

Deacidification of High Acid Olive Oil by Immobilized Lipase

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The acidity reduction of high acid oils prior to refining could be an attractive application for industry. In this study the enzymatic deacidification degummed and dewaxed olive oil with a high free fatty acid content, catalysed by an immobilized lipase was optimized (Novozym 435). The optimum amount of substrate for synthesizing triglycerides was the necessary stoichiometric amount of fatty acid and glycerol. The mixing was performed with a mechanical stirrer and the dehydration was performed by vacuum pump or by bubbling dry nitrogen. The dehydration rate was faster than the deacidification rate. The four parameters chosen for this experimental plan: pressure, temperature, and the amounts of glycerol and enzyme in the reaction mixture. The use of glycerol more than theoretical amount did not affect the rate of deacidification. The stoichiometric amount of glycerol was sufficient for the deacidification of high acid olive oil. Maintaining low pressure is very important for the removal of water which is formed during the esterification reaction. Either, the reaction is conducted at 20 mm Hg or the dehydration is performed by bubbling dry nitrogen. The optimum amount of immobilized enzyme necessary for the deacidification reaction was determined and 10:1 ratio of oil: immobilized enzyme appeared to be satisfactory. The effect of temperature on the deacidification of high acid olive oil was investigated (between 40- 70° C). The reaction temperature influenced the rate of deacidification. The optimum operation temperature was found to be 60° C. At 70° C the deacidification decreased with increasing incubation time due to enzyme deactivation occurring at this temperature.

Key Words: High acid oils, immobilized lipase, Biorefining, Enzymatic deacidification

Introduction

Fats and oil with a high free fatty acid content after degumming and dewaxing can be converted into edible quality oil by biorefining with immobilized lipases. The acidity reduction of hyperacid oils and fats prior to refining could be an attractive application for industry. Because of their very high acidity, refining such oils and fats by conventional methods is indeed difficult. The large quantities of soaps obtained after neutralization induce the formation of irreducible emulsions, leading to significant losses.

Batchwise conversion of glycerol and partial glycerides with free fatty acid in hyperacid oils to

triglycerides using immobilized lipase has been reported by many authors¹⁻⁷. Reducing the acidity of these oils to about 5% would be compatible with soda refining. The unique properties of some microbial lipases to synthesize triglyceride from a fatty acid and glycerol can be conceived for utilization to develop an alternative process for deacidifying a vegetable oil. The process may be useful in the deacidification of high acid vegetable oils. The present study makes an effort to investigate the potential of the enzymatic deacidification process for refining hyperacid olive oil by testing the enzymatic esterification reaction parameters namely reaction time and temperature, glycerol and enzyme concentrations.

Experimental

Materials

Lipase(EC 3.1.1.3) (Novozym 435) was generously given by Novo Nordisk A/S Bagsvaered, Denmark. In the manufacture of Novozym 435, recombinant DNA technology has been used. The gene coding for the lipase has been transferred from a selected strain of *Candida antrartica* to the host organism, *Aspergillus oryzae*. The enzyme produced by this host organism is immobilized on a macroporous acrylic resin.

Hyperacid olive oil was purchased from a local oil and soap factory. All of the chemicals were obtained from E. Merck (Darmstadt, Germany).

Degumming and Dewaxing of Hyperacid Oil

The oil was degummed by 0.1% phosphoric acid at 60° C for 30 minutes⁸, and then dewaxed by treatment with 0.2 % $CaCl_2$ at 15° for 4 hrs⁸. The degummed, dewaxed high acid olive oil and refined oils were analysed for free fatty acids⁹ and unsaponifiable matter¹⁰.

Enzyme Assay

The enzymatic activity is based on a batch ester synthesis assay. *Substrates*: n-propanol and lauric acid, *Temperature*: 60° C, *Time*: 15 minutes. The ester formation was calculated on the acid values of the reaction mixture measured before and after the above mentioned incubation. The acid values were determined by titration.

Analytical Methods

The acidity of the high acid olive oil and reaction mixture and the amount of glycerol present were determined according to IUPAC norms¹¹. The products at varying reaction times were analysed by thin layer chromatography(Merck plates, 60 G, 0.25 mm silica gel). The polarity differences in the various components of the mixture required the use of distinct solvent systems(hexane-ethylether-acetic acid; 70:30:1 and chloroform-acetone-acetic acid; 90:10:1).

The plates were dried and sprayed with a mixture of copper acetate saturated in water and orthophosphoric acid at 85% (50:50 v/v). Spots appeared by carbonization in the drying over at 180° C for five minutes.

For the quantitation of glycerides and free fatty acid fractions, the plate is removed from TLC tank and dried in a stream of nitrogen. The developed plate is sprayed lightly with 2',7'-dichlorofluorescein solution (0.1% in ethanol-water, 96:4) and viewed under ultraviolet light to locate the various components. The bands

are marked and, with a microspatula, scraped into a small glass tube. The lipid fraction is extracted with n-hexane from silica gel and quantitated by means of rhodamine 6G reagent^{12,13}.

Water Removal Through Different Means

For these studies, the synthesis reactions were carried out in a thermostated beaker at 60°C, in which 40 g of high acid olive oil and 1.5 g of glycerol were mixed with 4 g of Novozym 435. Mixin was accomplished with a mechanical stirrer. When water was allowed to freely evaporate, the reaction vessel was left uncovered. When a vacuum was applied to the vessel, the reaction medium was covered and a vacuum pump was used to create the vacuum.

In the reaction where dry nitrogen is used to remove the water, the reaction vessel is left uncovered. This nitrogen bubbling is also used as a mixing device in this type of reactor.

Results and Discussion

High acid olive oil not only contains free fatty acids and triglycerides, it also contains mono- and diglycerides. The deacidification reaction occurs according to the scheme given in Figure 1. The lipase reaction is reversible so that hydrolysis and resynthesis of glycerides occur when lipases are incubated with oils and fats. The breakdown and resynthesis cause acyl migration between glyceride molecules and give interesterified products.

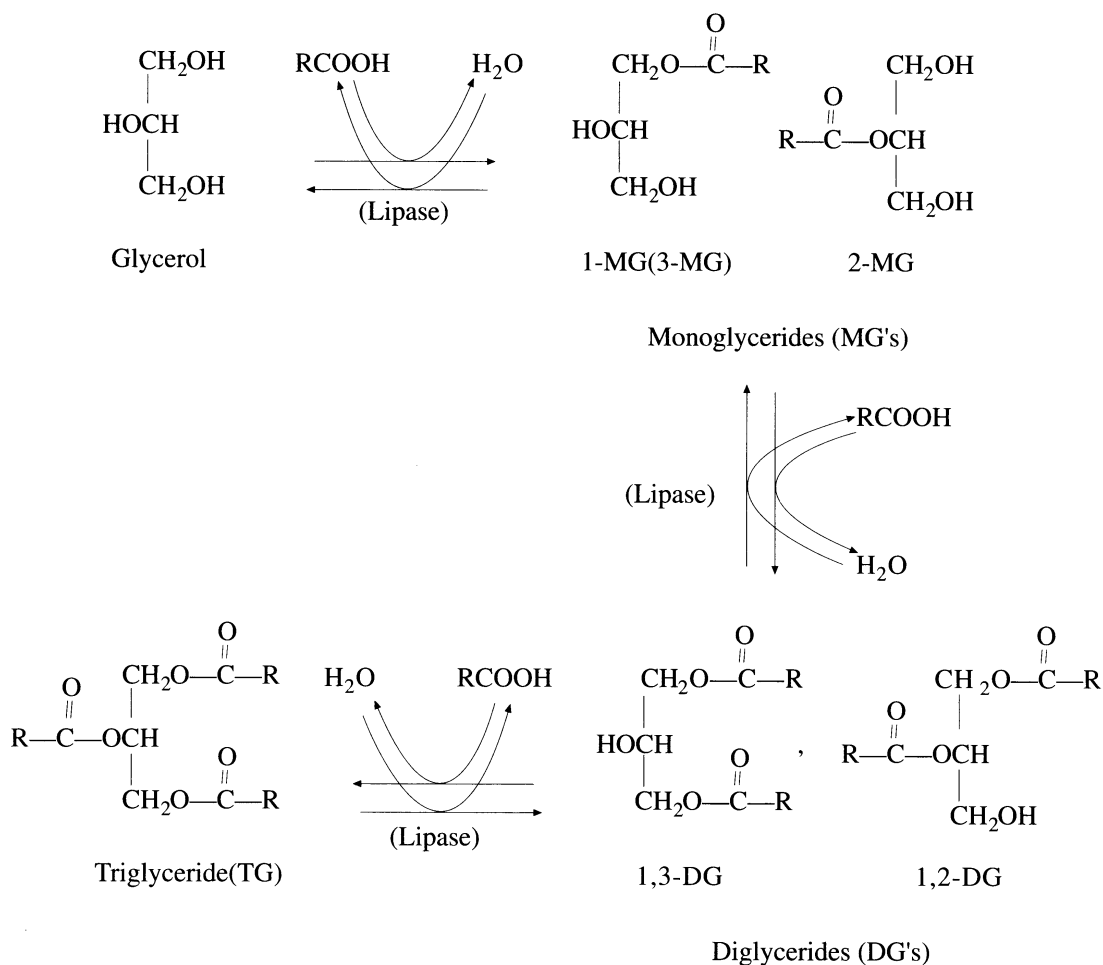


Figure 1. Esterification reactions of glycerols, mono- and diglycerides with free fatty acids

Under conditions in which the amount of water in the reaction system is restricted, hydrolysis of oil can be minimized so that lipase catalysed esterification becomes the dominant reaction. It was found that the esterification reaction was very low when water content was low and that the enzyme dissolved in pure glycerol exhibited no synthesis activity. This loss of synthetic activity is probably due to the fact that a certain amount of water is essential for the enzyme's catalytic activity. On the other hand at higher water contents the esterification decreased gradually. Since the hydrolysis reaction in other words the backward the reaction of the ester synthesis becomes significant. The esterification reaction was the highest at about one percent of water. Therefore an initial water content of one percent in the reaction medium was employed^{14,15}.

The optimum amount of substrate for synthesizing triglycerides was the necessary stoichiometric amount of fatty acid and glycerol. The deacidification reaction was carried out in the reactor system in Figure 2. The mixing was performed with a mechanical stirrer and the dehydration was performed by vacuum pump or by bubbling dry nitrogen. The dehydration rate was faster than the deacidification rate.

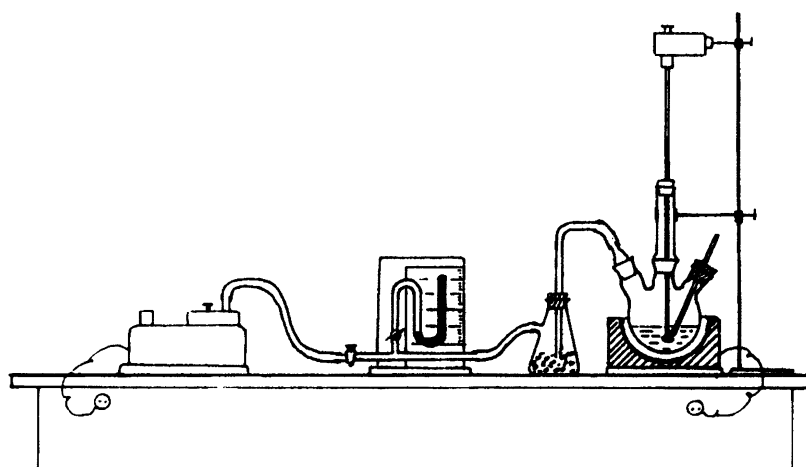


Figure 2. The outline of the biorefining reactor system

For the optimization of the deacidification conditions four parameters were chosen: pressure, temperature, and the amount of glycerol and enzyme in the reaction medium.

For the removal of the water formed during the reesterification reaction low pressure is essential. The reaction should be conducted at approximately 20 mm Hg (Table 1). The rate of free fatty acid decrease is enhanced when the reaction is conducted at 50 mm Hg instead of atmospheric pressure.

The deacidification of high acid olive oil was studied at varying temperatures ranging between 50-70°C. Table 2 shows that the reaction temperature has an important effect on the rate of reesterification or deacidification. When the reaction is conducted at 70°C the decrease in the activity is due less to the expected enzyme deactivation at the temperature.

Theoretically one mole of glycerol requires three moles of fatty acid. The data in Table 3 shows that by using stoichiometric amounts of glycerol, the acidity of the high acid olive oil decreases significantly compared to emissions of glycerol. The addition of more than the theoretical amount glycerol did not affect the rate of deacidification. High acid olive oil contains not only free fatty acid and triglycerides, but also contains mono- and diglycerides. According to the reaction scheme in Figure 1 it also requires free fatty acids for the formation of triglycerides from mono- and diglycerides. Therefore addition of glycerol less than the theoretical amount is sufficient.

The effect of the amount of Novozym 435 on the deacidification reaction was also investigated (Table 4). An enzyme: oil ratio of 1:10 appeared to be better than the 0.5:10 and 2:10 ratios, in the reduction of the free fatty acid level of high acid olive oil.

Table 1. The effect of the pressure on the extent of deacidification of high acid olive oil (60° C, Imm. enzyme: 10 %, Glycerol: theoretical amount)

Pressure	Incubation time (hrs)	Free fatty acid in the reaction medium(%)
Atmospheric	0	32.0
	1	20.4
	2	16.2
	4	13.1
	6	11.6
	10	10.4
	15	10.1
Atmospheric, under dry nitrogen flow	0	32.0
	1	15.3
	2	11.3
	4	7.0
	6	6.0
	10	5.3
	15	4.9
50 mm Hg	0	32.0
	1	16.1
	2	12.0
	4	7.3
	6	6.2
	10	3.8
	15	5.4
20 mm Hg	0	32.0
	1	13.4
	2	10.2
	4	5.9
	6	4.5
	10	3.8
	15	3.7

Table 2. The effect of the pressure on the extent of deacidification of high acid olive oil (20 mm Hg, Imm. enzyme: 10 %, Glycerol: theoretical amount)

Temperature	Incubation time (hrs)	Free fatty acid in the reaction medium(%)
50° C	0	32.0
	1	18.8
	2	14.1
	4	9.4
	6	8.4
	10	8.2
	15	8.1
60° C	0	32.0
	1	13.4
	2	10.2
	4	5.9
	6	4.5
	10	3.8
	15	3.7
70° C	0	32.0
	1	12.1
	2	10.5
	4	9.2
	6	8.7
	10	7.4
	15	8.2

Table 3. The effect of glycerol amount on the deacidification degree of high acid olive oil (20 mm Hg, 60° C, Immobilized Enzyme: 10%)

Amount of added glycerol in the reaction medium	Incubation time (hrs)	Free fatty acid level in the reaction medium (%)
—	0	33.2
	1	27.2
	2	24.5
	4	23.0
	6	22.1
	10	21.8
	15	21.7
Theoretical amount (36 mg/g HAOO*)	0	32.0
	1	13.4
	2	10.2
	4	5.9
	6	4.5
	10	3.8
	15	3.7
2x Theoretical amount (72 mg/g HAOO*)	0	30.9
	1	13.1
	2	9.9
	4	5.6
	6	4.3
	10	3.7
	15	3.7

*HAOO: High acid olive oil

Table 4. The effect of the enzyme amount on the deacidification degree of high acid olive oil (20 mm Hg, 60° C, Glycerol: theoretical amount)

Immobilized enzyme amount in the reaction	Incubation time (hrs)	Free fatty acid in the reaction medium(%)
5%	0	32.0
	1	15.2
	2	11.8
	4	7.2
	6	6.1
	10	5.9
	15	5.6
10%	0	32.0
	1	13.4
	2	10.2
	4	5.9
	6	4.5
	10	3.8
	15	3.7
20%	0	32.0
	1	13.0
	2	9.8
	4	5.8
	6	4.5
	10	3.7
	15	3.8

After the optimization of pressure, temperature, amount of enzyme and glycerol, the change of the concentration of reaction components in the reaction medium were monitored for 20 hours (Figure 3). In the first hour, the 1,2-and 1,3-diglycerides(DG) and the triglycerides(TG) rapidly increased, whereas

the monoglycerides(MG) and free fatty acid (FFA) contents decreased rapidly, the glycerol disappeared completely.

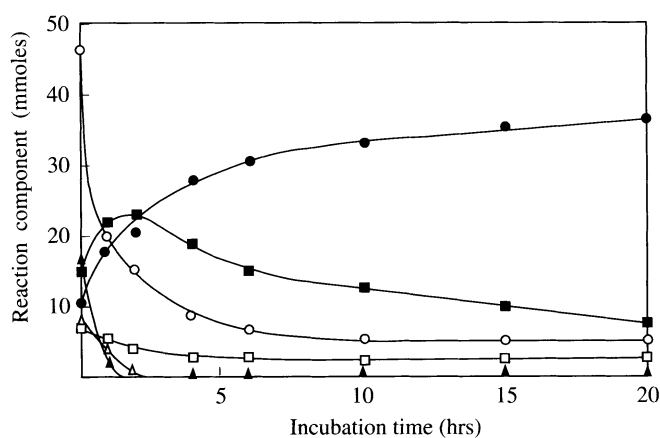


Figure 3. Changes of the concentration of reaction components during the biorefining reaction. (○) : FFA, (●): TG, (□):1,3-DG, (Δ): MG and (▲): Glycerol

Before the reesterification reaction the mixture composition in mmoles was: Glycerol: 16.3(3.6%), FFA: 46.5(31.6 %), MG: 8.3(7.2 %), 1,2-DG: 7.4(11.5 %), 1,3-DG: 15.3 (23.7 %) and TG: 10.5(22.4 %). Under the optimum conditions of reesterification, the deacidified olive oil contained 5.4 mmoles of FFA (3.7 %), no glycerol and MG, 8 mmoles of 1,3-DG(12.8 %), 2.5 mmoles of 1,2-DG(4%), and 36 mmoles of TG (79.6 %). After 15 hours, the reaction medium underwent virtually no further change, as regards to both the FFA reduction and the TG increase. The optimum reaction time was therefore fixed at 15 hours.

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