ENZYMATIC INCORPORATION OF FATTY ACID INTO TRIACYLGLYCEROLS OF SUNFLOWER OIL RESULTING IN STRUCTURAL LIPIDS

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ABSTRACT:

The paper describes the results, obtained by enzymatic incorporation of free fatty acid (FFA) into triacylglycerols of sunflower oil. The enzymatic reaction was the transesterification reaction at normal pressure operation (NPO) at different temperatures, as well as in supercritical carbon dioxide (SC CO_2) using a batch reactor. Transesterification process conditions (temperature, FFA concentration, stirrer speed, substrate molar ratio and incubation time) were optimised, performing experiments in NPO and, for comparison, in SC CO_2. The immobilised form of the lipase (immobilized lipase from *Rhizomucor miehei*) was used. The enzymatic system could be described by a set of parallel and consecutive chemical reactions. A temperature of 353 K, stirring rate of 400 rpm, euricic acid concentration 0.05 g/cm^3, enzyme concentration 0.25 g/cm^3 and incubation time of 6h were found to be the optimum reaction conditions. Under these conditions a maximum FFA conversion of 81.15 % was attained in NPO and 68.15 % in SC CO_2 at 10 MPa. Optimising all these parameters could modify the lipids structure by acidolysis processes and oils with predictable composition could be obtained. An overview of our future research on various types of enzymatic reactors in supercritical fluids, which can further give data on the productivity and the economy of the process, is also given.

INTRODUCTION

Recent heightened awareness of interest in pharmaceutical products associated with a large part of established chemical processes has led to the application of considerable pressures on the non food industry, to adopt a cleaner and greener approach to manufacture. Lipases are powerful tools for the syntheses of structured lipids (SLs) which are triacylglycerols (TAG) having particular fatty acid (FA) at the specific position (Yugo et all, 2000). Consequently, when designing SLs with particular chemical structure it is possible to control the behaviour of TAG thereby improving the nutritional and pharmaceutical properties of TAG. Methods for production of structured triglycerides by the acidolysis of cod liver oil (B. Camacho Paez et all, 2002 and 2003) have been previously reported. Most of the reasearch about the synthesis of SLs is directed toward studying the enzymatic acidolysis with a palmitic-stearic acid mixtuire or caprilic acid by Lipozyme IM (Fayez Hamam et all, 2005 et 2008, Fernando Camachob et all, 2007, Marıa E. Carrın et all, 2008). A lot of enzymatic processes were studied in the frame of supercritical fluids (SCFs). The factors affecting the enzyme stability in supercritical fluids (effect of water activity, effect of pressure and temperature, number of pressurisation–depressurisation steps) and inhibition of enzymes are well documented with experimental results on hydrolases (Željko Knez, Maja Habulin, 2002). Studies of kinetics and thermodynamics in lipase catalysed long chain fatty ester synthesis in SCFs (Chiara Giulia Laudani et all, 2007) have been previously reported. Structural lipids as fatty esters play important roles in the

different applications: food (human and animal feeds), nonfoods application (pharmaceutical field, cosmetics bio-detergent) and also to obtain the vegetable bio-combustible – biodiesel by transesterfication processes. Application of SC CO_2 as an alternative reaction medium for enzyme-catalysed acidolysis by enzymatic incorporation of monounsaturated fatty acid into triacylglycerol of sunflower oil is presented. From economic point of view sunflower oil is a very good source for valorization, especially by the variety of fatty esters content. The enzymatic incorporation of free fatty acid was carried out in a batch reactor, where the influence of temperature, pressure and of free fatty acid concentration on the incorporation of monounsaturated fatty acid (euricic fatty acid EA) into triacylglycerol s from sunflower oil was studied. The goal of the study is the engineering of improved sunflower oil quality by triacylglycerols transesterification by immobilized enzymes in NPO and SC CO_2, emphasising the pharmaceutical properties of TAG.

MATERIALS AND METHODS

I.1 Materials

I.1.1 Sunflower oil
The sunflower oil from Tovarna.olja GEA d.d., Slovenia, was used in transesterification reactions. This was purchased from a local grocery store.

I.1.2 Enzyme
Lipase (Lipozyme RM IM) from *Rhzomucor miehei* immobilized on a macroporous anion exchange resin was obtained from NOVO Nordisk A/S, Bagsvaerd, Danmark.

I.1.3 Gases
Carbon dioxide 4.5 was supplied by Messer MG Ruše, Slovenia

I.1.4 Other chemicals
Free fatty acid standards, as well as euricic acid were purchased from Sigma-Aldrich (Germany). All reagents and solvent were of analytical or chromatographic grade.

I.2 Methods
The transesterification reaction in NPO was performed in a flask bioreactor. The reaction in SC CO_2 was performed in a batch reactor, previously described [3, 4, 5].

I.2.1 Enzymatic acidolysis
Acidolysis reaction with lipase was carried out at different conditions. The reactions were performed in the flask, in which preparation were: sunflower oil and different concentrations of euricic acid (EA), 3 ml n-hexan, and 0.25 g lipase. The mixture was stirred (400 rpm) and heated at different temperatures at normal pressure. The reaction began when the enzyme was added to the substrate. The kinetics of the reaction was followed by sampling at different time intervals. The samples were collected for two types of analysis, qualitative and quantitative. The first method (qualitative) was the detection of the percent of FFA by titration and the second method (quantitative) was gas chromatography analysis.

1.2.2 The acidolysis model
This model is based on the following hypothesis: (1) due to the 1:3 positional specificity of the lipase, only the fatty acids in positions 1 and 3 of the TAG participate in the reaction; (2) the exchange in position 1 does not depend on the nature of the fatty acid from position 3 and vice versa (Fernando Camachob, 2007). The system could be described by the set of reactions:

$$TAG_i + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} TAG_iE \underset{k_4}{\overset{k_3}{\rightleftarrows}} EAG_1 + DAG_i\text{-}OH$$

$$EAG_1 + AG^* \xrightarrow{k_5} EAG^* + AG_1$$

$$EAG^* + DAG_i - OH \xrightarrow{k_6} TAG_i\left(AG^*\right) + E$$

$$TAG_i + AG^* \xrightarrow{\text{LIPASE}} TAG_{i-1}\left(AG^*\right) + E$$

where TGA_i is triacylglycerol, E is lipase, TAG_iE is the complex with enzyme, DAG_1-OH is hydroxyl diacylglycerols, EAG_1 is enzyme complex with free fatty acid release, AG*is euricic acid, AG_1 is a free fatty acid released in system., $TAG_{i-1}(AG^*)$ is new product (modified triacylglycerol).

I.2.3 Transesterification reaction in SC CO_2

A batch reactor has been used to perform the bio-reaction tests in SC CO_2. The detailed description of the reactor has been given in the literature [3, 4, 5]. The reaction was carried out at 10 MPa and 353 K. Initially, the substrates were pumped into the reactor and then enzyme was added. Finally CO_2 was pumped into the reactor, up to the desired pressure.

RESULTS

Temperature effect

In order to investigate the temperature effect on the conversion, the reaction was performed at atmospheric pressure and different temperatures: 313 K, 333 K, 353 K, 363 K and 373 K. The experiments were performed during 6h of incubation. The maximum conversion of transesterification reaction was obtained for NPO, at 353 K, where the maximum enzymatic incorporation of FFA into triacylglycerols was obtained. The lowest conversion of transestreification reaction was observed at the temperature of 373 K.

The effect of temperature on the initial rate is shown in Figure 1. The optimum temperature for the enzymatic reaction in NPO was 353 K, followed by a decrease in the reaction rate with increasing temperature.

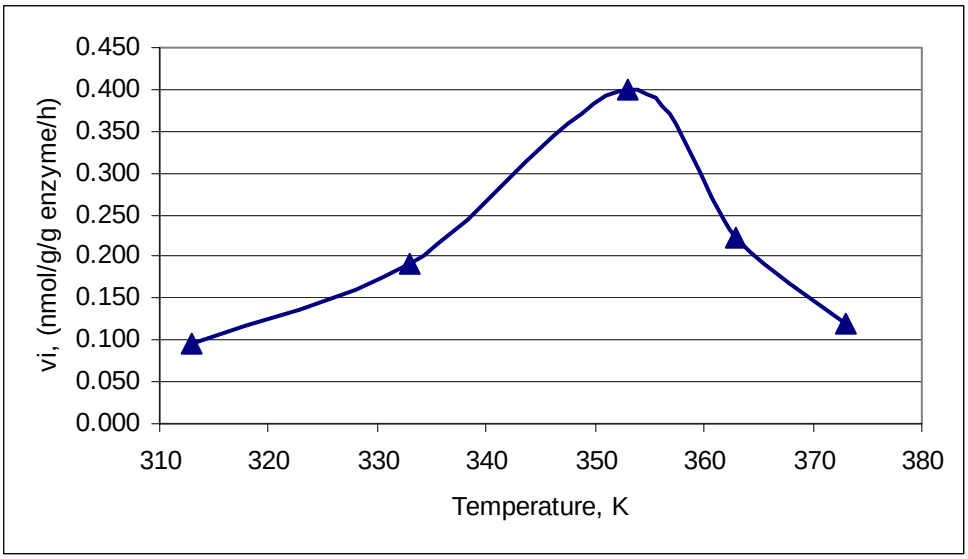

Figure 1: Effect of temperature on the initial rate of the enzymatic acidolysis reaction

From the Arrhenius plot of acidolysis reaction at NPO, the activation and deactivation energies, the equilibrium constant, the deactivation entropy and deactivation Gibb's free energy were estimated. Arrhenius plot for the acidolysis reaction at NPO is presented in Figure 2. Table 1 presents the calculated thermodynamic values.

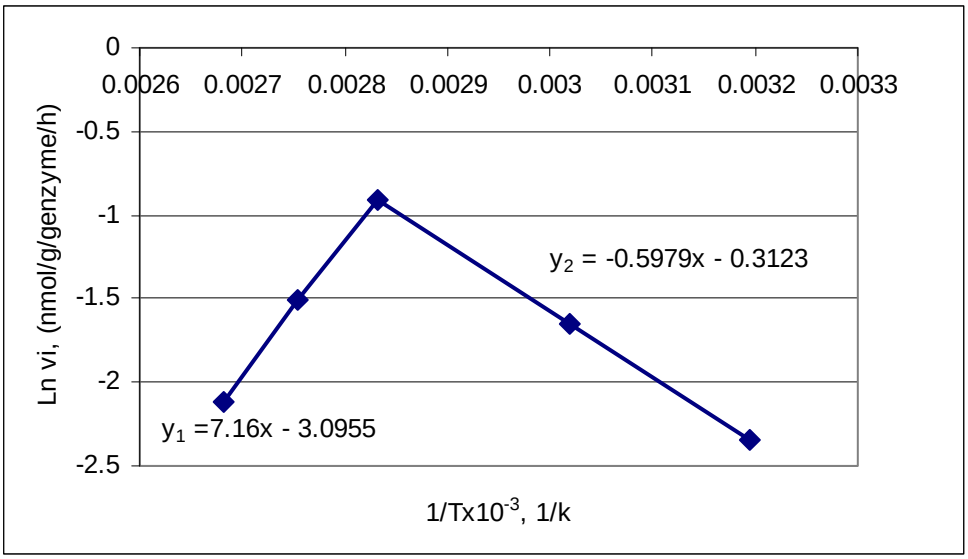

Figure 2: Arrhenius plot for the euricic acid incorporation into tryacilglycerols of sunflower oil.

Table 1: Thermodynamic properties: deactivation of lipase
during the euricic acid incorporation into triacylglycerols from sunflower oil

Magnitude	**Value**
Ea (kJ/mol)	4.970
ΔH_d (kJ/mol)	54.557
K_d	1.018
ΔG_d (kJ/mol)	54.9765
ΔS_d (J/mol*K)	138.98

Ea is activation energy; ΔH_d is deactivation enthalpy; K_d is equilibrium constant; ΔG_d is Gibb's free energy of deactivation; and ΔS_d is deactivation entropy.
The optimal reaction parameters were: 353 K; sunflower oil/organic solvent ratio (n-hexan) 1:5; euricic acid concentration of 0.02 g/ml sunflower oil and 0.05 g/ml sunflower oil respectively; the enzyme concentration of 0.25 g/ml sunflower oil; stirring rate of 400 rpm.

Comparison between the reactions performed in NPO *and* SC CO_2

SC CO_2 is an alternative reaction medium for enzyme-catalysed acidolysis reactions by enzymatic incorporation of euricic acid into triacylglycerol from sunflower oil.

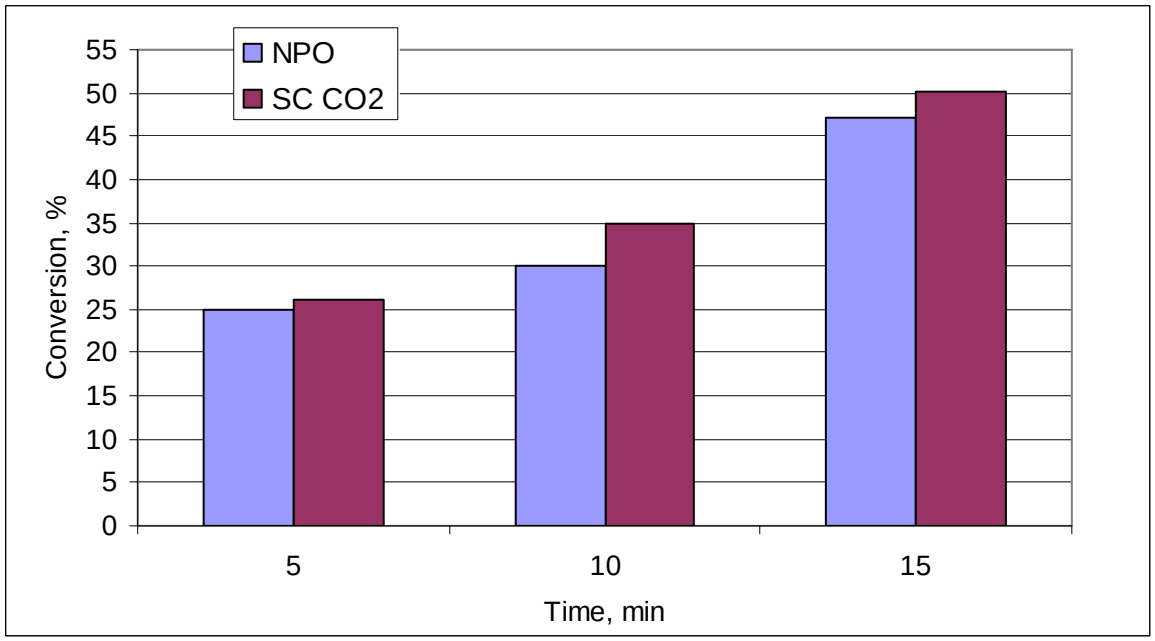

Figure 3: Comparative results between the conversion of reaction, performed in NPO and SC CO_2

The preliminary reactions show that for the same conditions of temperature and concentration of substrates as employed previously for the NPO, the conversion of the enzymatic reaction in SC CO_2 (10MPa) is slightly higher in the first 15 min after the beginning of the reaction (Figure 3). These results represent an encouraging first step for future studies regarding the optimization of enzymatic transesterification by using SCF media.

CONCLUSIONS

This study represents a preliminary research on improving sunflower oil quality by enzymatic transesterification. The obtained results are encouraging regarding the possibility of replacing the monounsaturated acids from triacylglycerols, with potential pharmaceutical applications.
Further studies are still necessary for optimizing the process both in NPO and in SCFs.
In SC CO_2 the reaction parameters: pressure, temperature, enzyme concentration, stirring rate and oil/free fatty acid ratio, require additional consideration.

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