Scientific paper

Enzymatic Esterification of Glycerol and Stearic Acid in Non-conventional Media

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Abstract

Ionic liquids as trihexyl-tetradecyl-phophonium-dicyanamide (Cyphos 105) and cocosalkyl-pentaethoxi-methyl-ammonium-methosulfate (Ammoeng 100) were applied for the esterification of stearic acid and glycerol using *Candida antarctica* lipase (Novozyme 435). When only ILs were applied as solvents at 1:15 initial substrate molar ratio the conversion was 76 and 78 % in the case of two kinds of ILs, respectively. Mixed the ILs and supercritical CO_2 the conversion reached 79 and 86 %. The conversion was found highest in supercritical CO_2, reached 90 %. Moreover formation of glycerol-di-stearate is much lower in the case of ILs comparing with $SCCO_2$.

Keywords*:* Glycerol-mono-stearate, Lipase, Ionic liquids, Supercritical CO_2

1. Introduction

The monoesters of glycerol and long chain fatty acids, the monoglycerides, like glycerol-mono-stearate, constitute the most important food-grade emulsifiers and they are widely used in the pharmaceutical, cosmetic industry and detergents chemistry, as well. They are traditionally produced at high temperature (220–250 °C) in the presence of alkaline catalysts, which procedure yields 40–60 % monoglycerides depending on the glycerol excess. Combination of this process with membrane separation results better yield values[1]. These conventional catalysts – besides the environmental problems – cause side-reactions by degradation of the fatty acid (e.g. oxidation, dimerisation) or the glycerol (e.g. polymerisation, dehydration, oxidation)[2]. For this reason the use of less corrosive catalysts should improve the efficiency.

Enzymatic esterification of glycerol-mono-stearate from glycerol and stearic acid offers an alternative method because it works under milder reaction conditions and with higher selectivity[3]. The main problem occurring during the realization of this reaction is the distinct solubility of the substrates. Glycerol and stearic acid are both soluble in organic solvents, for example hexane, toluene, chloroform, dioxane, acetone, etc., but these media are toxic, carcinogen, flammable and not environmental friendly. For this reason alternative new media for the realization of the esterification process: ionic liquids (ILs)[4] and supercritical fluids (SCFs)[5,6] have been studied.

Biocatalysis in non-conventional media has received growing interest in recent years. Several researches focused on environmental friendly alternative solvents as ILs and SC CO_2.

Ionic liquids offer new possibilities for the application of biocatalysts and enzymatic reactions[7]. Their application has several advantages. They possess no vapour pressure, they are able to dissolve many compounds and they have high thermal and chemical stability. In recent years, room-temperature ILs (compounds that consist only of ions and are liquid at room temperature) have increasingly attracted attention as the green, high-tech reaction media of the future. This is mainly because of extremely low vapour pressure, their thermal stability and their widely tunable properties with regard to polarity, hydrophobicity and solvent miscibility behaviour through appropriate modification of the cation and anion.

Supercritical fluids are promising green solvents. They offer several advantageous properties at temperatures and pressures slightly above the critical points (e.g. 31 °C and 7.38 MPa for CO_2). They have high compressibility, very low viscosity and high diffusivity. Supercritical CO_2 is non-toxic and carbon dioxide is a by-product of other industrial processes[8]. Furthermore separation of the reaction components from the substrates is easier than in traditional solvents.

In the literature we can find many studies on esterification reaction in ILs. Among of these the first paper on lipase catalyzed reaction in IL was published in 2000[9]. In these studies ILs such as [BMIM][PF_6] and [BMIM][PF_4] were examined to replace organic solvents and *Candida antarctica* lipase B enzyme was investigated and showed the same activity in IL compared with the organic solvent.

Equilibrium of reversible esterification reactions can be shifted and conversion can be improved by product removal. In other works successful experiments and increased reaction conversion results have been reported in the field of enhanced esterification reaction in ionic liquids with membrane assisted removal of products[10,11].

In this paper we report our experimental results obtained during the esterification of stearic acid and glycerol using *Candida antarctica* lipase (Novozyme 435) in nonconventional reaction media: ILs and SC CO_2.

2. Materials and Methods

2. 1. Materials

Novozyme 435, a commercial grade *Candida antarctica* lipase B immobilized onto macroporous polyacrylate resin beads) was purchased from Novozymes A/S (Bagsvaerd, Denmark).

ILs as trihexyl-tetradecyl-phophonium-dicyanamide (Cyphos 105) and cocosalkyl-pentaethoxi-methyl-ammonium-methosulfate (Ammoeng 100) were commercial grade. Cyphos 105 was purchased from IoLiTec Ionic Liquids Technologies GmbH & Co. KG, Denzlingen, Germany and Ammoeng 100 was from Solvent Innovation GmbH, Köln, Germany.

Standards of analytical grade were purchased from Sigma-Aldrich Co. (glycerol-mono- and di-stearate). All other chemicals were reagent grade.

2. 2. Methods

Analysis of stearic acid and the formed product as glycerol-mono- and di-stearate was carried out by HPLC under the following circumstances: Merck-Hitachi L-6000A type HPLC apparatus, Merck RI-71 type refractive index detector, AGILENT 79911GP-500 PLgel column (5µm, reverse phase), tetrahydrofuran (THF) mobile phase, 0.5 cm^3 min^{-1} flow rate, 30 °C, sample volume: 20 µl.

Extraction of substrates and products from ILs was carried out with hexane as extraction material in the case of Cyphos 105 and with diethyl-ether in the case of Ammoeng 100 in two extraction steps. During the procedure 100 µl sample + 500 µl hexane/ diethyl-ether (1st extraction step) were mixed. Centrifugation of the solution was carried out for separation of sample and dilution. This procedure was repeated again (2nd extraction step). Evaporation of hexane/diethyl-ether from the dilution was realized and the remained sample was dissolved in THF for HPLC analysis.

Calculation of the activation energy

The activation energy of the reaction was calculated from the Arrhenius-equation (Eq. 1), where reaction rate is proportional to the activation energy. After logarithmization (Eq. 2) it can be seen that representation of ln v *vs.* ln T gives the value of E_a/RT from which Ea can be counted.

$$v = A \times \exp(-E_a/RT) \qquad (1)$$

$$\ln v = \ln A - E_a/RT \qquad (2)$$

where, v = reaction rate
A = Arrhenius constant
$R = 8.314\ J \times mol^{-1} \times K^{-1}$
E_a = activation energy, $J \times mol^{-1}$
T = temperature, K

Experimental procedure in ILs

The batch enzyme reactions were carried out in shaken flasks. In each reaction mixture 2 cm^3 ionic liquid, 100 mg cm^{-3} Novozyme 435 and different amount of glycerol and stearic acid (mmol cm^{-3}) were mixed, incubated on different temperatures and 150 rpm. Conversion was calculated as the amount of the consumed stearic acid (mmol) referred to the amount of the initial stearic acid (mmol) (%).

Experimental procedure in SC CO_2

These experiments were carried out in a batch stirred reactor with 60 cm^3 volume, thermostated on 70 °C. The applied pressure was 100 bar and 150 rpm stirring. Initial amount of substrates (mg) refer to the total volume of the reactor (60 cm^3). As biocatalyst 100 mg cm^{-3} Novozyme 435 was used.

In this experiments solid phase samples were analysed. Results are given in mg g^{-1} reaction mixture referred to the total weight of reaction mixture. Conversion was calculated as earlier described.

Experimental procedure in IL-SC CO_2 mixtures

In this case experiments were carried out in the same batch stirred reactor at same parameters (70 °C, 100 bar) as described in experimental procedure in SC CO_2 · 30 cm^3 ionic liquid was applied in each reaction.

Liquid phase samples with very high viscosity were analysed. Results are given in mg g^{-1} reaction mixture referred to the total weight of reaction mixture. Conversion was counted as the amount of the consumed stearic acid (mmol) referred to the amount of the initial stearic acid (mmol) (%).

3. Results and Discussion

3. 1. Experiments in Ionic Liquids

Cyphos 105

Firstly the effect of glycerol molar ratio (1:3, 1:7, 1:15) on the conversion of the reaction and product composition in the former described IL: Cyphos 105 was examined. In Figure 1 the progress curves (conversions as a function of reaction rate) are presented.

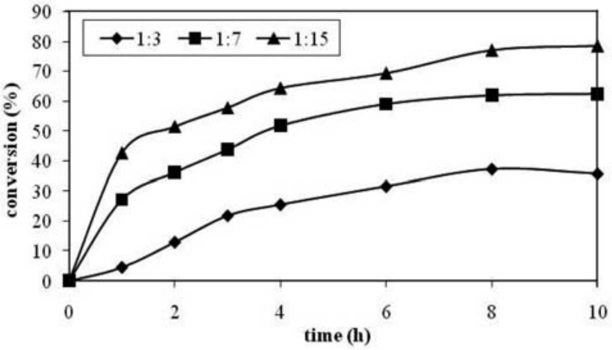

Figure 1: Conversions at 50 °C and different initial substrate molar ratio in Cyphos 105

The results showed that quite high conversions were achieved after 10 hours in cases of 1:7 and 1:15 molar ratio. We found that in the case of 1:3 and 1:7 molar ratio glycerol-di-stearate was also formed in the reaction, while in the case of 1:15 molar ratio it wasn't formed. Final conversion of 78 % was achieved under these circumstances.

Based on this result only the substrate composition of 1:15 was applied in the further experiments and the effect of the applied temperature was examined. Experiments were carried out at 30 °C, 40 °C and 50 °C. The conversion data as a function of time are presented in Figure 2. As it was expected, higher conversion values were achieved during the reaction (especially in the beginning) at higher temperatures. Regarding the selectivity of mono-stearate formation the ratio of amount of stearic acid built in mono-stearate compared to the overall amount of stearic acid in both esters was calculated. As an example ratio of 2.7:1 was found in the case of 1:3 initial substrate molar ratio. Under different reaction conditions the ratio is altered. The excess of glycerol has significant effect on the mono-stearate content of the formed products.

Reaction rate at enzymatic esterification – as in all reactions – can be enhanced by increased temperature. In this case thermal activation of the lipase can be observed (Figure 2). Thermostability of Novozyme 435 lipase is higher than other lipase preparations, its activity and productivity is still very high at 50 °C, deactivation occurs only above 60 °C.

At 1:15 initial substrate molar ratio and 30 °C the conversion after 10 h was ~69%, at 40 °C ~74% and at 50 °C ~78 %. From the results it can be seen that temperature had a significant effect on the initial reaction rate, but the final conversion was not highly influenced by it.

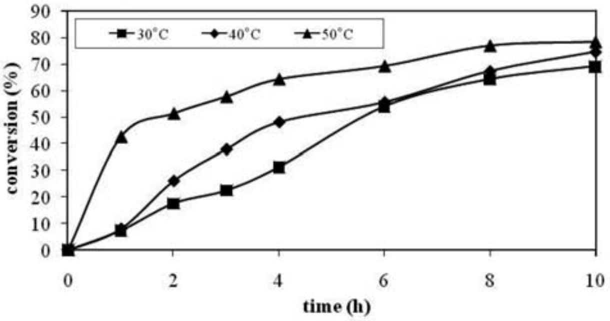

Figure 2: Conversions at different temperatures and 1:15 initial substrate molar ratio in Cyphos 105

Based on the conversion vs. time data, reaction rates were calculated and plotted against temperature data. Then activation energy was determined from the ln v – 1/T diagrams (not shown) using the well-known Arrhenius-equation. Activation energy for the esterification reaction of stearic acid and glycerol in Cyphos 105 at 1:15 initial substrate molar ratio was calculated to be 43.23 kJ mol^{-1} value.

Ammoeng 100

The effect of the stearic acid : glycerol ratio (1:3, 1:7, 1:15) and temperature (30 °C, 40 °C, 50 °C) on the

Table 1: Influence of the substrate ratio on product composition at 50 °C

Initial substrate molar ratio	Time (h)	Formed glycerol-mono-stearate (mg cm^{-3})	Formed glycerol-di-stearate (mg cm^{-3})
1:3	10	6.82	5.02
1:7	10	17.64	4.03
1:15	10	28.42	–

Table 2: Product composition in case of different molar ratio and temperature

Initial substrate molar ratio	Temperature (°C)	Time (h)	Formed glycerol-mono-stearate (mg cm^{-3})	Formed glycerol-di-stearate (mg cm^{-3})
1:3	30	10	12.36	6.91
1:3	40	10	14.12	8.44
1:3	50	10	14.55	8.22
1:7	30	10	13.94	2.58
1:7	40	10	15.58	1.56
1:7	50	10	18.98	4.59
1:15	30	10	16.08	1.65
1:15	40	10	19.30	1.28
1:15	50	10	21.19	0.84

conversion of the reaction and product composition in the case of the other IL: Ammoeng 100 was studied. In Figure 3, 4 and 5 the progress curves (conversions as a function of reaction rate) are presented, moreover composition of the final mixtures obtained under various circumstances are summarised in Table 2.

Results showed (Table 2) that the higher the initial glycerol excess and applied temperature the less glycerol-di-stearate and more glycerol-mono-stearate were formed.

Reaction temperature had a definite effect on the conversion of the esterification reaction under the applied circumstances. The higher the temperature was the better conversion could be achieved.

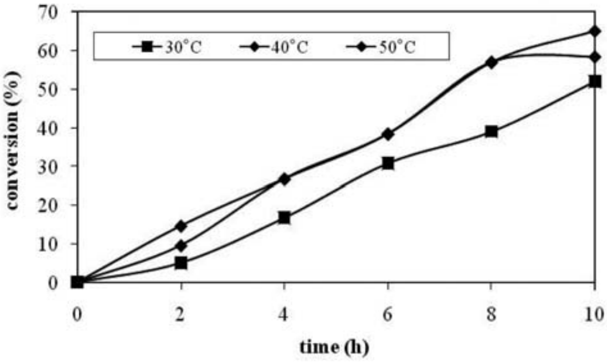

Figure 3: Conversions at various temperatures and 1:3 initial substrate molar ratio in Ammoeng 100

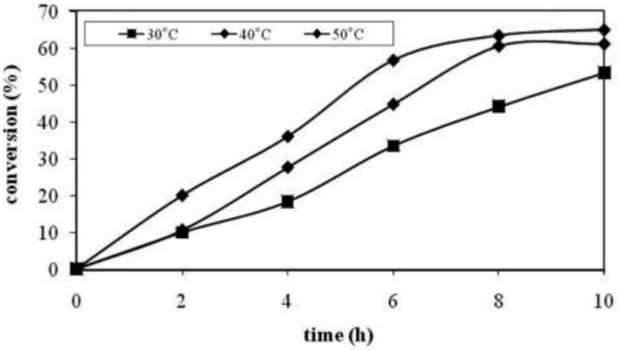

Figure 4: Conversions at various temperatures and 1:7 initial substrate molar ratio in Ammoeng 100

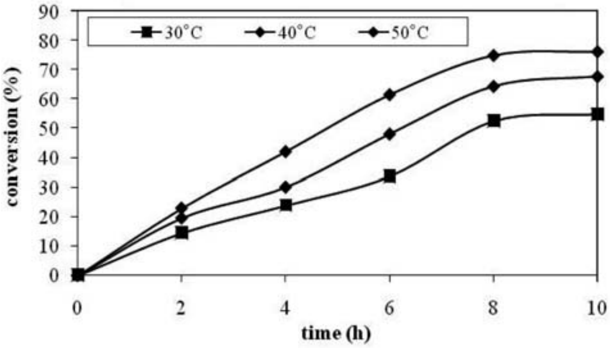

Figure 5: Conversions at various temperatures and 1:15 initial substrate molar ratio in Ammoeng 100

Activation energy of the reaction in Ammoeng 100 at 1:15 initial substrate molar ratio was counted 18.93 kJ mol^{-1} value.

3. 2. Experiments in SC CO_2

In these experiments the effect of SC CO_2 solvent on the conversion and product composition with different initial substrate molar ratio (1:3, 1:7, 1:15) was studied. Results are summarized in Table 3.

From the high conversion values it can be seen that the SC CO_2 is a very appropriate solvent for this esterification reaction. The reason could be supposedly the good solubility and diffusivity of each substrate and product at the applied temperature and pressure (70 °C, 100 bar). At the same time it can be seen that the relative amount of the formed glycerol-di-stearate is considerably high, but proportionally decreasing with the initial substrate molar ratio. Since the amount of di-stearate formed was considerable less in ionic liquids, we thought that simultaneous application (a combination) of SC CO_2 and ionic liquids might result in better experimental data: high conversion and less amount of di-stearate formed.

Table 3: Conversions and product composition at different initial substrate molar ratio in SC CO_2

Initial molar ratio (stearic acid:glycerol)	Formed glycerol-mono-stearate (mg g^{-1} reaction mixture)	Formed glycerol-mono-stearate (mg g^{-1} reaction mixture)	Conversion (%)
1:3	347.32	473.80	98.0
1:7	283.03	160.98	97.2
1:15	183.89	87.15	94.2

Experiments were performed at only one temperature and pressure. With changes in temperature or pressure or both we can change the properties of SC CO_2 (e. g. solubility, viscosity) and activity of the enzyme and consecutively achieve different results. At higher pressure the solubility of substrates and products can be higher in connection with the higher fluid density. Applying higher temperature the reaction rate increases and higher solubility can be achieved.

3. 3. Experiments in Ionic Liquid-SC CO_2 Mixtures

In these experiments we studied the effect of SC CO_2-ILs solvent mixtures on the conversion and product composition at 1:15 initial substrate molar ratio. Results are shown in Table 4.

Table 4: Conversions and product composition at 1:15 initial substrate molar ratio in ionic liquid-SC CO_2 mixtures

Ionic liquid	Initial molar ratio	Formed glycerol-mono-stearate (mg g^{-1} reaction mixture)	Formed glycerol-di-stearate (mg g^{-1} reaction mixture)	Conversion (%)
Cyphos 105	1:15	28.96	11.37	78.9
Ammoeng 100	1:15	30.36	19.48	86.8

In this case conversion values were lower than when only SC CO_2 was applied as a solvent. The reason could be the worse solubility, mixing and diffusion conditions of the substrates and the applied biocatalyst. The added IL could have positive effect on the enzyme stability and so this way on the conversion. The established negative effect was supposedly due to the worse changed solubility conditions. Accordingly the amount of the formed glycerol-di-stearate is markedly lower compared to the case of SC CO_2.

The combination of IL with supercritical fluids is particularly useful if the IL is not soluble in them and it has been proven that reactions are quite effective when IL and SC CO_2 are combined. When using SC CO_2 as a solvent or in the combination with ILs it is very important to define temperature and pressure, since these parameters have a significant influence on solubility and diffusivity of both the substrates and products of the reaction; moreover it may result in formation of two-phase system which influences the reaction rate, as well.

4. Conclusions

The enzymatic production possibilities of glycerol-mono-stearate were studied during our work. We realized the reaction in non-conventional media as two kind of ILs and SC CO_2 and studied the effect of the initial substrate molar ratio and the temperature on the product composition and conversion of the reaction.

From the results it can be appointed that solubility and mixing of the applied substrates and biocatalyst is a very important parameter of the reaction and has a strong effect on the conversion. For this reason SC CO_2 is a very appropriate and advantageous solvent for this esterification reaction, where substrate's solubility is the weak point of the reaction. The conversion in this solvent media is above 90 %.

The tested ILs also offer an available alternative method as "green solvent". The achieved conversions (under the applied circumstances) are lower than in the case of SC CO_2, but they are very promising. When only ILs were applied beside 1:15 initial substrate molar ratio the conversion was 76 and 78 % in the case of two kind of ILs respectively. Mixed the ILs and SC CO_2 the conversion reached 79 and 86 %.

The effect of initial substrate molar ratio on the product composition was also studied. Results show that the higher the initial glycerol excess is, the less glycerol-di-stearate and the more glycerol-mono-stearate are formed. Formation ratio of glycerol-di-stearate is much lower in the case of ILs comparing with $SCCO_2$.

The results could lead to further examinations in the future in the field of production possibilities of glycerol-mono-stearate in non conventional media.

5. Acknowledgement

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Povzetek

Ionski tekočini (ILs) triheksil-tetradecil-fofonijev-dicianamid (Cyphos 105) in kokosalkil-pentaetoksi-metil-amonijev-metosulfat (Ammoeng 100) smo uporabili kot topili pri reakciji esterifikacije stearinske kisline in glicerola z lipazo iz *Candida antarctice* (Novozyme 435). Pri začetni množinski koncentraciji substrata 1:15 in v primeru, ko smo uporabili ILs kot samostojni topili, je bila dosežena presnova 76 in 78 %. Višjo, 90 % presnovo smo dosegli v superkritičnem CO_2. V primeru uporabe ILs je nastalo več glicerol distearata kot v primeru uporabe $ScCO_2$.