = 0.05$ and DF = 3. The detailed calculations of the AAD value and the chi-square test of the model generated for TG conversion are given in Appendix P.

Using the response optimizer function of the software, following conditions were found to be the optimum ones: 52°C temperature, 1.95 mole glycerol, and 7.95% [TOMA].[Tf_2N]. Predicted response at these conditions was 91.5% TG conversion; however, the reaction resulted in 78.6% TG conversion.

5.5.3 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 summarized 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 those obtained with the other ILs used for optimization in the previous steps. Pure AMMOENG 102 had resulted in 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.

RSM was used to generate models for both DG yield and TG conversion responses. 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 55-60^{o}C, glycerol mole between 1.8-2 mmol, and [TOMA].[Tf_2N] concentration less than 10% can be recommended for high DG yield (~70%) and TG conversion (~90%).

6. CONCLUSION

The aim of the present project was to develop an efficient glycerolysis system to produce DG, which has not been reported so far. Screening of 19 different ILs, with comparison to conventional organic solvents, leaded to the choice of six of them for further investigation. One-variable-at-a-time approach was used to see the effects of ratio between the substrates and IL (substrates/IL), temperature (T), ratio between oil and glycerol (oil/glycerol), and the type of lipase. It was seen that the effects of the factors varied in a wide range. To generalize the results are quite difficult, since each IL has its own properties due to greatly differring structures. One should consider the needs, such as desired product, cost issues depending on the reaction time, temperature, and IL amount to be used, to choose the most suitable IL. Generally speaking, it can be said that the effect of temperature was positive for all ILs tested; DG yield and TG conversion increased with increasing temperature.

To improve the results further, two different approaches were tested, namely *n*-hexane/IL system and binary IL system. Use of *n*-hexane/IL system in different ratios generally improved DG yield and TG conversion, again the effect was dependent on the IL type. Temperature effect on this system was again found to be well correlated with the results.

The ILs to be included in binary IL systems were chosen with the aim of increasing DG yield and TG conversion while decreasing MG concentration, the latter of which was a drawback for a few promising ILs. Indeed, this approach was found to be effective. A binary IL system consisting of [TOMA].[Tf_2N]/AMMOENG 102 was chosen for optimization by response surface methodology. Considering the optimum levels for both parameters, it can be concluded that a temperature between 55-60^oC, glycerol mole between 1.8-2 mmol, and [TOMA].[Tf_2N] concentration less than 10%, 24 h reaction time with 2 mmol oil and lipase in the amount of 15 wt % based on oil mass can be recommended for the glycerolysis reaction with expected DG yield at ~70% and TG conversion ~90%.

The present study showed that ILs can be used for selective biocatalytic reactions. The important point is that the reaction conditions should be carefully chosen. As observation for [MTOA].[TFA], for example, greatly varied results may be obtained when the reaction conditions are slightly changed.

The use of ILs for enzyme-catalyzed synthetic reactions represents a field of interest which is in a primary stage. For future research, the recyclability and reusability of ILs and enzyme can be investigated. There exist two approaches for this purpose: recycling the IL itself, or recycling the IL together with the enzyme, since immobilized enzyme particles are suspended in the IL phase. Both procedures were shown to be very effective (Fehér *et al.*, 2008). In addition, kinetics of the enzymatic reaction in IL medium can be investigated.

In conclusion, this study described a first attempt to produce DGs by enzymatic glycerolysis of oils employing neoteric green solvents (ILs) as reaction media. The results indicated that better product selectivity and higher conversion using ILs, over the reactions in most efficient conventional solvents, could be obtained through judicious selection of proper ILs or/and tuning property by mixing binary IL system. The results of this work will doubtless provide the practical experiences for molecular design of ILs with desired properties, specifically for enzymatic production of DGs.

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Appendix A. List of ILs used in glycerolysis reactions.

Table A.1: List of ILs used in glycerolysis reactions.

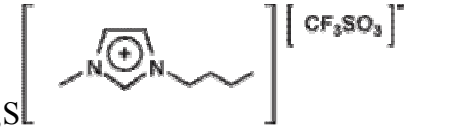
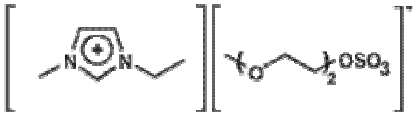
Name	Abbreviation	Formula	Structure
1-Butyl-3-methylimidazolium tetrafluoroborate	[BMIM].[BF_4]	$C_8H_{15}F_4N_2B$	
1-Butyl-3-methylimidazolium hexafluorophosphate	[BMIM].[PF_6]	$C_8H_{15}F_6N_2P$	
1-Butyl-3-methylimidazolium trifluoromethanesulfonate	[BMIM].[CF_3SO_3]	$C_9H_{15}F_3N_2O_3S$	
1,3-Dimethylimidazolium dimethylphosphate	[DMIM].[DMP]	$C_7H_{15}N_2O_4P$	
1-Ethyl-3-methylimidazolium 2(2-methoxyethoxy)ethylsulfate	[EMIM].[MDEG.SO_4]	$C_{11}H_{22}N_2O_6S$	

Table A.1: List of ILs used in glycerolysis reactions (continued).

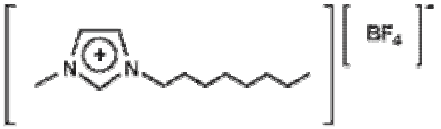
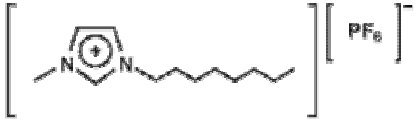
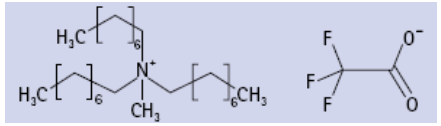
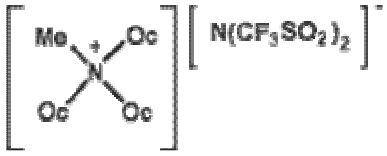
Name	Abbreviation	Formula	Structure
1-Ethyl-3-methylimidazolium n-octylsulfate	[EMIM].[OctSO$_4$]	$C_{14}H_{28}N_2O_4S$	
1-Methyl-3-octylimidazolium tetrafluoroborate	[OMIM].[BF$_4$]	$C_{12}H_{23}F_4N_2B$	
1-Methyl-3-octylimidazolium hexafluorophosphate	[OMIM].[PF$_6$]	$C_{12}H_{23}F_6N_2P$	
Methyltrioctylammonium trifluoroacetate	[MTOA].[TFA]	$C_{27}H_{54}F_3NO_2$	
Trioctylmethylammonium bis(trifluoromethylsulfonyl)imide	[TOMA].[Tf$_2$N]	$C_{11}H_{20}F_6N_2O_4S_2$	

Table A.1: List of ILs used in glycerolysis reactions (continued).

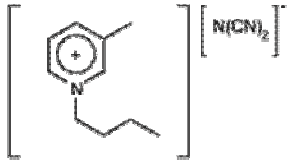
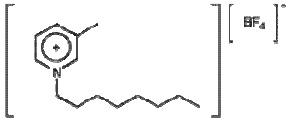
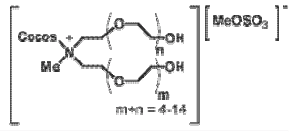
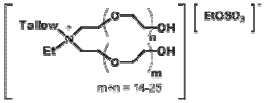
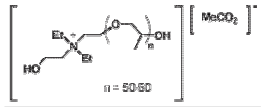
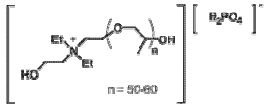
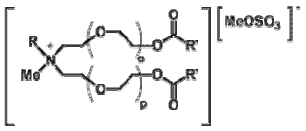
Name	Abbreviation	Formula	Structure
1-Butyl-3-methylpyridinium dicyanamide	[3-MBP][$N(CN)_2$]	$C_{10}H_{16}NBr$	
1-Butyl-3-methylpyrrolidinium dicyanamide	[MeBuPyO]. [$N(CN)_2$]	$C_{12}H_{16}N_4$	
1-Ethyl-3-methylpyridinium perfluorobutanesulfonate	[MeEtPy].[$C_4F_9SO_3$]	$C_{12}H_{12}F_9NO_3S$	
3-Methyl-1-octylpyridinium tetrafluoroborate	[MeOcPy]. [BF_4]	$C_{14}H_{24}BF_4N$	
AMMOENG^{TM} 100 Cocosaklyl pentaethoxi methyl ammonium methylsufate	-	not available	
AMMOENG^{TM} 102 Tetraalkyl ammonium sulfate	-	notavailable	

Table A.1: List of ILs used in glycerolysis reactions (continued).

Name	Abbreviation	Formula	Structure
AMMOENG^{TM} 111 Poly[oxy(methyl-1,2-ethanediyl)]-alpha-[2-(diethylhydroxyethylammonio)ethyl]-acetate	-	notavailable	
AMMOENG^{TM} 112 Poly[oxy(methyl-1,2-ethanediyl)]-alpha-[2-(diethylhydroxyethylammonio)ethyl]-dihydrogenphosphate	-	notavailable	
AMMOENG^{TM} 120 Quaternary ammonium sulfate	-	not available	

Appendix B. Time courses of the glycerolysis reactions performed for screening of ILs.

Reaction conditions: 1 mmol of oil, 0.5 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 48 h, 700 rpm

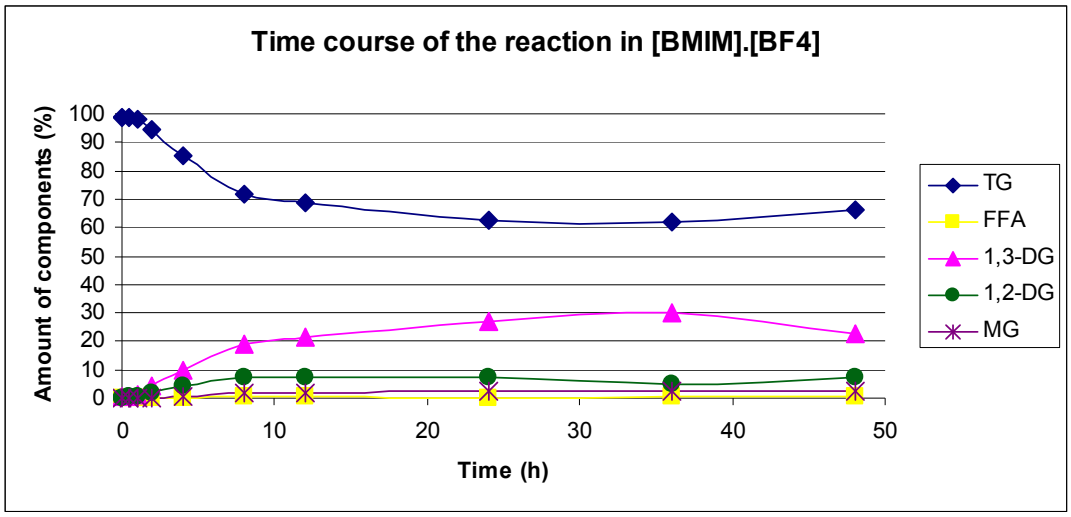


Figure B.1: Time course of the reaction in [BMIM].[BF_4].

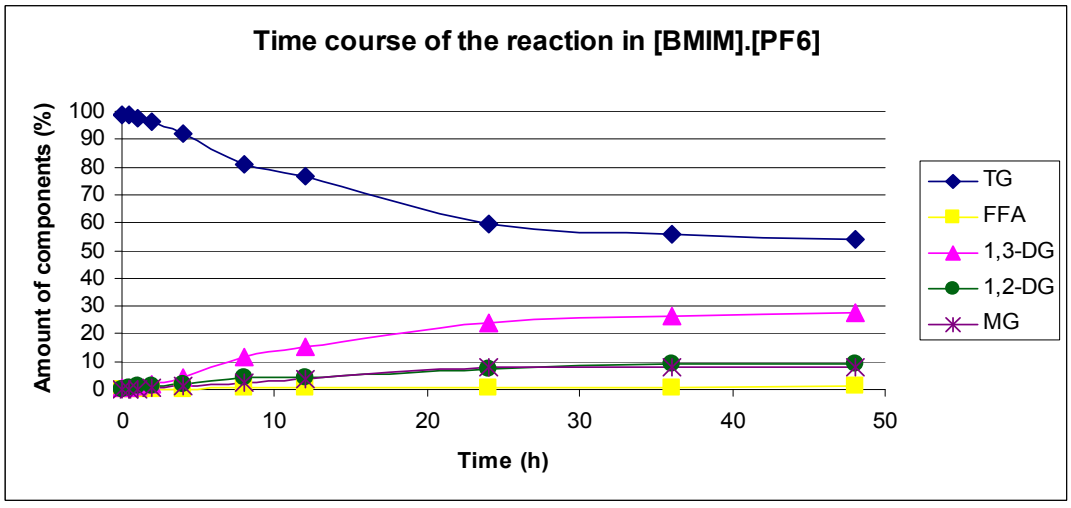


Figure B.2: Time course of the reaction in [BMIM].[PF_6].

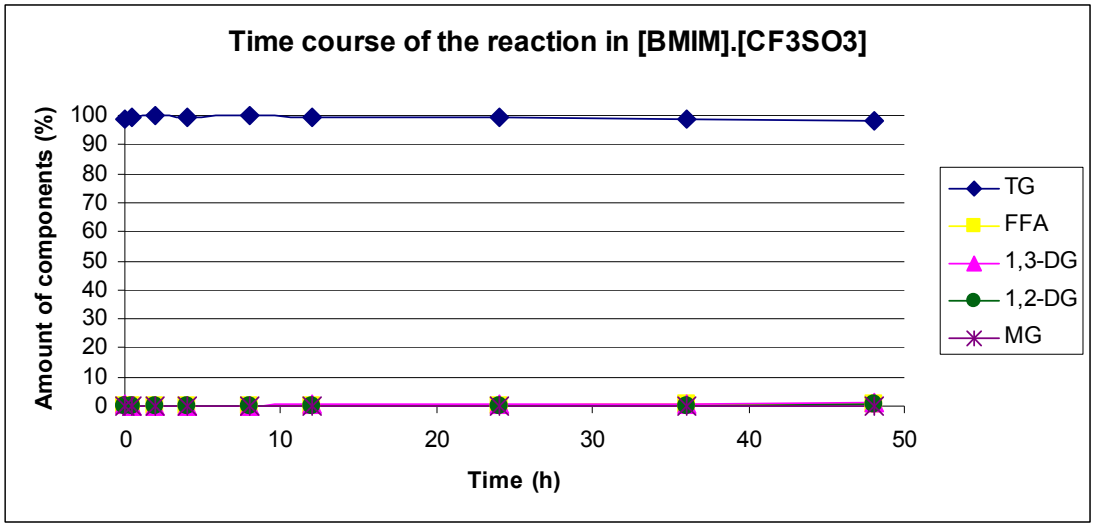


Figure B.3: Time course of the reaction in [BMIM].[CF_3SO_3].

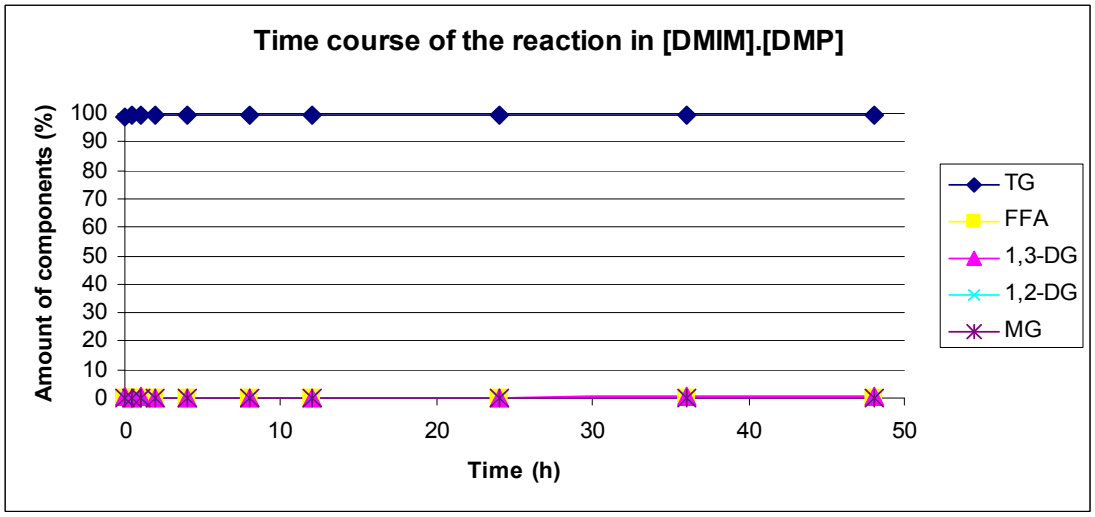


Figure B.4: Time course of the reaction in [DMIM].[DMP].

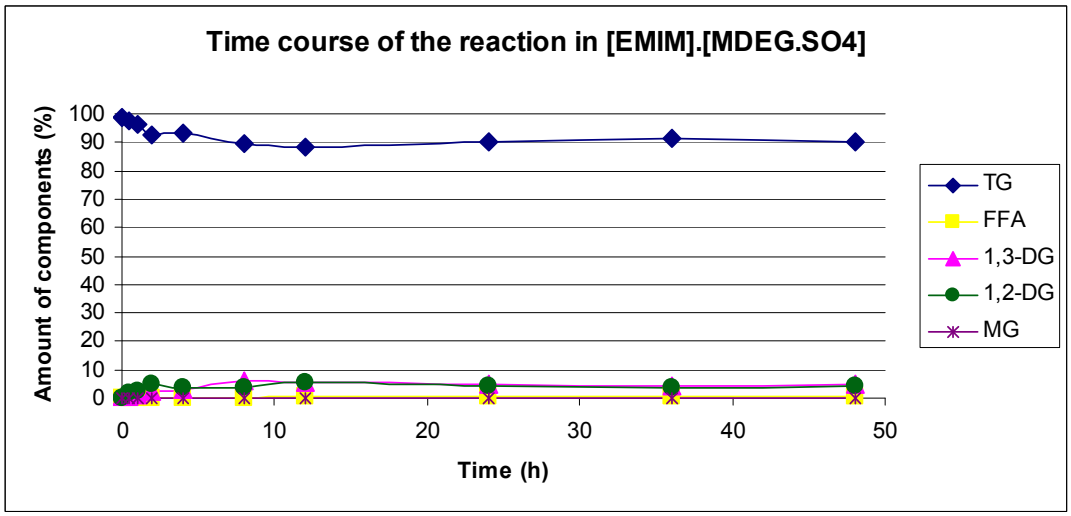


Figure B.5: Time course of the reaction in [EMIM].[$MDEG.SO_4$].

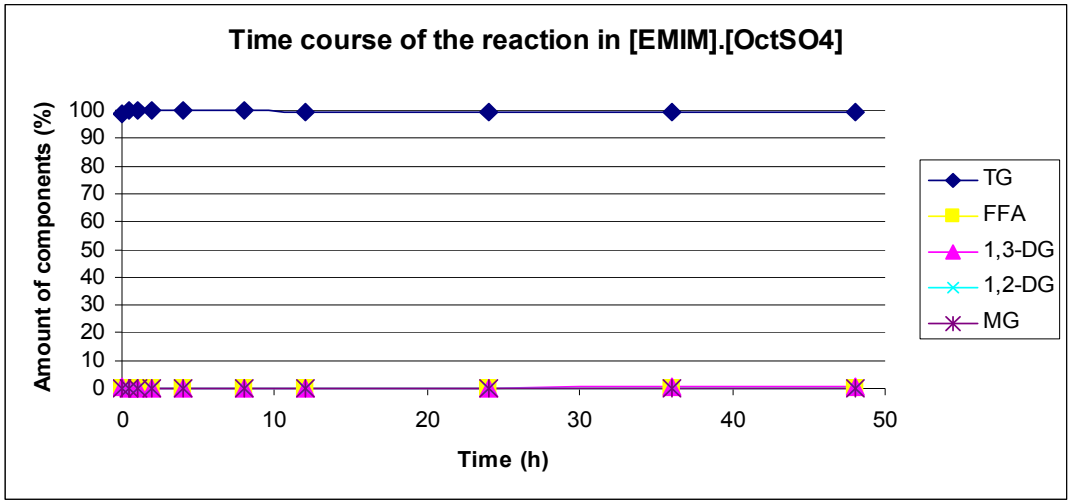


Figure B.6: Time course of the reaction in [EMIM].[$OctSO_4$].

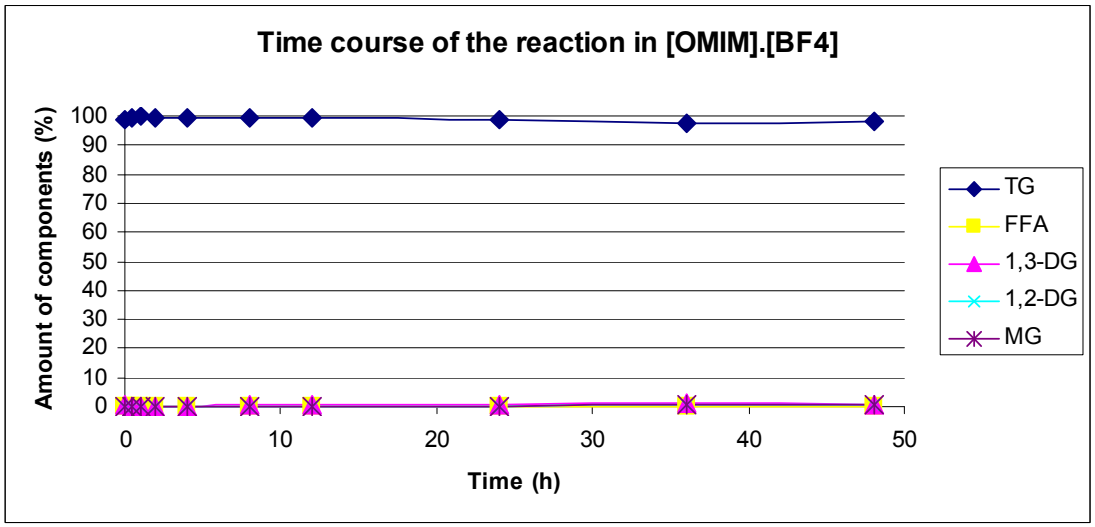


Figure B.7: Time course of the reaction in [OMIM].[BF_4].

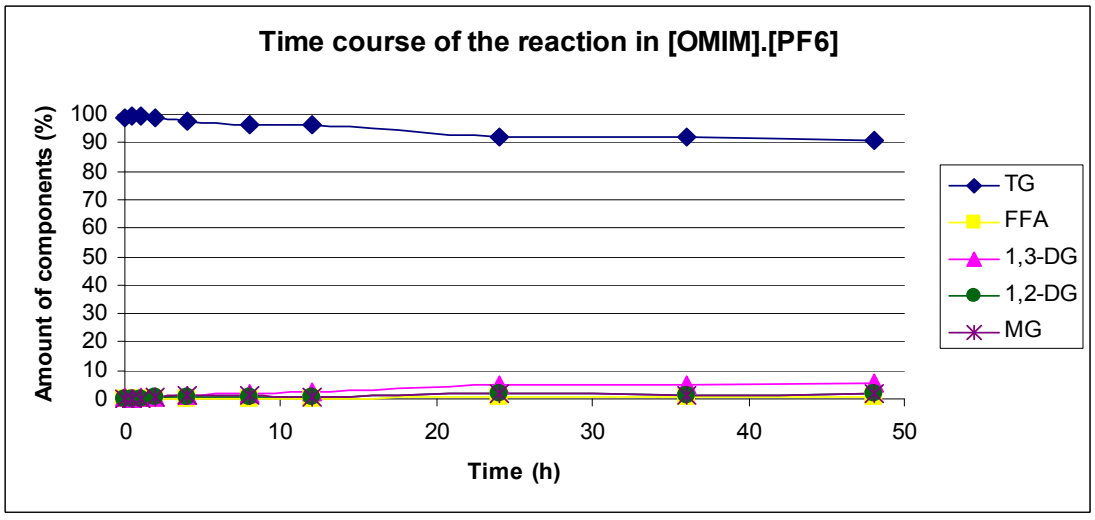


Figure B.8: Time course of the reaction in [OMIM].[PF_6].

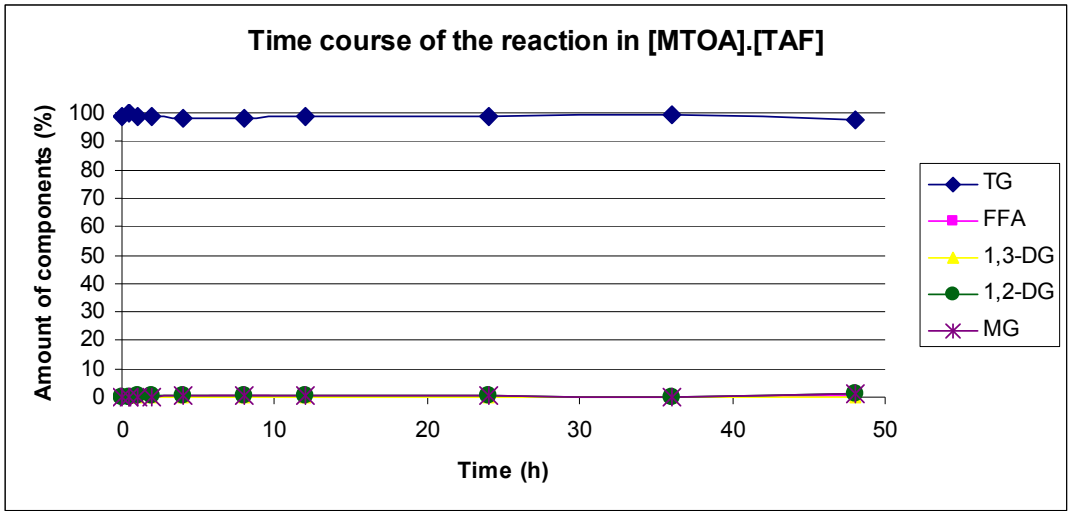


Figure B.9: Time course of the reaction in [MTOA].[TFA].

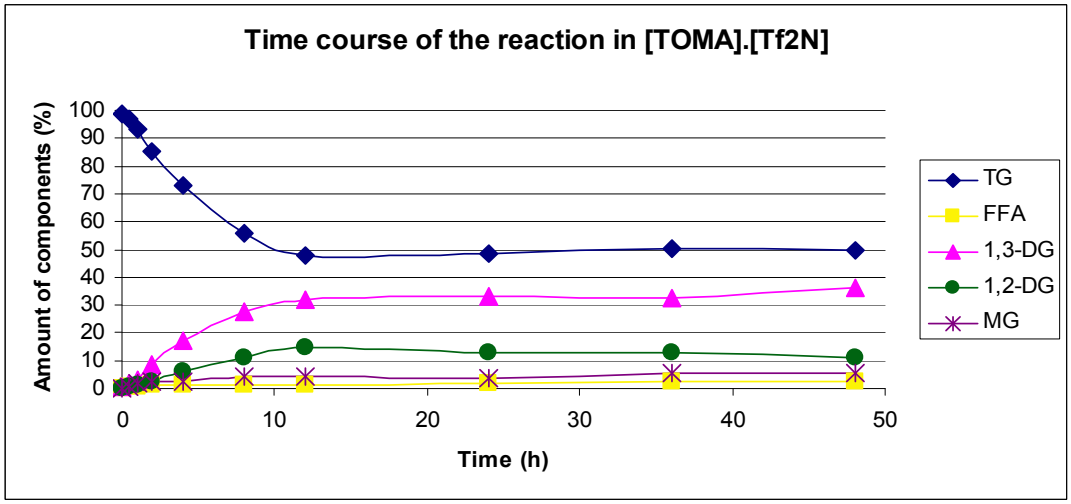


Figure B.10: Time course of the reaction in [TOMA].[Tf_2N].

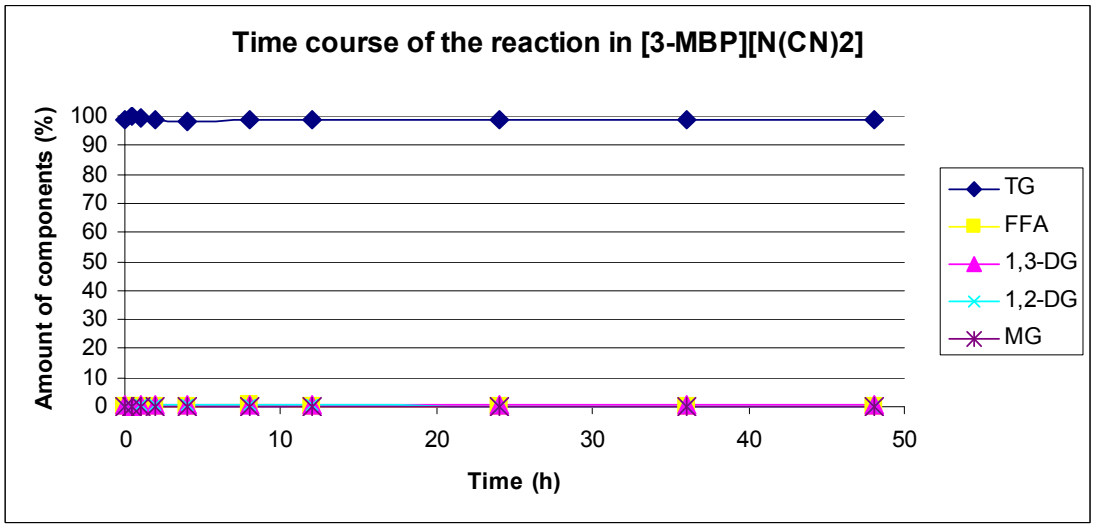


Figure B.11: Time course of the reaction in [3-MBP][$N(CN)_2$].

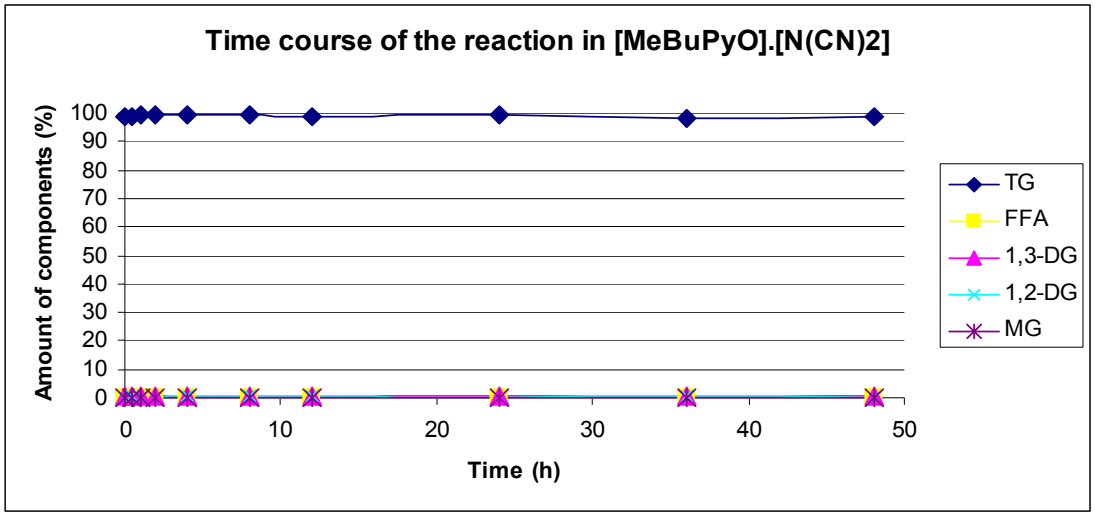


Figure B.12: Time course of the reaction in [MeBuPyO]. $[N(CN)_2]$.

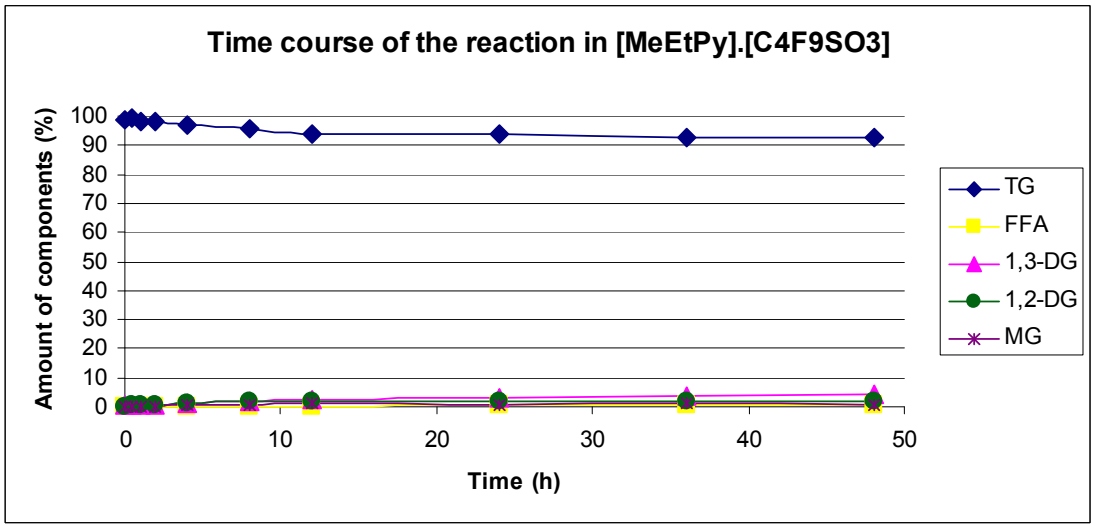


Figure B.13: Time course of the reaction in $[MeEtPy].[C_4F_9SO_3]$.

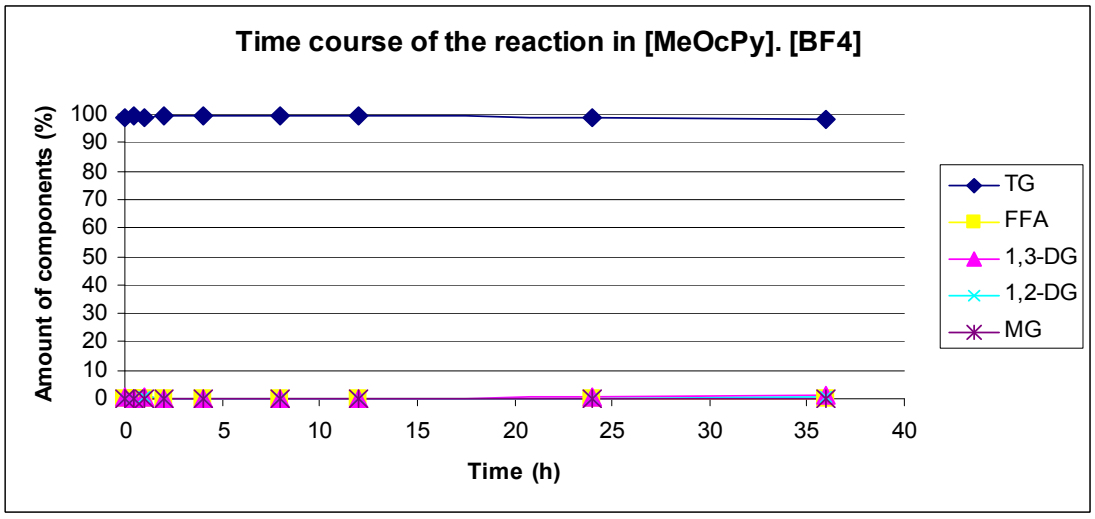


Figure B.14: Time course of the reaction in $[MeOcPy].[BF_4]$.

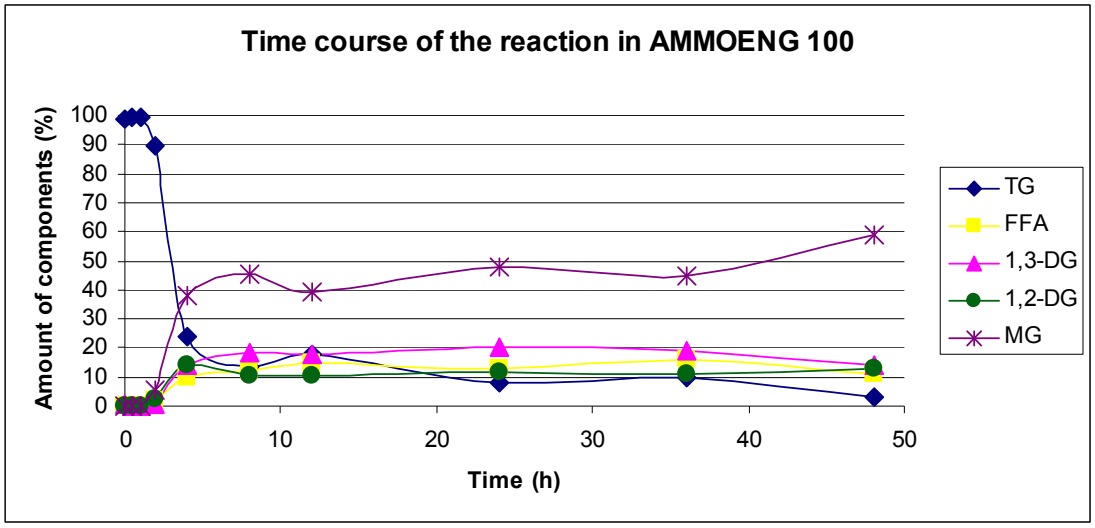


Figure B.15: Time course of the reaction in AMMOENG 100.

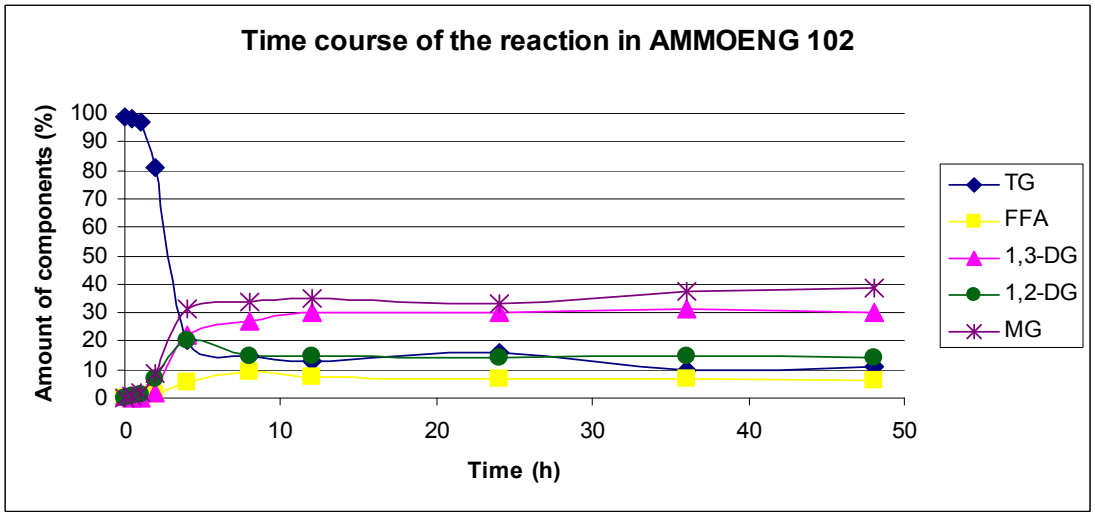


Figure B.16: Time course of the reaction in AMMOENG 102.

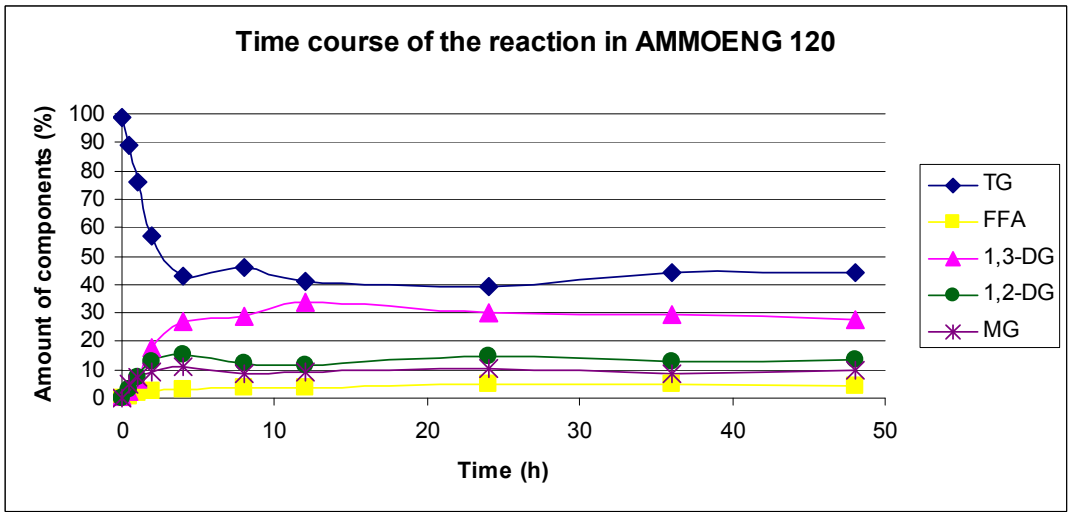


Figure B.17: Time course of the reaction in AMMOENG 120.

AMMOENG 111

Reaction medium was very viscous. Starting from the first half an hour, unusual peaks occurred so that none of the products or TG could be defined. A blank reaction without enzyme addition was run for 48 h to seperate the peaks of impurities, but there were almost no peaks other than TG for the whole period.

AMMOENG 112

As the previous one, this IL resulted in unusual peaks as well. Since the blank reaction didn't help for AMMOENG 111, no blank was run for AMMOENG 112. Both ILs need to be analyzed by a more precise equipment other than TLC-FID.

Appendix C.1. Time courses of the glycerolysis reactions performed to investigate the effect of substrates/IL ratio.

Reaction conditions: 0.5 mmol of oil, 0.25 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 24 h, 700 rpm

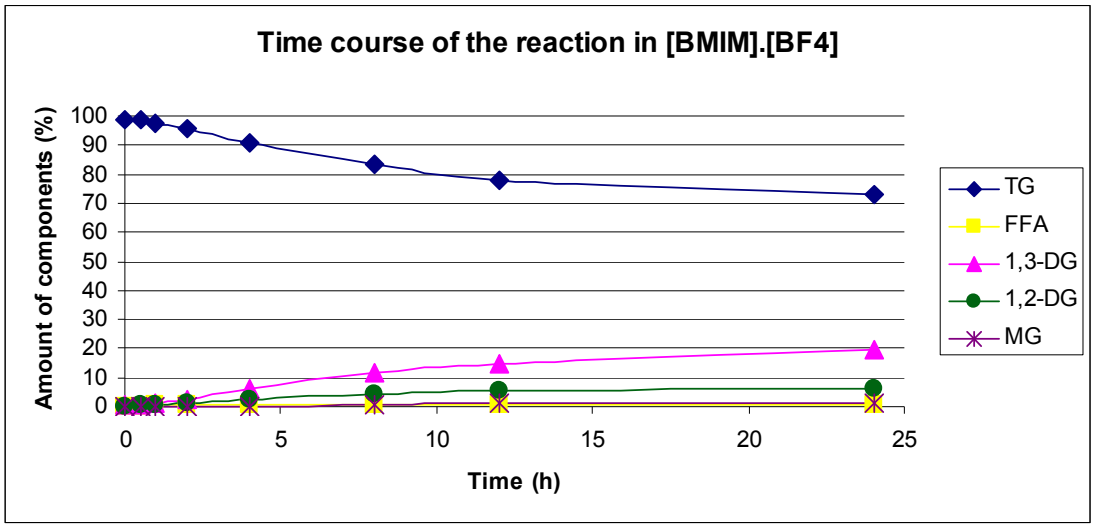


Figure C.1.1: Time course of the reaction in [BMIM].[BF_4].

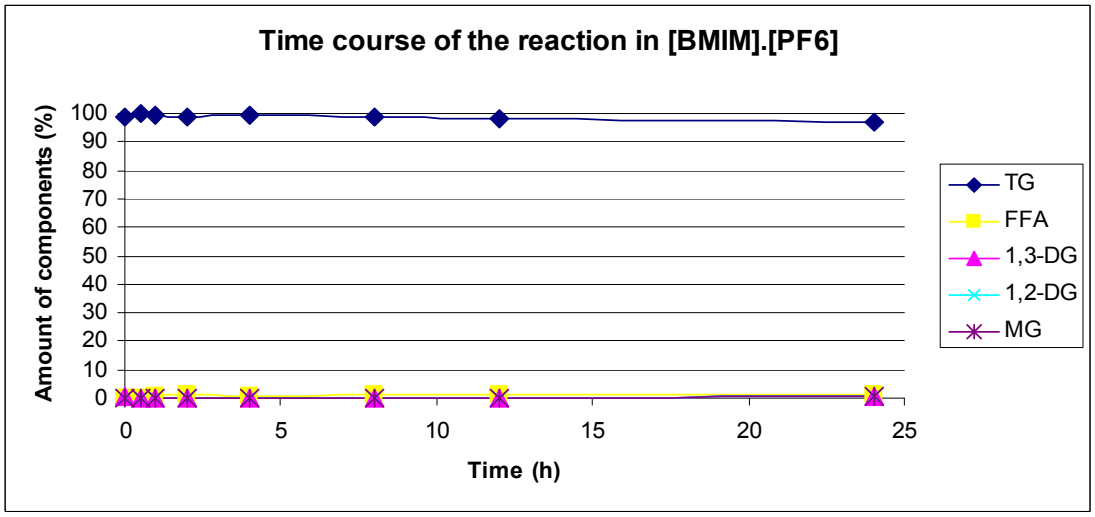


Figure C.1.2: Time course of the reaction in [BMIM].[PF_6].

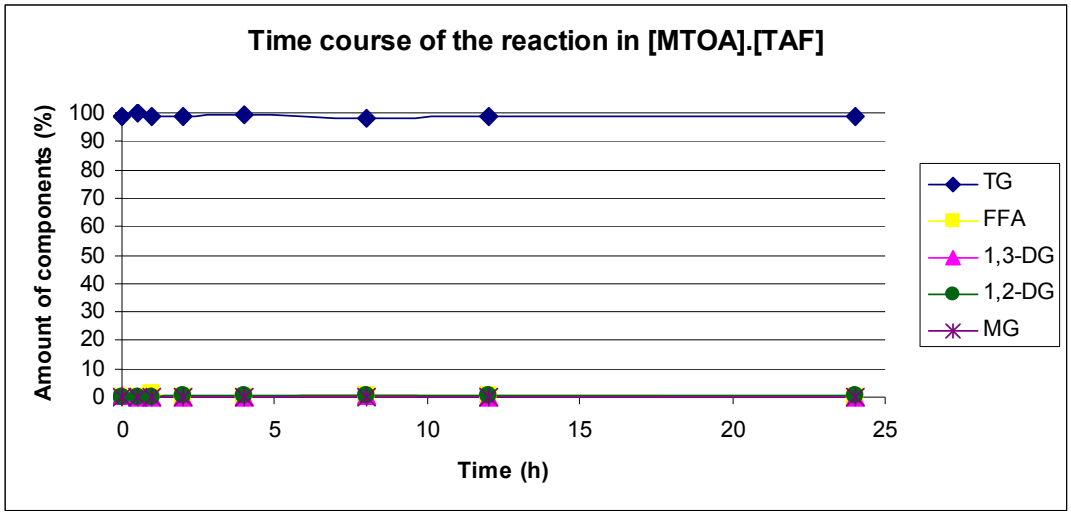


Figure C.1.3: Time course of the reaction in [MTOA].[TFA].

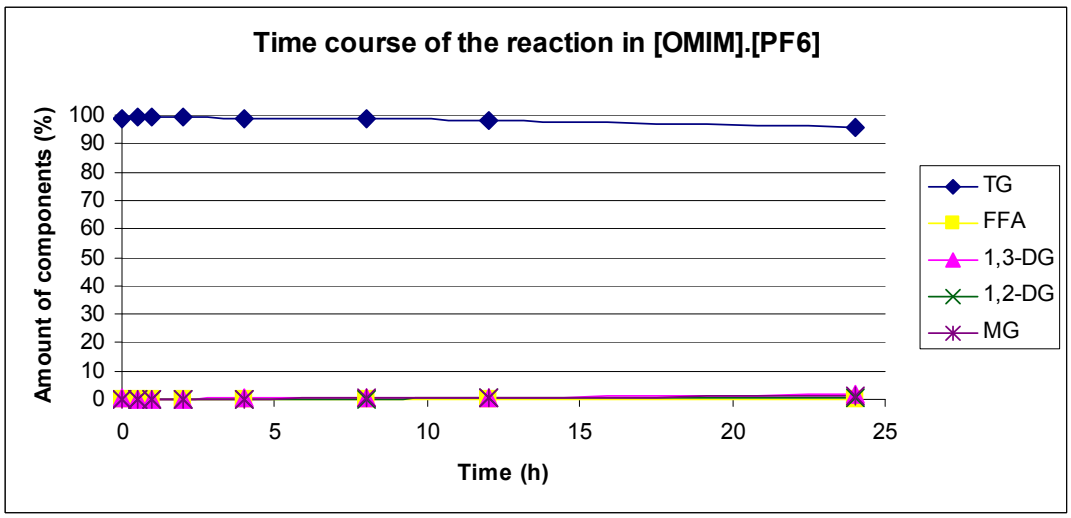


Figure C.1.4: Time course of the reaction in [OMIM].[PF_6].

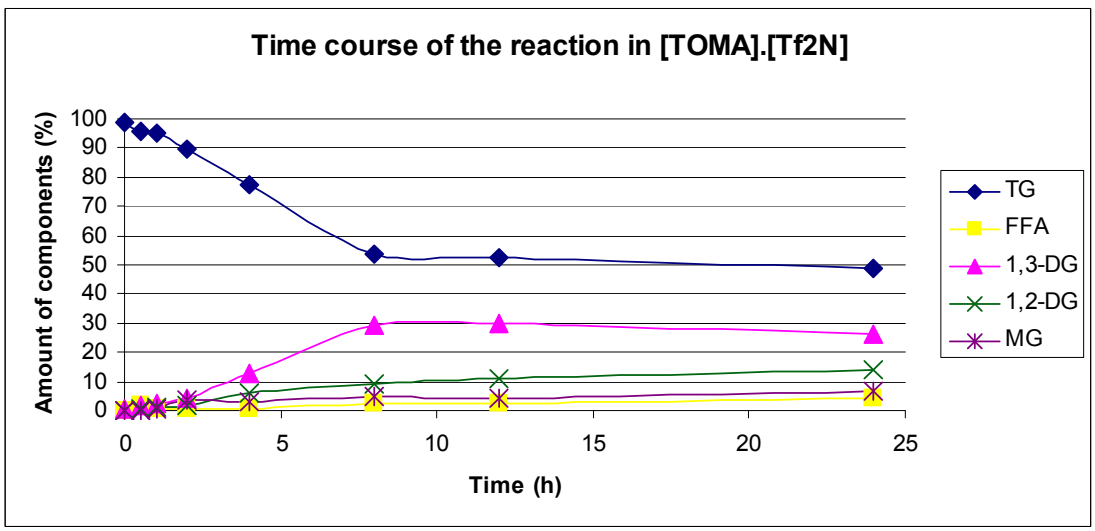


Figure C.1.5: Time course of the reaction in [TOMA].[Tf_2N].

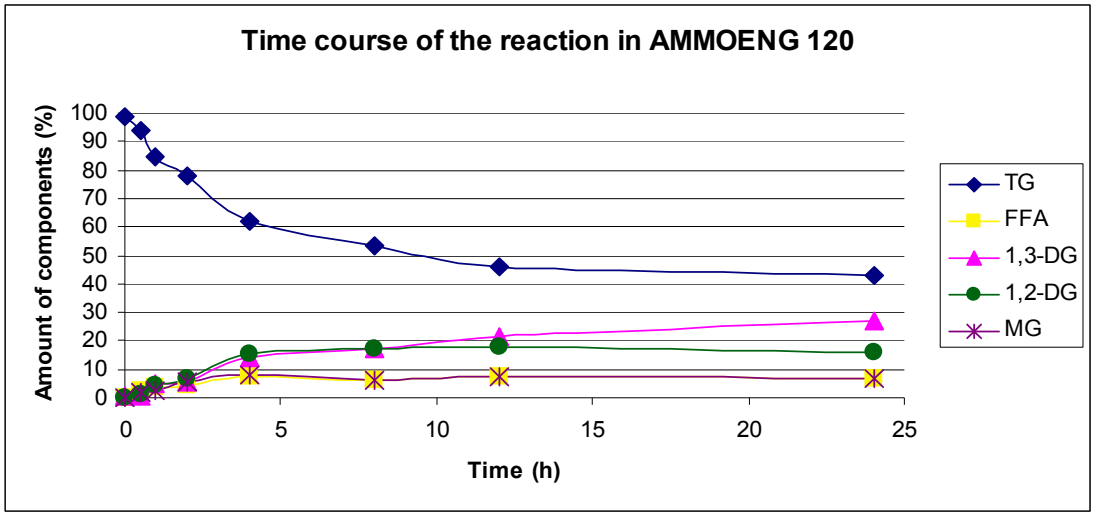


Figure C.1.6: Time course of the reaction in AMMOENG 120.

Appendix C.2. Time courses of the glycerolysis reactions performed to investigate the effect of substrates/IL ratio.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 24 h, 700 rpm

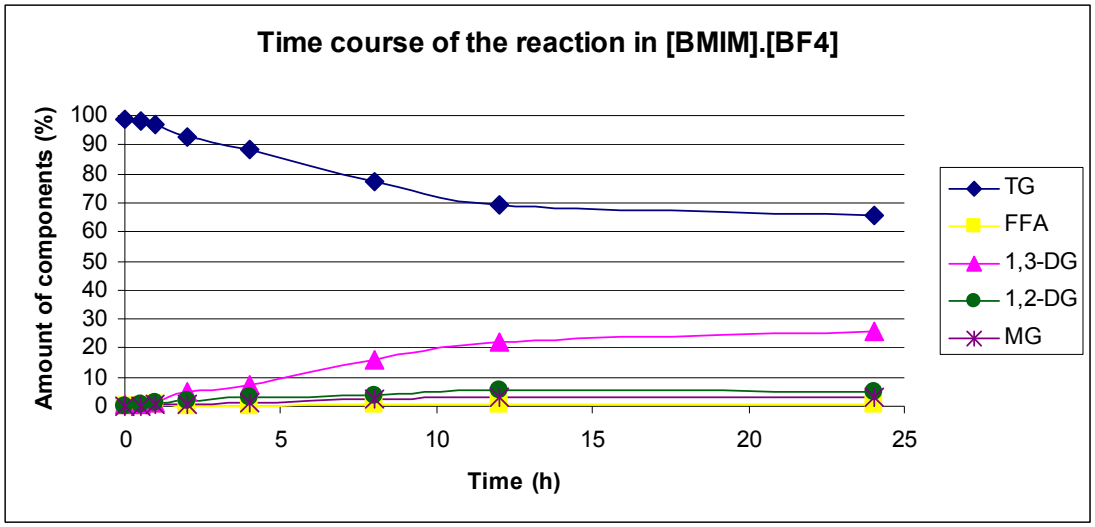


Figure C.2.1: Time course of the reaction in [BMIM].[BF_4].

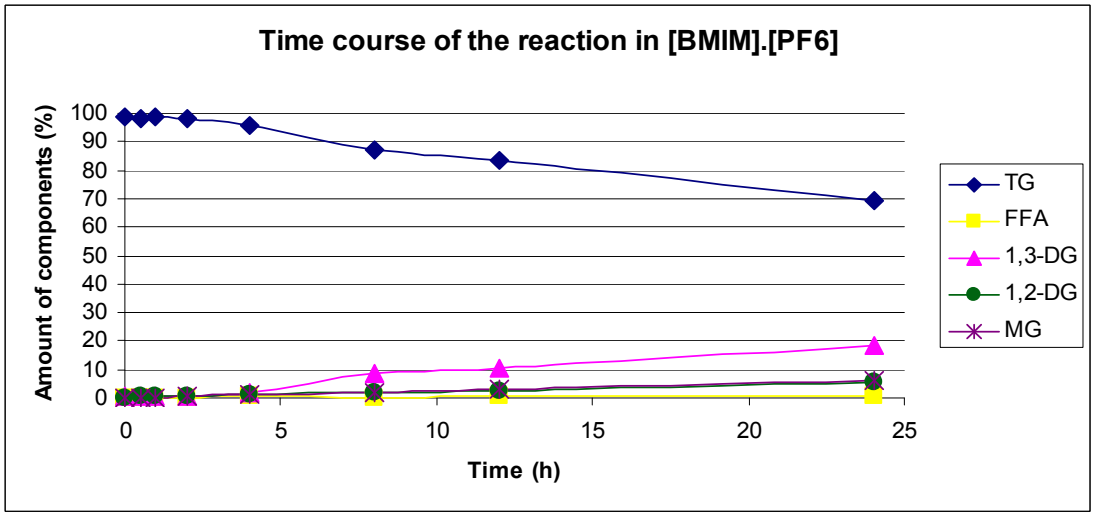


Figure C.2.2: Time course of the reaction in [BMIM].[PF_6].

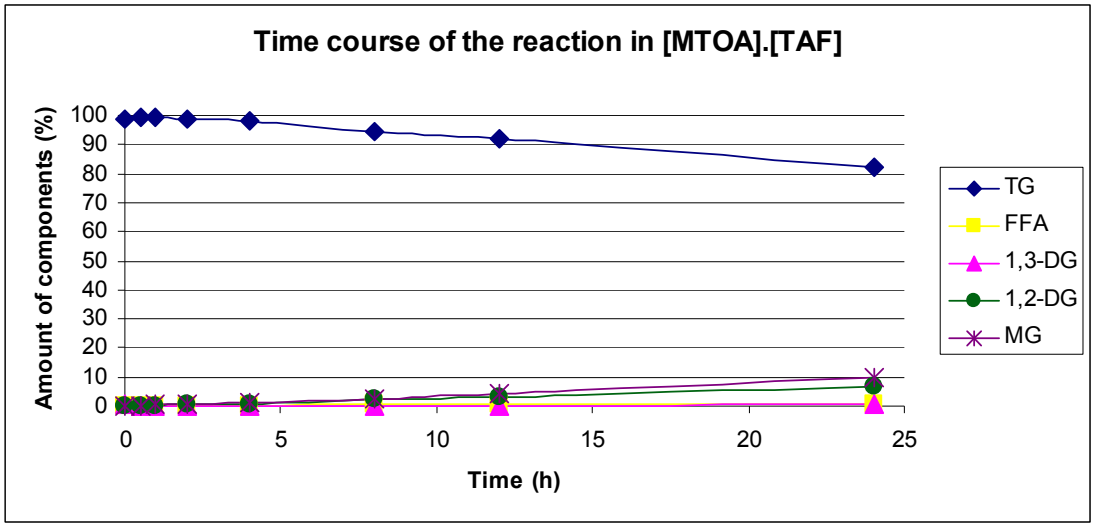


Figure C.2.3: Time course of the reaction in [MTOA].[TFA].

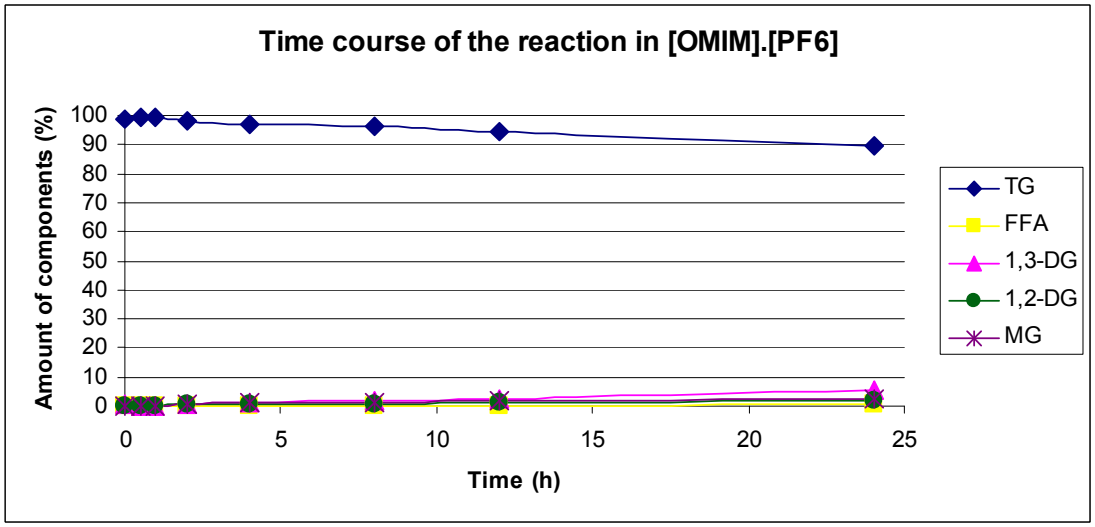


Figure C.2.4: Time course of the reaction in [OMIM].[PF_6].

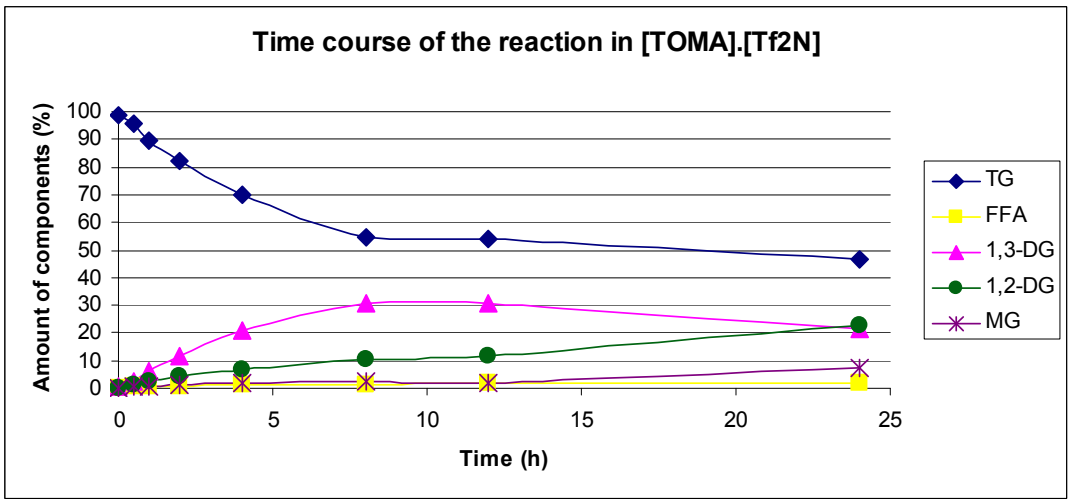


Figure C.2.5: Time course of the reaction in [TOMA].[Tf_2N].

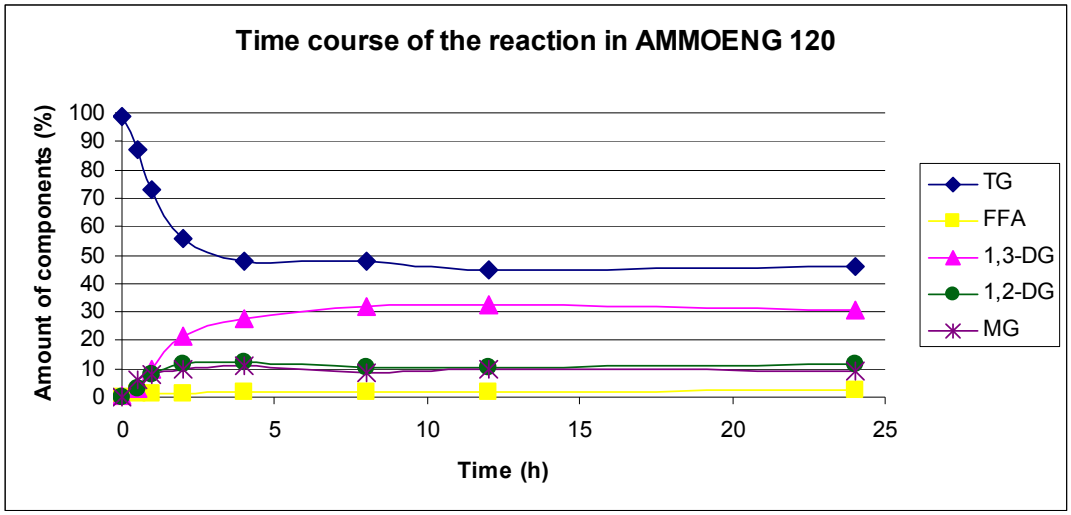


Figure C.2.6: Time course of the reaction in AMMOENG 120.

Appendix C.3. Comparison of the three different levels used to investigate the effect of substrates/IL ratio.

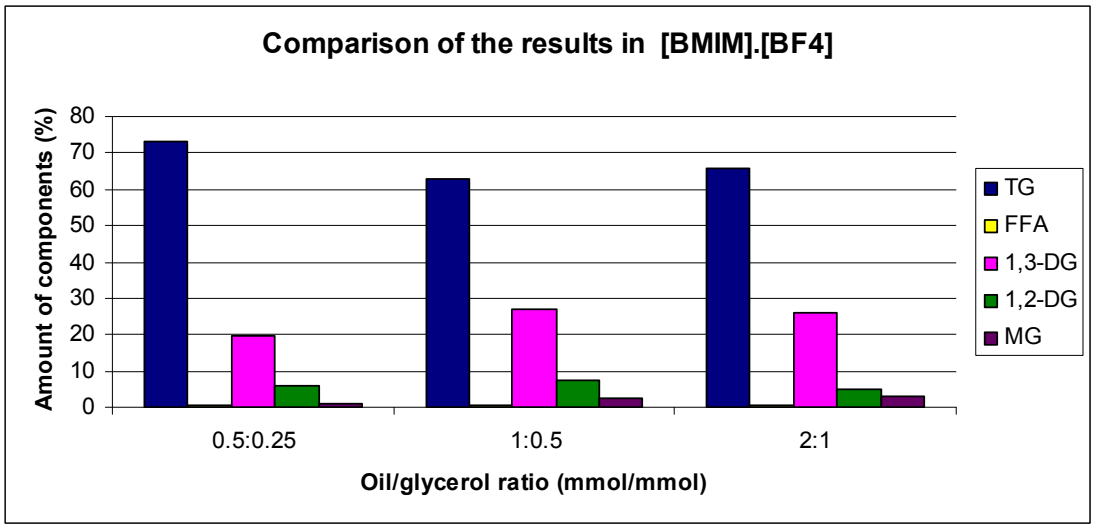


Figure C.3.1: Comparison of the results in [BMIM].[BF_4].

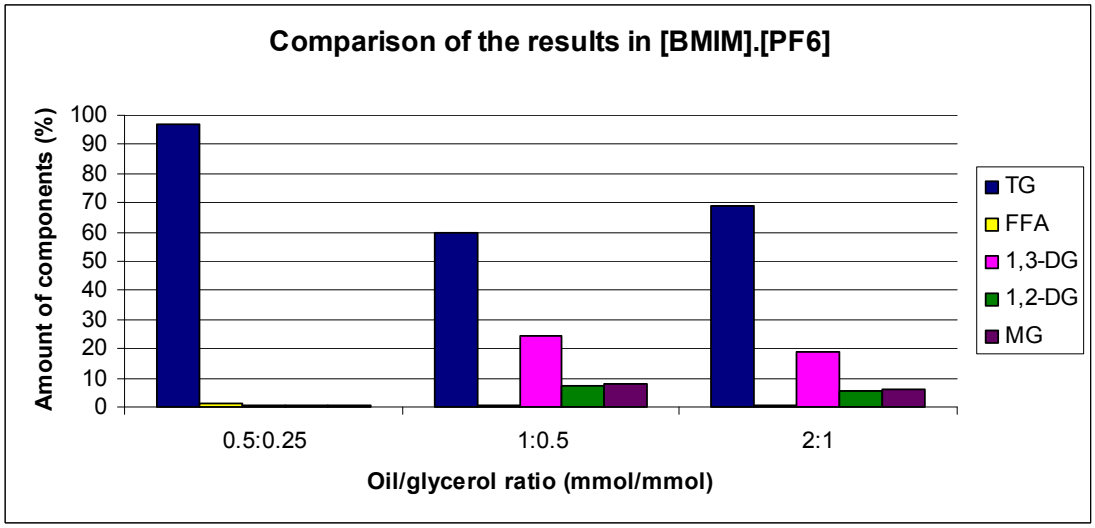


Figure C.3.2: Comparison of the results in [BMIM].[PF_6].

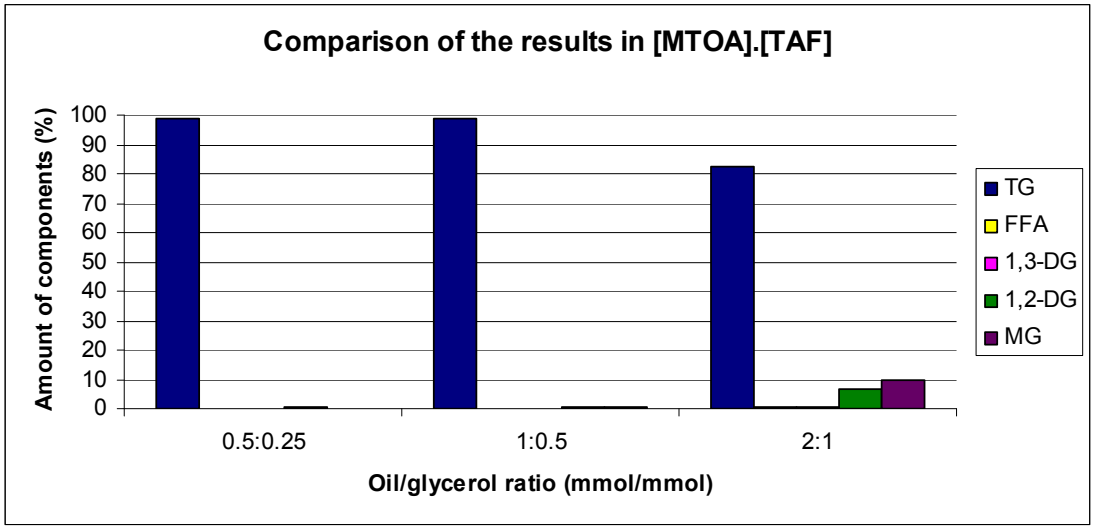


Figure C.3.3: Comparison of the results in [MTOA].[TFA].

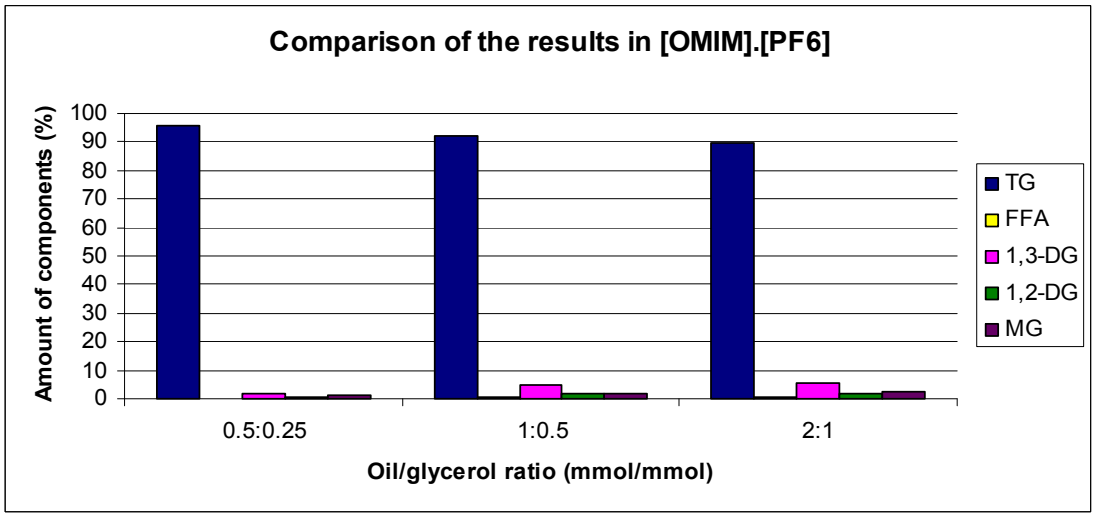


Figure C.3.4: Comparison of the results in [OMIM].[PF_6].

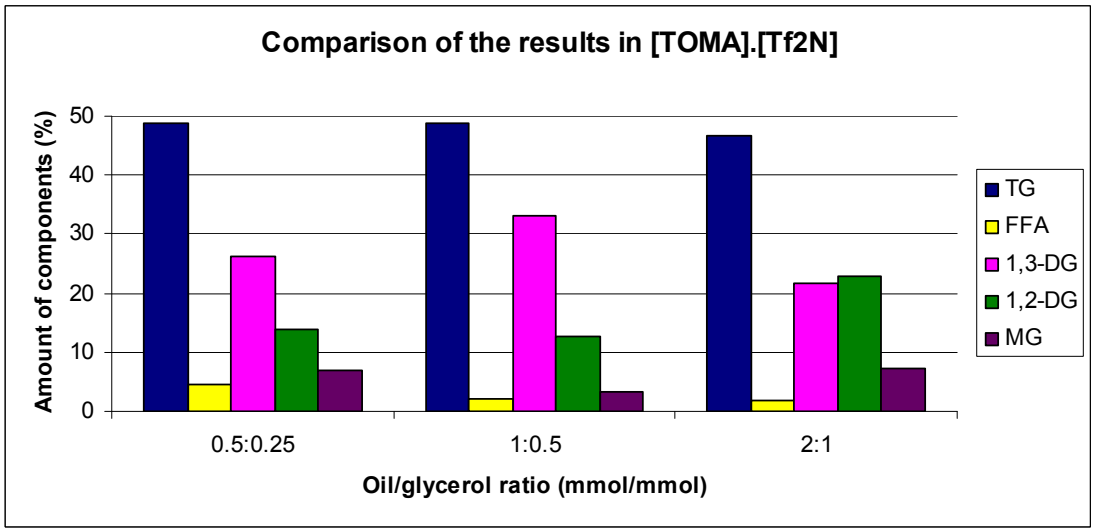


Figure C.3.5: Comparison of the results in [TOMA].[Tf_2N].

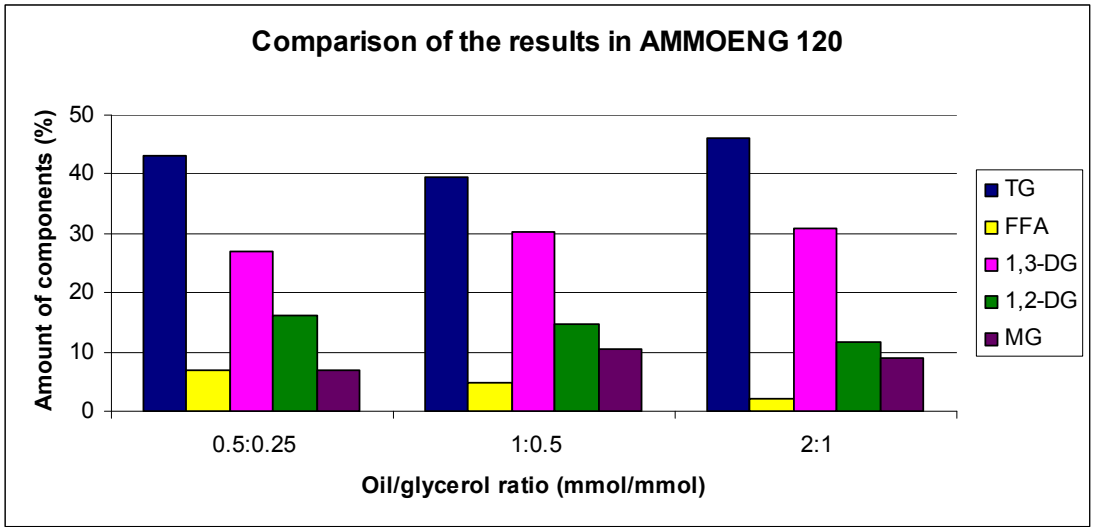


Figure C.3.6: Comparison of the results in AMMOENG 120.

Appendix D.1. Time courses of the glycerolysis reactions performed to investigate the effect of temperature.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 50°C, 24 h, 700 rpm

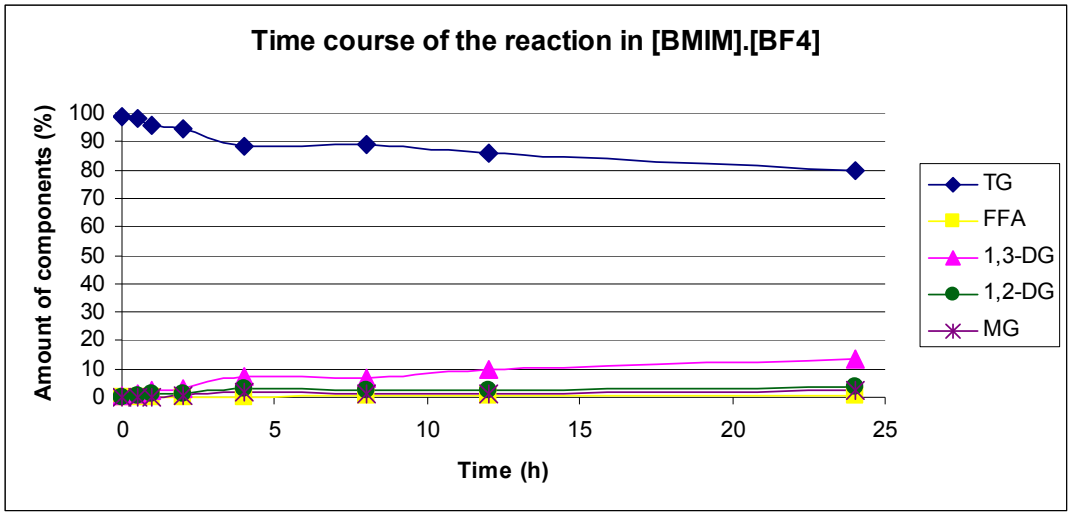


Figure D.1.1: Time course of the reaction in [BMIM].[BF_4].

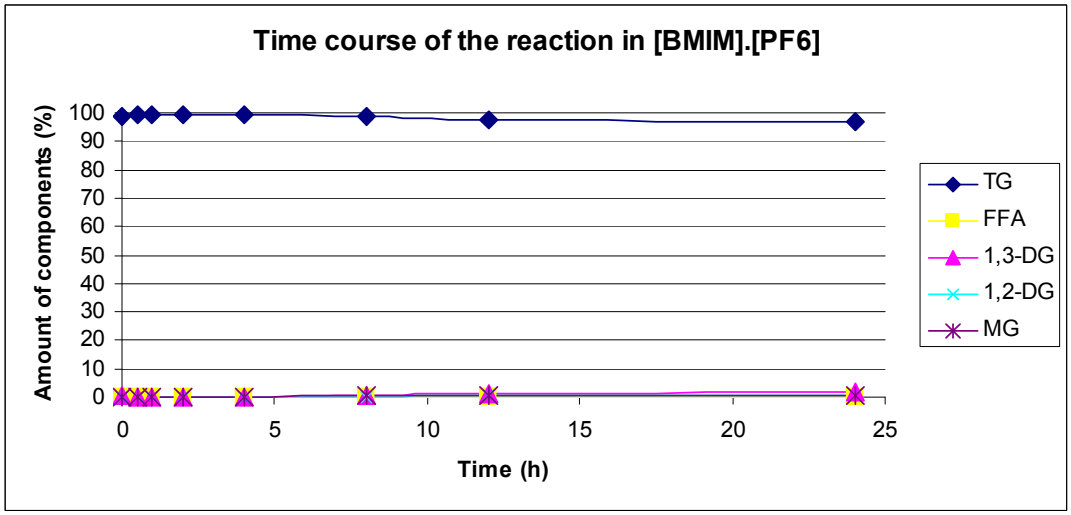


Figure D.1.2: Time course of the reaction in [BMIM].[PF_6].

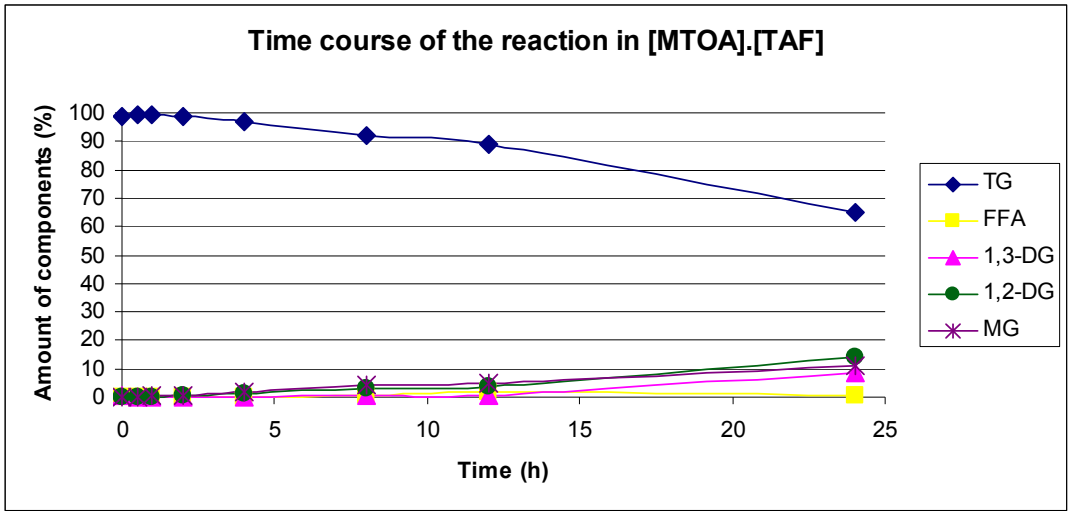


Figure D.1.3: Time course of the reaction in [MTOA].[TFA].

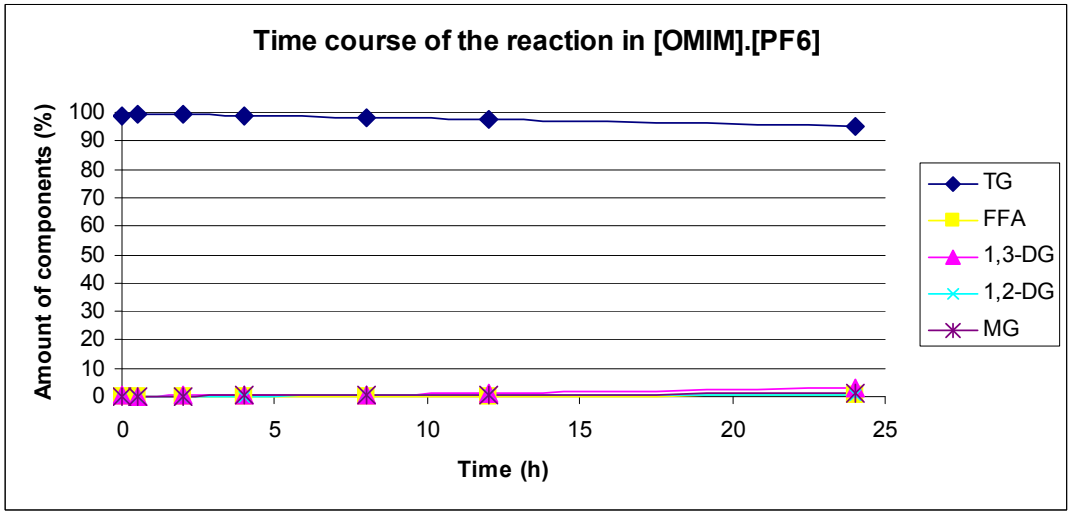


Figure D.1.4: Time course of the reaction in [OMIM].[PF_6].

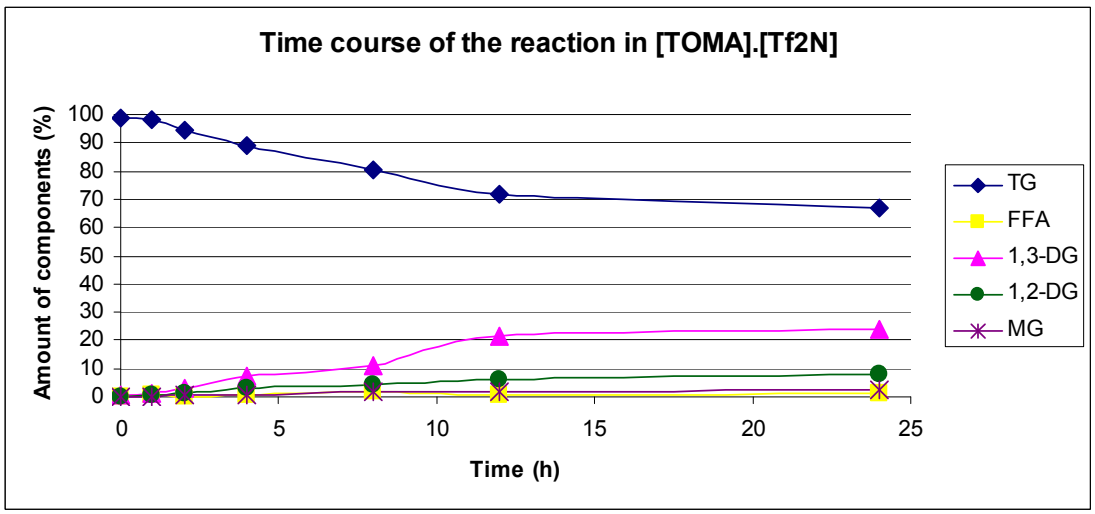


Figure D.1.5: Time course of the reaction in [TOMA].[Tf_2N].

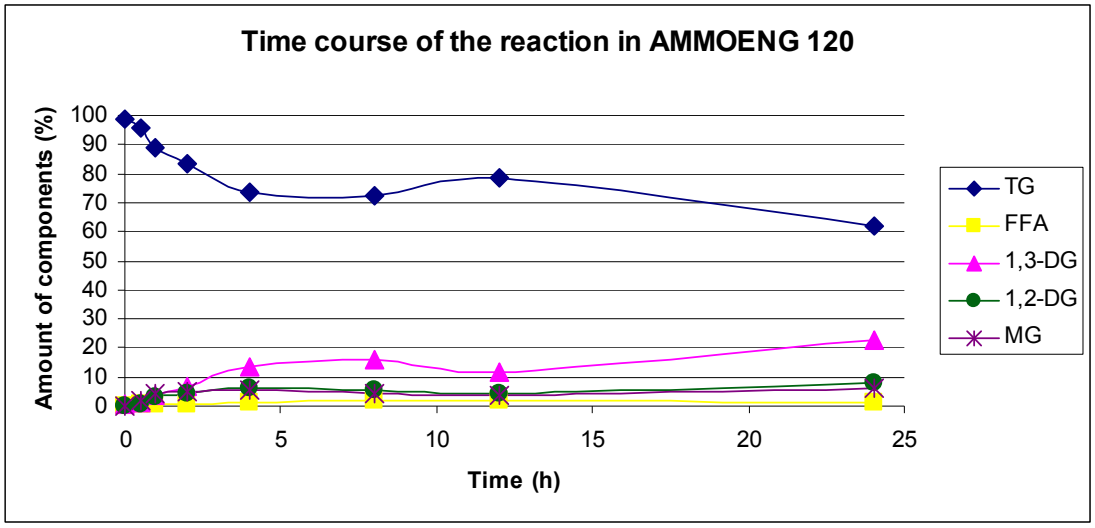


Figure D.1.6: Time course of the reaction in AMMOENG 120.

Appendix D.2. Time courses of the glycerolysis reactions performed to investigate the effect of temperature.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 70°C, 24 h, 700 rpm

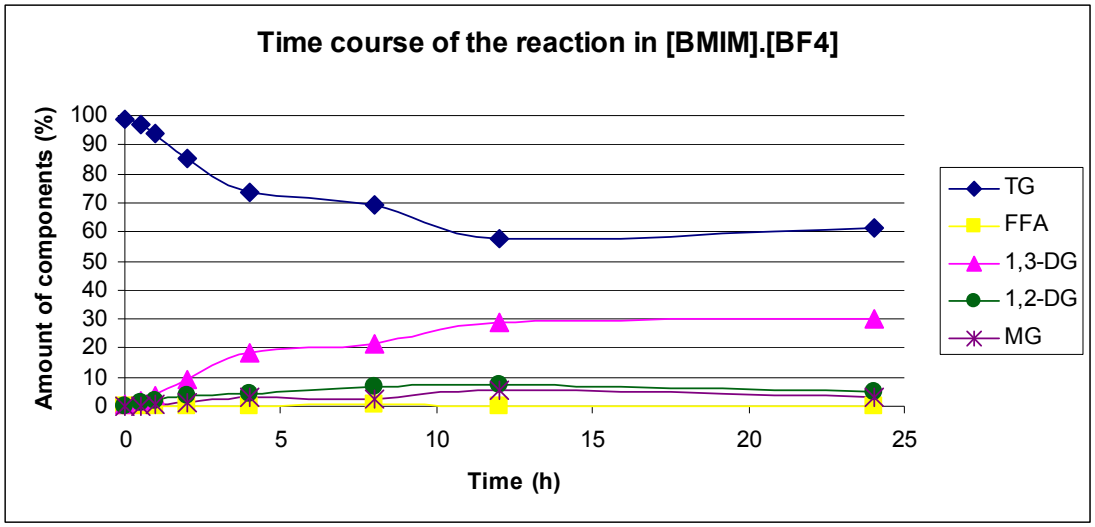


Figure D.2.1: Time course of the reaction in [BMIM].[BF_4].

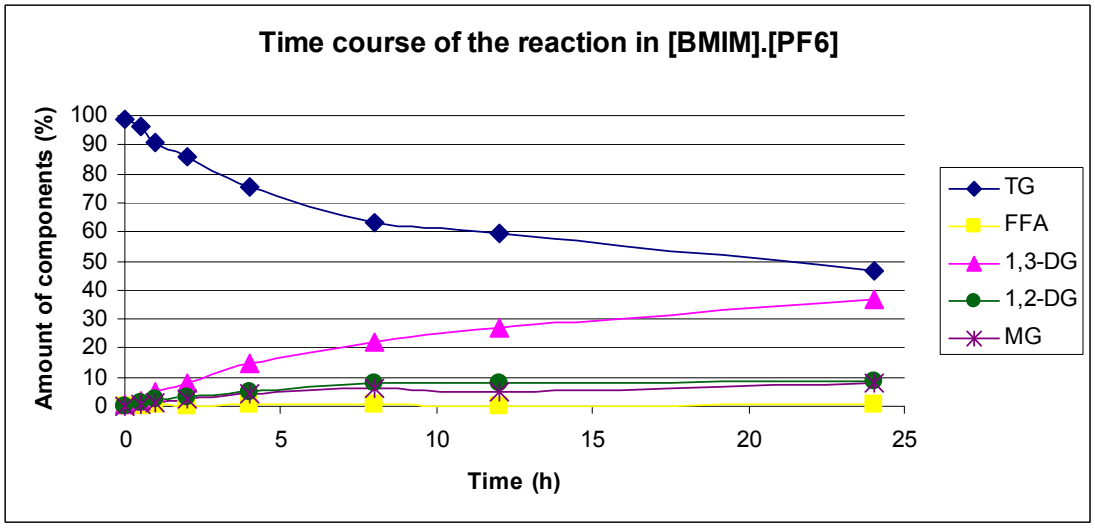


Figure D.2.2: Time course of the reaction in [BMIM].[PF_6].

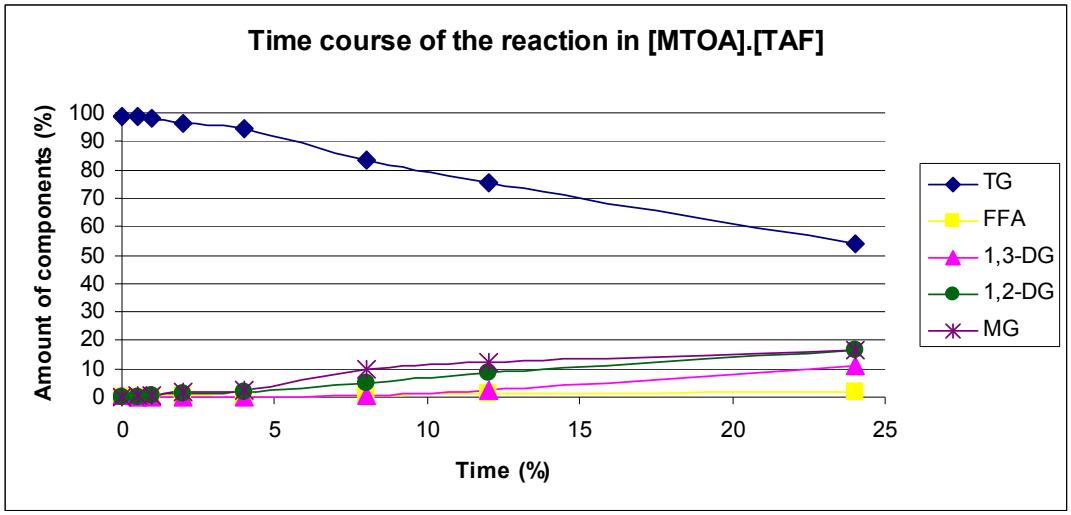


Figure D.2.3: Time course of the reaction in [MTOA].[TFA].

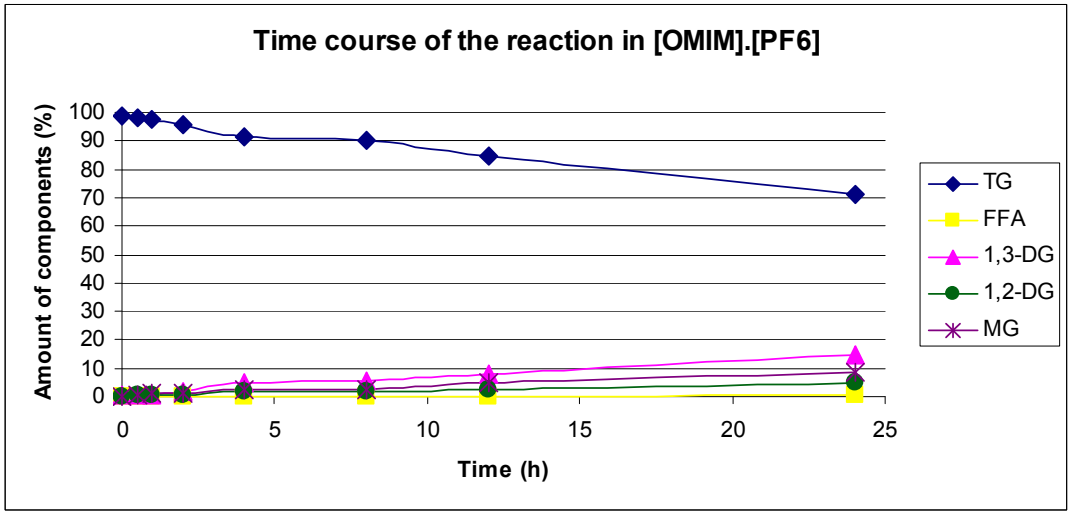


Figure D.2.4: Time course of the reaction in [OMIM].[PF_6].

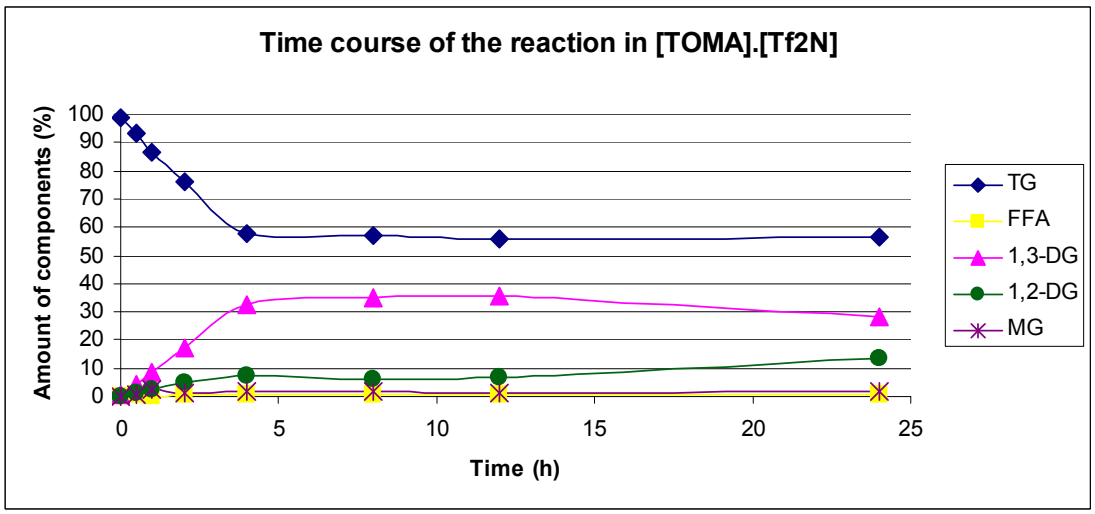


Figure D.2.5: Time course of the reaction in [TOMA].[Tf_2N].

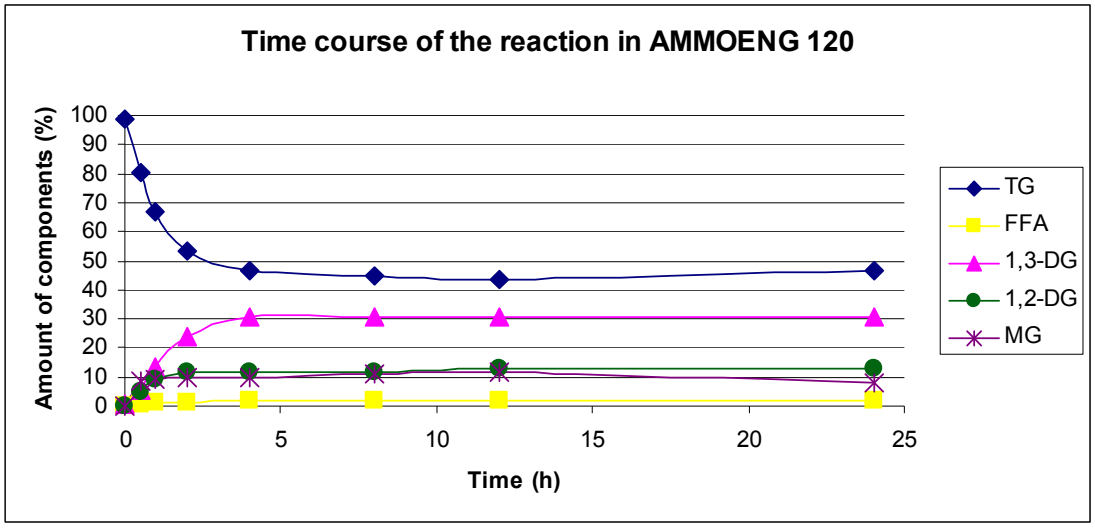


Figure D.2.6: Time course of the reaction in AMMOENG 120.

Appendix D.3. Comparison of the three different levels used to investigate the effect of temperature.

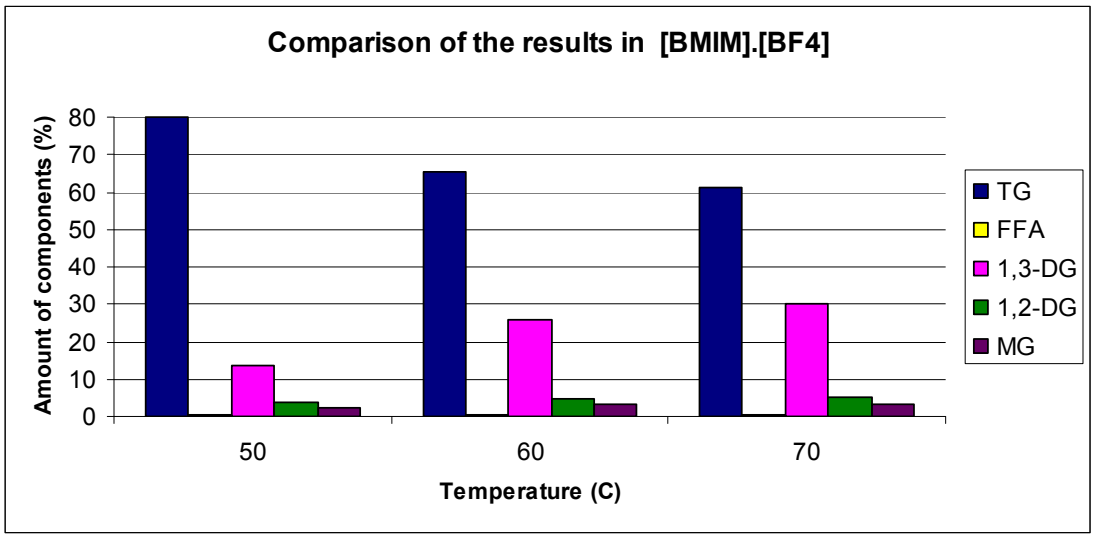


Figure D.3.1: Comparison of the results in [BMIM].[BF_4].

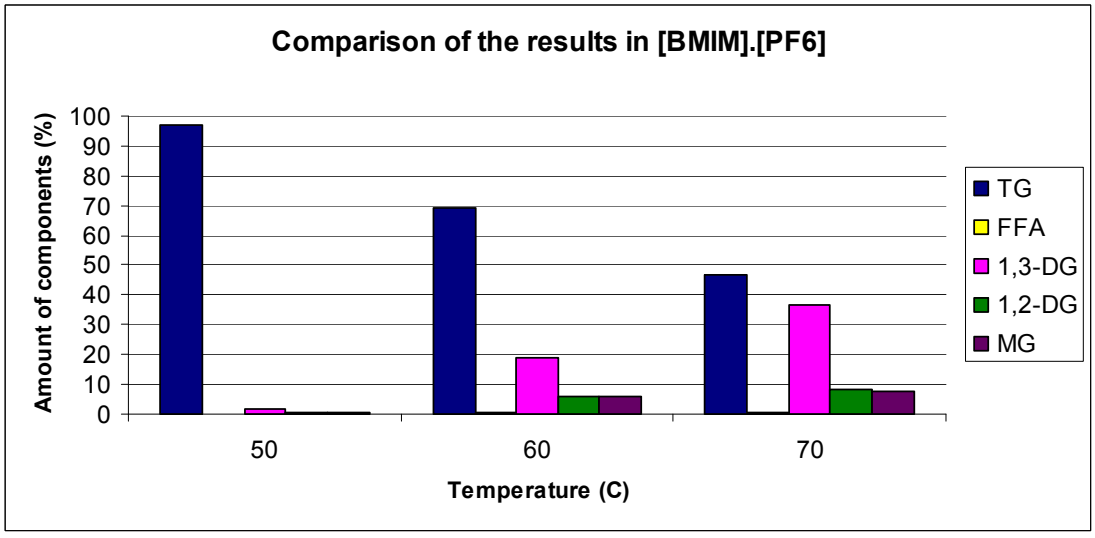


Figure D.3.2: Comparison of the results in [BMIM].[PF_6].

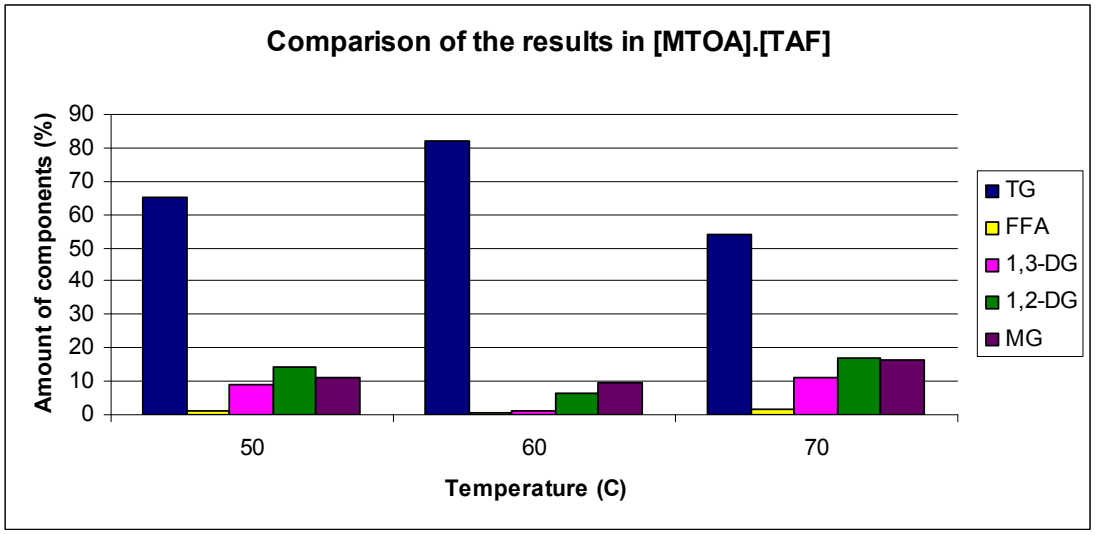


Figure D.3.3: Comparison of the results in [MTOA].[TFA].

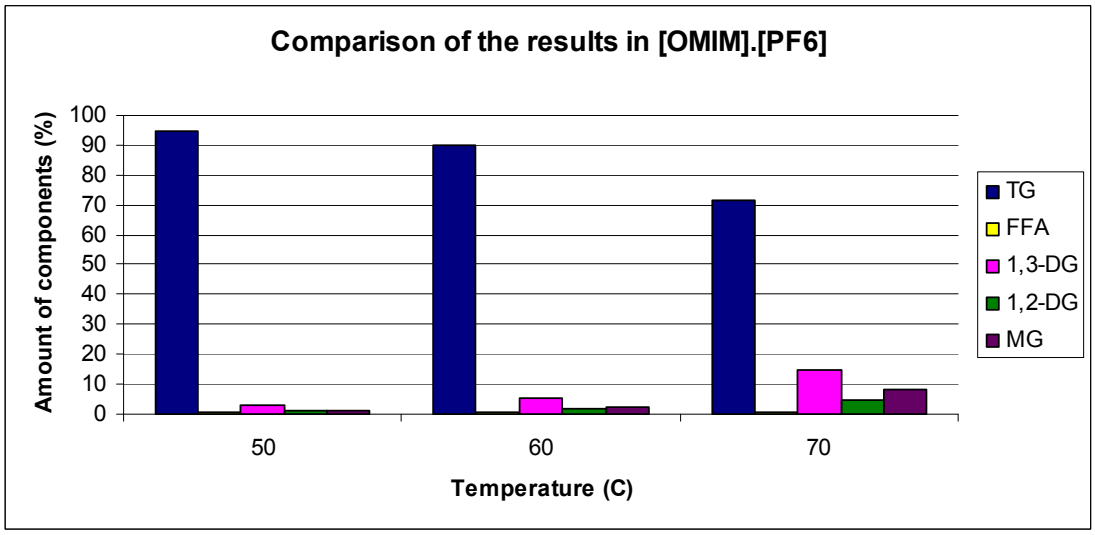


Figure D.3.4: Comparison of the results in [OMIM].[PF_6].

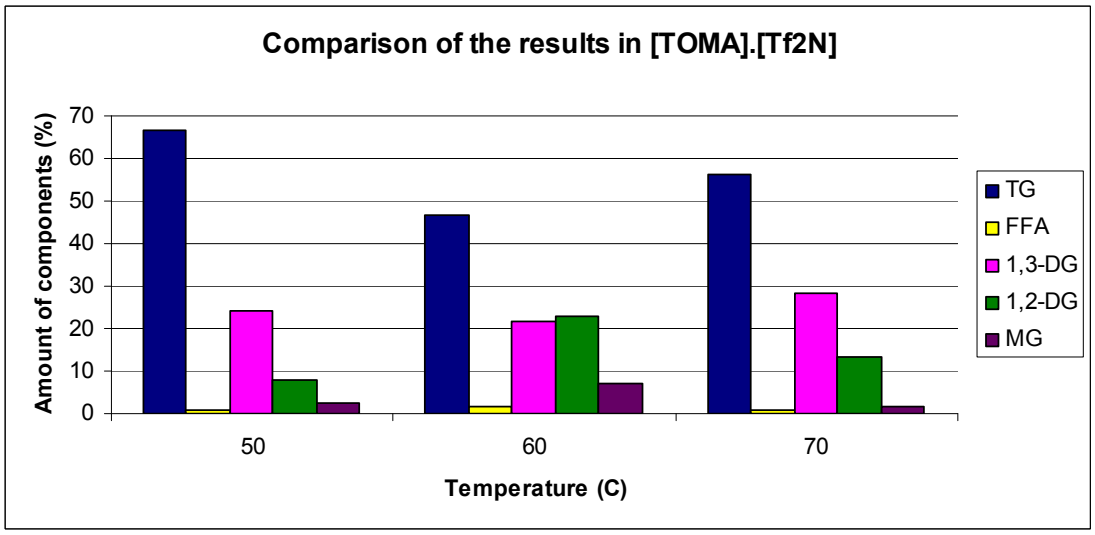


Figure D.3.5: Comparison of the results in [TOMA].[Tf_2N].

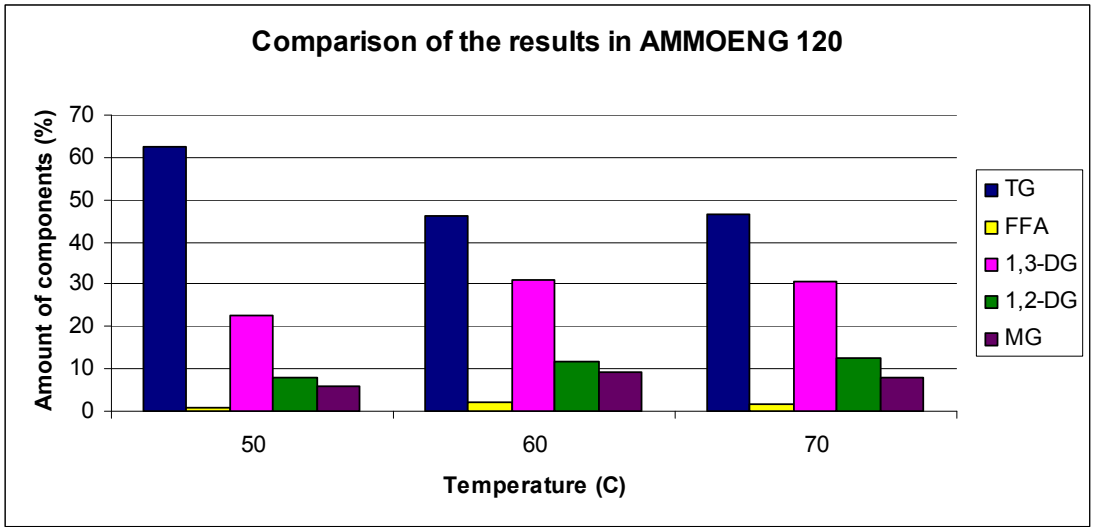


Figure D.3.6: Comparison of the results in AMMOENG 120.

Appendix E.1. Time courses of the glycerolysis reactions performed to investigate the effect of oil/glycerol ratio.

Reaction conditions: 2 mmol of oil, 1.25 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 24 h, 700 rpm

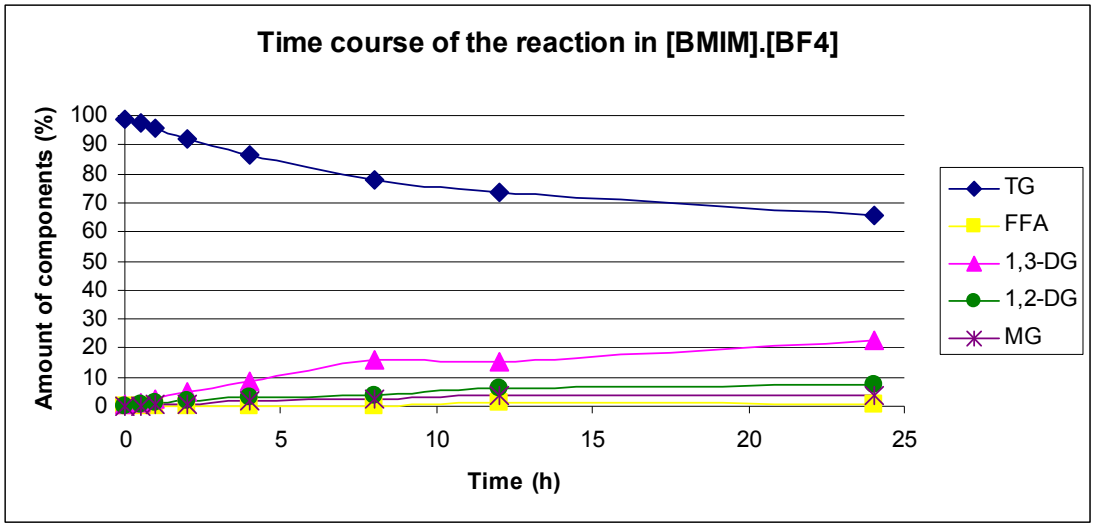


Figure E.1.1: Time course of the reaction in [BMIM].[BF_4].

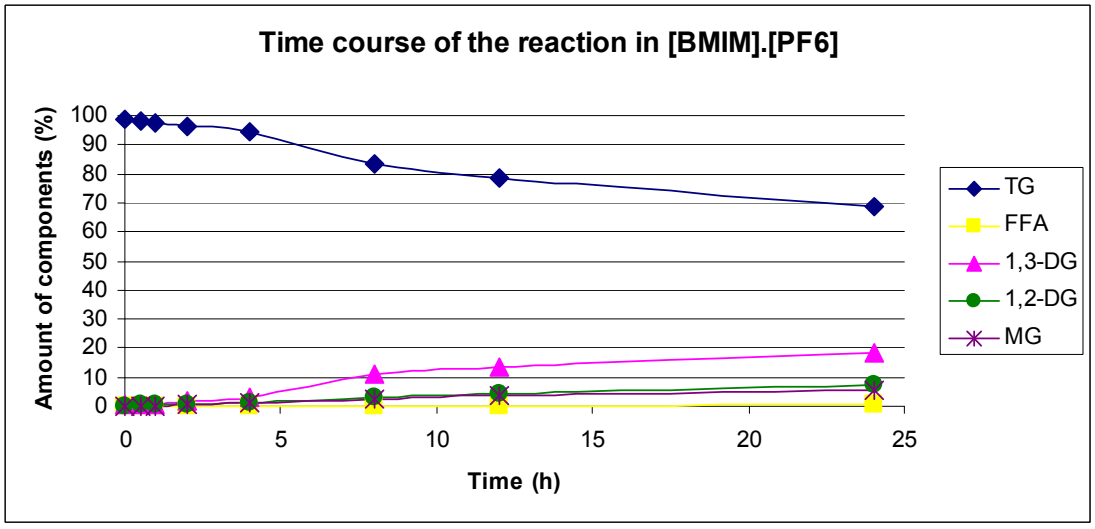


Figure E.1.2: Time course of the reaction in [BMIM].[PF_6].

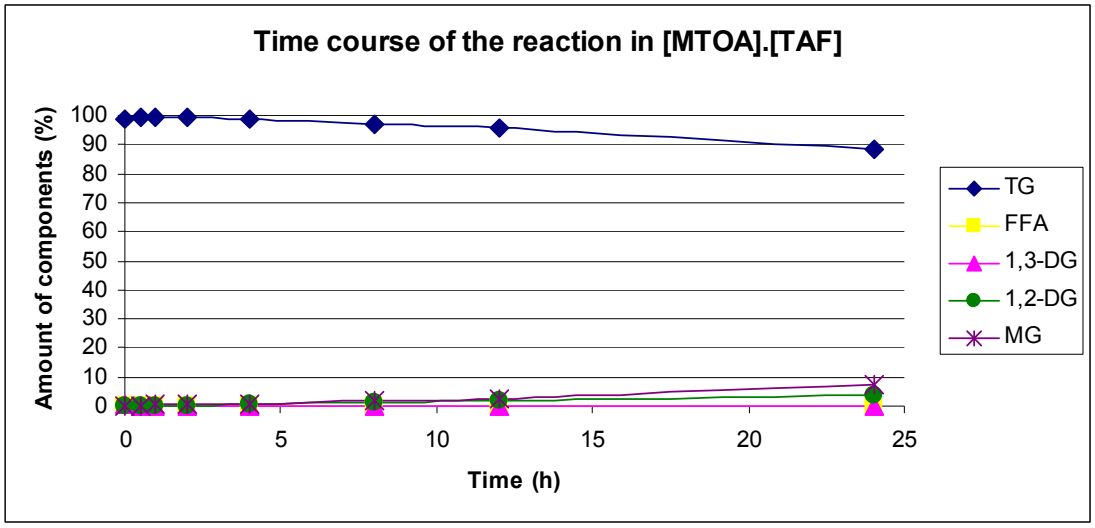


Figure E.1.3: Time course of the reaction in [MTOA].[TFA].

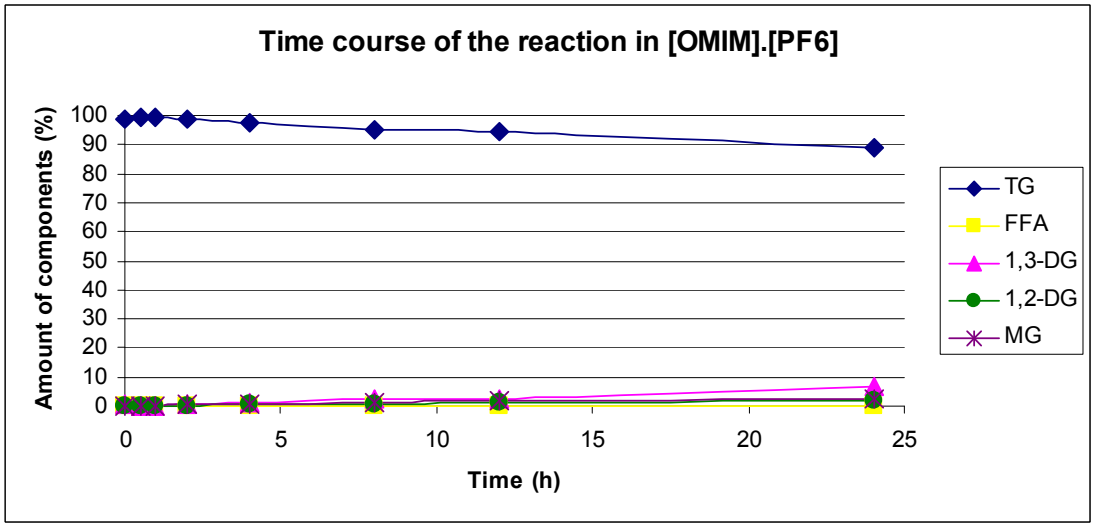


Figure E.1.4: Time course of the reaction in [OMIM].[PF_6].

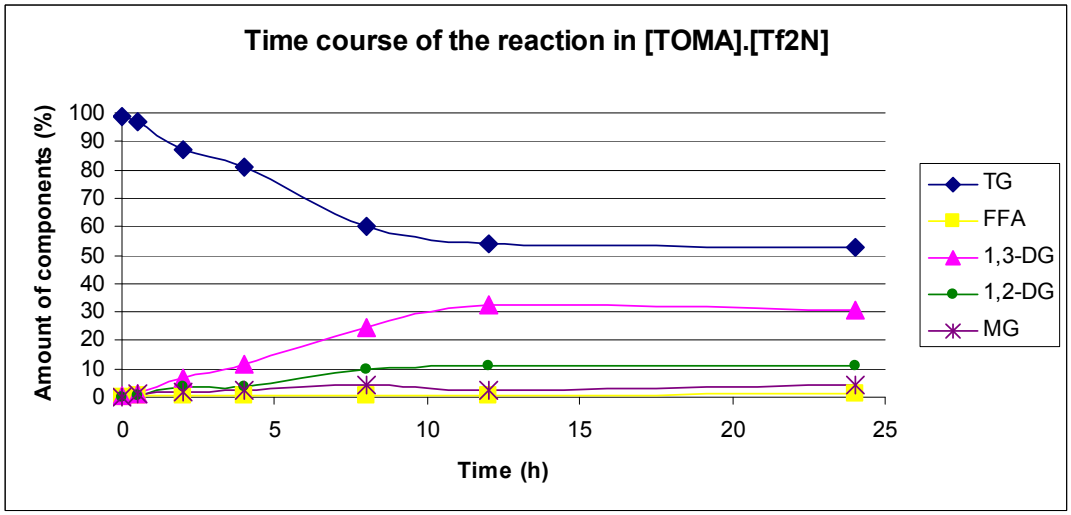


Figure E.1.5: Time course of the reaction in [TOMA].[Tf_2N].

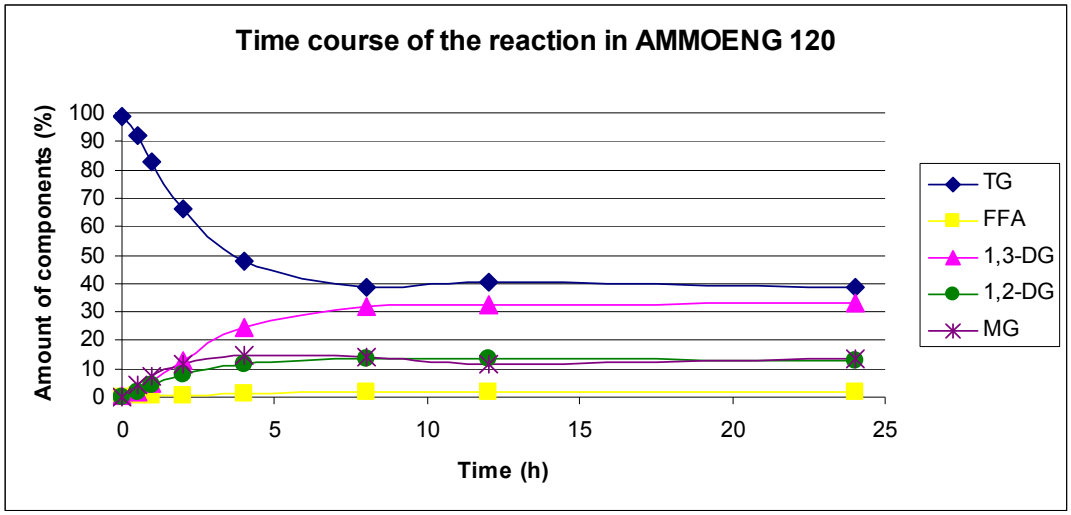


Figure E.1.6: Time course of the reaction in AMMOENG 120.

Appendix E.2. Time courses of the glycerolysis reactions performed to investigate the effect of oil/glycerol ratio.

Reaction conditions: 2 mmol of oil, 1.5 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 24 h, 700 rpm

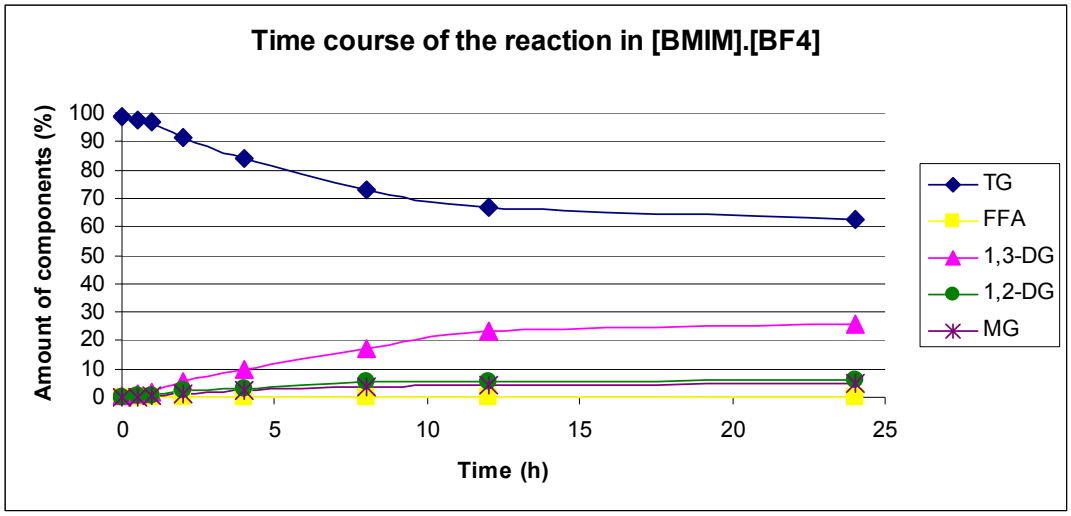


Figure E.2.1: Time course of the reaction in [BMIM].[BF_4].

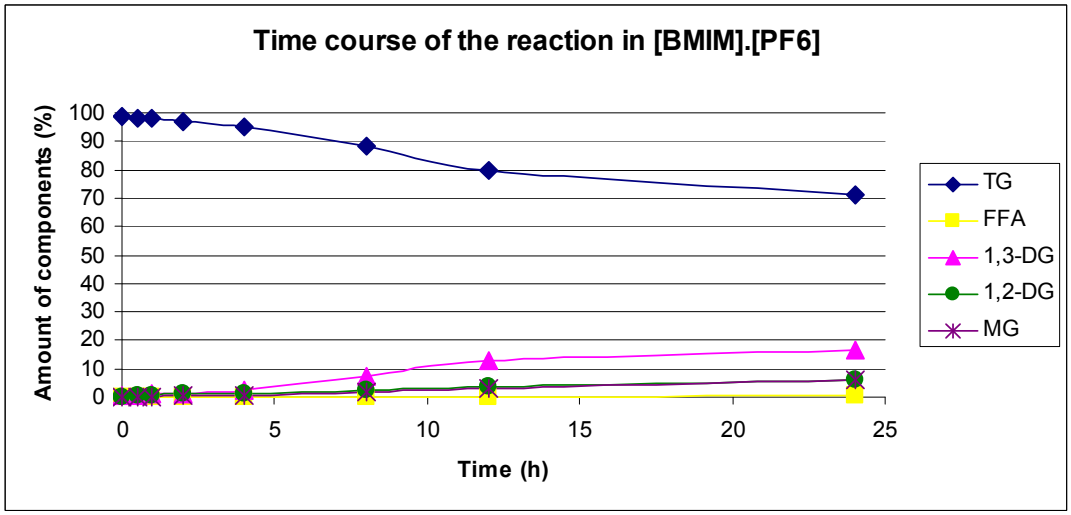


Figure E.2.2: Time course of the reaction in [BMIM].[PF_6].

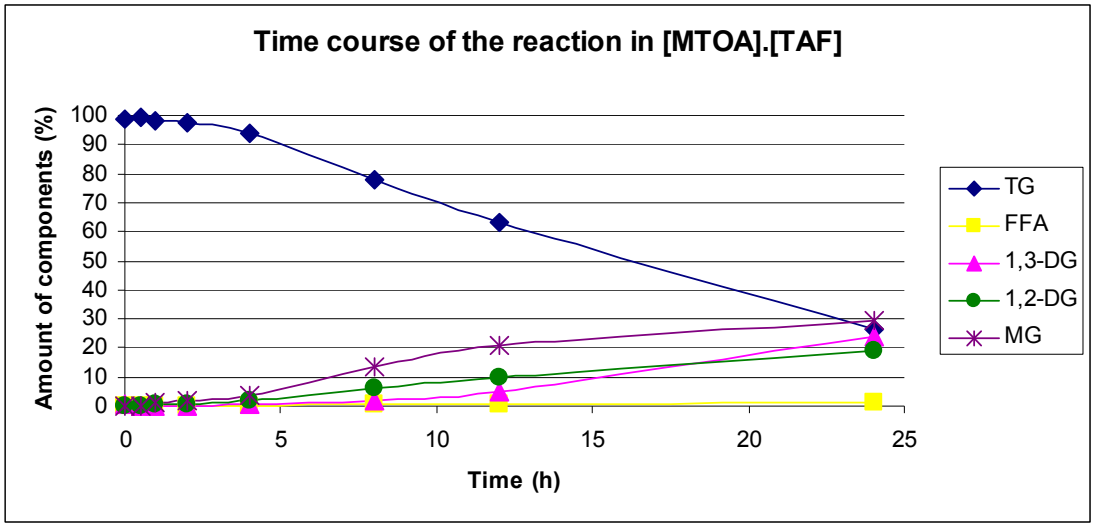


Figure E.2.3: Time course of the reaction in [MTOA].[TFA].

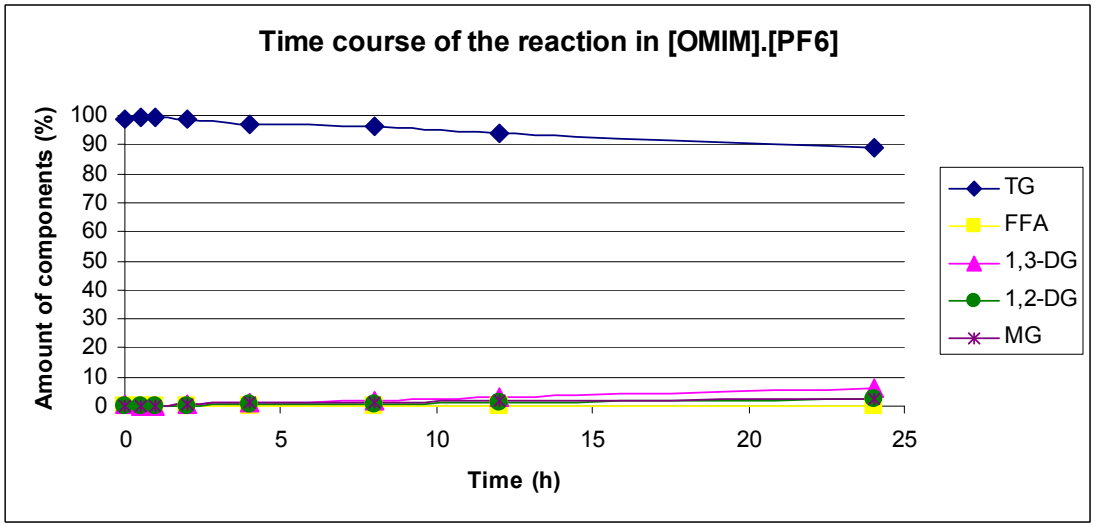


Figure E.2.4: Time course of the reaction in [OMIM].[PF_6].

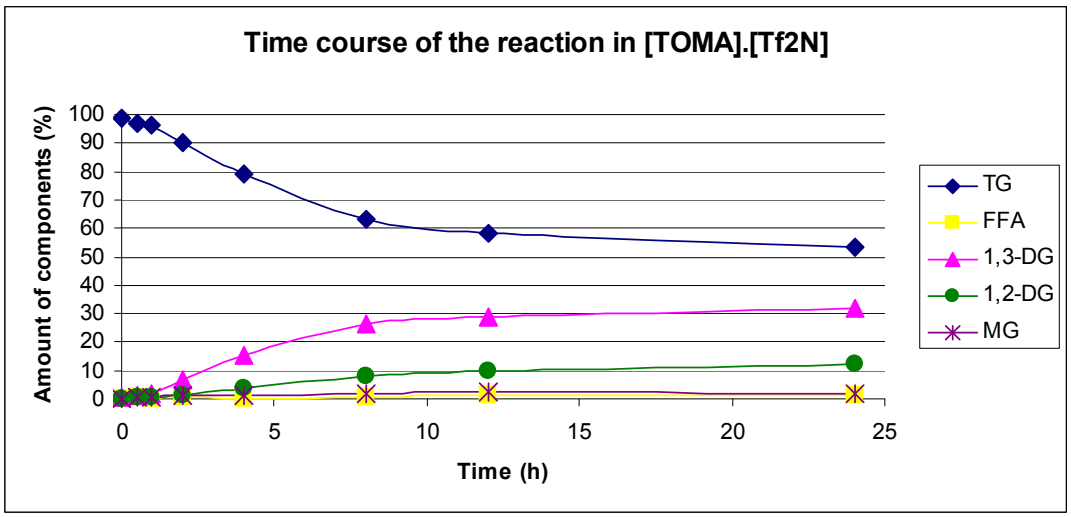


Figure E.2.5: Time course of the reaction in [TOMA].[Tf_2N].

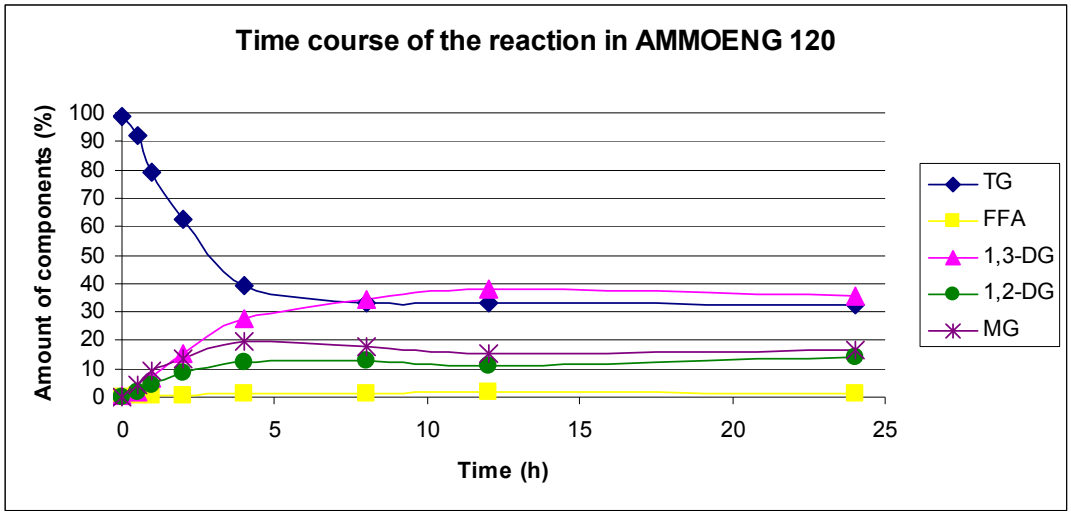


Figure E.2.6: Time course of the reaction in AMMOENG 120.

Appendix E.3. Comparison of the three different levels used to investigate the effect of oil/glycerol ratio.

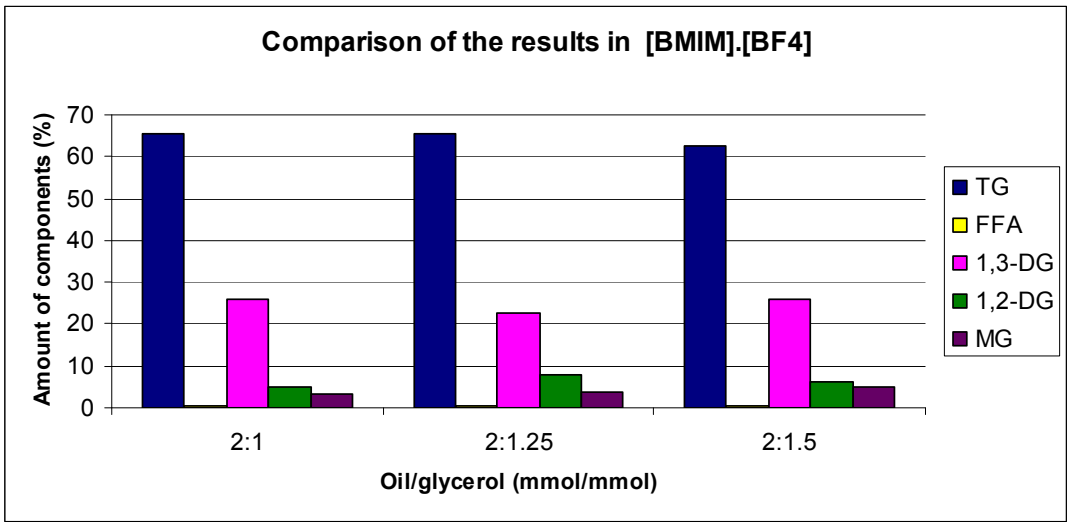


Figure E.3.1: Comparison of the results in [BMIM].[BF_4].

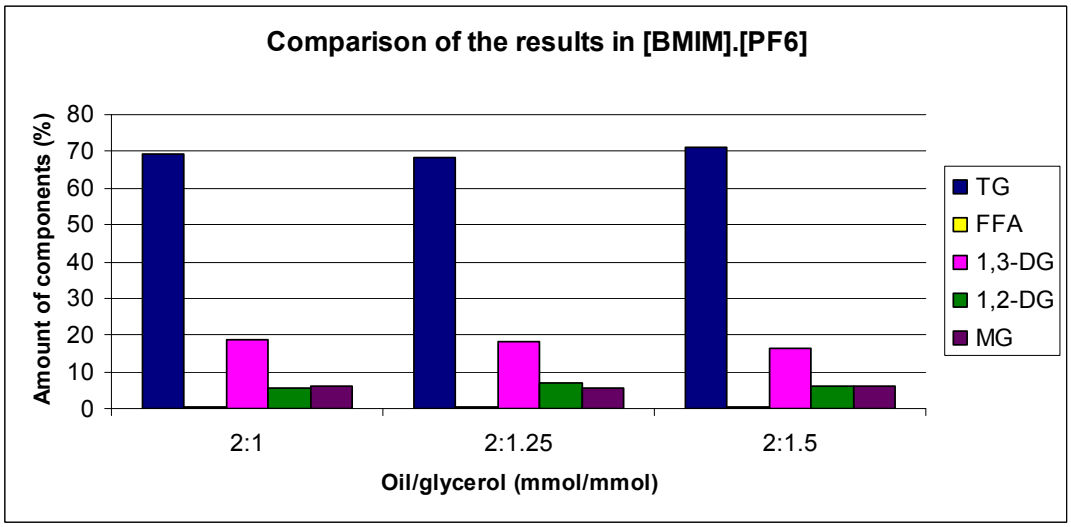


Figure E.3.2: Comparison of the results in [BMIM].[PF_6].

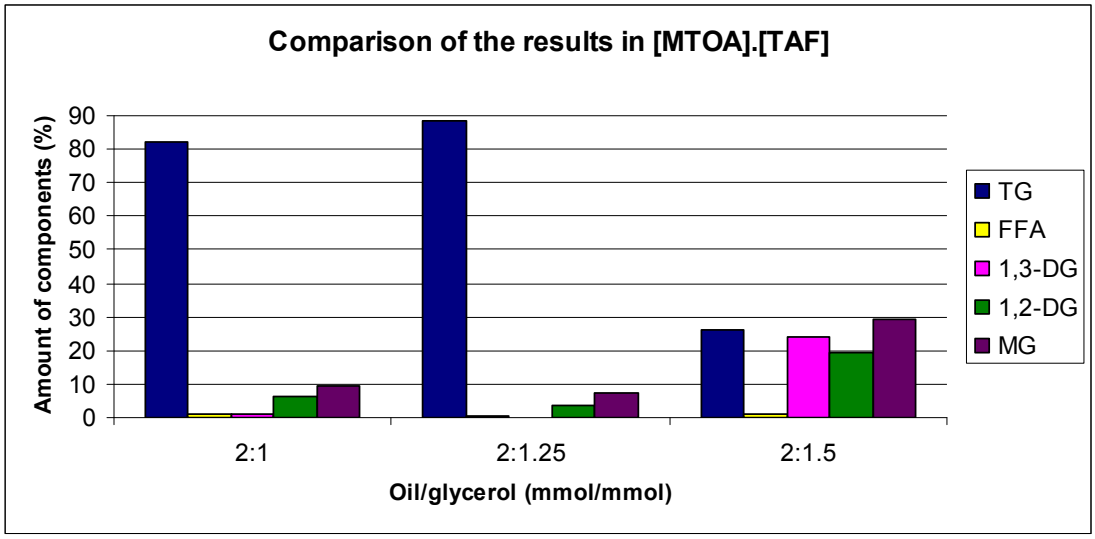


Figure E.3.3: Comparison of the results in [MTOA].[TFA].

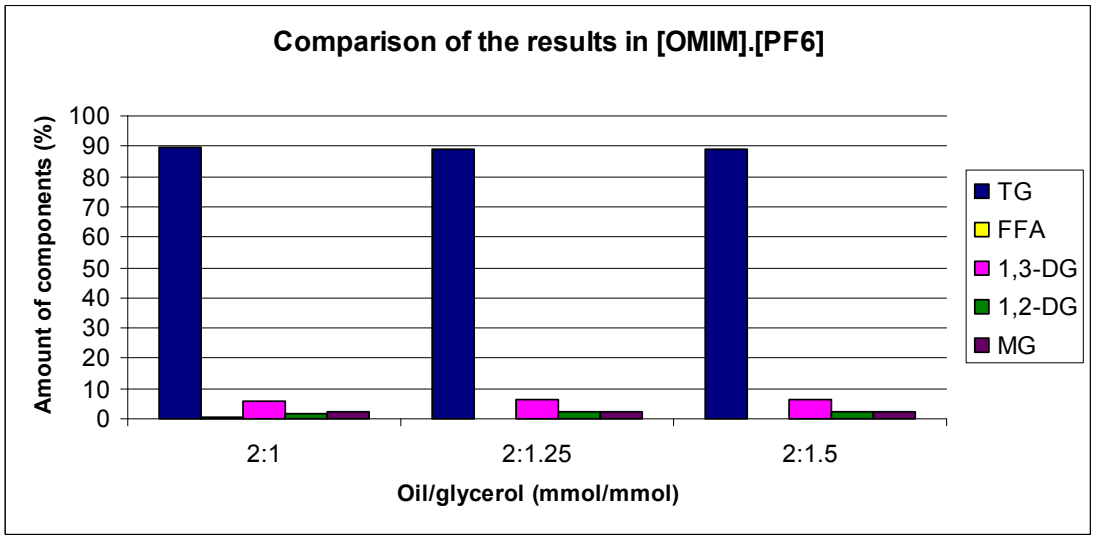


Figure E.3.4: Comparison of the results in [OMIM].[PF_6].

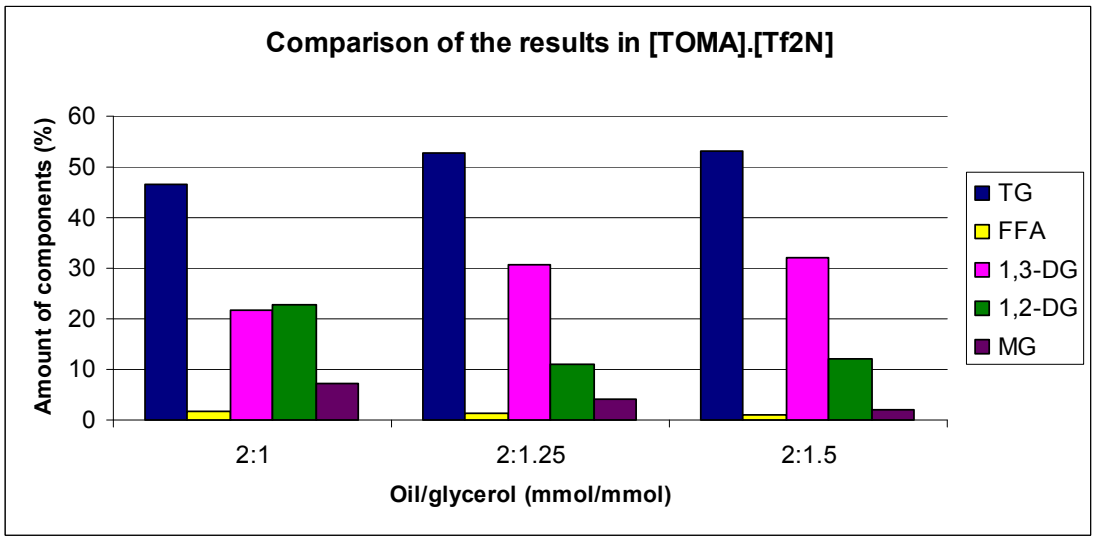


Figure E.3.5: Comparison of the results in [TOMA].[Tf_2N].

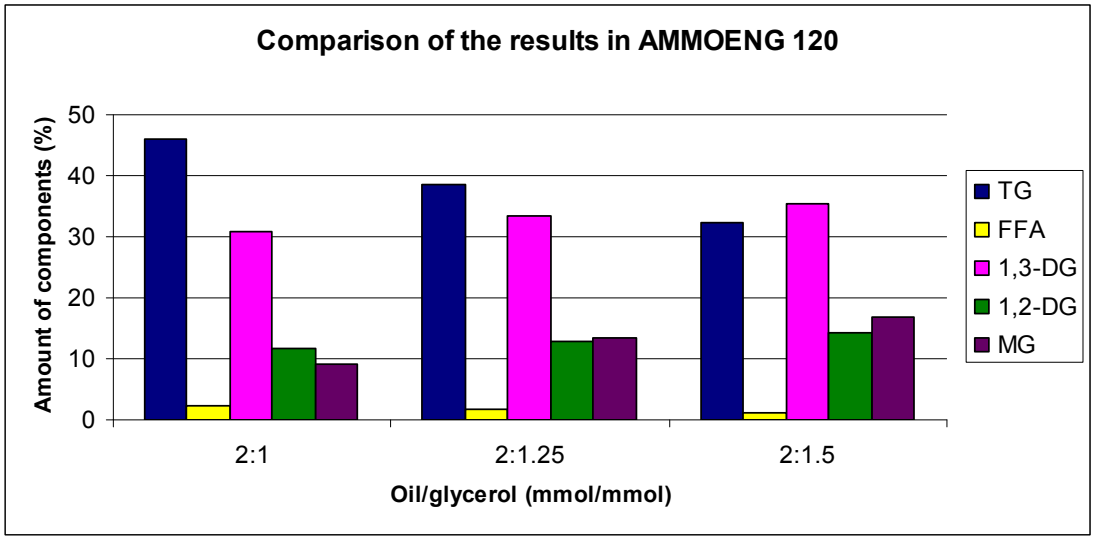


Figure E.3.6: Comparison of the results in AMMOENG 120.

Appendix F.1. Time courses of the glycerolysis reactions performed to investigate the effect of lipase type.

Reaction conditions: 2 mmol of oil, 1.5 mmol of glycerol, Lipozyme RM IM in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 24 h, 700 rpm

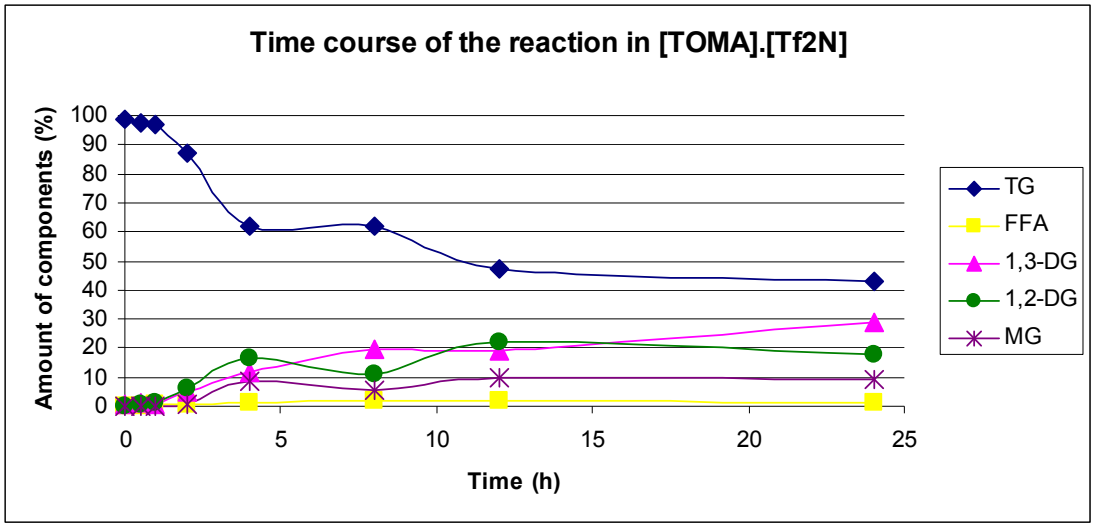


Figure F.1.1: Time course of the reaction in [TOMA].[Tf_2N].

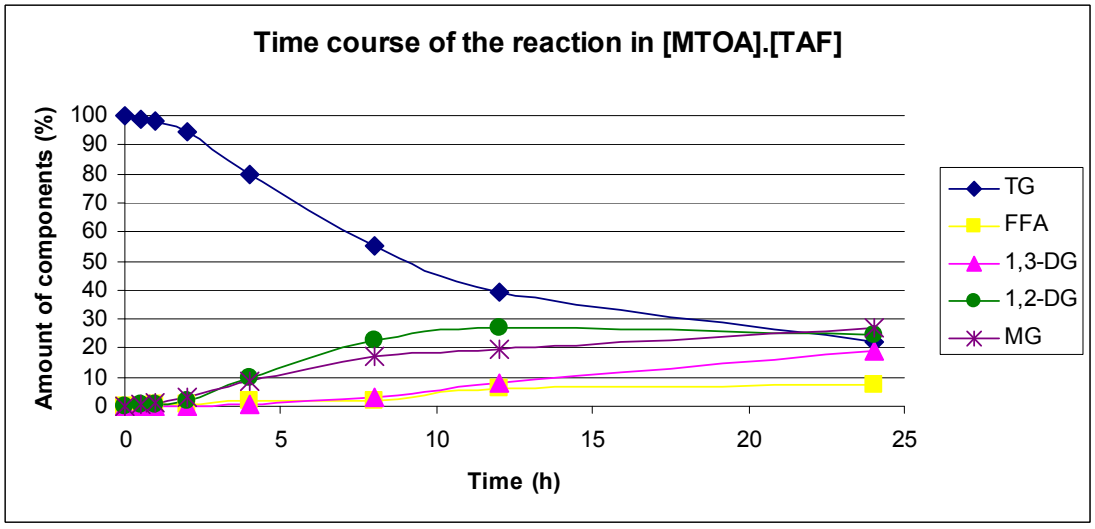


Figure F.1.2: Time course of the reaction in [MTOA].[TFA].

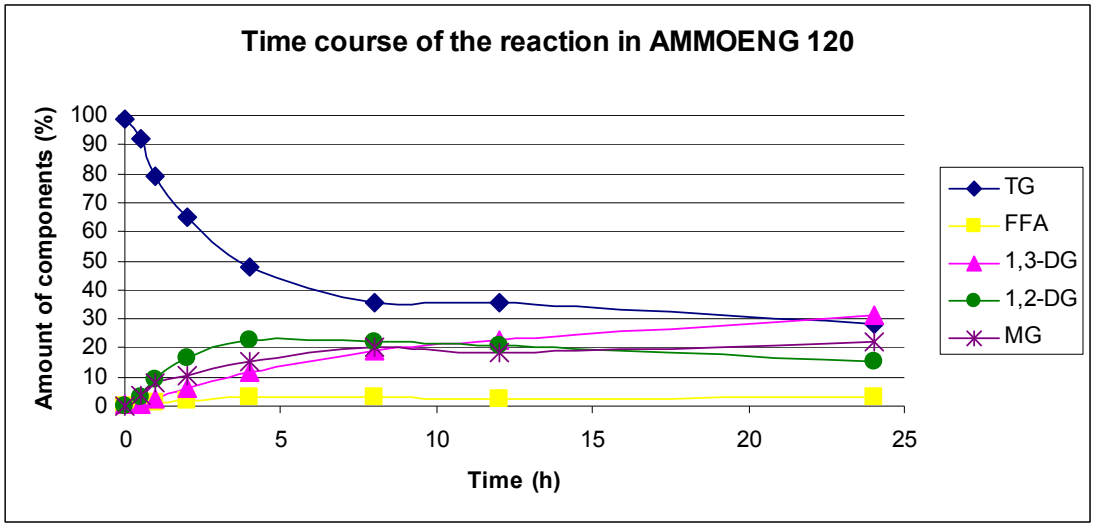


Figure F.1.3: Time course of the reaction in AMMOENG 120.

Appendix F.2. Time courses of the glycerolysis reactions performed to investigate the effect of lipase type.

Reaction conditions: 2 mmol of oil, 1.5 mmol of glycerol, Lipozyme TL IM in the amount of 10 wt % based on the oil mass, 1 g IL, 60°C, 24 h, 700 rpm

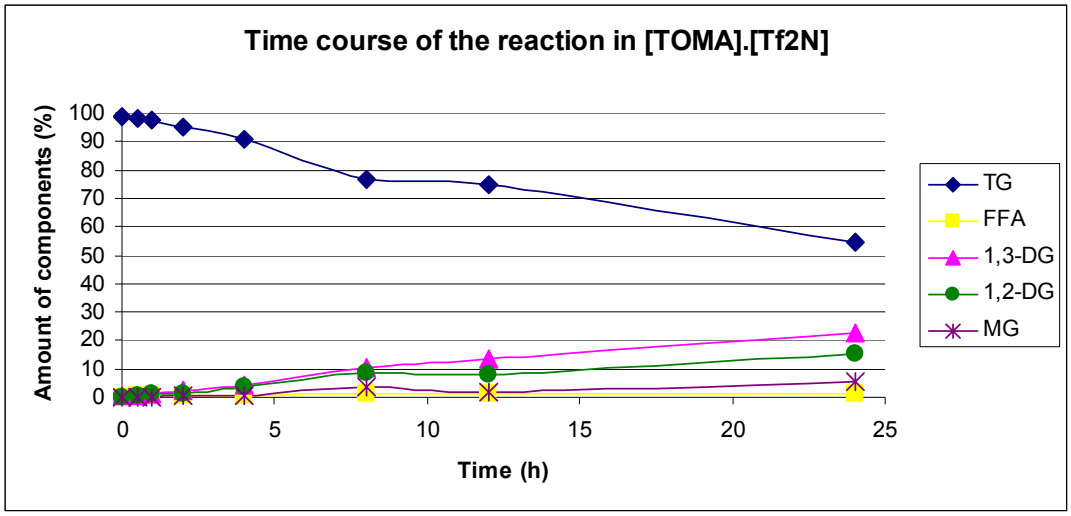


Figure F.2.1: Time course of the reaction in [TOMA].[Tf_2N].

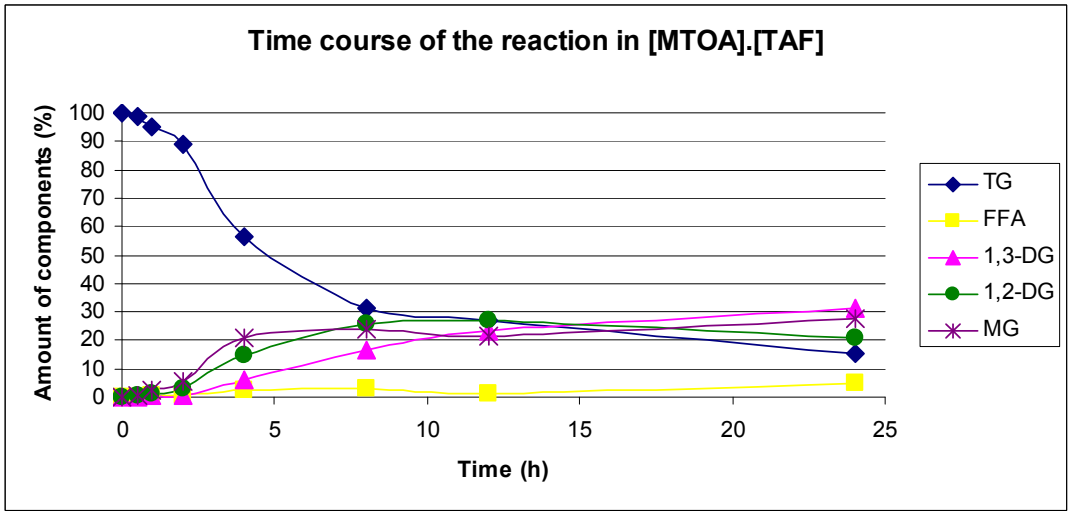


Figure F.2.2: Time course of the reaction in [MTOA].[TFA].

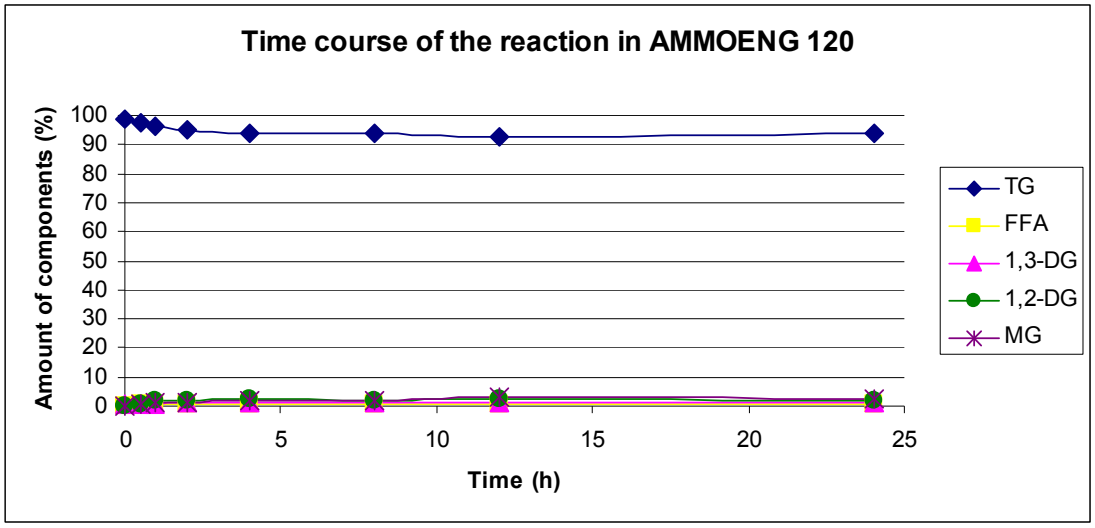


Figure F.2.3: Time course of the reaction in AMMOENG 120.

Appendix F.3. Comparison of the three different levels used to investigate the effect of lipase type.

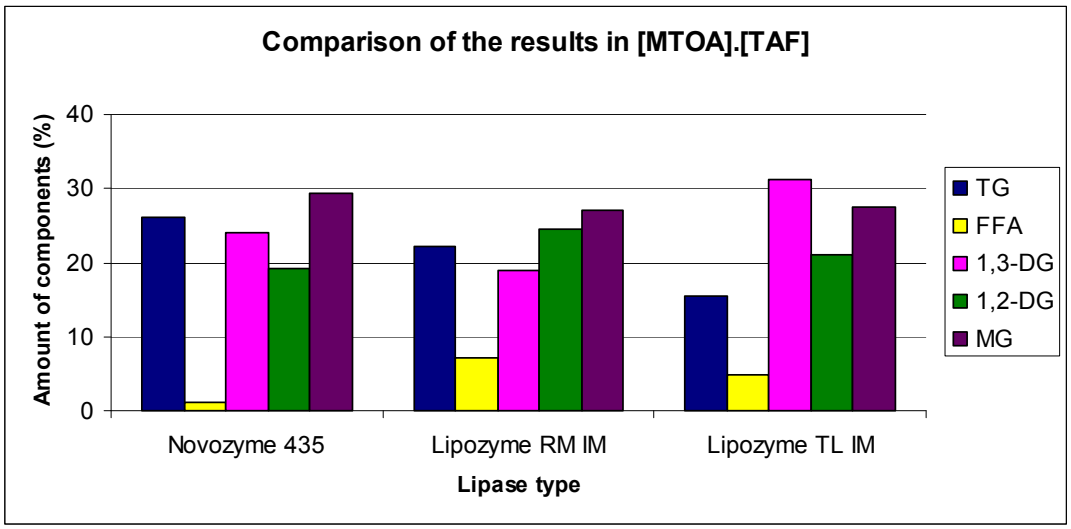


Figure F.3.1: Comparison of the results in [MTOA].[TFA].

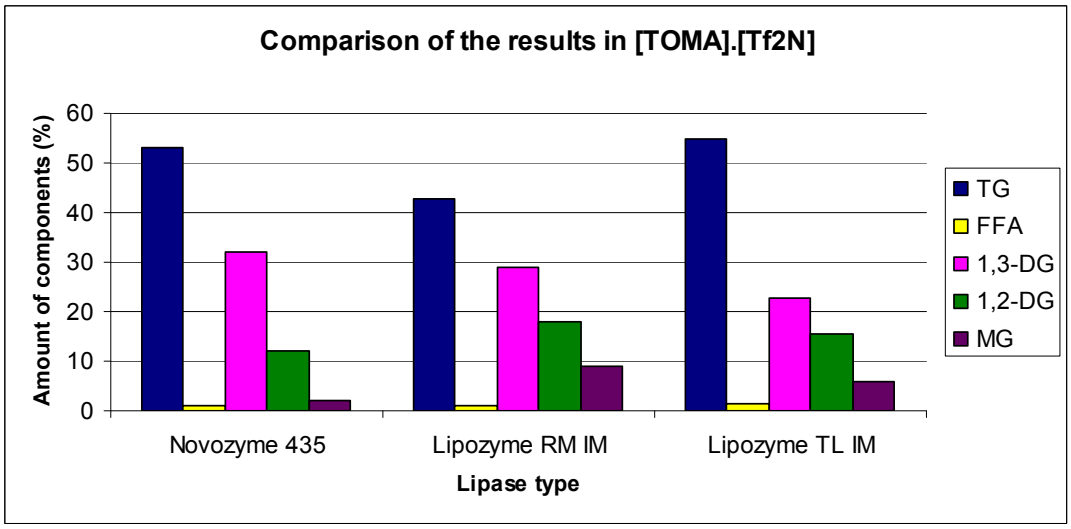


Figure F.3.2: Comparison of the results in [TOMA].[Tf_2N].

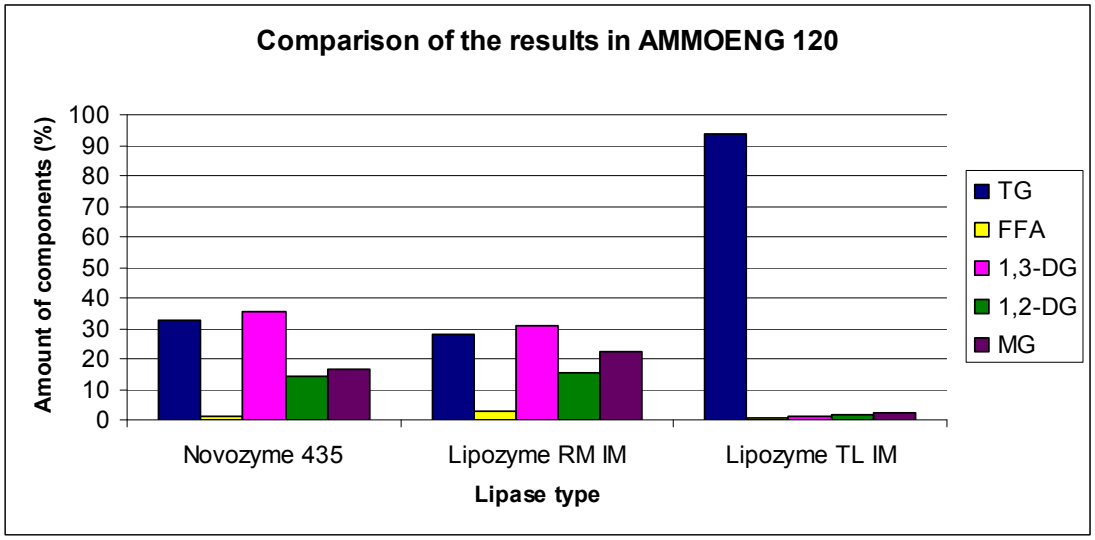


Figure F.3.3: Comparison of the results in AMMOENG 120.

Appendix G.1. Time courses of the glycerolysis reactions performed in *n*-hexane/IL system.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.5 mL *n*-hexane, 0.5 mL IL, 50°C, 24 h, 700 rpm

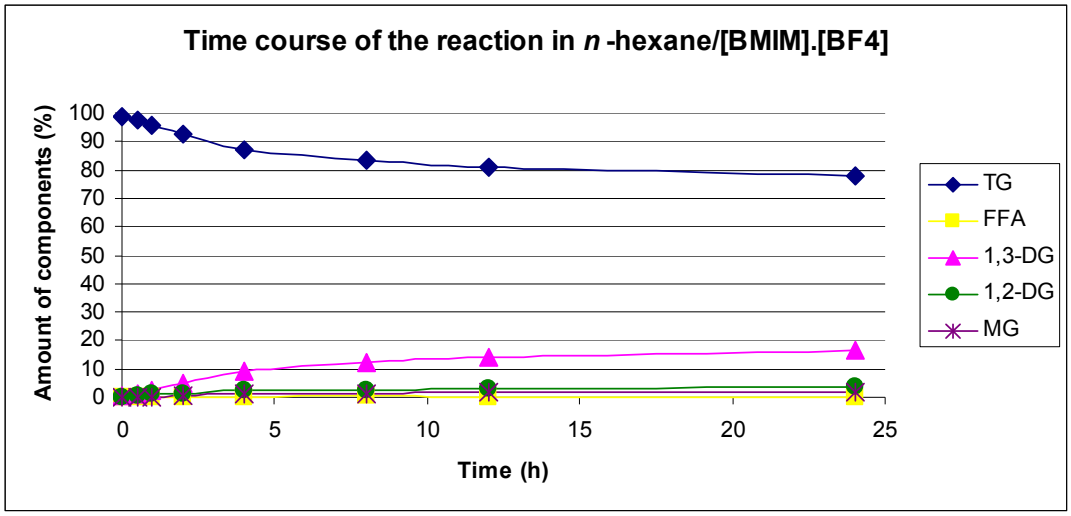


Figure G.1.1: Time course of the reaction in *n*-hexane/[BMIM].[BF_4].

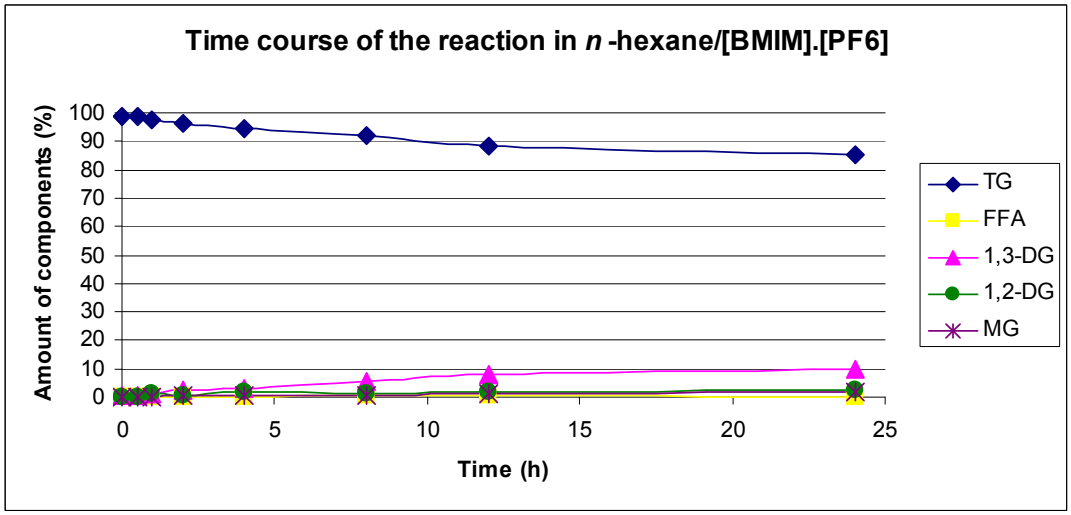


Figure G.1.2: Time course of the reaction in *n*-hexane/[BMIM].[PF_6].

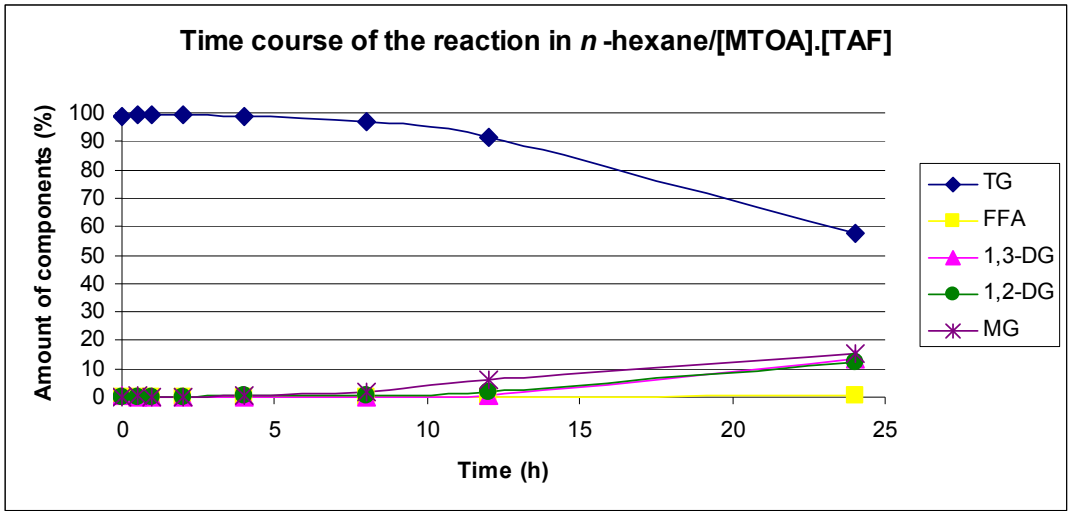


Figure G.1.3: Time course of the reaction in *n*-hexane/[MTOA].[TFA].

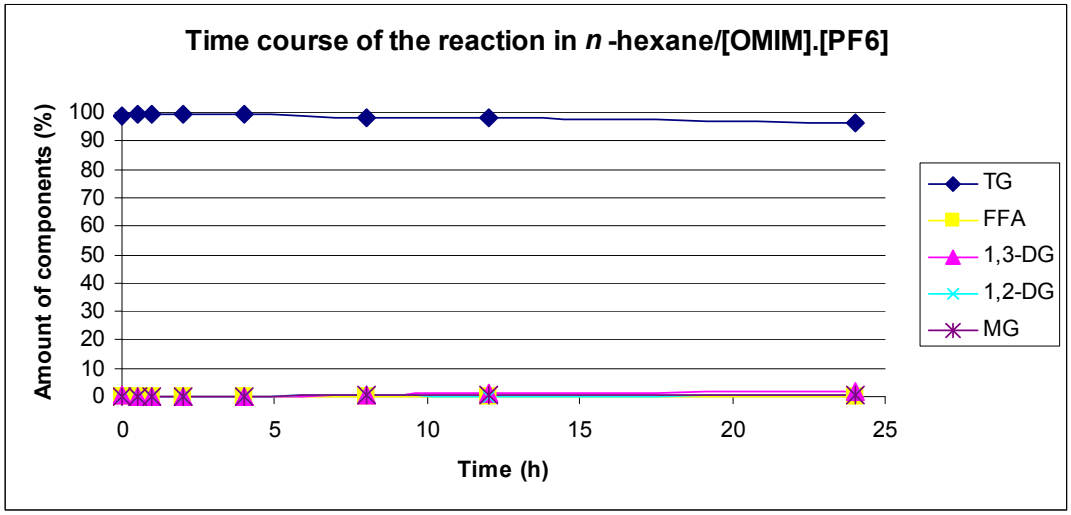


Figure G.1.4: Time course of the reaction in *n*-hexane/[OMIM].[PF_6].

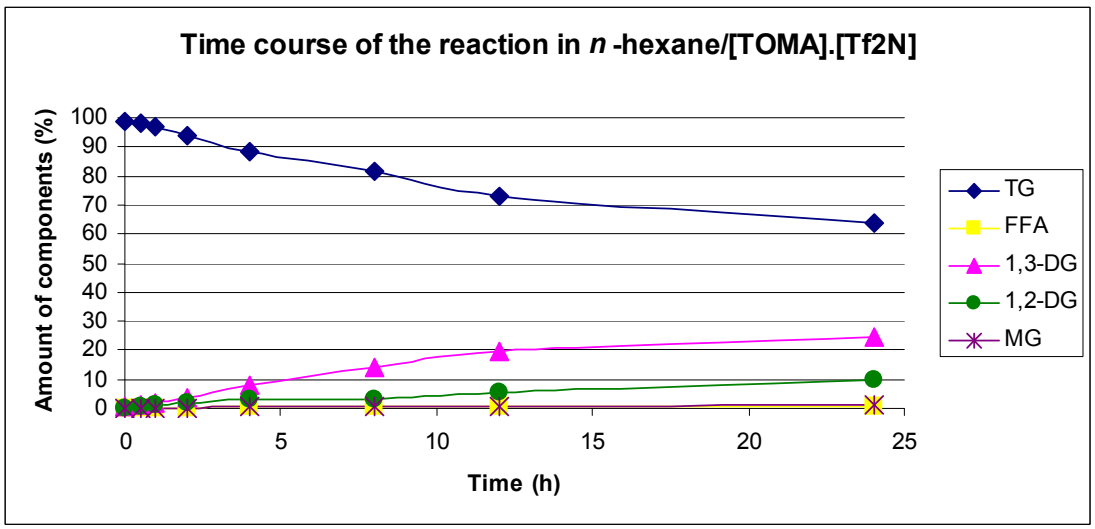


Figure G.1.5: Time course of the reaction in *n*-hexane/[TOMA].[Tf_2N].

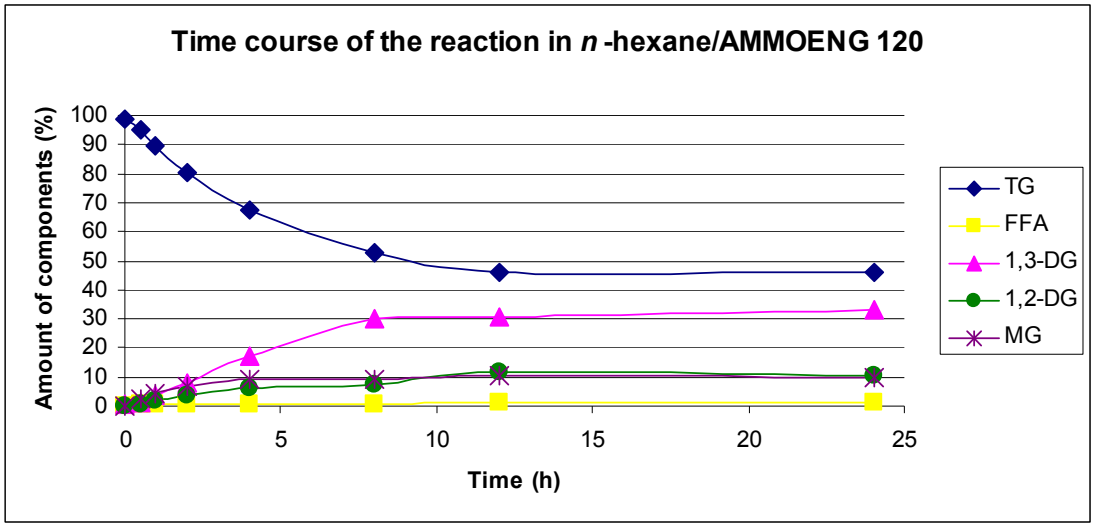


Figure G.1.6: Time course of the reaction in *n*-hexane/AMMOENG 120.

Appendix G.2. Comparison of the pure IL systems with *n*-hexane/IL systems.

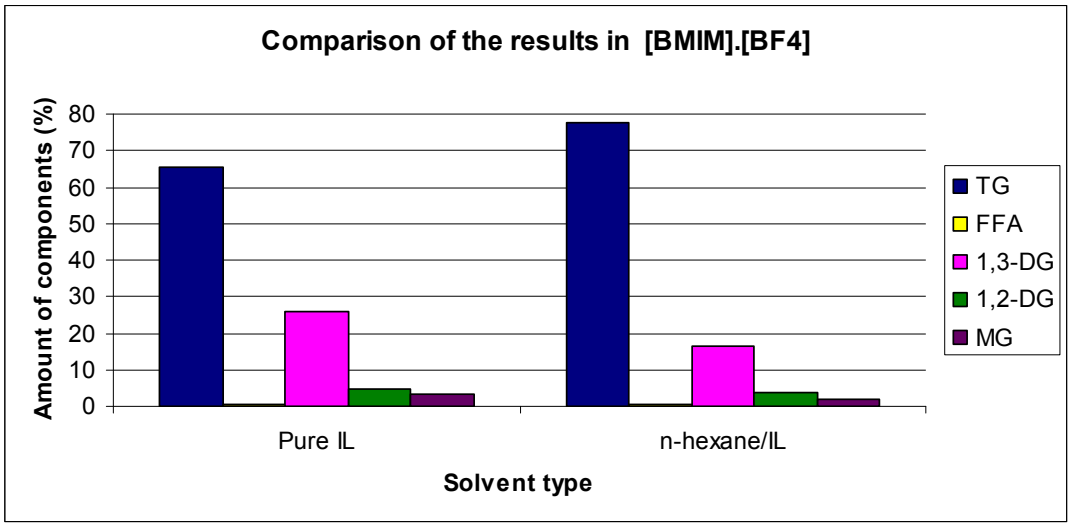


Figure G.2.1: Comparison of the results in [BMIM].[BF_4].

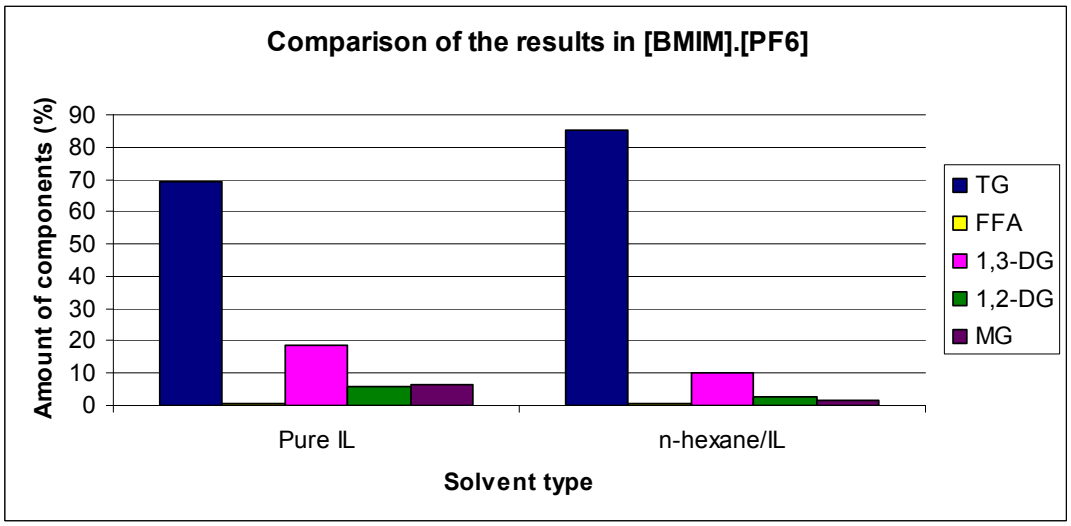


Figure G.2.2: Comparison of the results in [BMIM].[PF_6].

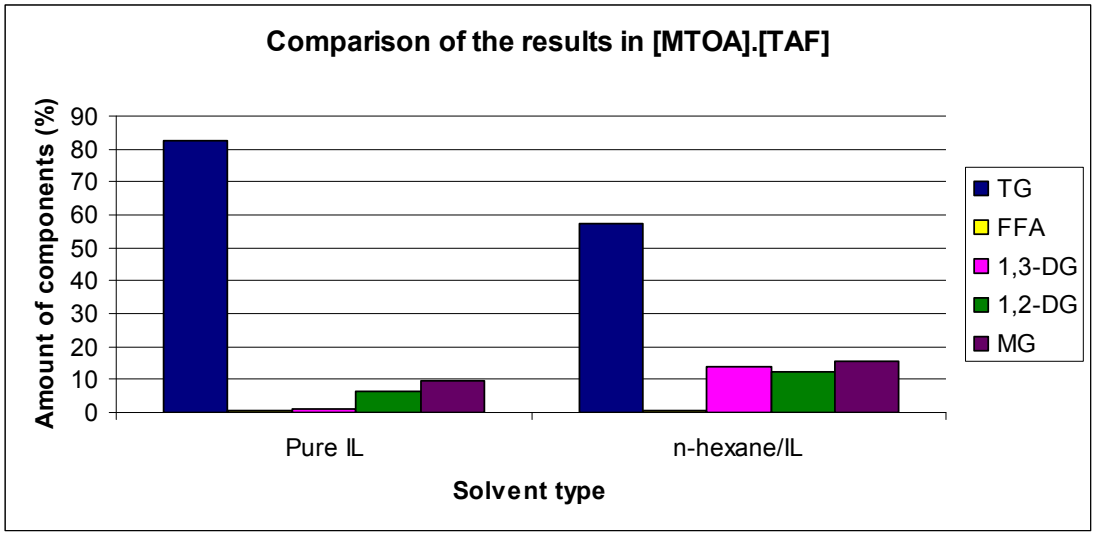


Figure G.2.3: Comparison of the results in [MTOA].[TFA].

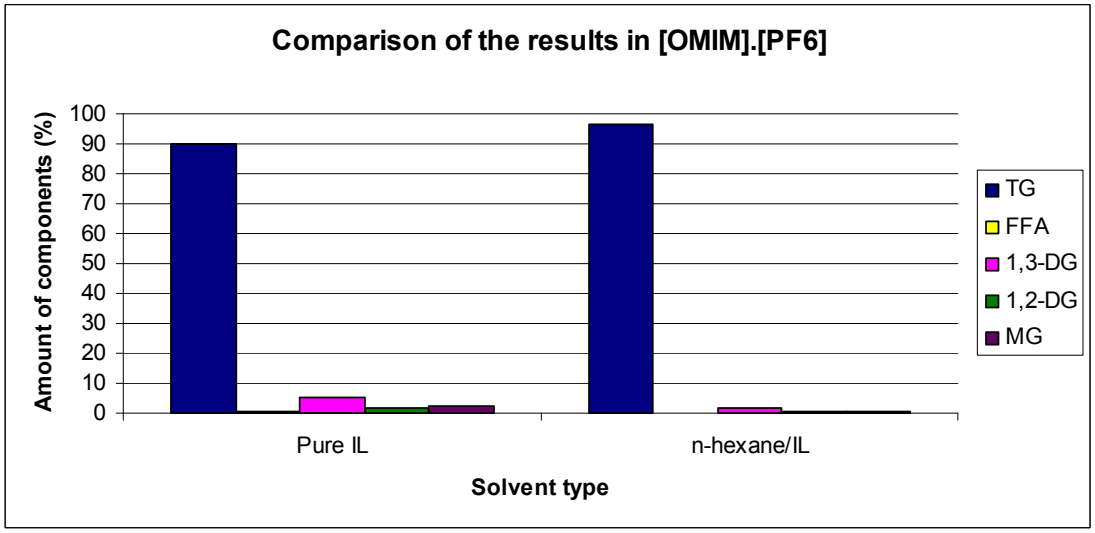


Figure G.2.4: Comparison of the results in [OMIM].[PF_6].

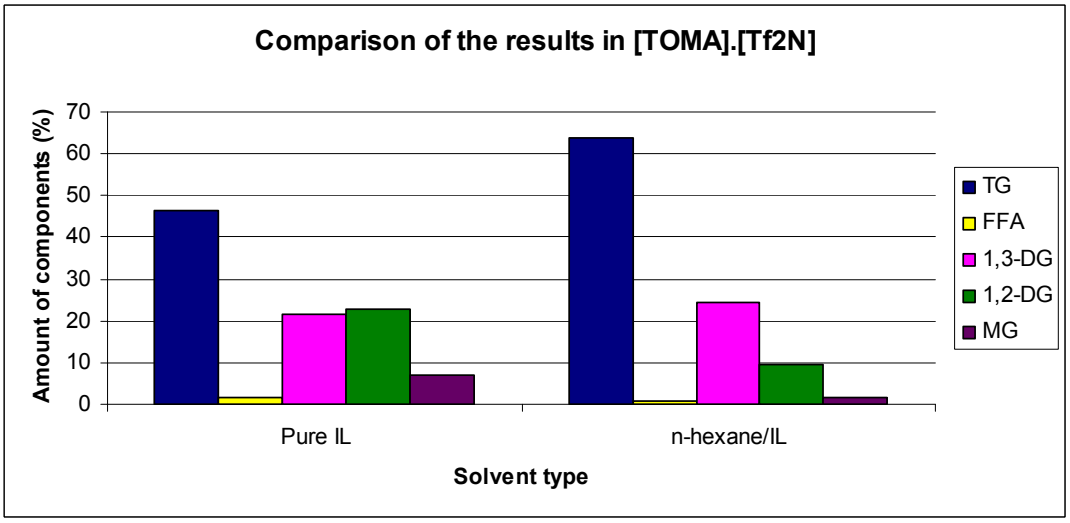


Figure G.2.5: Comparison of the results in [TOMA].[Tf_2N].

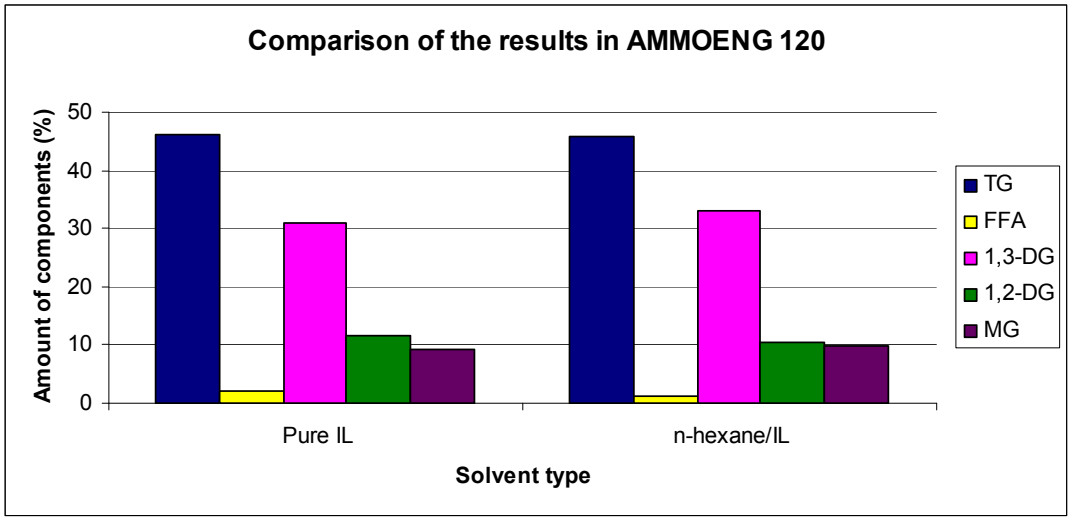


Figure G.2.6: Comparison of the results in AMMOENG 120.

Appendix H.1. Time courses of the glycerolysis reactions performed to investigate the effect of temperature on *n*-hexane/IL system.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.5 mL *n*-hexane, 0.5 mL IL, 40°C, 24 h, 700 rpm

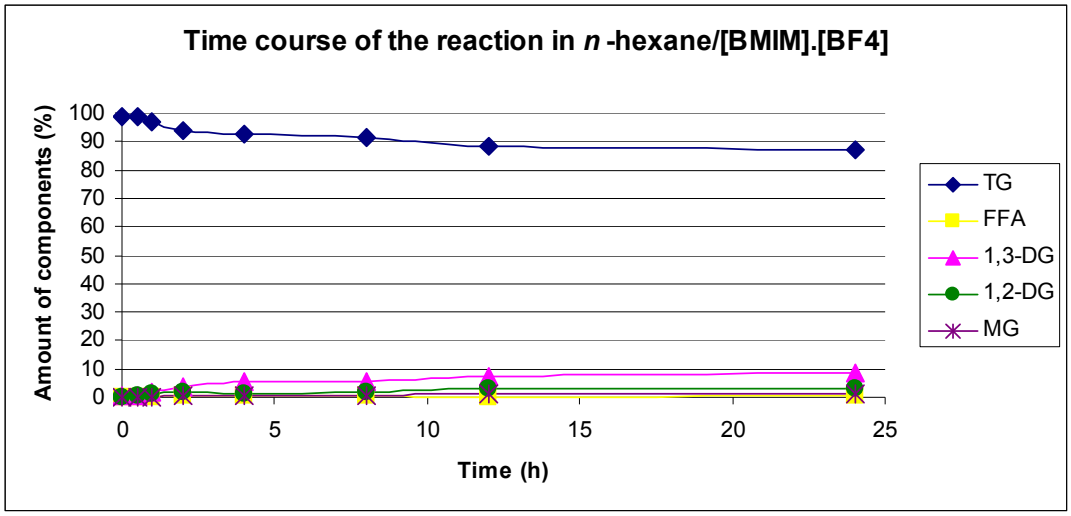


Figure H.1.1: Time course of the reaction in *n*-hexane/[BMIM].[BF_4].

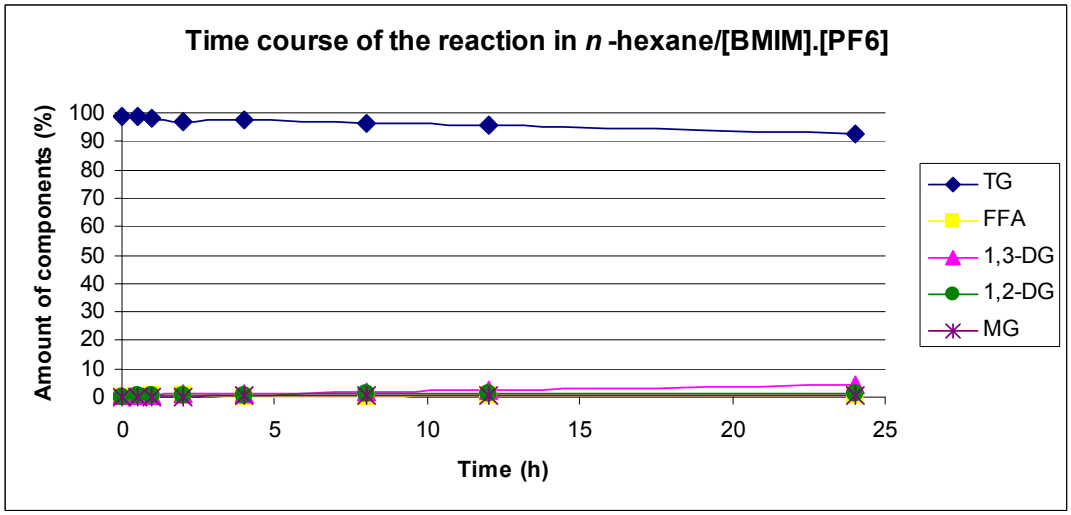


Figure H.1.2: Time course of the reaction in *n*-hexane/[BMIM].[PF_6].

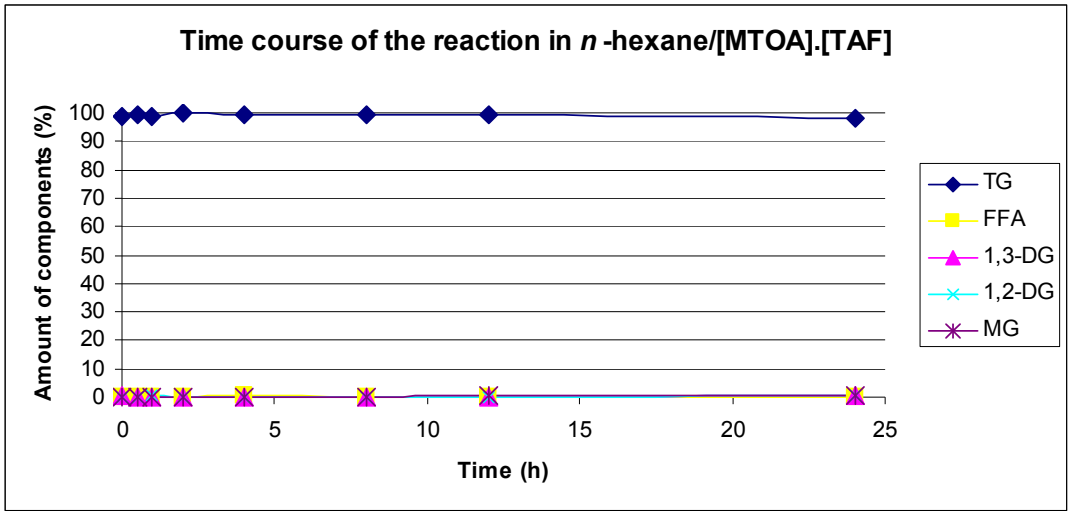


Figure H.1.3: Time course of the reaction in *n*-hexane/[MTOA].[TFA].

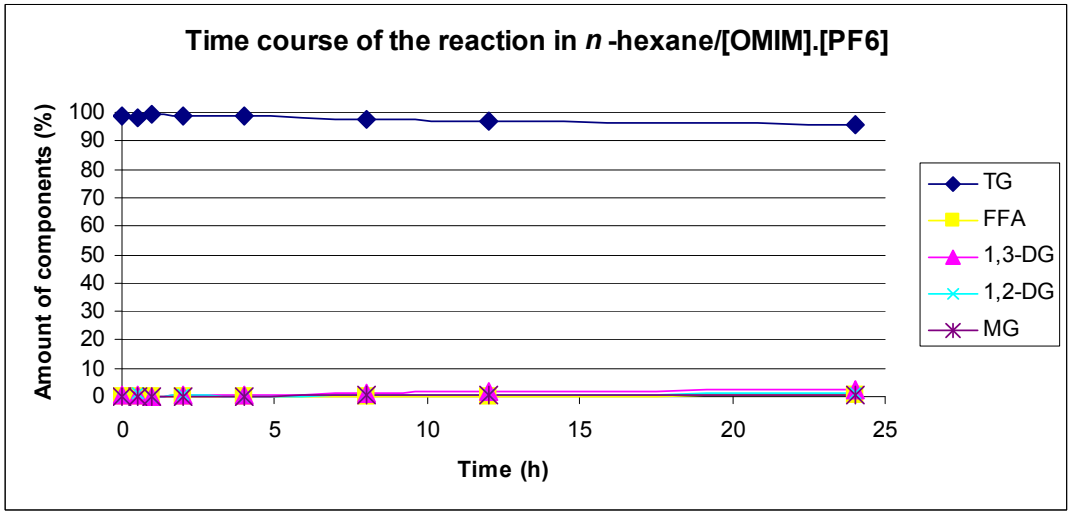


Figure H.1.4: Time course of the reaction in *n*-hexane/[OMIM].[PF_6].

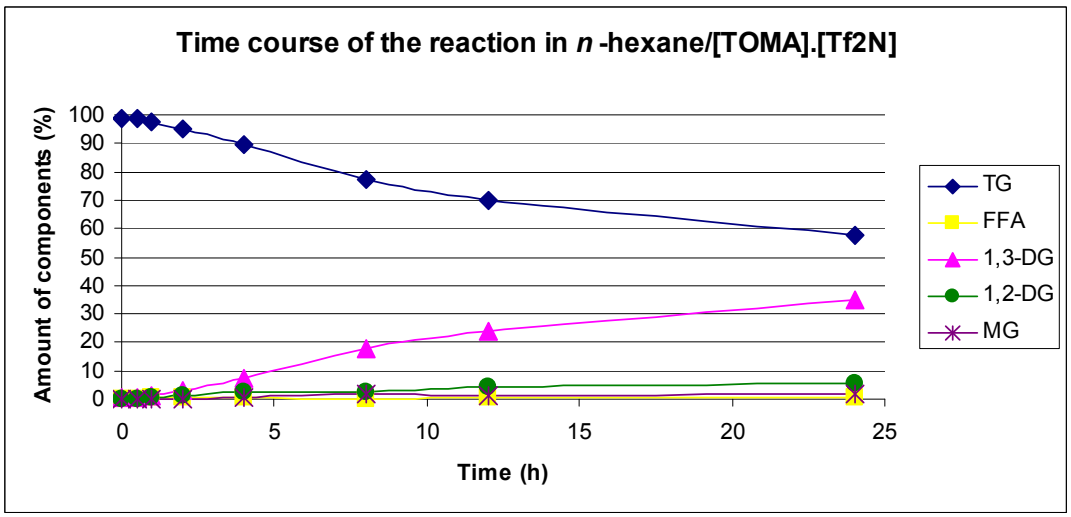


Figure H.1.5: Time course of the reaction in *n*-hexane/[TOMA].[Tf_2N].

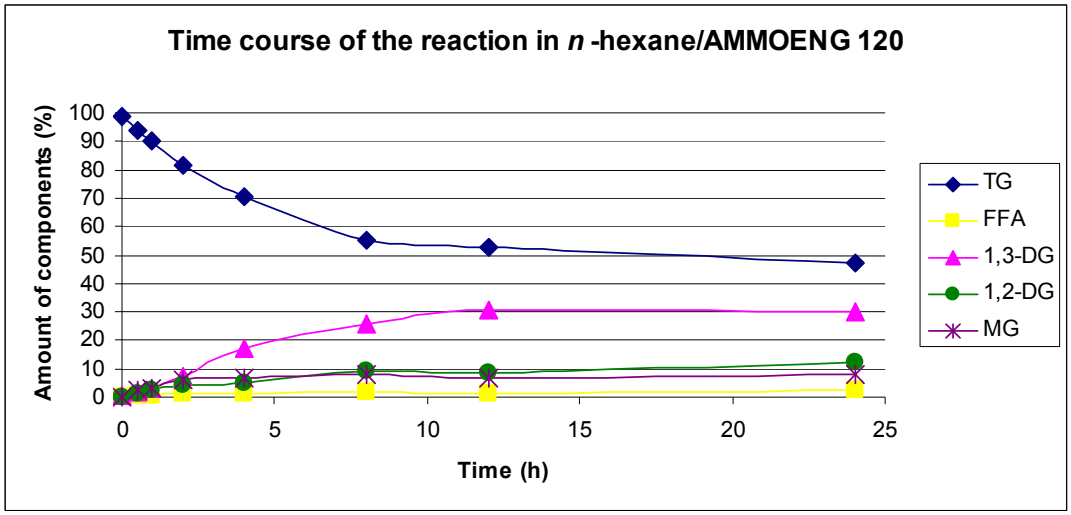


Figure H.1.6: Time course of the reaction in *n*-hexane/AMMOENG 120.

Appendix H.2. Time courses of the glycerolysis reactions performed to investigate the effect of temperature on *n*-hexane/IL system.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.5 mL *n*-hexane, 0.5 mL IL, 60°C, 24 h, 700 rpm

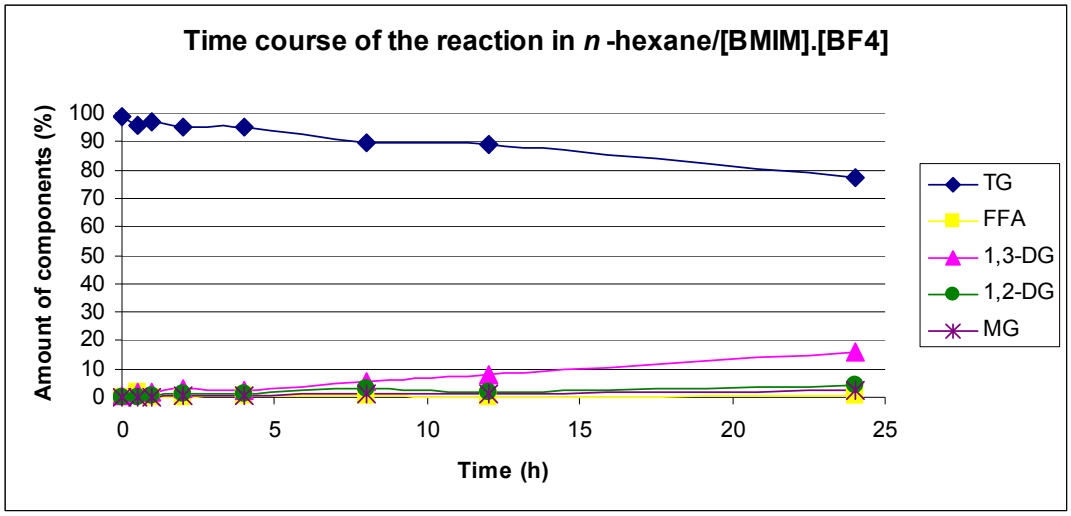


Figure H.2.1: Time course of the reaction in *n*-hexane/[BMIM].[BF_4].

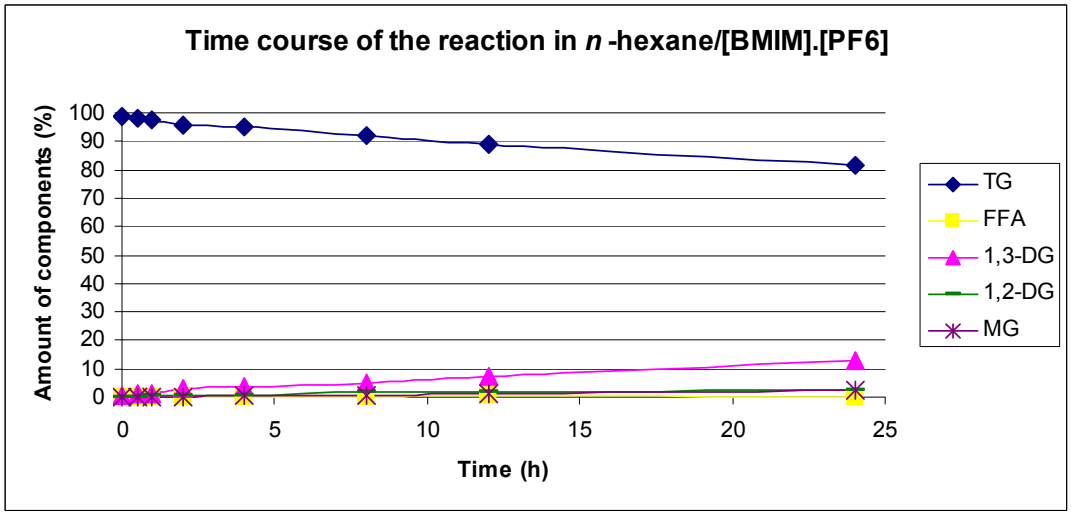


Figure H.2.2: Time course of the reaction in *n*-hexane/[BMIM].[PF_6].

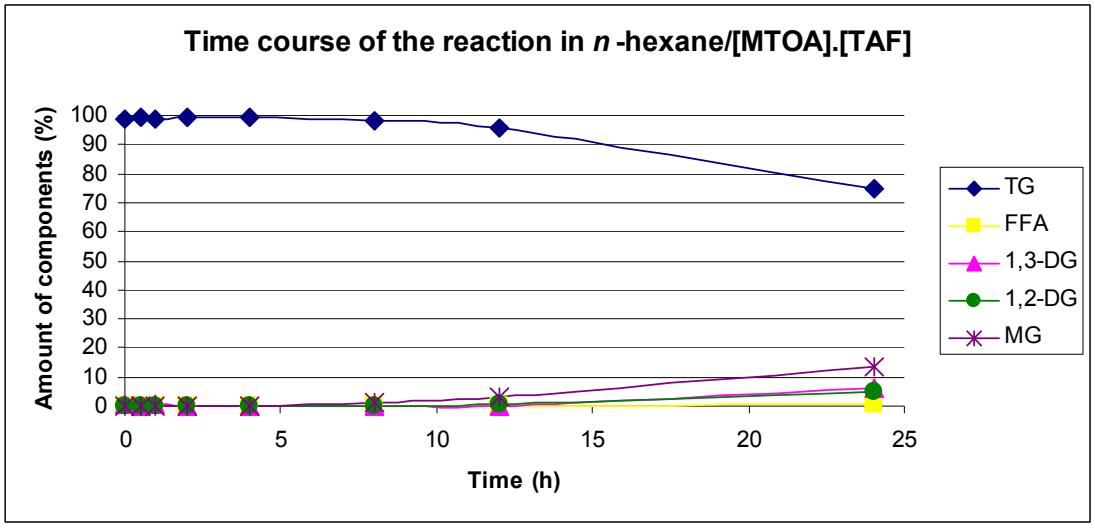


Figure H.2.3: Time course of the reaction in *n*-hexane/[MTOA].[TFA].

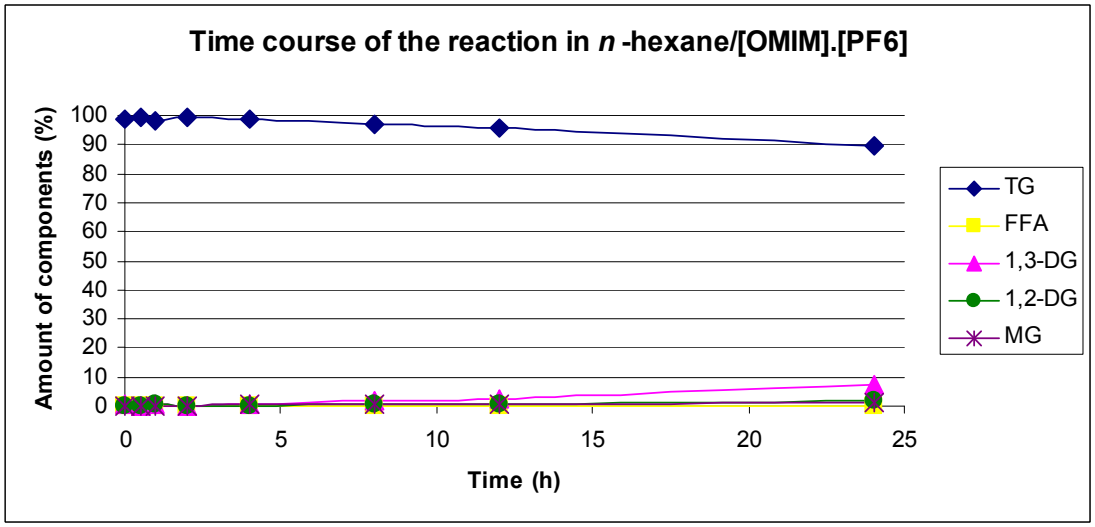


Figure H.2.4: Time course of the reaction in *n*-hexane/[OMIM].[PF_6].

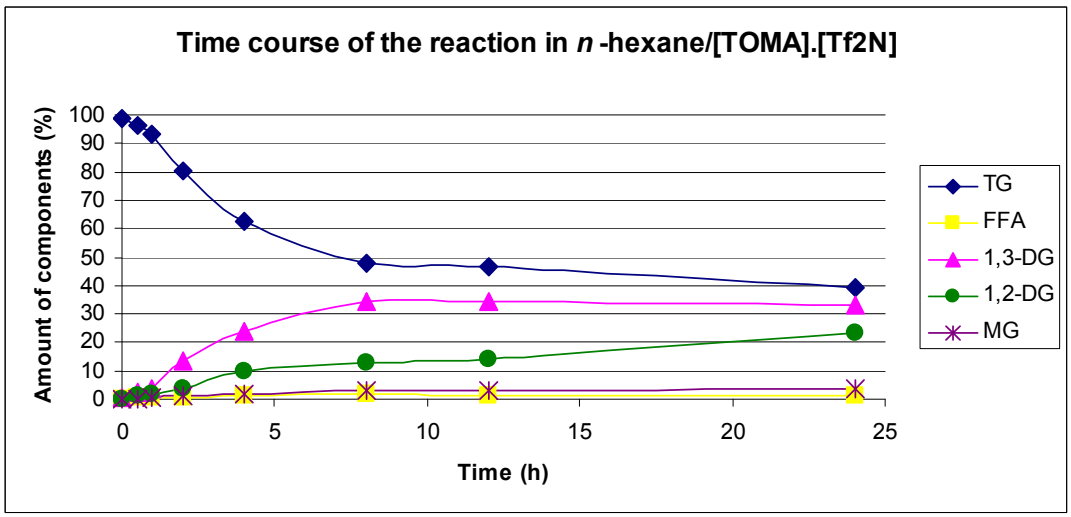


Figure H.2.5: Time course of the reaction in *n*-hexane/[TOMA].[Tf_2N].

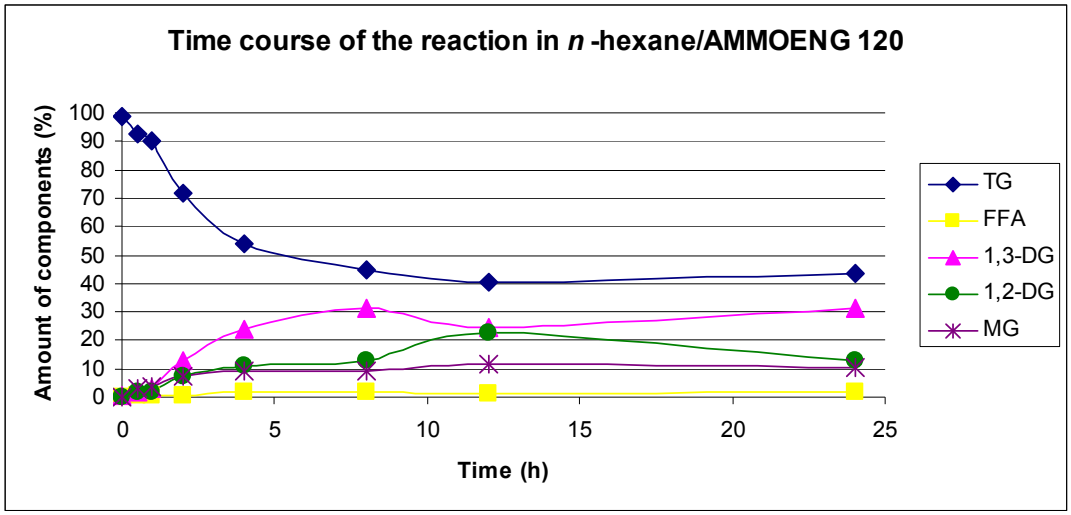


Figure H.2.6: Time course of the reaction in *n*-hexane/AMMOENG 120.

Appendix H.3. Comparison of the three different levels used to investigate the effect of temperature on *n*-hexane/IL system.

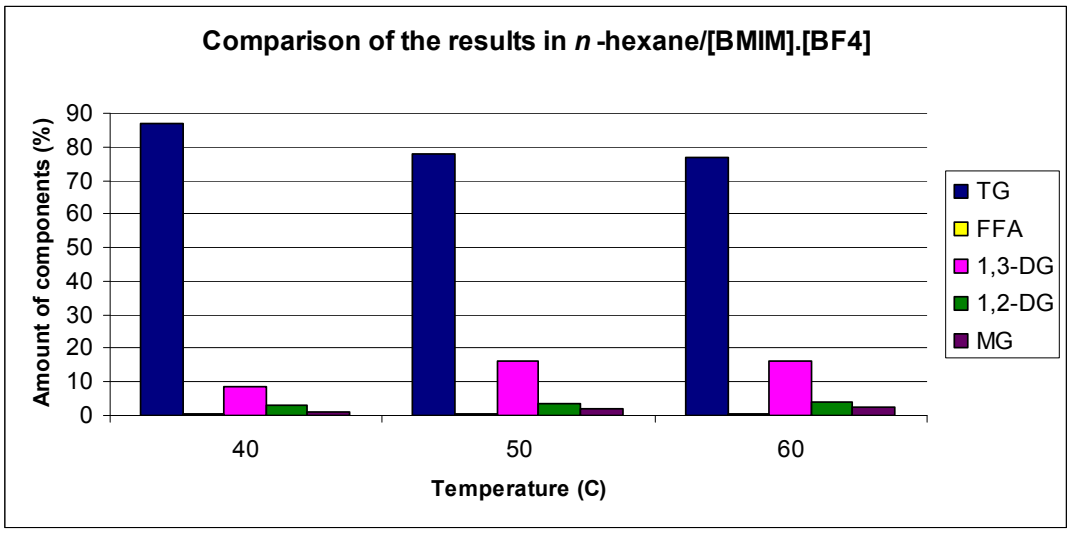


Figure H.3.1: Comparison of the results in *n*-hexane/[BMIM].[BF_4].

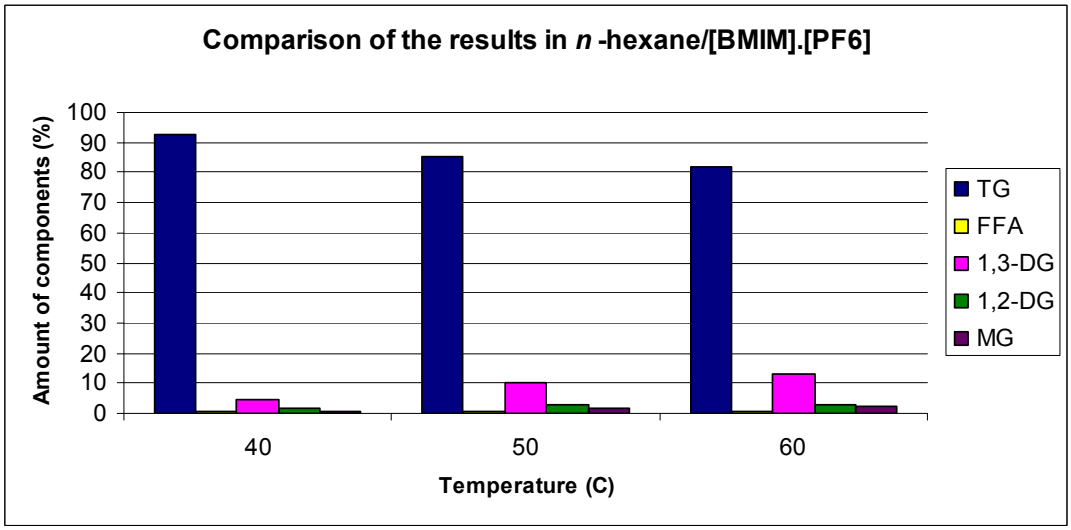


Figure H.3.2: Comparison of the results in *n*-hexane/[BMIM].[PF_6].

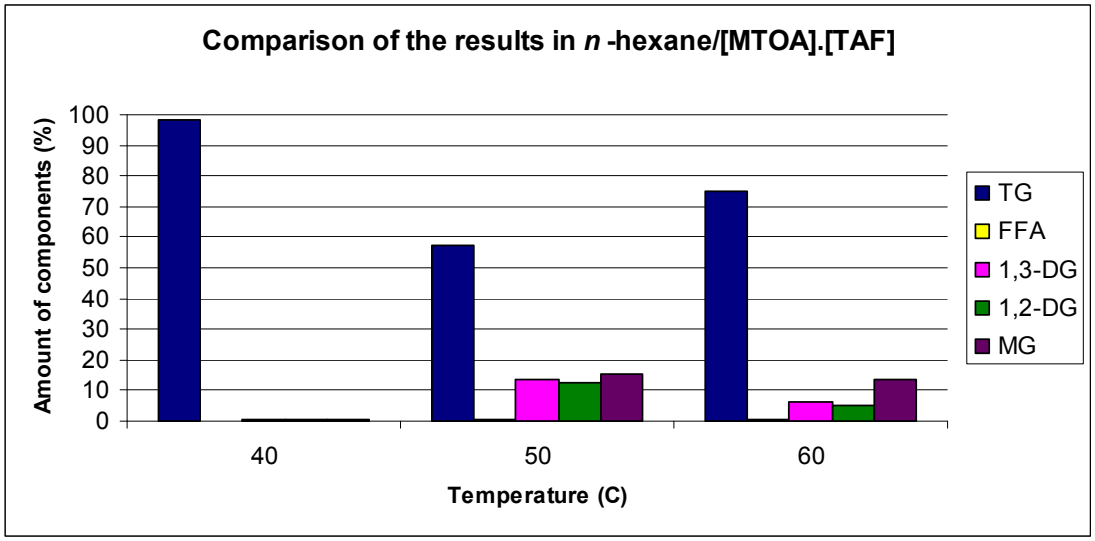


Figure H.3.3: Comparison of the results in *n*-hexane/[MTOA].[TFA].

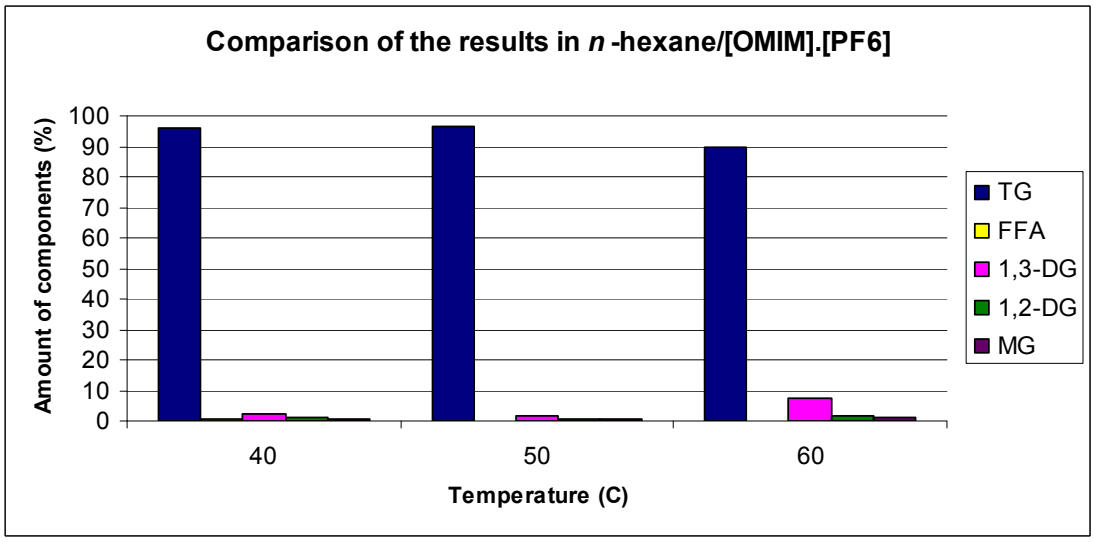


Figure H.3.4: Comparison of the results in *n*-hexane/[OMIM].[PF_6].

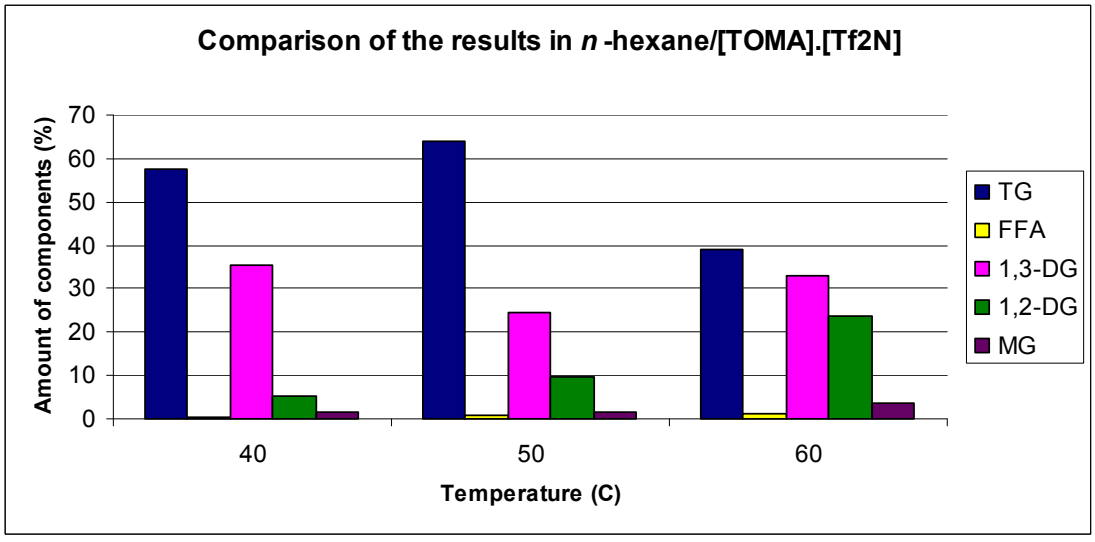


Figure H.3.5: Comparison of the results in *n*-hexane/[TOMA].[Tf_2N].

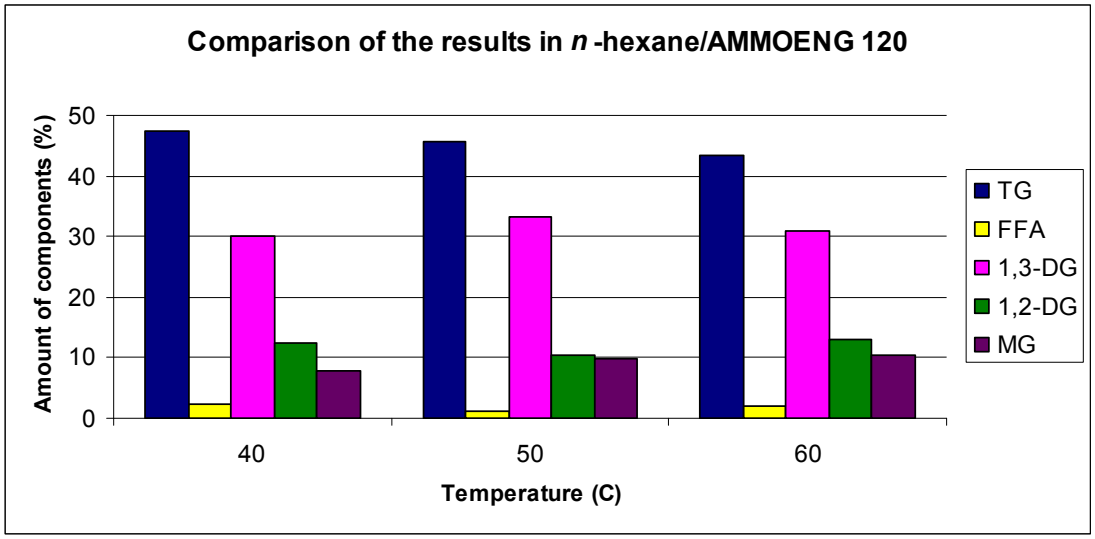


Figure H.3.6: Comparison of the results in *n*-hexane/AMMOENG 120.

Appendix I.1. Time courses of the glycerolysis reactions performed to investigate the effect of *n*-hexane/IL ratio on the *n*-hexane/IL system.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.335 mL *n*-hexane, 0.665 mL IL, 50°C, 24 h, 700 rpm

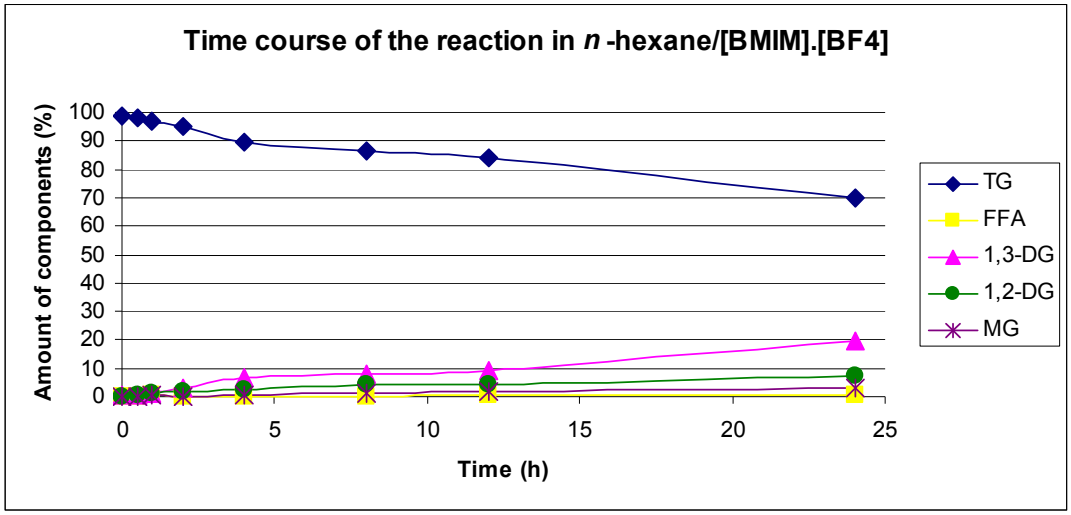


Figure I.1.1: Time course of the reaction in *n*-hexane/[BMIM].[BF_4].

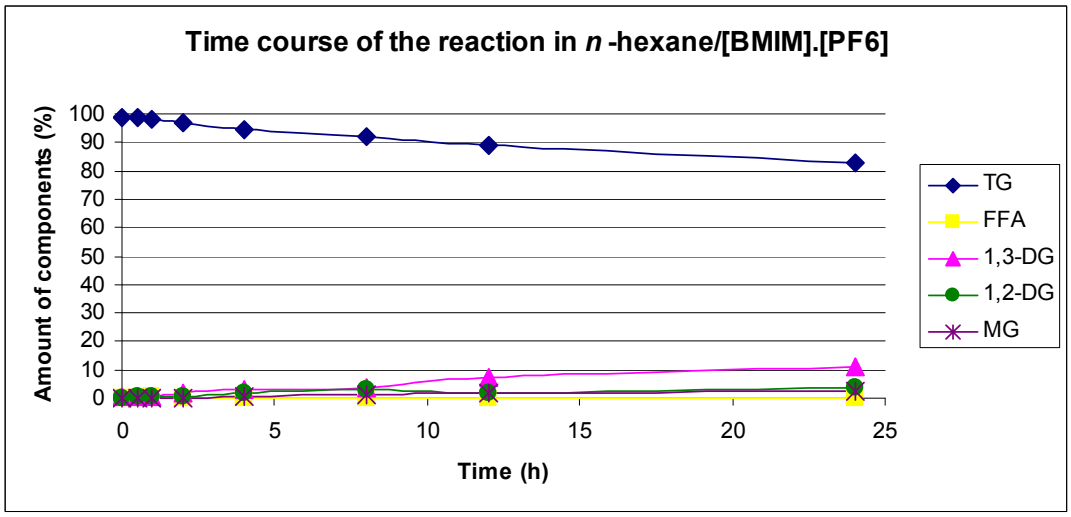


Figure I.1.2: Time course of the reaction in *n*-hexane/[BMIM].[PF_6].

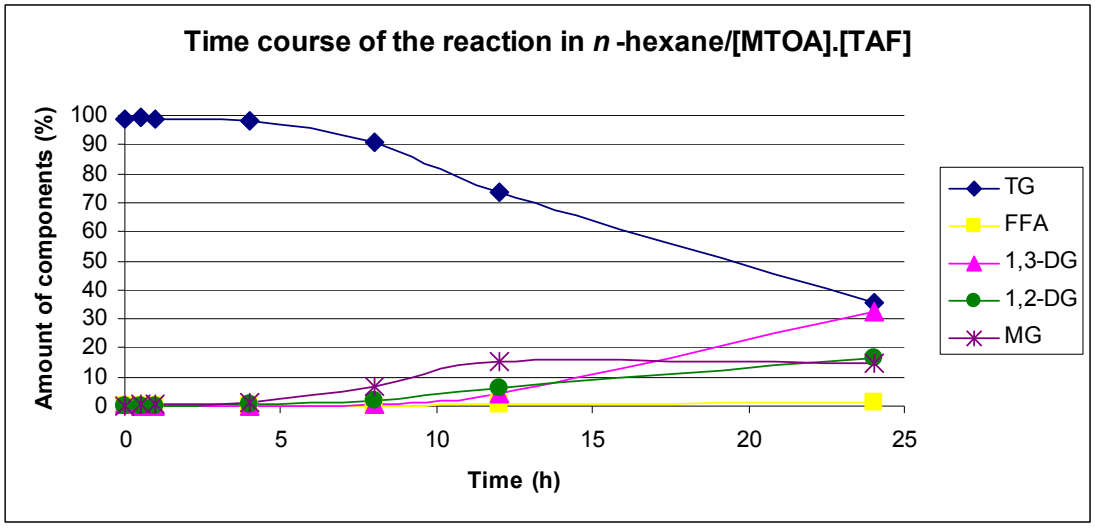


Figure I.1.3: Time course of the reaction in *n*-hexane/[MTOA].[TFA].

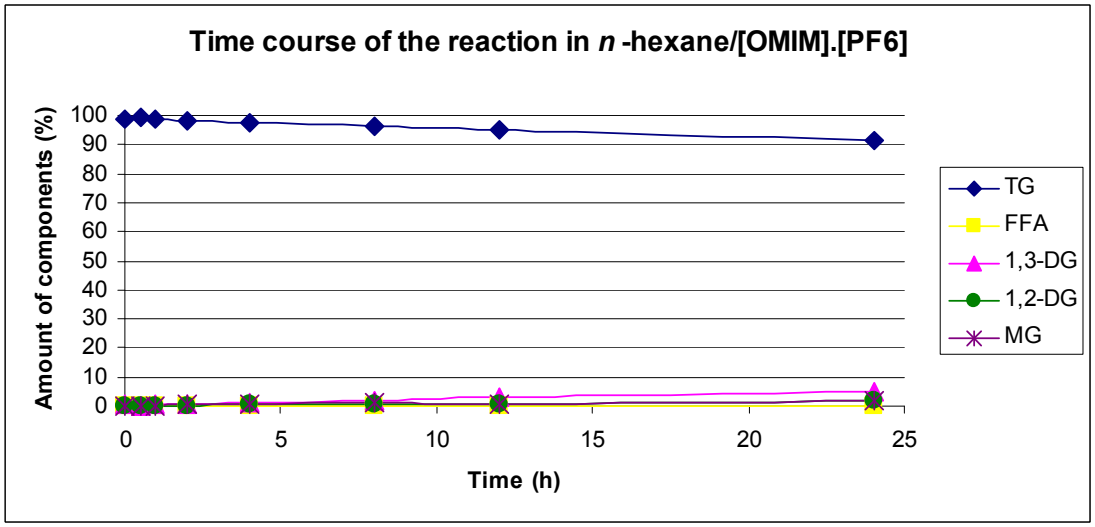


Figure I.1.4: Time course of the reaction in *n*-hexane/[OMIM].[PF_6].

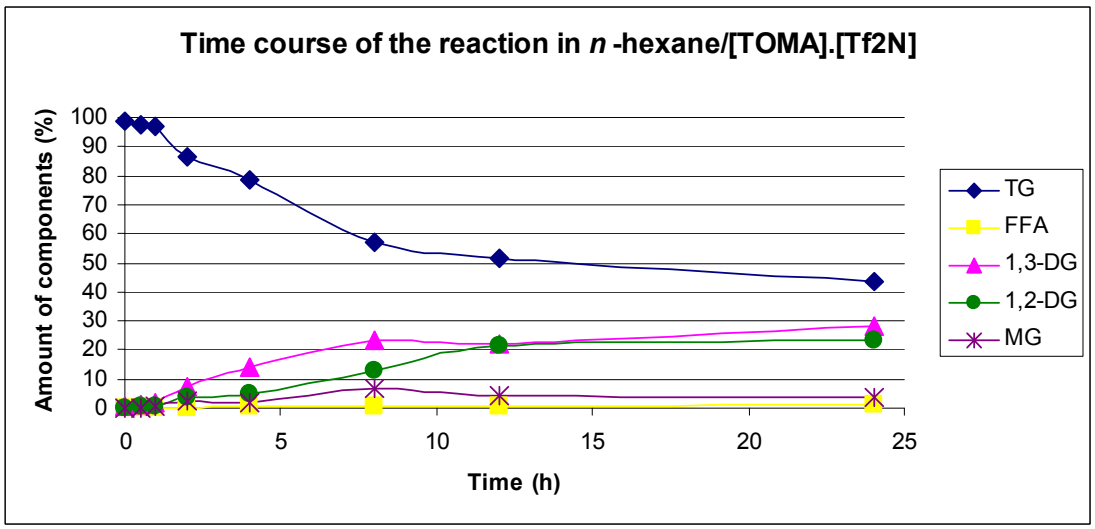


Figure I.1.5: Time course of the reaction in *n*-hexane/[TOMA].[Tf_2N].

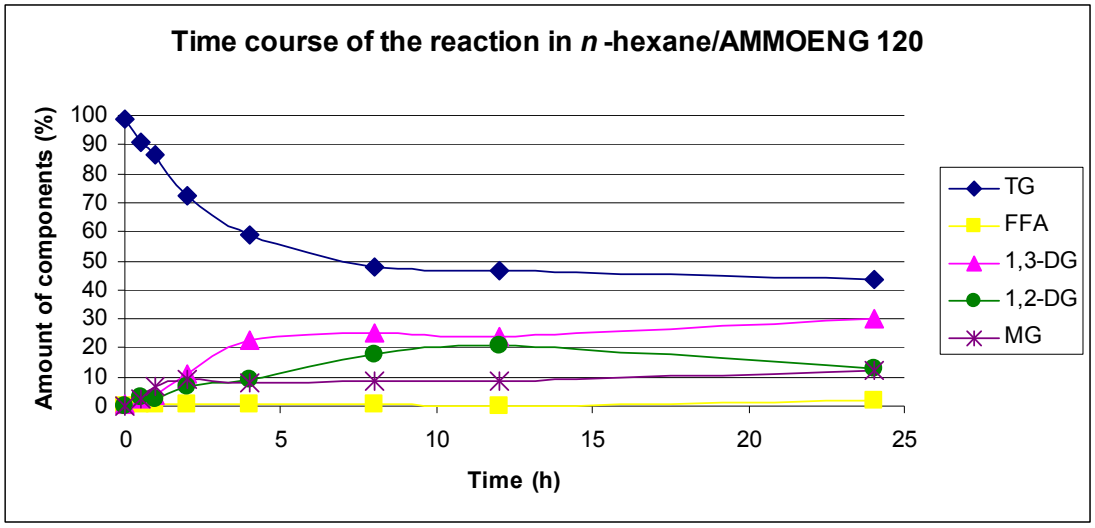


Figure I.1.6: Time course of the reaction in *n*-hexane/AMMOENG 120.

Appendix I.2. Time courses of the glycerolysis reactions performed to investigate the effect of *n*-hexane/IL ratio on the *n*-hexane/IL system.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.665 mL *n*-hexane, 0.335 mL IL, 50°C, 24 h, 700 rpm

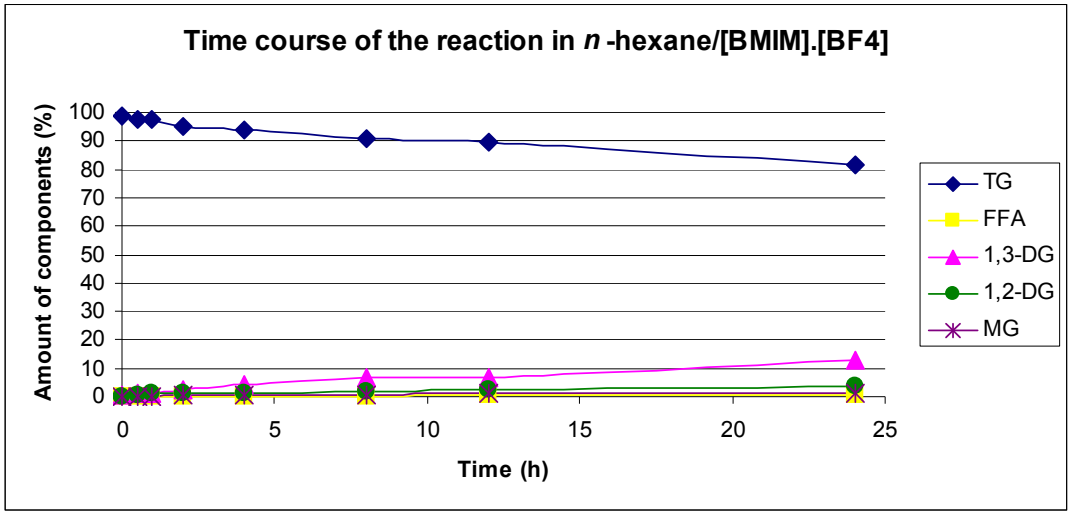


Figure I.2.1: Time course of the reaction in *n*-hexane/[BMIM].[BF_4].

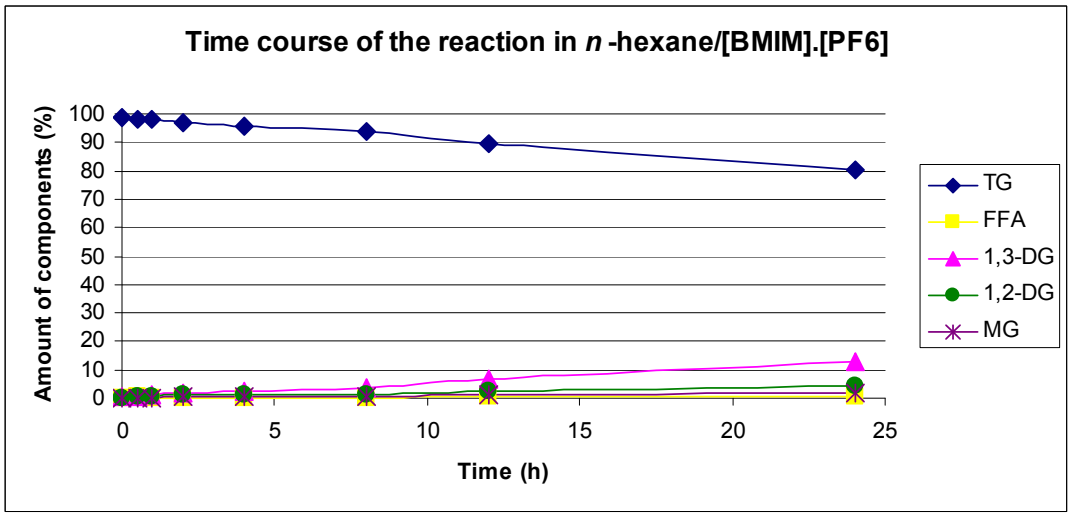


Figure I.2.2: Time course of the reaction in *n*-hexane/[BMIM].[PF_6].

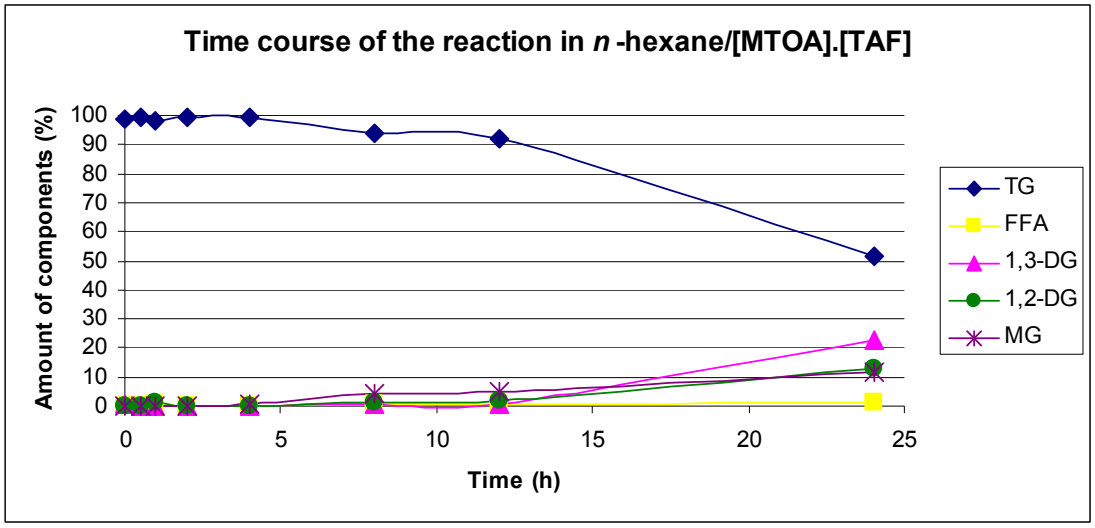


Figure I.2.3: Time course of the reaction in *n*-hexane/[MTOA].[TFA].

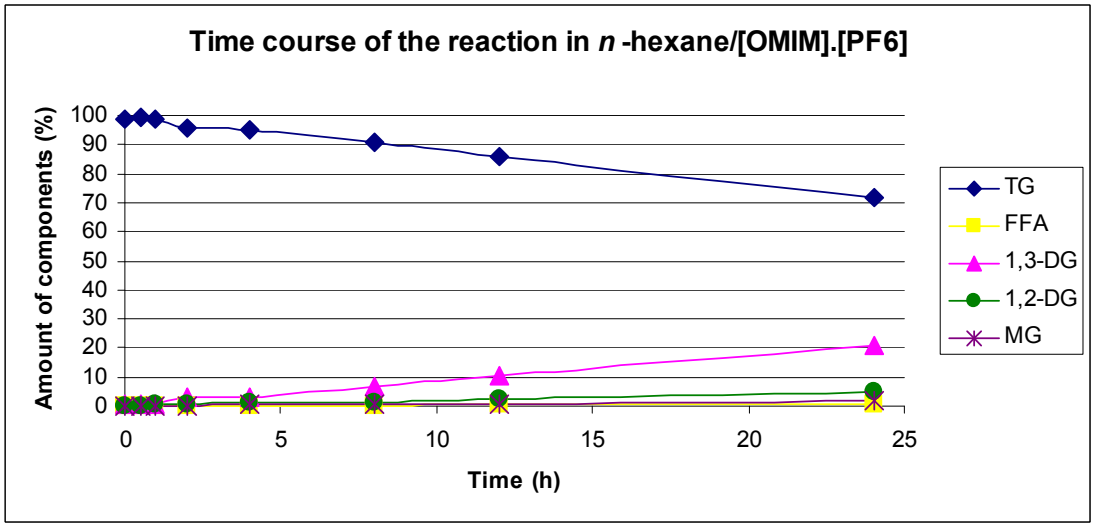


Figure I.2.4: Time course of the reaction in *n*-hexane/[OMIM].[PF_6].

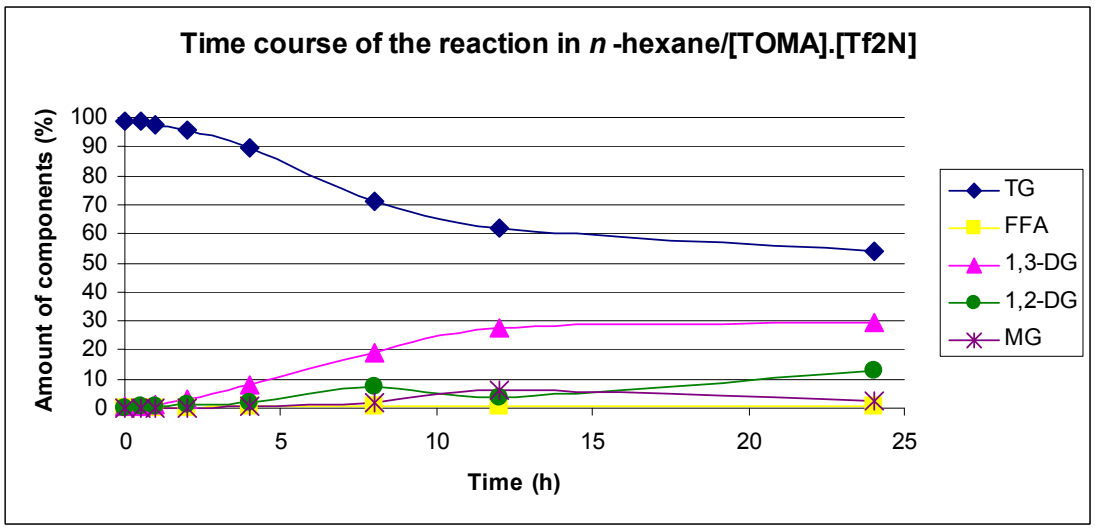


Figure I.2.5: Time course of the reaction in *n*-hexane/[TOMA].[Tf_2N].

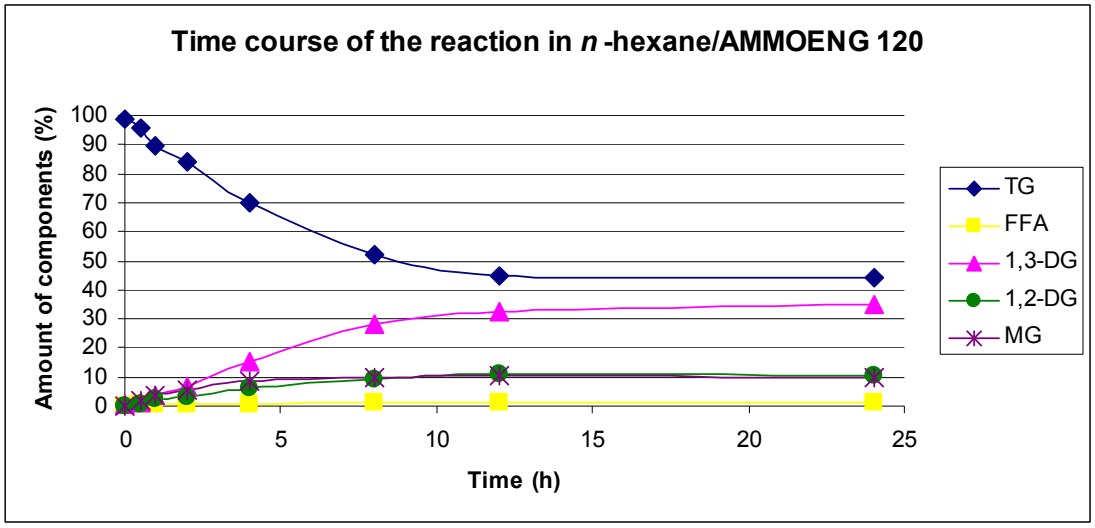


Figure I.2.6: Time course of the reaction in *n*-hexane/AMMOENG 120.

Appendix I.3. Comparison of the three different levels used to investigate the effect of *n*-hexane/IL ratio on the *n*-hexane/IL system.

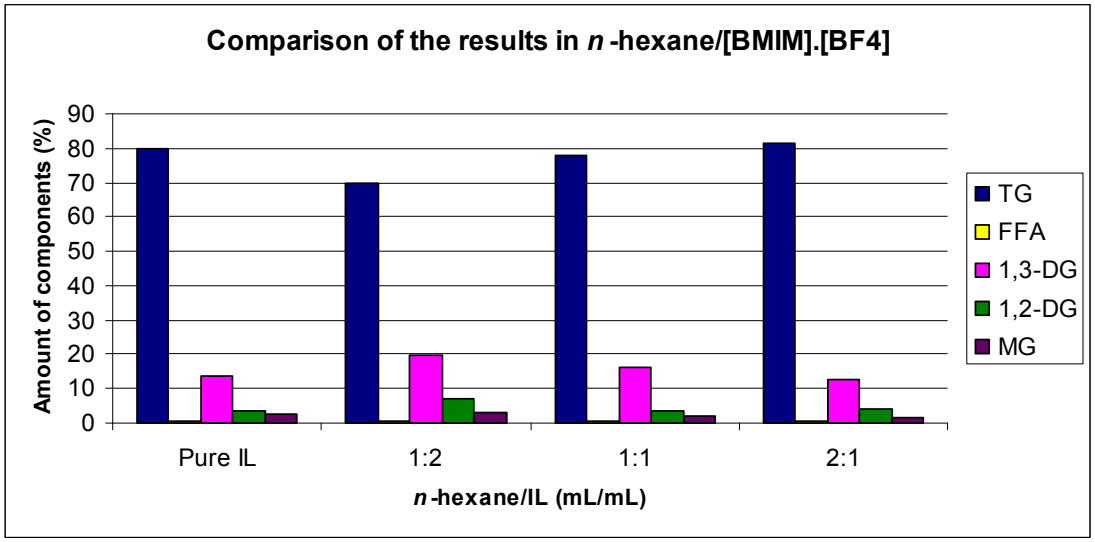


Figure I.3.1: Comparison of the results in [BMIM].[BF_4].

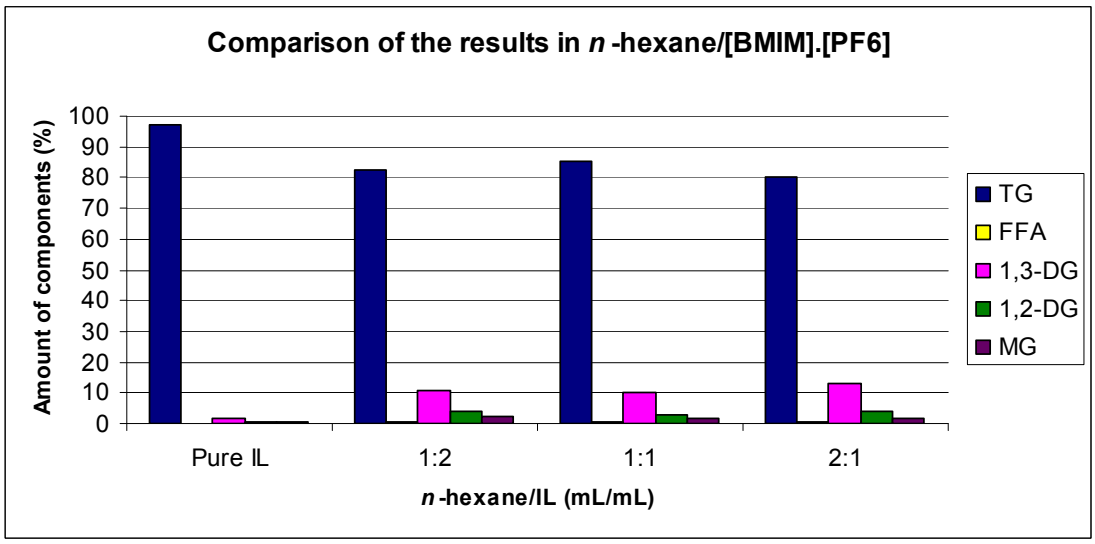


Figure I.3.2: Comparison of the results in [BMIM].[PF_6].

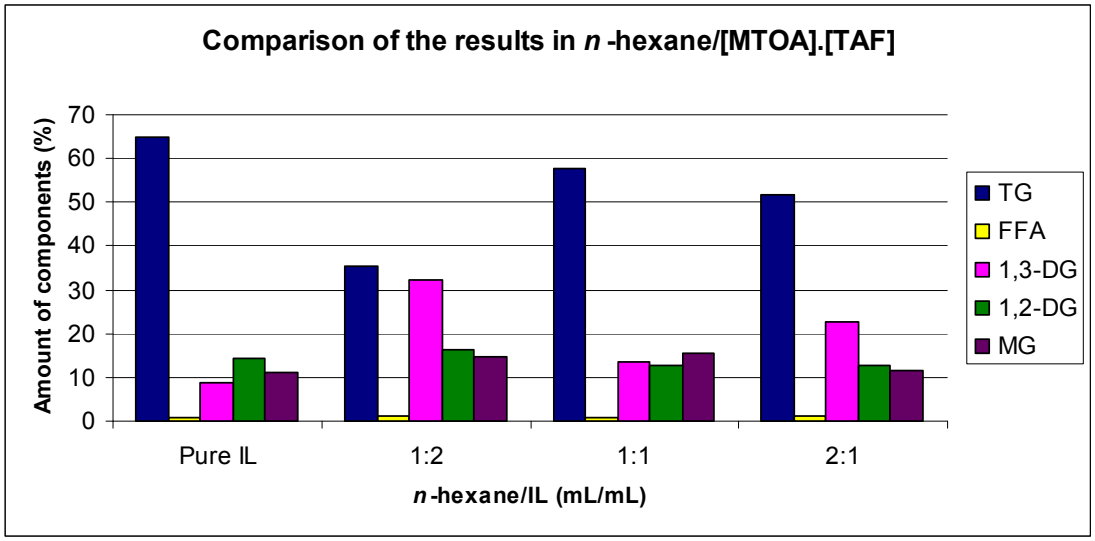


Figure I.3.3: Comparison of the results in [MTOA].[TFA].

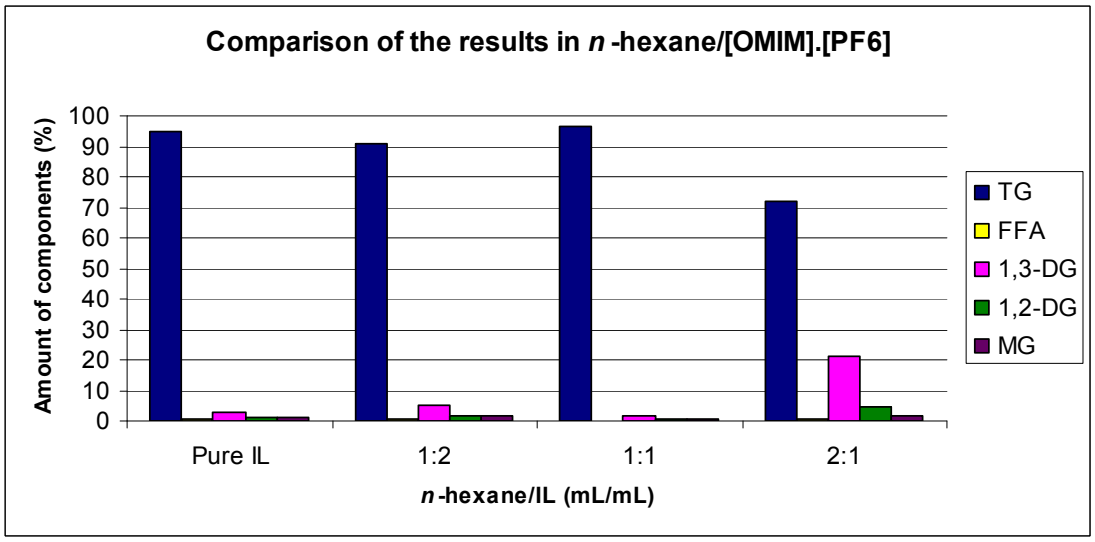


Figure I.3.4: Comparison of the results in [OMIM].[PF_6].

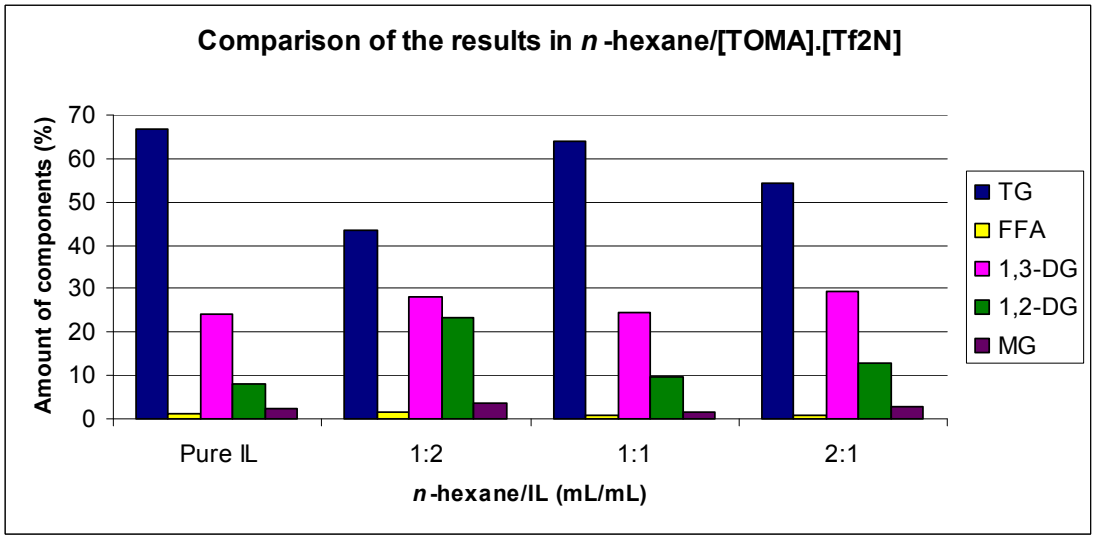


Figure I.3.5: Comparison of the results in [$TOMA$].[Tf_2N].

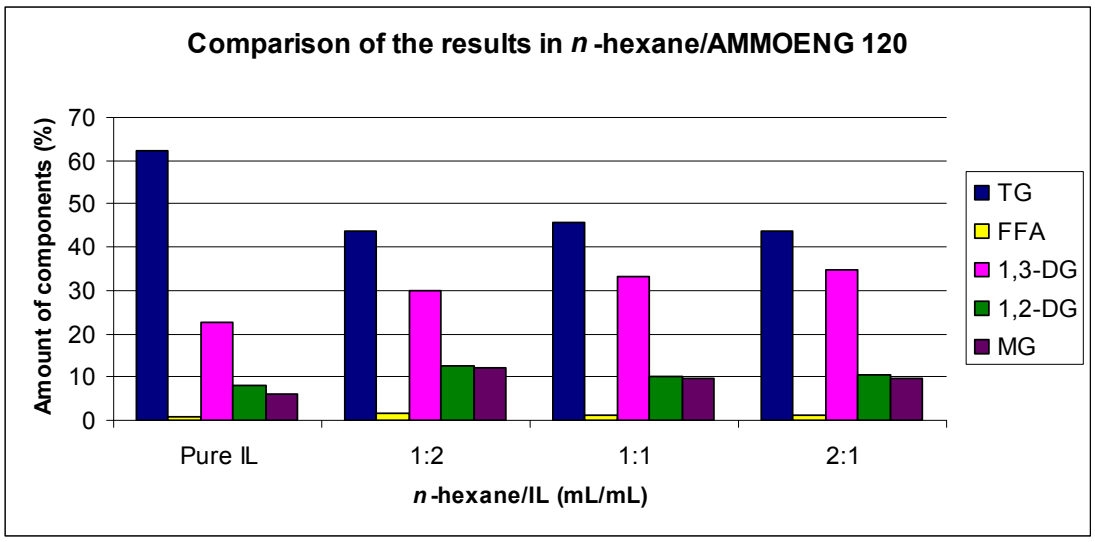


Figure I.3.6: Comparison of the results in AMMOENG 120.

Appendix J.1. Time courses of the glycerolysis reactions performed in binary IL system including AMMOENG 100.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.5 mL AMMOENG 100, 0.5 mL IL, 60°C, 24 h, 700 rpm

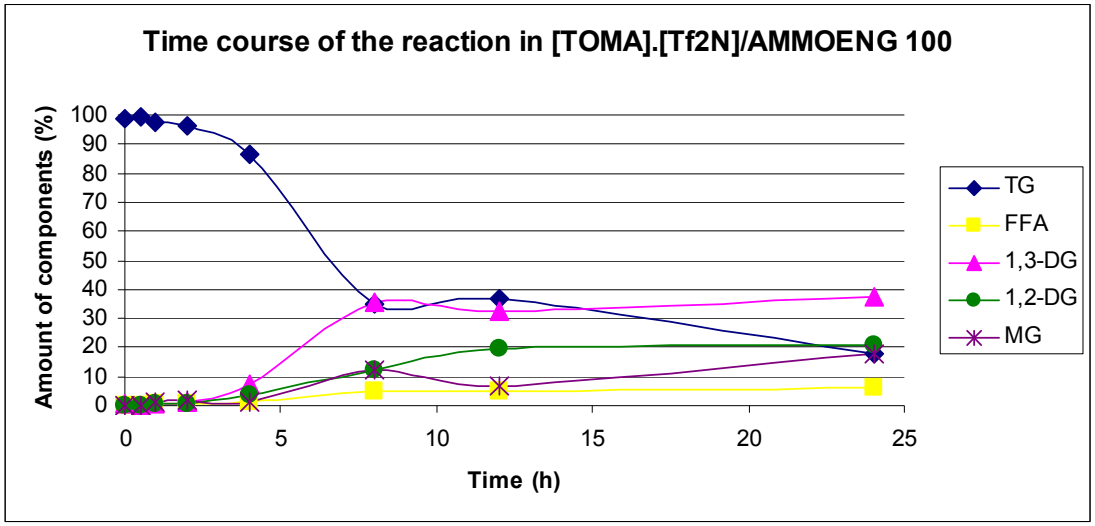


Figure J.1.1: Time course of the reaction in [TOMA].[Tf_2N]/AMMOENG 100.

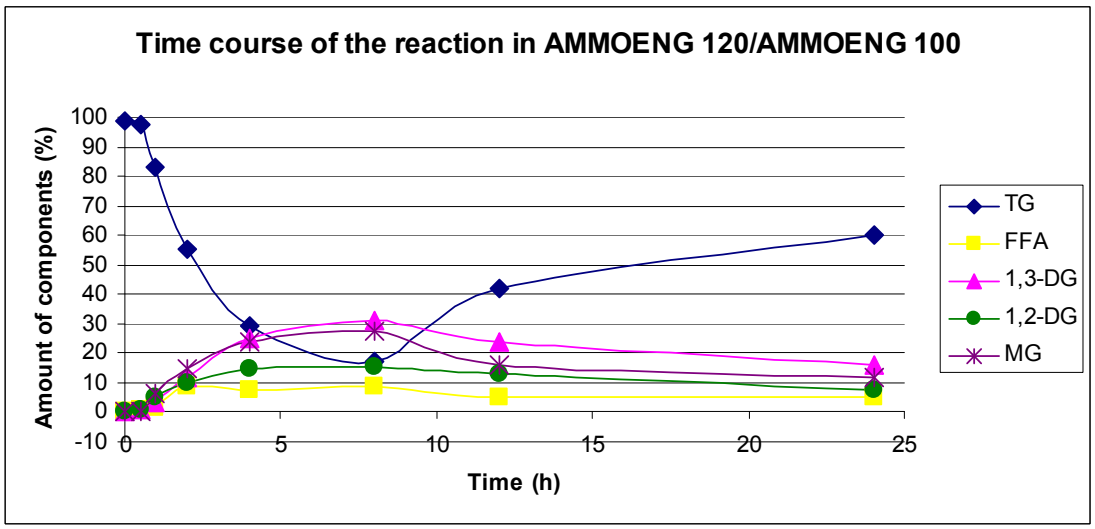


Figure J.1.2: Time course of the reaction in AMMOENG 120/AMMOENG 100.

Appendix J.2. Time courses of the glycerolysis reactions performed in binary IL system including AMMOENG 102.

Reaction conditions: 2 mmol of oil, 1 mmol of glycerol, Novozym 435 in the amount of 10 wt % based on the oil mass, 0.5 mL AMMOENG 102, 0.5 mL IL, 60°C, 24 h, 700 rpm

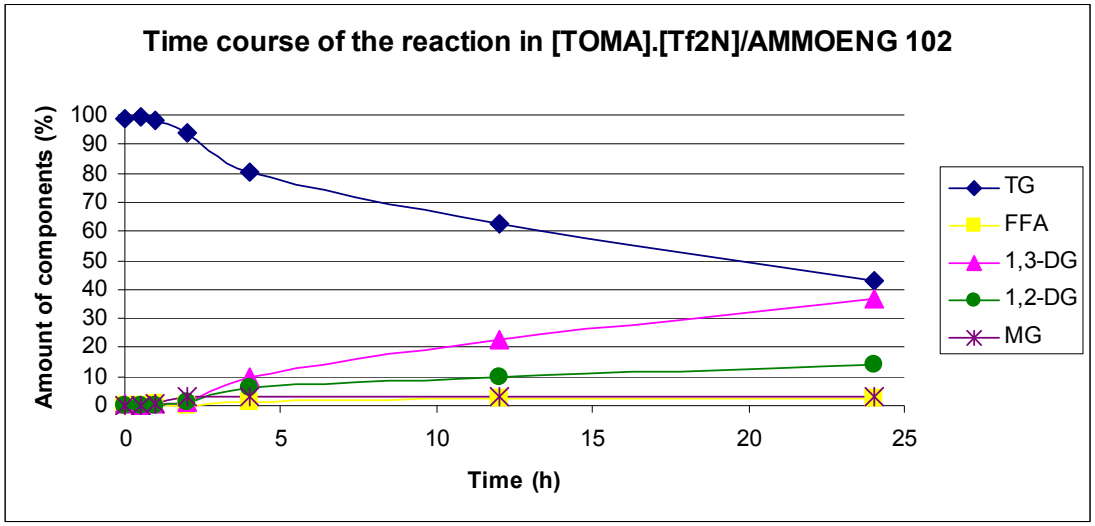


Figure J.2.1: Time course of the reaction in [TOMA].[Tf_2N]/AMMOENG 102.

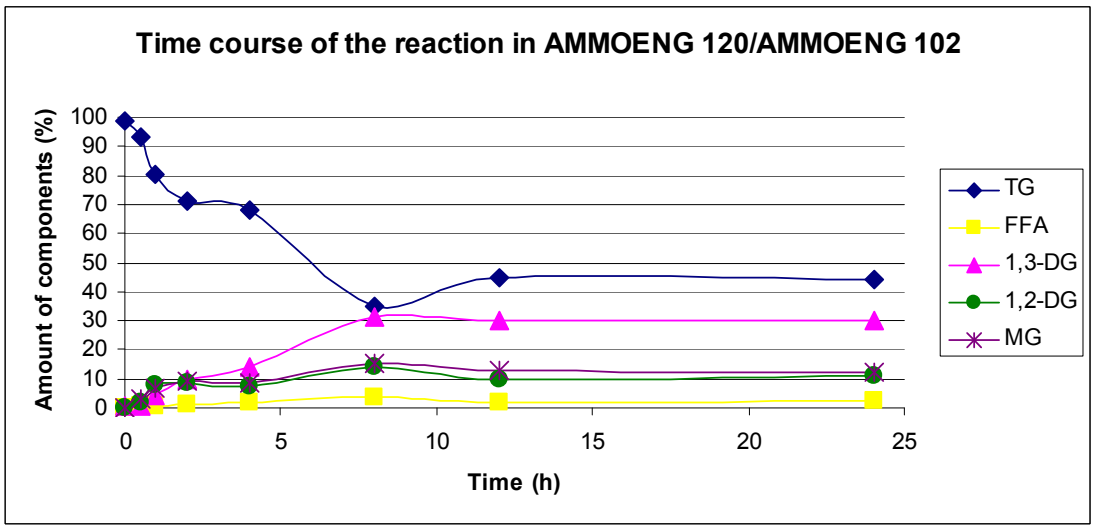


Figure J.2.2: Time course of the reaction in AMMOENG 120/AMMOENG 102.

Appendix J.3. Comparison of binary IL systems with pure ILs.

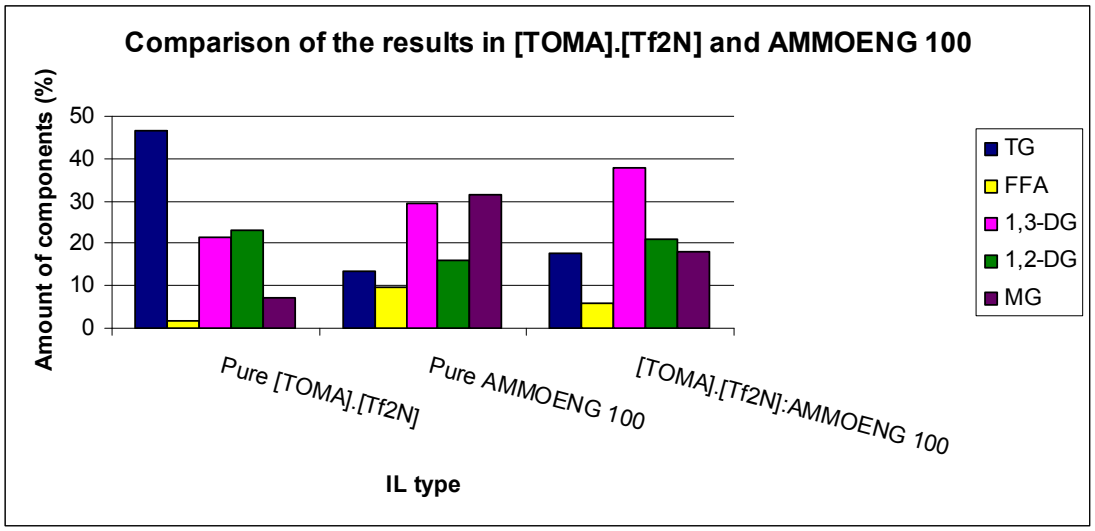


Figure J.3.1: Comparison of the results in [TOMA].[Tf_2N] and AMMOENG 100.

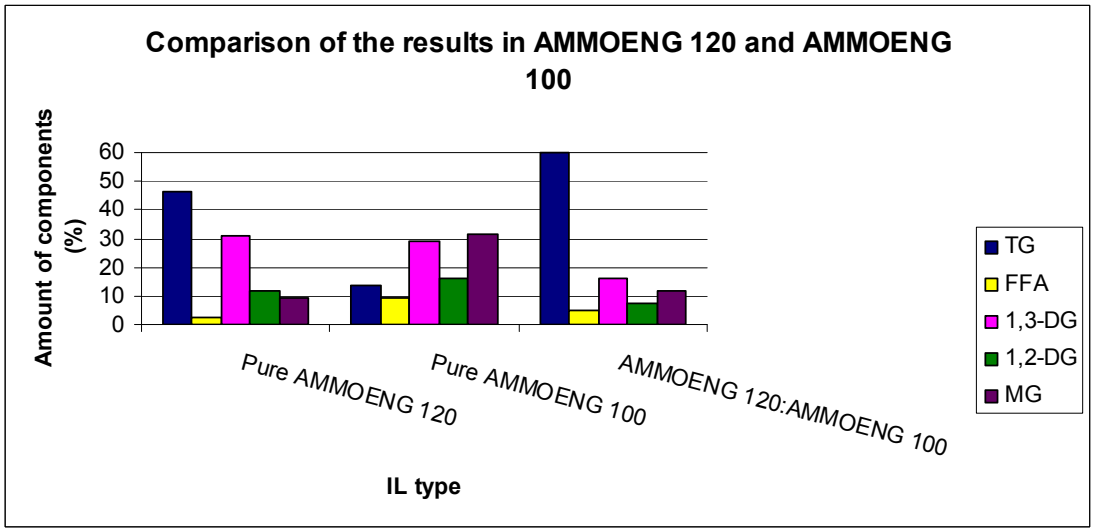


Figure J.3.2: Comparison of the results in AMMOENG 120 and AMMOENG 100.

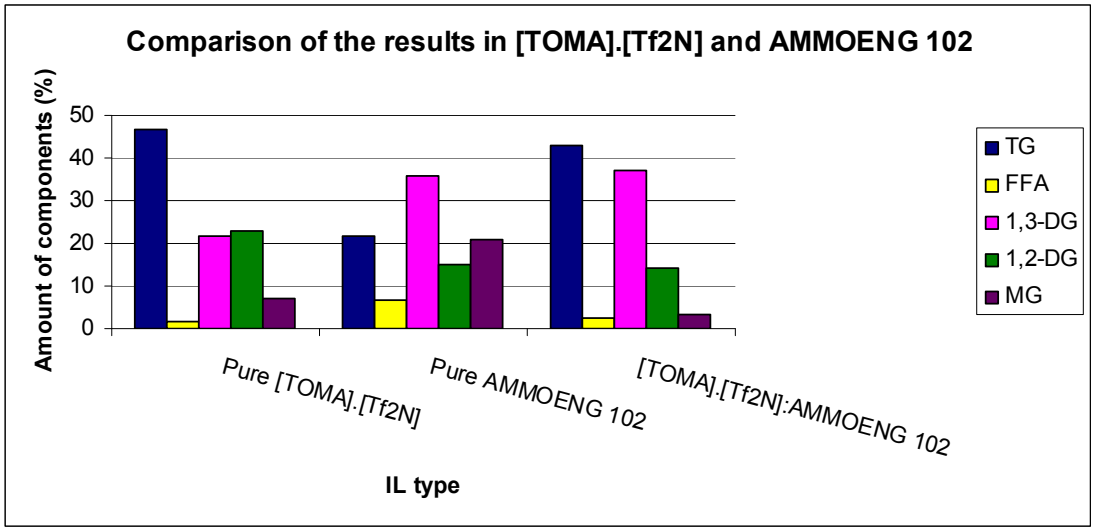


Figure J.3.3: Comparison of the results in [TOMA].[Tf_2N] and AMMOENG 102.

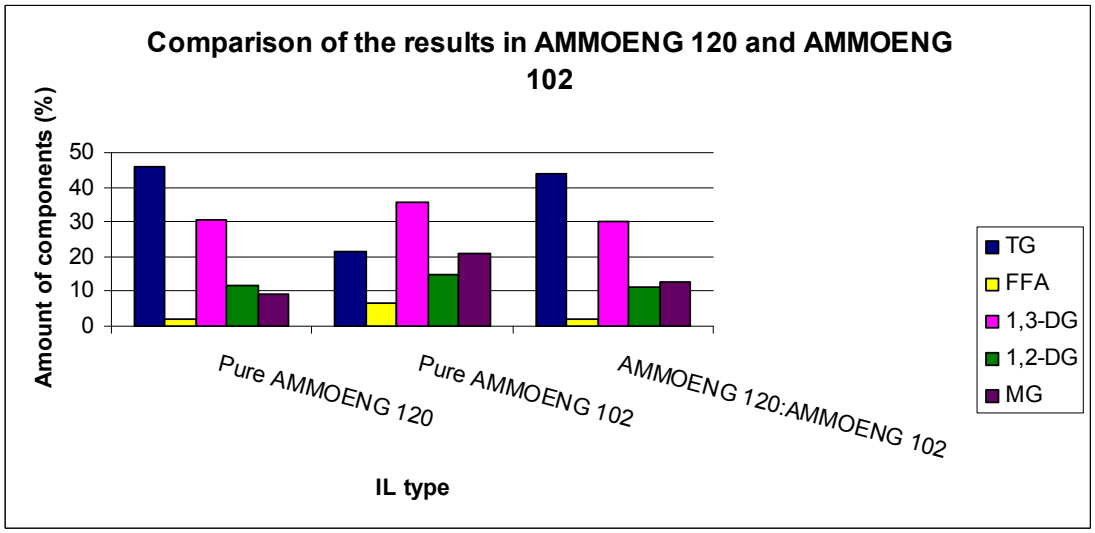


Figure J.3.4: Comparison of the results in AMMOENG 120 and AMMOENG 102.

Appendix K. ANOVA table and regression results for DG yield.

Table K.1: ANOVA table for DG yield.

Source	DF	SS	MS	*F*	*P*
Regression	9	624.83	69.43	1.9	0.206
Linear	3	506.74	16.04	0.44	0.733
Square	3	63.02	21.01	0.57	0.65
Interaction	3	55.07	18.36	0.5	0.693
Residual error	7	256.45	36.64		
Lack-of-fit	5	155.73	31.15	0.62	0.713
Pure error	2	100.71	50.36		
Total	16	881.27			

DF: degree of freedom; SS: sum of squares; MS: mean square.

Table K.2: The regression coefficients and *P* values for DG yield.

Term	Regression coefficient	*P* value
Constant	11.1289	0.889
Temperature	1.1328	0.385
Glycerol mole	-0.0825	0.999
TT percent	0.3727	0.562
Temperature*Temperature	-0.0079	0.356
Glycerol mole*Glycerol mole	10.6331	0.612
TT percent*TT percent	-0.0002	0.941
Temperature*Glycerol mole	-0.0184	0.970
Temperature*TT percent	-0.0003	0.954
Glycerol mole*TT percent	-0.3492	0.26

TT percent: [TOMA].[Tf_2N] percent.

Appendix L. Statistical plots of the model generated for DG yield.

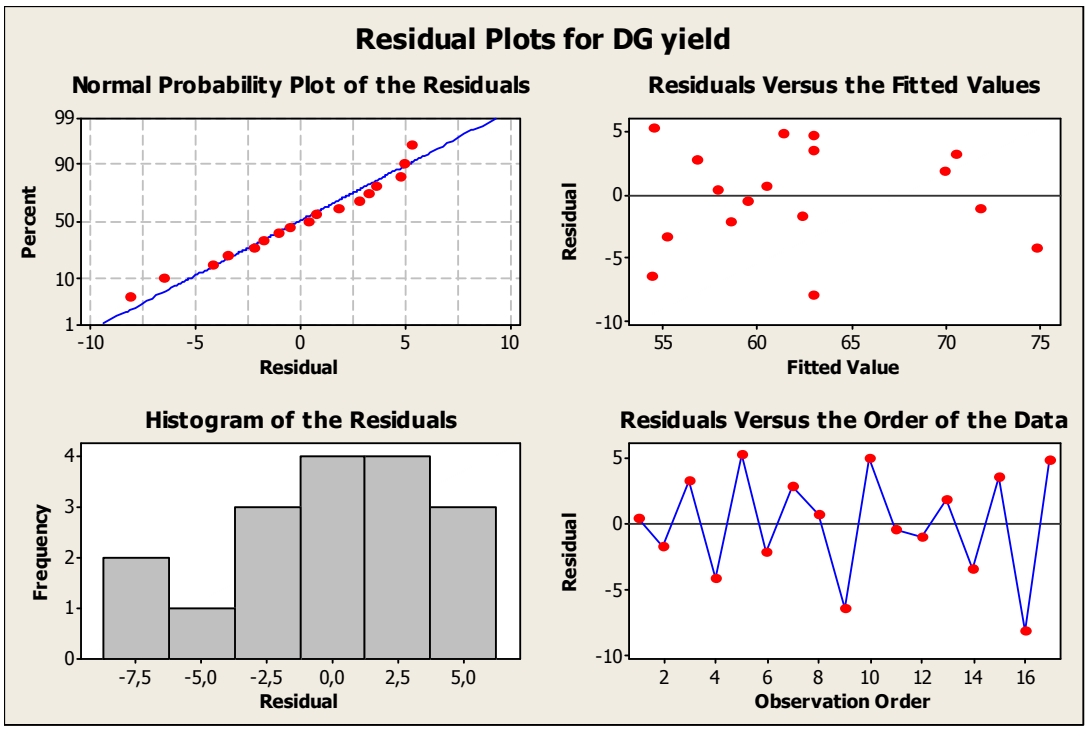


Figure L.1: Residual plots for DG yield.

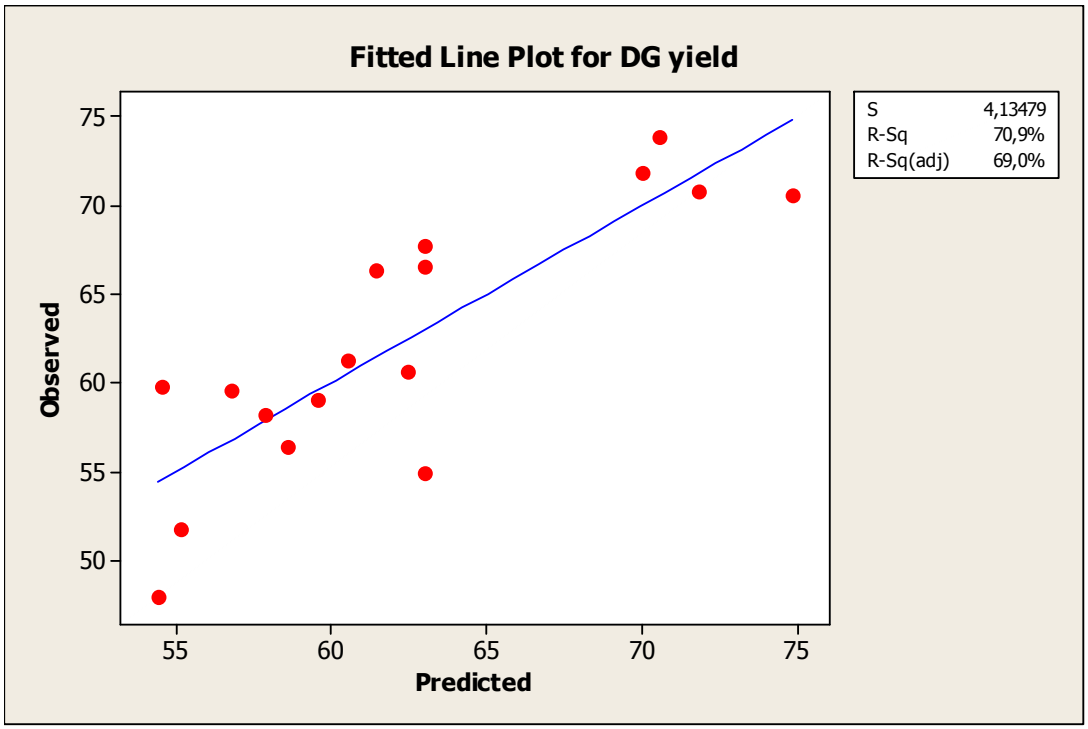


Figure L.2: Fitted line plot for DG yield.

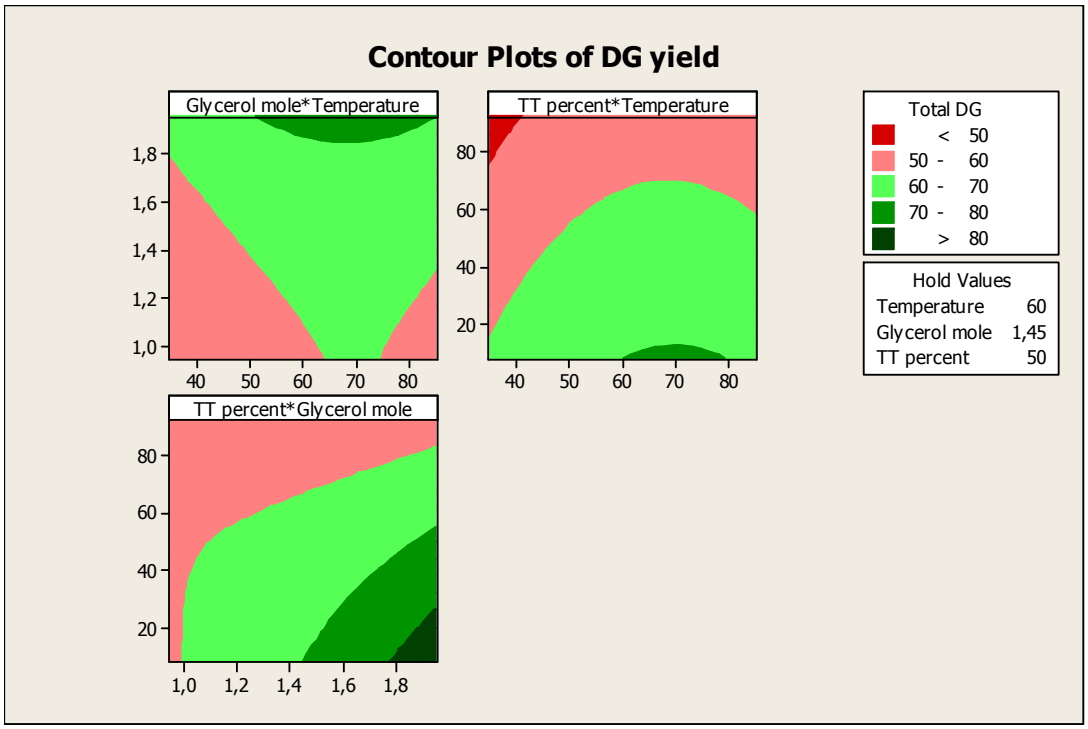


Figure L.3: Contour plots for DG yield.

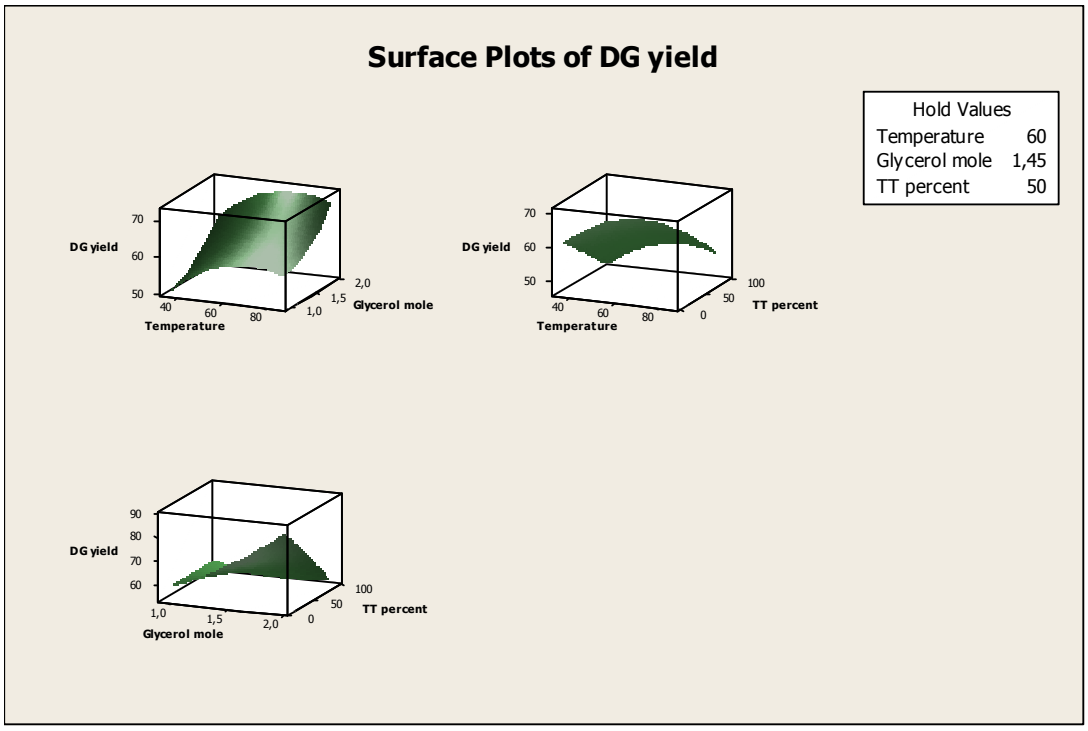


Figure L.4: Surface plots for DG yield.

Appendix M. AAD calculation and chi-square test of the model generated for DG yield.

AAD is calculated by the following equation:

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

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively (Table 14.1), and p is the number of experimental run.

$$AAD = \frac{0.92}{17} \times 100 = 5.42\ \%$$

Table M.1: Experimental and calculated responses for the model.

Exp. no	Experimental response	Calculated response
1	58.19	57.83
2	60.67	62.41
3	73.85	70.55
4	70.6	74.79
5	59.8	54.51
6	56.36	58.58
7	59.57	56.75
8	61.21	60.48
9	47.94	54.42
10	66.34	61.4
11	59	59.51
12	70.75	71.81
13	71.85	69.97
14	51.74	55.15
15	66.54	62.95

Table M.1: Experimental and calculated responses for the model (continued).

Exp. no	Experimental response	Calculated response
16	54.87	62.95
17	67.7	62.95

Table M.2: Verification of the model generated for DG yield by chi-square (χ^2) test

Exp. no	Factors			Responses	
	T, ^oC	TT percent, %	Glycerol, mmole	Observed	Predicted*
1	52	7.95	1.95	72.09	84.68
2	69	7.95	1.95	72.4	87
3	85	7.95	0.94	65.15	58.36
4	85	92	0.94	46.02	58.1
					$\chi^2 = 7.62^{**}$

*: calculated according to the equation generated by the regression coefficients , see Appendix K, Table K.2.

**: $$x^2 = \sum \left[\frac{(\text{Observed value} - \text{Predicted value})^2}{\text{Predicted value}} \right]$$

cutoff point = 7.82 at $\alpha = 0.05$ and DF = 3

Appendix N. ANOVA table and regression results for TG conversion.

Table N.1: ANOVA table for TG conversion.

Source	DF	SS	MS	*F*	*P*
Regression	9	943.54	104.83	10.92	0.004
Linear	3	867.14	9.04	0.94	0.477
Square	3	22.73	7.57	0.79	0.543
Interaction	3	53.67	17.89	1.86	0.237
Residual error	6	57.6	9.6		
Lack-of-fit	4	33.48	8.37	0.69	0.662
Pure error	2	24.12	12.05		
Total	15	1001.14			

DF: degree of freedom; SS: sum of squares; MS: mean square.

Table N.2: The regression coefficients and *P* values for TG conversion.

Term	Regression coefficient	*P* value
Constant	80.9614	0.09
Temperature	-0.3123	0.689
Glycerol mole	21.8008	0.552
TT percent	-0.3921	0.26
Temperature*Temperature	-0.0055	0.321
Glycerol mole*Glycerol mole	-11.0804	0.331
TT percent*TT percent	0.0002	0.92
Temperature*Glycerol mole	0.4373	0.123
Temperature*TT percent	0.004	0.217
Glycerol mole*TT percent	-0.099	0.523

TT percent: [TOMA].[Tf_2N] percent.

Appendix O. Statistical plots of the model generated for TG conversion.

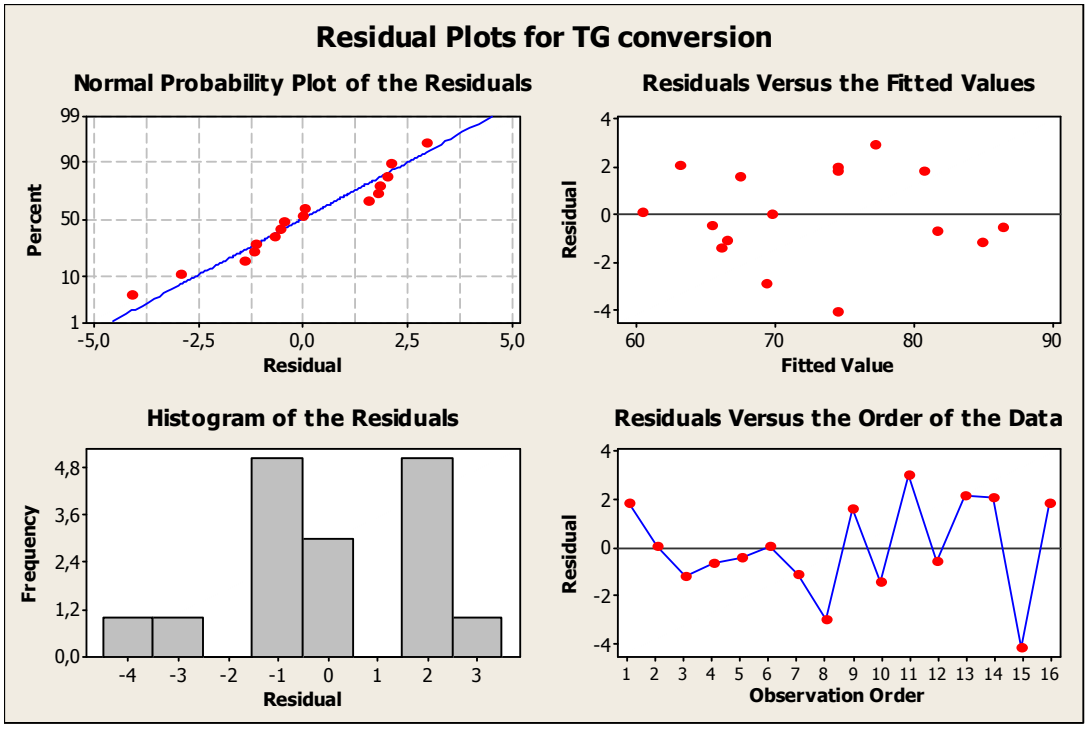


Figure O.1: Residual plots for TG conversion.

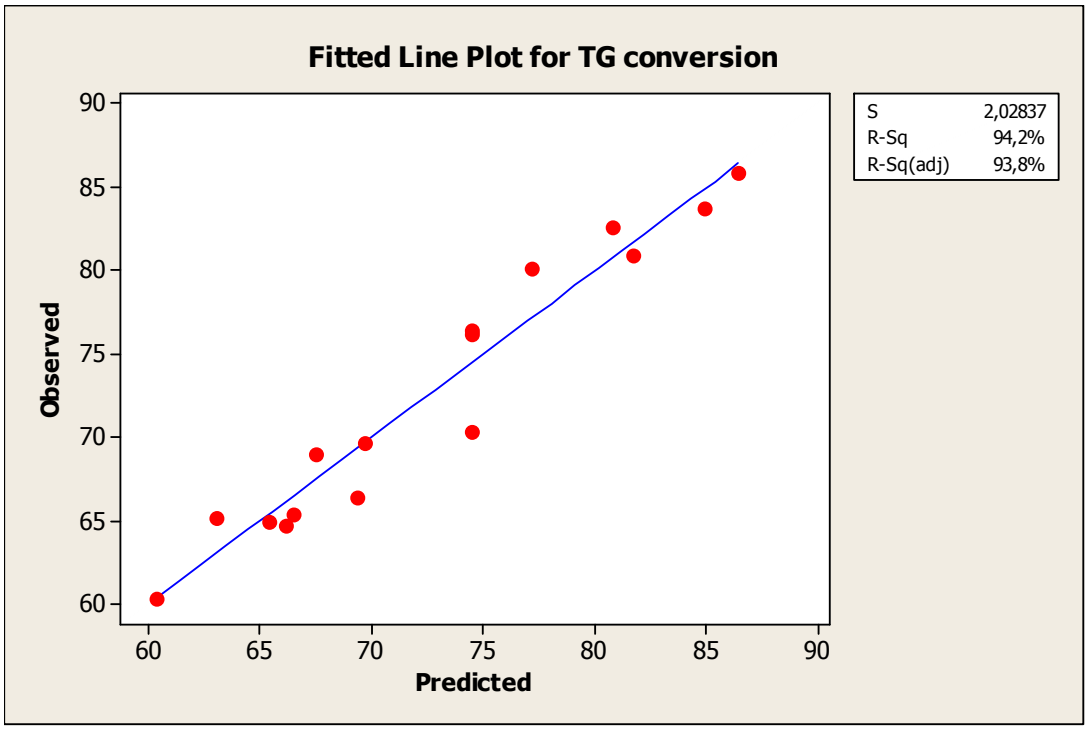


Figure O.2: Fitted line plot for TG conversion.

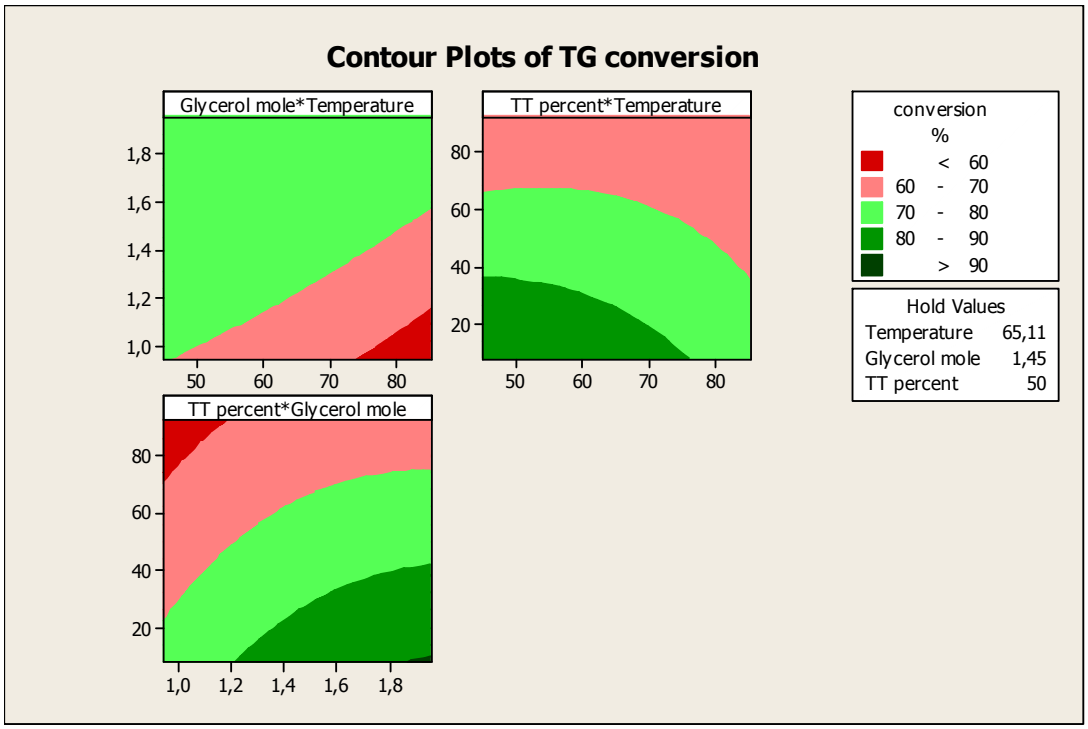


Figure O.3: contour plots for TG conversion.

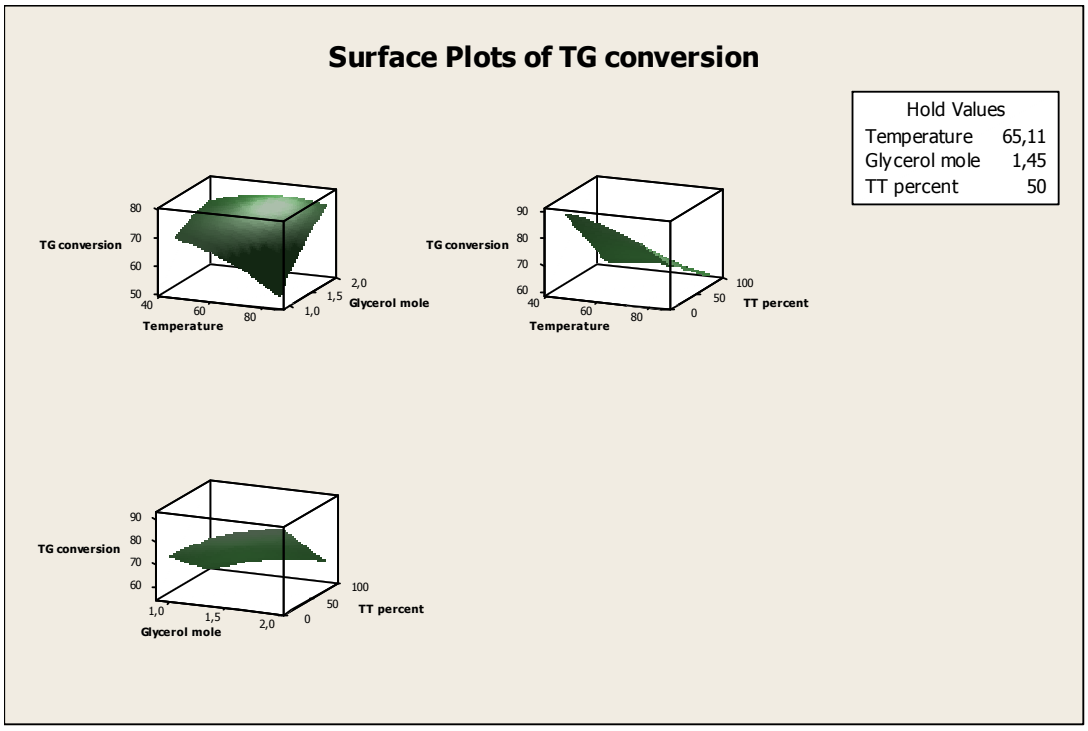


Figure O.4: Surface plots for TG conversion.

Appendix P. AAD calculation and chi-square test of the model generated for TG conversion.

AAD is calculated by the following equation:

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

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively (Table 17.1), and p is the number of experimental run.

$$AAD = \frac{0.53}{17} \times 100 = 3.35\ \%$$

Table P.1: Experimental and calculated responses for the model.

Exp. no	Experimental response	Calculated response
1	82.6	77.59
2	69.69	70.53
3	83.72	81.71
4	80.98	82.53
5	64.9	62.15
6	60.33	61.14
7	65.35	63.31
8	66.36	70.17
9	57.99	64.43
10	69	64.27
11	64.67	67.73
12	80.13	78.78
13	85.87	88
14	65.14	64.66
15	76.46	74.25

Table P.1: Experimental and calculated responses for the model.

Exp. no	Experimental response	Calculated response
16	70.34	74.25
17	76.24	74.25

Table P.2: Verification of the model generated for TG conversion by chi-square (χ^2) test

Exp. no	Factors			Responses	
	T, ^oC	TT percent, %	Glycerol, mmole	Observed	Predicted*
1	52	7.95	1.95	78.63	91.58
2	69	7.95	1.95	77.54	90
3	85	7.95	0.94	67.19	59.2
4	85	92	0.94	50	48.71
				χ^2 =	4.67**

*: calculated according to the equation generated by the regression coefficients , see Appendix N, Table N.2.

**: $$x^2 = \sum \left[\frac{(\text{Observed value} - \text{Predicted value})^2}{\text{Predicted value}} \right]$$

cutoff point = 7.82 at α = 0.05 and DF = 3

BIOGRAPHY

Derya Kahveci was born in Sivas at 1984. She graduated from Beşiktaş Anatolian High School at 2001. She completed her undergrad education at Food Engineering Department of Istanbul Technical University at 2005. She spent 8 months at Denmark Technical University as an exchange student by the Erasmus Exchange Programme. She has been a master's student at Food Engineering Department of Istanbul Technical University since September 2005, and also a research assistant at the same department since December 2005.