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Low-Pressure Lipase-Catalyzed Production of Mono- and Diglycerides with and Without *N*-Butane and AOT Surfactant

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Abstract The aim of this work is to report the production of mono- and diglycerides from olive oil at ambient condition and in pressurized *n*-butane as solvent medium. For this purpose, a commercial immobilized lipase (Novozym 435) was employed as catalyst and sodium (bis-2-ethyl-hexyl) sulfosuccinate (Aerosol-OT or AOT) as surfactant. The experiments were conducted in batch mode varying the temperature, pressure, and AOT concentration. Results showed that lipase-catalyzed glycerolysis either with compressed *n*-butane or in solvent-free system with AOT as surfactant might be a potential alternative route to conventional methods, as high contents of reaction products, especially monoglycerides (~ 60 wt.%), were achieved at mild temperature and pressure with a relatively low solvent to substrates mass ratio (4:1) in short reaction times (2 h).

Keywords Glycerolysis · Olive oil · Lipase · AOT · *n*-Butane · Solvent-free system

Introduction

Over the last 10 years, there has been a growing interest in the development of alternative processes for the production of mono- (MAG) and diglycerides (DAG). The establishment of worldwide biodiesel government programs, with the expected glycerol surplus, together with the well-known drawbacks of the conventional chemical glycerolysis technique [1–3] (energy intensive, provides low yields (30–40%), dark-colored, burn-tasted products, and the need of product post-purification by molecular distillation) have prompted many attempts to produce MAG and DAG by enzyme-catalyzed reactions.

Monoglycerides are widely employed in the food industry as emulsifiers for bakery products, margarines, confectionery, and anti-staling agents in bread. In addition, owing to

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their excellent lubricant, emulsifying, and plasticizing properties, MAG are used in textile processing, production of plastics, and formulation of oil for different types of machinery [4–7]. Also, production of MAG from enzymatic glycerolysis has been alleged to offer industrial potential as ingredients or compounds with improved functionality or a healthier nutritional profile [4, 8].

Diglycerides are naturally occurring minor constituents of edible fats and oils, mainly constituted by triacylglycerols and have attracted much attention over the last years due to their several important beneficial properties to human health [9]. Recently, it has been found that DAG, particularly *sn*-1,3-diacylglycerols, may have beneficial effects on obesity and lipemia prevention [10, 11], despite having a similar energy value and digestibility as known for triacylglycerols.

Glycerolysis of vegetable oils has been conducted with lipases, in organic medium [4–7], in solvent-free systems [12–14], with free or immobilized enzymes [4–7, 12–14], in ionic liquids [15] or using compressed fluids as reaction media [9, 16].

Considering the very limited mutual solubility of glycerol–oil mixtures, the fact that the use of organic solvents may produce various undesirable physicochemical effects on enzyme molecules and also the high costs associated with solvent removal [7, 17], it seems reasonable to consider the use of compressed fluids to conduct enzyme-catalyzed reactions as a promising route to completely eliminate solvent traces from reaction products, bringing many advantages in terms of energy consumption, favorable transport properties, easier recovery of the products, adjustable solvation ability, and minimizing side reactions to prevent the formation of undesired compounds [18–21].

Recently, it has been shown that propane and, due to the low-vapor (saturation) pressure, especially *n*-butane, may also be convenient to conduct enzyme-catalyzed bioconversions [21–24]. Besides, the use of a surface-active agent with or without a compressed fluid has proved to be a potential route to the production of MAG and/or DAG, in the so-called reverse micelle systems, because microemulsions allow both hydrophilic and hydrophobic substances to be dissolved in high concentrations and provide an enormous interfacial area, which favors lipase-catalyzed reactions [21, 24–31].

This work makes part of a broader project aiming at developing new processes for the production of emulsifiers through enzyme-catalyzed glycerolysis reaction [21, 24, 30, 31]. Here, the main objective is to investigate the enzymatic glycerolysis of olive oil for the production of MAG and DAG using AOT as surfactant in low-pressure *n*-butane and at ambient pressure condition.

Materials and Methods

Materials

The substrates used in the glycerolysis reactions were commercial olive oil (Arisco, Brazil), Novozym 435 (immobilized lipase obtained by *Candida antarctica*) (Novozymes S.A., Araucária, PR, Brazil), glycerol (Merck, 99.5%). AOT (sodium (bis-2-ethyl-hexyl) sulfosuccinate, Aerosol-OT or AOT, Sigma-Aldrich, 99%) was used as surfactant and *n*-butane (White Martins S.A., 2.5–99.5 mol% purity) as solvent. Authentic standards of 1-(*cis*-9-octadecenoyl)-*rac*-glycerol (Sigma), glycerol-1,2 and 1,3-dioleate (Sigma) and glyceryl trioleate (Sigma-Aldrich) were used for reaction conversion determination. Acetonitrile (99.9%) and acetone (99.8%) were purchased from Merck.

Apparatus and Experimental Procedure

The experimental apparatus and procedure used for mono- and diglyceride production from olive oil with immobilized lipases in pressurized *n*-butane was presented in previous, recent, works [21, 24]. Briefly, precise amounts of the substrates (at specified glycerol-to-oil molar ratio, typically 5 g), enzyme (by weight of substrates) and AOT were weighed on a precision scale balance (Ohaus Analytical Standard with 0.0001 g accuracy) and loaded into the reaction vessel, which was immediately closed and the temperature control (accuracy of 0.5 °C) was turned on. The charge of a known amount of *n*-butane was performed with the help of the syringe pump (Isco 260 D, resulting accuracy of ±0.01 g in n-butane loadings) until the pre-established pressure was achieved. With known values of pressure and temperature in the syringe pump reservoir, solvent density was estimated using the HBT (P-V-T) correlation for compressed liquids [32] or taken from experimental literature values [33], making possible to estimate the mass of solvent charged into the reaction vessel (jacketed 50 mL reactor, Parr, model 48 43). Typically, around 3 min were sufficient to feed and pressurize the reactor to the desired value, and once the system pressure had been reached, the agitation was turned on and the reaction time was set to zero. Based on the uncertainty in *n*-butane loadings, substrates weighing and predictions in solvent feed, *n*-butane-to-substrate ratio varied approximately 5% of the desired value.

With respect to the experiments carried out at room pressure, enzymatic glycerolysis reactions were carried out using 30 g of the substrates in a mechanically stirred (IKA-RW 20 digital stirrer) jacketed flask (60 mL) equipped with a sampling pipette and a PT-100 probe (0.1 °C accuracy) for temperature monitoring—additional details can be found elsewhere [30, 31].

Throughout this work, at room condition, and with pressurized *n*-butane, the agitation was kept constant at 600 rpm and based on the phase behavior of the substrates, olive oil + glycerol, in compressed *n*-butane, the solvent to substrates mass ratio was fixed at 4:1 [21, 24, 30, 31, 34]. At the end of the reaction, the immobilized lipase was removed by vacuum filtration, and the products were recovered for further analysis. It may be important to emphasize that in all cases, at ambient pressure and with pressurized n-butane, destructive experiments, considering the whole content of reactor vessel, without sampling, were carried out.

Analytical Methods

Quantitative analyses of the products were conducted using an HPLC system from Agilent 1100 Series, with refractive index detector. The following instrumentation and conditions were used: Zorbax C_{18} column (4.6 m×250 mm, 5 μm), flow rate of 1.0 mL/min, column temperature of 35 °C; detector temperature of 40 °C; the mobile phase, acetonitrile/acetone (1:1, *v*/*v*). acetonitrile/acetone (1:1, *v*/*v*) was used as a sample dissolving solvent, and the injection volume was 20 μL. The quantification of reaction products was carried out using authentic standards of MAG, DAG, and TAG. The calibration curves were built with the following concentrations 300, 800, 1,000, 2,000, 5,000, 8,000, and 10,000 ppm. The content of reaction products was expressed in terms of the whole amount of MAG, DAG, and TAG, as weight percent of the total sample, in surfactant-free basis. All analyzes were replicated at least three times. Before injection, samples were carefully handled with slight warming and gentle agitation to avoid phase separation. Based on previous works and experience with the system investigated, experimental uncertainties are estimated to be less than 5 % in terms of MAG, DAG and TAG contents [21, 24, 30, 31].

Sequential Strategy of the Experimental Designs

On the basis of a previous research [24], a full experimental design comprising the variable temperature (30–70 °C), pressure from ambient to 20 bar, and AOT concentration from 2.5 to 7.5 wt.% was conceived. Then, in an attempt to investigate possible technical and economic compatibility, another full design was carried out keeping fixed the temperature at 30 °C and varying the working pressure from ambient to 20 bar and AOT concentration from 0.1 to 2.5 wt.%.

For the two experimental designs mentioned, it was adopted that the glycerol to olive oil molar ratio of 2:1, 7.5 wt.% of Novozym 435, 600 rpm, solvent to substrates mass ratio of 4:1 (in the case of *n*-butane) and 2 h of reaction time. The Software Statistica® 6.0 (Statsoft Inc) was used to assist the design and the statistical analysis of experimental information, adopting in all cases studied a confidence level of 95% ($p<0.05$).

Results and Discussion

In a very recent work, we have investigated the effects of temperature (30–70 °C), pressure (10 to 160 bar), and AOT concentration (2.5 to 7.5 wt.%), keeping fixed the other parameters exactly as mentioned above, on the production of MAG and DAG from Novozym 435 glycerolysis of olive oil in *n*-butane [24]. In that work, high contents of MAG, ~70 wt.%, with appreciable production of DAG was observed in 2 h of reaction and, at a confidence level of 95%, the surfactant had a pronounced positive effect on the production of MAG, while temperature and pressure presented a negative effect on the reaction conversion.

Thus, in attempt to meet possible technical viability and improve potential economic feasibility of enzymatic glycerolysis reaction, and taking into account such previous results, we have decided to proceed with the investigation executing an experimental design, now varying the pressure from 1 (solvent free) to 20 bar (with compressed *n*-butane), while maintaining the ranges for the other two variables at temperature and AOT concentration, 30 to 70 °C and 2.5 to 7.5 wt.%, respectively. These ranges were adopted because increase of AOT concentration could be thought as inadequate in terms of economic aspects while keeping the original temperature range could be interesting due to the optimum working temperature of the enzyme [35].

Results of the first experimental design are presented in Table 1 and Fig. 1 shows the related variable effects. Clearly, lower MAG and DAG content values were found compared to the previous investigation [24]. At this time, temperature was not significant whereas again AOT concentration had a positive effect and pressure had a significant, negative, influence on MAG production. It is also interesting to observe from Fig. 1 the negative cross interactions verified for temperature and pressure, temperature, and AOT concentration and, finally, pressure and AOT concentration.

Although the maximum content of MAG + DAG found here,~74 wt.% is not so high compared to that earlier reported (MAG + DAG about 80 wt.%), it can still be considered relevant if compared with the yields obtained from the conventional alkali-catalyzed process. In pursuit of economic aspects, another experimental design was built, fixing the temperature at the lowest level (30 °C), while lowering the AOT concentration (0.1 to 2.5 wt.%) and keeping the pressure range of the later design (1 to 20 bar). For this case, as shown in Table 2, the maximum MAG content found was around 46 wt.%, while for MAG + DAG, it was found ~65 wt.%, lower, but not negligible values compared to those

Table 1 Matrix of the first experimental design, showing real and coded (between parenthesis) values, with response in terms of MAG, DAG, TAG, MAG, and DAG contents.

Run	*T* (°C)	*P* (bar)	AOT (wt.%)	MAG (wt.%)	DAG (wt.%)	MAG + DAG (wt.%)	TAG (wt.%)
1	30 (−1)	1 (−1)	2.5 (−1)	30.6	9.3	39.9	35.5
2	30 (−1)	20 (1)	2.5 (−1)	29.9	13.6	43.5	29.9
3	70 (1)	1 (−1)	2.5 (−1)	37.6	13.8	51.4	31.2
4	70 (1)	20 (1)	2.5 (−1)	37.6	7.8	45.4	17.6
5	30 (−1)	1 (-1)	7.5 (1)	43.3	12.0	55.3	25.1
6	30 (−1)	20 (1)	7.5 (1)	48.9	10.0	58.9	20.6
7	70 (1)	1 (-1)	7.5 (1)	58.3	16.1	74.4	8.5
8	70 (1)	20 (1)	7.5 (1)	20.5	7.2	27.7	48.1
9	50 (0)	10 (0)	5 (0)	42.3	13.9	56.2	17.7
10	50 (0)	10 (0)	5 (0)	40.1	10.3	50.4	16.6
11	50 (0)	10 (0)	5 (0)	40.6	14.7	55.3	17.8

Effect of temperature, pressure and AOT in the glycerolysis of olive oil with (10 and 20 bar) and without (1 bar, ambient pressure) compressed *n*-butane. Reaction condition: glycerol to olive oil molar ratio of 2:1, 7.5 wt.% of Novozym 435, 600 rpm, solvent to substrates mass ratio kept constant at 4:1 (in the case of *n*-butane) and 2 h of reaction time

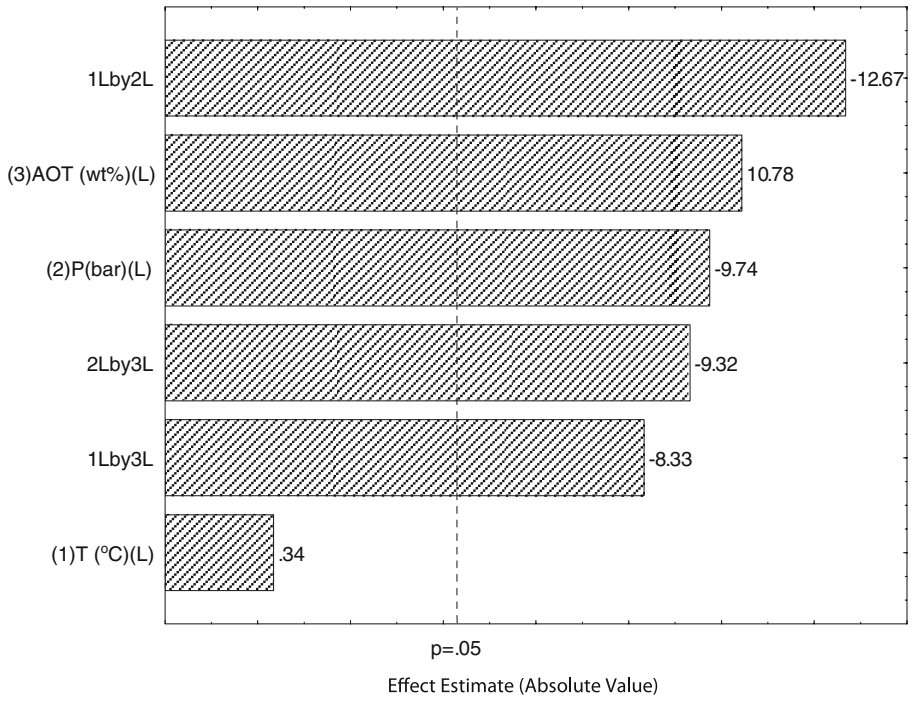

Fig. 1 Pareto chart of the effects of temperature, pressure, and AOT concentration on MAG enzymatic production ($p<0.05$). Experimental data and conditions shown in Table 1

Table 2 Matrix of the second experimental design, showing real and coded (between parenthesis) values, with response in terms of MAG, DAG, TAG, MAG, and DAG contents.

Run	*P* (bar)	AOT (wt.%)	MAG (wt.%)	DAG (wt.%)	MAG + DAG (wt.%)	TAG (wt.%)
1	1 (−1)	0.1 (-1)	3.3	4.9	8.2	58.9
2	20 (1)	0.1 (-1)	0.0	4.5	4.5	54.8
3	1 (−1)	2.5 (1)	31.7	10.2	41.9	32.5
4	20 (1)	2.5 (1)	32.8	18.0	50.8	32.2
5	10 (0)	1.3 (0)	45.3	20.1	65.4	18.3
6	10 (0)	1.3 (0)	46.3	18.1	64.4	20.3
7	10 (0)	1.3 (0)	45.0	18.0	63.0	19.9

Effect of pressure and AOT in the glycerolysis of olive oil with (10 and 20 bar) and without (1 bar) compressed n-butane. Reaction condition: 30 °C, glycerol to olive oil molar ratio of 2:1, 7.5 wt.% of Novozym 435, 600 rpm, solvent to substrates mass ratio kept constant at 4:1 (in the case of *n*-butane) and 2 h of reaction time

observed for some conditions in the first design. As one should expect for this case, AOT concentration had a remarkable positive effect on MAG production (Fig. 2). One should also note from Table 2 that, except for runs 1 and 2, in all other cases, the use of *n*-butane was equivalent or beneficial for the MAG production, thus pointing the importance of minimizing possible mass-transfer restrictions. For these two experimental conditions, one should call attention to the fact that greater experimental errors in TAG content are expected to occur, since phase separation becomes more important and the fact that chromatogram picks also become not so well definite.

Inspection of literature shows that high contents of MAG (around 60 wt.%) were recently found by Esmelindro et al. [21] in conducting lipase-glycerolysis in compressed propane in the presence of AOT at mild conditions of temperature and pressure. In that case, the authors verified a significant negative effect of temperature, while system pressure seemed not to influence significantly the reaction conversion. The negligible influence of pressure was explained in that work in terms of two aspects: the homogeneous one-phase system observed for the systems formed by glycerol + olive oil + propane + AOT found for

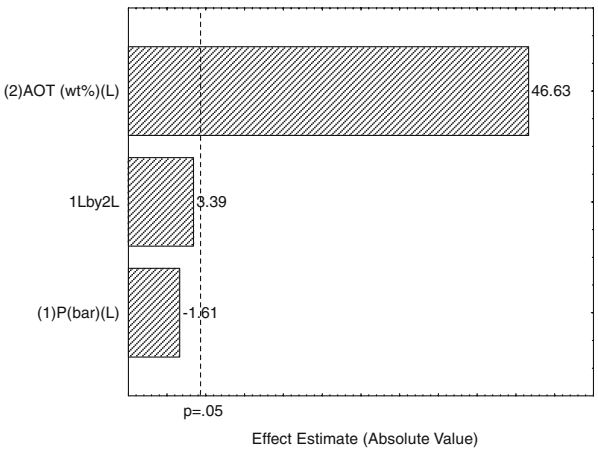

Fig. 2 Pareto chart of the effects of pressure and AOT concentration on MAG enzymatic production ($p<0.05$). Experimental data and conditions shown in Table 2

all conditions studied [36] and due to the fact that no significant differences in Novozym 435 activity were observed when treating the enzyme in propane [37].

In the temperature and pressure ranges investigated in this work, *n*-butane behaves like a compressed liquid and thus exhibits small density variations. Also, as noted for propane, one-phase homogeneous systems were observed for the systems formed by glycerol + olive oil + *n*-butane + AOT for all conditions investigated here [34]. On the other hand, a positive effect was observed when Novozym 435 was treated in compressed *n*-butane in a wide temperature (35–75 °C) and pressure (10–250 bar) [37].

Taking into account the works available in the literature and results found in the present report, one may conclude that glycerolysis is, in fact, a complex reaction system, involving, in the present case, glycerol, oil, *n*-butane as solvent, and AOT surfactant. It is believed that the surfactant promotes the reaction to occur in the interface glycerol/oil, and as a consequence, the reaction conversion may be improved. In this case, it seems plausible that microemulsions contribute to the formation of reverse micelles, with consequent increase of the interfacial area to the substrate to be catalyzed, which would demonstrate the vital role of AOT to provide higher MAG and DAG conversions. Regarding the use of solvent-free systems, a good discussion on such subject may be found elsewhere [30, 31].

Finally, it may be relevant to mention that measurements of enzyme activity [37] before (fresh) and after (used) reaction experiments revealed no important changes in residual lipase activity, thus making possible enzyme reuse. Further experiments are underway within our working group, mainly focusing on other types of surfactants and compressed fluids.

Conclusions

In this work, the effect of some process variables on the production of mono- and diacylglycerols from olive oil in compressed *n*-butane and in solvent-free system using AOT as surfactant. Results showed that lipase-catalyzed glycerolysis using AOT surfactant in pressurized *n*-butane might be a promising alternative to conventional alkali and enzyme-catalyzed reaction in organic solvents, as high contents were achieved at mild temperature and pressure conditions with a relatively low solvent to substrates mass ratio (4:1) in short reaction times (2 h).

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