Comparison of glycerolysis of sardine oil by Lipozyme 435 in solvent free and SC-CO_2 media

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ABSTRACT

The efficiency of lipase catalyzed glycerolysis of sardine oil in solvent free and supercritical carbon dioxide media has been investigated. The immiscibility between substrates, glycerol and oil, is an important drawback to reach good high conversions of triglycerides (TAG) into monoglycerides (MAG) and diglycerides (DAG) in short reaction times. To improve mass transfer rates, emulsification of both reactants as reverse micelles (glycerol-in-oil) has been carried out. Enzyme-catalyzed reaction is an attractive alternative since the reaction can be carried out under mild conditions avoiding the oxidation of omega-3 fatty acids. In this work, a commercial immobilized lipase (Lipozyme 435) was employed as biocatalyst. The effects of SC-CO2 density on reaction rates and oxidation stability has been compared with those obtained in solvent free system at atmospheric pressure. The effect of temperature and pressure on reaction yield, oxidation state of reaction products and enzyme stability was studied. Good stability of Lipozyme 435 has been observed in both systems proving no thermal deactivation at temperatures higher than its optimum.

INTRODUCTION

Fish oil is rich in omega-3 (n-3) polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid. The importance of omega-3 PUFAs in human nutrition and disease prevention was scientifically recognized some decades ago because they are involved in many important biological processes in the human body. Among the different types of lipid derivatives containing PUFA concentrates, MAG and DAG have good bioavailability. In addition, MAG or its mixtures with DAG account for 75% of worldwide emulsifier production. The well-known drawbacks of the conventional chemical glycerolysis technique (energy intensive, low yields (30–40%), oxidized products) have prompted a growing interest in the development of alternative processes for the production of MAG and DAG rich in n-3 PUFAs. Enzyme-catalyzed reaction is an attractive alternative since the reaction can be carried out under mild conditions. To overcome the problem of the immiscibility of glycerol and oil, different approaches have been used in the literature to improve the contact between the reactants and hence reduce mass transfer limitation. Lipase-catalyzed glycerolysis has been carried out in different reaction media such as organic solvents, compressed fluids, and ionic liquids, in order to improve the mass transfer. Recently, the uses of different surfactants to increase the interfacial area, and ultrasound irradiation have been also proposed to reduce mass transfer limitation. Although the best conversions are reached when the glycerolysis reaction is carried out in organic solvent medium, the cost, toxicity and energy required for removal from the product mixture, are important aspects to be considered when dealing with conventional solvent systems [1].

Enzymatic reactions in supercritical fluids (SCFs) rise as an alternative to organic solvents. This technology is based on the use of lipases which are able to catalyze different reactions in CO_2 supercritical media. Supercritical carbon dioxide (SC-CO_2) is probably the most used

SCF when choosing environmental replacement for organic solvents. Some previous studies of enzymatic reactions of different lipid sources in SC-CO_2 have been reported in the literature. However, in case of enzymatic glycerolysis, other compressed fluids as propane, n-butane, and acetone, have been used. Some studies of glycerolysis of soybean oil in SC-CO_2 can be found, but without using enzymatic catalyst at high temperatures [2].

On the other hand, biocatalytic processing in the presence of surfactants has also received attention in order to increase the miscibility of the substrates. Surfactants are molecules with amphipatic characteristics, and they are able to form micellar systems. In addition, lipases demonstrate high interfacial activity in micellar system, because the formation of the active site during the reaction occurs at the interface between the substrate and the enzyme. Nevertheless, it must be taken into account that food grade surfactants could be modified by lipases. For instance, the lipase Novozym 435 presented activity toward some surfactants as soy lecithin, Tween 65, Tween 80 and Tween 85 in glycerolysis reactions. To avoid this problem, other kind of synthetic surfactants have been used, such as sodium (bis-2-ethyl-hexyl) sulfosuccinate (aerosol-OT or AOT). This anionic surfactant has the ability to form reverse micelles in a great number of non-polar organic substances as oils, and several other polar solvents such as glycerol. In this case, good results have been obtained in glycerolysis systems when adding more that 7.5% of AOT. However, the surfactant has to be removed and it may generate problems during downstream processes [3-4].

In this work a comparison of glycerolysis of sardine oil in solvent free and SC-CO_2 media have been studied. In both systems, to improve the mass transfer limitation, avoiding the used of any organic solvent or surfactant, the formation of a microemulsion just with the reactants as reverse micelles (glycerol-in-oil) has been carried out. These microemulsions exhibit relatively ordered structure and provide an enormous interfacial area, which favors lipase-catalyzed reactions. This emulsification process has been carried out just before the addition of the lipase. Agitation and MAG and DAG formation, as emulsifiers, help to create a good contact among reactants. The commercial immobilized lipase Lipozyme 435 from *Candida antarctica* B was employed as biocatalyst. The reaction kinetics have been determined at different operating pressures (0.1, 15, 20 and 25 MPa) and temperatures (40, 50, 65, 80, 90ºC). The experiments were conducted in a batch mode keeping constant the enzyme concentration at 5 wt% (by weight of substrates) and agitation of 800 rpm. Finally the oxidative status in the final reaction products was evaluated through the peroxide and anisidine values. MAG yields and the oxidation stability of the reaction products were compared for both systems.

MATERIALS AND METHODS

Materials

Refined sardine oil was kindly provided by Industrias Afines S.L. (Spain) with 18.3% of EPA and 7% of DHA and a water content of 0.2%. Glycerol was purchased from Sigma Aldrich with a purity of ≥ 99.5% and a water content of 0.18%. The food grade lipase Lipozyme® 435 from *Candida antarctica* (immobilized on a macroporous hydrophobic acrylic resin) was kindly donated by Novozymes A/S (Bagsvaerd, Denmark). CO_2 (99.9%) was supplied by Air Liquide S.A. (Spain). All other chemicals used were of analytical or HPLC grade.

Microemulsions preparation

To prepare the microemulsion of the substrates as reverse micelles, the appropriate amount of glycerol was added drop by drop to the suitable amount of oil (molar ratio: 3:1) while are completely mixed at high speed. High speed blender (Miccra D9 equipped with a DS-20/PF EMR rotor–stator) at different speed from 16000 to 35000 rpm was used by pulses during 3 minutes. The characterization of the emulsions was performed 10 min after emulsification to

avoid any creaming or coalescence effect. Droplet size distribution, mean droplet diameter and polydispersity index (PDI) of samples were measured by dynamic light scattering (DLS), using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., UK) to evaluate the best conditions to produce a stable emulsion with small (or the smallest) droplet size.

Glycerolysis of sardine oil by Lipozyme 435

On one hand, the glycerolysis reaction in SC-CO_2 has been performed in a high pressure batch stirred tank reactor made of stainless steel and having an internal volume of 100 mL. Emulsion was charged into the reactor and it was then closed, placed in a thermostatic water bath and connected to the pressure circuit. Subsequently, SC-CO_2 was fed into the reactor by means of a high pressure pump (ISCO 260 D) up to the desired pressure, which was maintained by a digital pressure controller. Operating temperature and pressure have been varied in the range between 40-90ºC and 15-25 MPa, respectively. Samples were taken periodically during 8 h through a siphoned capillary equipped with a microfilter made of sintered steel, which prevented the withdrawal of the enzyme from the reaction mixture and, thus, stopped the reaction. On the other hand, the glycerolysis reaction, solvent free system, has been carried out in a 100 mL jacketed batch reactor at different temperatures. All the samples were stored at -18ºC prior to analysis.

Analysis of the reaction products

The neutral lipid profile (TAG, DAG, MAG and FFA) was analysed and quantified by a normal phase high performance liquid chromatography (NP-HPLC). The method and calibration procedure were previously reported [5].

Measurement of lipid oxidation

The oxidation status can be estimated using two assays: the peroxide value (PV) and the anisidine value (AnV). The PV measures the concentration of hydroperoxides formed in the initial stages of lipid oxidation (primary oxidation). The AnV is an estimation of the concentration of secondary oxidation products. Determinations of these values for the samples before and after the experiments have been performed in order to evaluate potential lipid oxidation processes during the glycerolysis reaction. Total oxidation of the oil can be estimated by the formula: TOTOX = 2PV + AnV. All determinations were performed according to standard methods [6-7].

RESULTS

Emulsification process

Some previous experiments have been performed to define the optimum speed in which the microemulsion presents the best droplet size distribution. As a reverse micelle system, the oil behaves as the continuous phase and glycerol represents the discontinuous one. The results showed that the smallest glycerol droplet diameter was obtained at 29000 rpm. In addition, the polydispersity index was low at this speed, around 0.4.

To evaluate the effect substrates emulsification in the reaction rate, results have been compared with those obtained without substrates emulsification at the same conditions in SC-CO_2 media and in solvent free system. Lipase-catalyzed glycerolysis of sardine oil in SC-CO_2 media is presented in Figure 1, but the same tendency was observed for solvent free system. As it can be observed, mass transfer limitations lead to lower reaction rate when no previous emulsification of reactants was carried out. For the reverse micelle system, higher interfacial area is provided which favors lipase-catalyzed reactions. At longer reaction times, similar conversion was reached, probably due to the MAG and DAG formation that can act as emulsifiers.

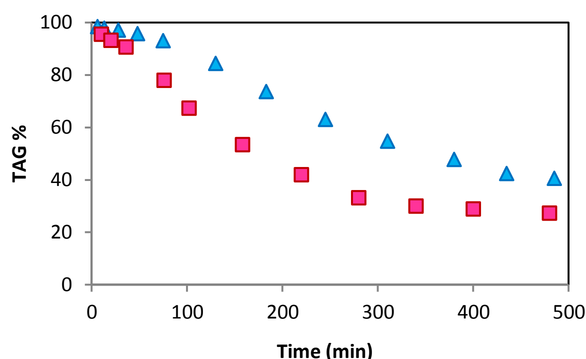

Figure 1. TAG conversion by lipase-catalyzed glycerolysis of sardine oil in SC-CO_2 media with (■) and without (▲) substrates emulsification. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates and 15 MPa.

Pressure effect on glycerolysis of sardine oil by Lipozyme 435

Loss *et al.* [2] have recently reviewed different applications of supercritical fluids as alternative solvent for biocatalysis processes, concluding that there seems to be no "rule of thumb" for predicting the effect of pressure on enzyme activity in SC-CO_2. On the one hand, operating pressure can affect the reaction rate constant, but also, density-related changes in the physical parameters of SC-CO_2 may indirectly affect the enzyme catalytic activity, and thus the reaction performance. Figure 2 presents TAG conversion at different operating pressures (15 to 25 MPa) at 50ºC, and the corresponding kinetic reaction at atmospheric pressure (0.1 MPa). Initial substrate molar ratio (3:1 glycerol:sardine oil), and enzyme loading (5% wt. of substrates) remained unchanged. The results show that pressure had no significant effect on TAG conversion in the pressure range studied. As it has been previously explained, in the reverse micelle system, sardine oil behaves as the continuous phase. According to the literature, solubility of CO_2 in fish oil slightly increase with pressure at constant temperature, for instance at 40ºC solubility at 10 MPa is 25.8% mass and at 25 MPa is 33.7% mass [8]. Therefore the improvement in the diffusivity in the reaction system is not significant by increasing operating pressure and not a significant effect of pressure in reaction rate has been observed.

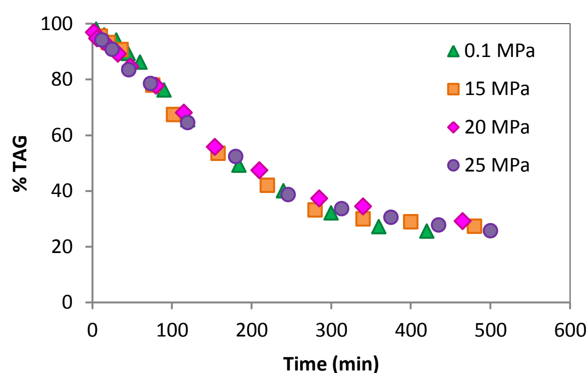

Figure 2. TAG conversion by lipase-catalyzed glycerolysis of sardine oil at different pressures. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates

Temperature effect on glycerolysis of sardine oil by Lipozyme 435

Operating temperature significantly influences enzyme-catalysed reactions by affecting both the enzyme activity and stability and the physical properties of the system such as viscosity and diffusivity [9]. To assess the effect of temperature on the kinetics of the glycerolysis of

sardine oil by Lipozyme 435 in SC-CO_2 media and in a solvent free system, operating temperature has been varied from 40 to 90ºC. Initial substrate molar ratio (3:1 glycerol:sardine oil) and enzyme loading (5% wt. of substrates) remained unchanged. For both reaction systems, the equilibrium conversion is essentially temperature independent, although, at 40ºC, 8 hours was not sufficient time to achieve equilibrium concentration. However, raising temperature from 40 to 90ºC resulted in an increase of the initial reaction rate, probably because of a higher kinetic energy of the molecules that leads to lower viscosity and higher diffusivity of the solvent and substrates. It must be highlighted that enzyme activity was not negatively affected by temperature even at 90ºC. Figure 3 compares the MAG production in a solvent free system and in SC-CO_2 at two different operating temperatures, 50ºC and 80ºC. Neither reaction rates nor equilibrium conversion seem to improve when the reaction is performed in SC-CO_2 media, probably due to the low solubility of SC-CO_2 in the continuous oily phase.

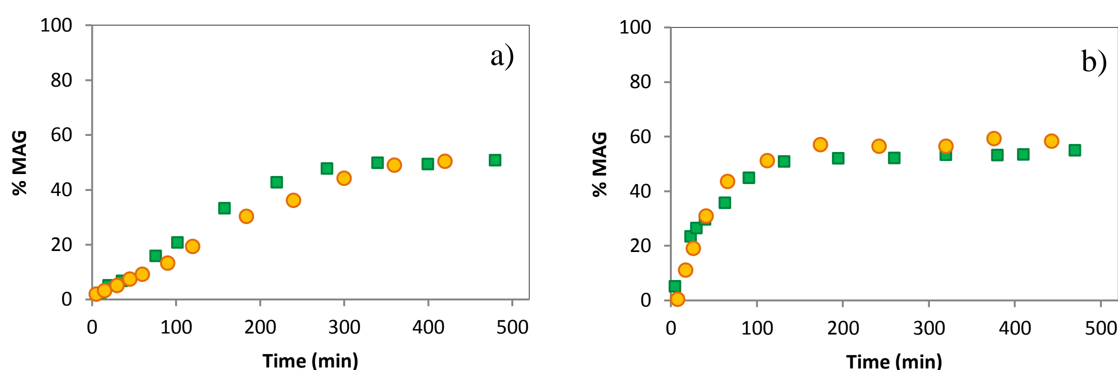

Figure 3. MAG production by lipase-catalyzed glycerolysis of sardine oil in (●) solvent free system and in (■) SC-CO_2 media at (a) 50ºC and (b) 80ºC

Comparison of lipid oxidation in both systems

In this work, peroxide and anisidine values have been determined for the supplied refined sardine oil and the reaction mixtures obtained at the different temperatures assayed after 8 hours of reaction in solvent free and in SC-CO_2 media. To separate the lipid phase from the lipase and the remained glycerol, the whole sample was centrifuged at 5000 rpm and 35ºC during 10 minutes. The upper phase formed by reaction products was collected under N_2 atmosphere and stored at -18ºC prior to analysis. In Table 1, it can be observed the oxidation status for the initial oil and glycerolysis products obtained from 40 to 90ºC in SC-CO_2 media. Anisidina values (around 24.0) are close to the limit but it can be considered "acceptable" and it seems to keep constant in the reaction products at different temperatures. Peroxide values however decreased from 40 to 90ºC. In fact, the lowest PV (3.6) was obtained at 90 ºC, the highest T assayed in this work. Further experiments should be done to verify this behaviour.

Table 1. Oxidation status of glycerolysis products obtained in SC-CO_2 media at different temperatures (3:1 as substrate molar ratio, 5% of Lipozyme 435 and 15.MPa)

			Reaction Temperature (ºC)				
	Legal Max.*	Initial oil	40	50	65	80	90
PV (meqO_2/Kg oil)	10	4.8	9.3	5.6	5.5	4.0	3.6
AnV	30	24.2	23.5	23.1	23.6	23.4	24.8
TOTOX	50	33.8	42.1	34.3	34.6	31.4	32

*According to EPS (European Pharmacopeia Standard).

Similar results regarding oxidation status have been obtained in solvent free system in the same temperature range.

CONCLUSION

The emulsification of glycerol and oil, to carry out a glycerolysis reaction, improves the initial contact of the substrates with the lipase providing higher reaction rates in solvent free and in SC-CO_2 media. The results show that pressure had no significant effect on conversion of TAG in the working pressure range. Otherwise, it has been demonstrated that an increase in temperature from 40 to 90 ºC produces higher reaction rates in both systems. It must be also highlighted that enzyme activity was not negatively affected by temperature even at 90 ºC. On the other hand, oxidation level of the products obtained under SC-CO_2 is very similar to those obtained in solvent free at the same temperatures. Further studies of oxidation are being done for obtaining sound conclusions.

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