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Lipase immobilized by different techniques on various support materials applied in oil hydrolysis

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Abstract: Batch hydrolysis of olive oil was performed by *Candida rugosa* lipase immobilized on Amberlite IRC-50 and Al_2O_3. These two supports were selected out of 16 carriers: inorganic materials (sand, silica gel, infusorial earth, Al_2O_3), inorganic salts ($CaCO_3$, $CaSO_4$), ion-exchange resins (Amberlite IRC-50 and IR-4B, Dowex 2X8), a natural resin (colophony), a natural biopolymer (sodium alginate), synthetic polymers (polypropylene, polyethylene) and zeolites. Lipase immobilization was carried out by simple adsorption, adsorption followed by cross-linking, adsorption on ion-exchange resins, combined adsorption and precipitation, pure precipitation and gel entrapment. The suitability of the supports and techniques for the immobilization of lipase was evaluated by estimating the enzyme activity, protein loading, immobilization efficiency and reusability of the immobilizates. Most of the immobilizates exhibited either a low enzyme activity or difficulties during the hydrolytic reaction. Only those prepared by ionic adsorption on Amberlite IRC-50 and by combined adsorption and precipitation on Al_2O_3 showed better activity, 2000 and 430 U/g support, respectively, and demonstrated satisfactory behavior when used repeatedly. The hydrolysis was studied as a function of several parameters: surfactant concentration, enzyme concentration, pH and temperature. The immobilized preparation with Amberlite IRC-50 was stable and active in the whole range of pH (4 to 9) and temperature (20 to 50 °C), demonstrating a 99 % degree of hydrolysis. In repeated usage, it was stable and active having a half-life of 16 batches, which corresponds to an operation time of 384 h. Its storage stability was remarkable too, since after 9 months it had lost only 25 % of the initial activity. The immobilizate with Al_2O_3 was less stable and less active. At optimal environmental conditions, the degree of hydrolysis did not exceed 79 %. In repeated usage, after the fourth batch, the degree of hydrolysis was reduced to 53.5 %.

Keywords: immobilization, lipase, *Candida rugosa*, hydrolysis, ion-exchange resin, Amberlite IRC-50.

INTRODUCTION

The steadily growing interest in lipases over the last two decades stems from their biotechnological versatility and the ability of these enzymes to catalyze a

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broad spectrum of bioconversion reactions with tremendous potential in various areas such as in food technology, biomedical sciences and chemical industry. Many of these applications are performed with immobilized lipases. The immobilization is an advantageous method that improves the stability of the biocatalyst and provides for its repeated use and the easy separation of the catalyst from the reaction medium.

Various techniques and even more support materials have been studied and consequently many immobilized preparations with a wide range of efficiency, stability and activity have been offered. Lipases have been covalently bound to activated poly(vinyl chloride),[1] nylon,[2] Eupergit[3] or to controlled pore silica.[4] Noncovalently attached or entrapped enzymes have been prepared in polyionic chitosan hydrogel[5] and alginate gels.[6] Adsorption on hydrophobic or hydrophilic supports, as a simple method, still attracts attention. Among the various supports, celite, cellulose, ethyl cellulose, carbon and synthetic polymers,[7] as well as rice straw[8] and alumina beads,[9] have served as carriers for this purpose. Recently, a novel form of lipase covalently immobilized on reversibly soluble polymers was proposed.[10] Another novel technique for the immobilization of lipase on colloidal gas aphrons, that is on spherical microbubbles, was described.[11]

Obviously, the problem of selecting the support material and the proper technique is very important and therefore the pursuit for suitable materials has not yet ceased. In the search for suitable and low cost materials, in this study sixteen inexpensive materials as supports using several immobilization techniques, among them simple adsorption, ion-exchange adsorption, combined adsorption and precipitation, adsorption and precipitation on zeolites and gel entrapment, were evaluated. The selection criteria were based on the enzyme loading onto or in the support, enzyme activity and immobilization efficiency. The activities of the immobilizates of commercial nonspecific *Candida rugosa* lipase were assessed in an aqueous medium by the hydrolysis of olive oil as a reaction system. Hereby, the factors influencing the course of the hydrolysis of olive oil as a reaction system. Hereby, the factors influencing the course of the hydrolysis of olive oil with immobilized enzyme on carriers with the best performances were studied. Among the factors investigated were the surfactant concentration, the enzyme concentration, the pH and the temperature of the reaction mixture, as well as the reusability and storage stability of the immobilizates.

EXPERIMENTAL

Materials

Lipase from *Candida rugosa* type VII containing 700–1500 U/mg lipase was purchased from Sigma Chemical Co. The enzyme powder containing 30 % lactose as a stabilizer, was α-amylases and proteases free. At the time of employment, this enzyme possessed 80 U/mg or 3200 units per mL of enzyme solution (4 % w/v).

Sixteen carriers for immobilization of the lipase were employed. Three synthetic ion-exchange resins, Amberlite IRC-50 (H) (beads of about 0.4 mm), Amberlite IR-4B (OH) (particles of 0.5 to 1

mm) and Dowex 2X8 (fine uniform round beads of 0.5 mm) were products from the British Drug Houses Ltd. In addition, another resin of natural origin was used, colophony (particles of 0.1 mm), which was a kind gift of Alkaloid. Two synthetic polymers, polypropylene (pulverized particles of maximum 0.2 mm, Hostalen PPH 1050) and polyethylene (particles of 0.2–0.4 mm, Hostalen LDf 4024) and one natural polymer, sodium alginate (Sigma Chem. Co.) were also used. Nine carriers of inorganic origin were tested: sand (particles of 0.1 mm), infusorial earth (powder), silica gel (granules of 1.5 mm), all from domestic sources, Al_2O_3 (beads of about 0.1 mm, Merck), $CaCO_3$ (powder, Alkaloid), $CaSO_4 \times 2H_2O$ (powder, Kemika) and three zeolite type carriers (very fine powders), one of them was a commercial product from the Union Carbide Corp., while zeolite X and zeolite M_{ox1} were synthesized at our Faculty.[12]

Olive oil (Estia), purchased from the local market, was used as the substrate for the determination of the lipolytic activity and in hydrolytic reactions. The other chemicals were of reagent grade and purchased from Merck and Alkaloid.

Immobilization of lipase

Unless otherwise stated, 3 mL of enzyme solution was added to 1 g of each support material. The enzyme solution was prepared by dissolving 4 g lipase powder in 100 mL distilled water or buffer. The activity of this solution was 3200 units per mL. The immobilization was carried out in 100 mL-glass vessel placed on a magnetic stirring plate (220 rpm) at 25 °C.[13]

Immobilization by simple adsorption. This procedure was employed with five carriers, three of inorganic origin (silica gel, sand and infusorial earth) and two synthetic polymers (polypropylene and polyethylene). The suspension of silica gel and water solution of lipase was incubated for 3 h on a stirring plate. After immobilization, the preparation was vacuum filtered through a Buchner funnel and rinsed 3 times with 10 mL portions of distilled water. The sand and the infusorial earth were rinsed with phosphate buffer (50 mM, pH 7.0), vacuum filtered and then the suspensions were stirred for 2 h under the above-mentioned conditions. In these cases the lipase was dissolved in the same phosphate buffer. The immobilizates were separated by vacuum filtration. The immobilized preparations on polyethylene and polypropylene carriers were obtained by a similar procedure, except that the carrires were preactivated with 2.5 mL ethanol, and the immobilization step lasted only 30 min.[13-16]

Adsorption followed by cross-linking. The polypropylene and polyethylene were mixed with lipase solution (4 % w/v) prepared with acetate buffer (0.2 M, pH 5.0) and stirred for 30 min. The immobilizates were then treated with glutaraldehyde; 50 mL of a 2.5 % (w/v) aqueous solution were added and the reaction was allowed to proceed for an additional 10 min. The immobilizates were separated by vacuum filtration.[17]

Ionic adsorption on resins. The resins, both types of Amberlite, Dowex 2X8 and the colophony, were first equilibrated in phosphate buffer (50 mM, pH 7.0) at room temperature over night. After vacuum filtration, the resins were incubated with the lipase dissolved in phosphate buffer at 4 °C for 1 h. The vacuum filtered immobilizates were used as obtained.[18] In some experiments with Amberlite IRC-50 (H), the time and temperature of immobilization were changed and the immobilizates were allowed to dry in a desiccator for 24 h.

Combined adsorption and precipitation. One gram of each of the powders of Al_2O_3, $CaCO_3$, $CaSO_4 \times 2H_2O$ without any previous treatment was added to a water solution of the lipase and incubated under stirring for 1 h. Then 10 mL of a chilled acetone were poured into the vessel and immediately vacuum filtered. The immobilizates were kept in a desiccator for 4 h to remove the acetone.[19] In some experiments with Al_2O_3, the time and temperature of immobilization were changed, as was the amount of the precipitating agent.

Immobilization by precipitation. Each mixture of zeolite and lipase solution was vortexed for 5 min. During vortexing, 8 mL of chilled acetone were poured into the suspension and the precipitation was allowed to preceed for an additional 30 min at 4 °C. The acetone was removed by keeping the immobilizates in a desiccator for 4 h.

Gel entrapment. The lipase powder was dissolved in Tris–HCl buffer (50 mM, pH 7.3) to obtain a 4 % w/v solution. This solution was added to sodium alginate to yield either a 1.5 % w/v or a 2.5 % w/v mixture which was left to deaerate over night at 25 °C. By dropping the lipase–alginate mixture through a syringe into an aqueous solution of $CaCl_2$ (0.1 M), gel beads of about 3-mm diameter were formed within 3 to 5 min. The beads were kept in $CaCl_2$ solution until used.

Evaluation of the immobilization techniques

Each of the techniques was evaluated by protein loading (mg protein/g immobilizate), lipase activity (expressed as U/g immobilizate and U/g immobilized lipase), immobilization efficiency and reusability.

The protein loading, P, was estimated as follows:

$$P = (c_0 V_0 - c_f V_f)/W_g \quad (1)$$

where the concentration of protein in the lipase solution before (c_0) and after immobilization (c_f) are given in mg/mL, while the volumes of the solution, V_0 and V_f, are in mL. W_g is the weight of the wet immobilizates in g.

The efficiency of the immobilization techiuques,[3] η, was estimated by calculating the lipolytic activity of the lipase solution before (E_0) and after immobilization (E_f), using the relation:

$$\eta = [(E_0 V_0 - E_f V_f)/V_0 E_0] \times 100 \quad (2)$$

The activities are given in U/mL, and the volumes are in mL.

Batch hydrolysis of olive oil

The hydrolysis of olive oil was carried out batchwise at 30 °C in 100 mL Erlenmeyer flasks under stirring at 220 rpm. The olive oil and distilled water in a 1:1 (v/v) ratio were vigorously stirred for 3 min to form a water emulsion. 10 mL of the emulsion were mixed with 5 g of the immobilized preparation (1 g immobilizate/1 g substrate) and the reaction was allowed to proceed for 24 h. The experiments were run under different environmental conditions. The emulsifying agent was Tween 80, the concentration of which was varied from 2 to 10 g/L. The enzyme concentration ranged from 10 to 800 U/g substrate. The influence of pH on the lipase performance was investigated with 3 types of buffer in 3 different pH intervals: acetate (pH 4, 5, 6), phosphate (pH 6, 7, 8) and TRIS–HCl (pH 7, 8, 9) buffer. Additionally, the influence of temperature (20 to 50 °C) on the activity of soluble and immobilized lipase was evaluated.

When used repeatedly, the suspension with the immobilized lipase preparation was centrifuged for 10 min at 2500 rpm after each batch. The solid phase was rinsed with 10 mL water and 10 mL isooctane to remove the remaining substrate and products from the surface of the immobilizate. The same oil-water emulsion replaced the supernatant and the hydrolysis reaction was again allowed to proceed for 24 h. The storage stability of the immobilizates was investigated by storing them in a refrigerator at 4 °C.

Analytical methods

The lypolitic activity was determined by the Kwon and Rhee method at 715 nm.[20] One unit of lipase activity was defined as an amount of enzyme that releases 1 μmol free fatty acids per minute in terms of NaOH under the assay conditions. The protein content was measured by the standard Lowry method. The total amount of free fatty acids was determined by the titration method against 0.05 M NaOH using phenolphthalein as the indicator. For this analysis, the oil samples were dissolved in a diethylether–ethanol mixture (1:1 v/v), in the ratio of 1:5 v/v. The oleic acid content was measured spectrophotometricaly.[20] A Knauer HPLC unit, equipped with a differential refractive index detector using an Aminex HPX-87H column (Bio-Rad) was used for monitoring the glycerol released. As the mobile phase, 5 mM H_2SO_4 (0.6 mL/min) was used at a working temperature of 50 °C.

RESULTS AND DISCUSSION

Selection of immobilization technique and matrix

In order to select an inexpensive support for optimal lipase immobilization, 16 carriers belonging to 3 types of support materials, adsorbents, ion-exchange resins and hydrogels, were tested. According to the chemical nature, they belong to silicates, alumosilicates, oxides, carbonates, sulfates, natural biopolymers of the polysaccharide type and synthetic polymers composed of propylene, ethylene and acrylic monomers. Lipase immobilization on these materials occurred by various binding mechanisms, from simple adsorption by van der Waals or ionic forces, to physical entrapment in the matrix. The results of the evaluation of the immobilization techniques and the support materials are given in Table I.

TABLE I. Activity and efficiency of the immobilizates prepared by various techniques

Technique/support material	Protein loading mg/g support	Lipolytic activity U/g support	Lipolytic activity U/g immobillized lipase	Immobilization efficiency/%
1. *Simple adsorption*				
Silica gel	9.3	14	1.5	53
Polypropylene	8.1	333	41.1	61
Polyethylene	1.5	n.d.*	–	88
SiO_2	15.7	95	6.1	76
Influsorial earth	20.0	17	0.9	89
2. *Adsorption followed by cross linking*				
Polypropylene + glutaraldehyde	n.d.	95	–	98
Polyethylene + glutaraldehyde	n.d.	n.d.	–	95
3. *Adsorption on resins*				
Amberlite IRC-50 (H)	14.9	600	40.3	75
Amberlite IR-4B (OH)	5.3	402	75.9	55
Dowex 2X8	7.1	280	39.4	50
Colophony	17.5	255	14.6	88
4. *Adsorption followed by precipitation*				
$CaSO_4 \times 2H_2O$	19.5	571	29.3	91
$CaCO_3$	19.7	529	26.9	92
Al_2O_3	19.7	181	9.2	96

TABLE I. Continued

Technique/support material	Protein loading mg/g support	Lipolytic activity U/g support	Lipolytic activity U/g immobillized lipase	Immobilization efficiency/%
5. *Precipitation*				
Zeolite M_{ox1}	6.1	12	2.0	62
Zeolite X	14.3	10	0.7	95
Zeolite UCC	16.7	4	0.2	93
6. *Entrapment*				
Sodium alginate	13.4	54	4.0	67

n.d.* – Not determined

Lipase immobilization

Immobilization by adsorption

Simple adsorption. The simple adsorption of the enzyme was carried out with five support materials: SiO_2, silica gel, infusorial earth, polypropylene and polyethylene. Except for the polypropylene immobilizates, the others demonstrated very low lipolytic activity. The protein loading ranged from 1.5 mg to 20 mg from an initial 20.1 mg protein. The infusorial earth retained 99.5 % protein with an immobilization efficiency of 89 %. Unfortunately, its activity was only 17 U/g support and did not correspond to the protein loading. Mustranta *et al.*[15] observed similar effects. Their celite–immobilizates showed insignificant lipolytic activity, although a high content of protein had been loaded. These inorganic supports were excluded from further investigations because of the poor results.

The polypropylene immobilizates exhibited a lipolytic activity of 333 U/g support at a protein loading of 8.1 mg/g (40.3 % protein adsorbed) and a moderate efficiency of 61 %. The better activity of this immobilizate might be due to the stronger adsorption of the hydrophobic enzyme onto the polypropylene surface. When Montero *et al.*[16] applied various kinds of polypropylene, the untreated polypropylene carrier led to a poor adsorption, usually less than 55 %.

Adsorption followed by cross-linking. Polypropylene and polyethylene were used again in a modified adsorption method in order to improve the immobilization efficiency. After coupling them with glutaraldehyde, the immobilization efficiency rose definately from 61 to 98 % for polypropylene and slightly for polyethylene, from 88 % to 95 % (Table I). However, the hindering effect of glutaraldehyde on polypropylene immobilizates resulted in a 3.5-fold decrease in its lipolytic activity, from 333 to 95 U/g support. Polyethylene was not a suitable support material because during the operation it turned into a gelatinous mass which was difficult to handle.

Lipase adsorption followed by precipitation. The solid inorganic salts, $CaSO_4$ and $CaCO_3$, exhibited moderate adsorptive properties while Al_2O_3 demonstrated very poor adsorption when a simple adsorption procedure was used. When the adsorption was assisted by precipitation of the enzyme onto the carriers, the protein adsorption on all three supports reached about 98 %. The activity of the enzyme per g $CaSO_4$ was 571 while per g $CaCO_3$ it was 529 U. Obviously, the activities of these samples were the best of all the immobilizates prepared by the adsorption technique. Al_2O_3, with the same amount of adsorbed protein, yielded about a 3 times lower specific activity (9.2 U/mg immobilized lipase) than the $CaSO_4$-immobilizate (29.3 U/mg immobilized lipase). Rosu *et al.*[19] reported that $CaCO_3$, due to its small crystalline particles, displayed a very good capacity for adsorption of 80 %, while $CaSO_4$ demonstrated a moderate capacity. In our case, $CaSO_4$ was the one that exhibited a higher adsorption capacity because its fine powder structure produced a larger adsorption area.

Immobilization by precipitation. The three zeolite were poor carriers for the enzyme when used as adsorbents, as well as when the enzyme was precipitated onto them. The protein loading was 3.4 to 14.0 mg/g support and the activity was very low, from 4 to 12 U/g support. On the other hand, the efficiency for the zeolites UCC and X was rather high, 93 and 95 % respectively (Table I). The efficiency of the zeolite carriers was closely connected to their degree of hydrophobicity. Lie and Molin,[21] working with hydrophobic and hydrophilic zeolites found that the hydrophilic zeolites exhibited very low activities of about 10 U/g, which is quite comparable to our results. Their hydrophobic zeolite yielded 45-times higher activity. Apparently, the zeolites used in this work, containing about 34.0 % SiO_2, 28.5 % Al_2O_3 and 21.3 % Fe_2O_3,[12] were not suitable carriers for hydrolytic reactions.

Ionic binding of lipase. Ionic binding gave immobilizates with a higher activity and, expectedly, with a better mechanical stability. The activity reached 600 U per gram support of Amberlite IRC-50, while for Amberlite IR-4B it was 402 U/g. The binding efficiency per g carrier was 74 % and 26 %, respectively. Yang and Rhee,[22] working with other types of Amberlite (IRA 94 and XAD 7) reported a binding efficiency between 46 and 56 %. In terms of specific activity, Amberlite IR-4B distinguished itself with the highest value of 75.9 U/mg immobilized lipase. The specific lipase activity of the immobilizates on Dowex 2X8 (40.3 U/mg immobilized lipase) was almost the same of that of Amberlite IRC-50, although the efficiency of the immobilization technique was 50 %. As for the protein loading, colophony loaded the highest amount of protein, 17.5 mg/g support, with an activity of 14.6 U/mg immobilized lipase and an efficiency of 88 %.

Gel entrapment of lipase. Using the entrapment technique, it was hoped that the stability of immobilizates prepared by the adsorption technique would be enhanced and that the limitations imposed by glutaraldehyde would be avoided. Unfortunately, this immobilizate showed a rather low activity of only 54 U/g support. Most of the enzyme was released from the gel into the $CaCl_2$ solution during

the 24 h of incubation. This leakage of lipase from the beads did not cease even when a higher alginate concentration of 2.5 g/L was used.

Based on the lipolytic activity as a first selection criterion, eight immobilizates (Amberlite IRC-50, $CaSO_4$, $CaCO_3$, Amberlite IR-4B, polypropylene, Dowex 2X8, colophony and Al_2O_3) were subjected to further evaluation of their hydrolytic activity and stability, *i.e.*, their reusability and durability.

Hydrolytic activity of the immobilized lipase

The immobilizates on inorganic carriers containing high amounts of precipitated lipase led to a similar degree of hydrolysis, of about 83 %. The Ca-based immobilizates were difficult to separate from the reaction system for reuse. A certain amount of the carrier was always lost. Of the immobilizates with organic carriers, the one with Amberlite IRC-50 hydrolyzed up to 93 % of the olive oil, while that with Dowex 2X8, only 61 % was hydrolyzed. The immobilizates on colophony, polypropylene and Amberlite IR-4B were also inconvenient to work with. Namely, the whole reaction system, including the immobilizates and products released turned into a gelationous cake, which made the separation of the hydrolysis products impossible.

Only the immobilizates on Al_2O_3, Amberlite IRC-50 and Dowex 2X8 demonstrated satisfactory behavior in the emulsion system. They remained stable in the first batch of hydrolysis and offered an easy separation of the products from the medium. In the second batch with Dowex 2X8 immobilizates, the degree of hydrolysis dropped by 77 %, whereas with Amberlite IRC-50 and aluminum oxide the reduction was 18 and 4 %, respectively. However, after the third batch, the immobilizate on Al_2O_3 lost its activity more rapidly, so that in the last batch it retained only 16.7 % of its initial activity (Fig. 1).

Although the immobilizates on Amberlite IRC-50 and Al_2O_3 displayed acceptable stability, their activities were still rather modest. Thus, a few factors that

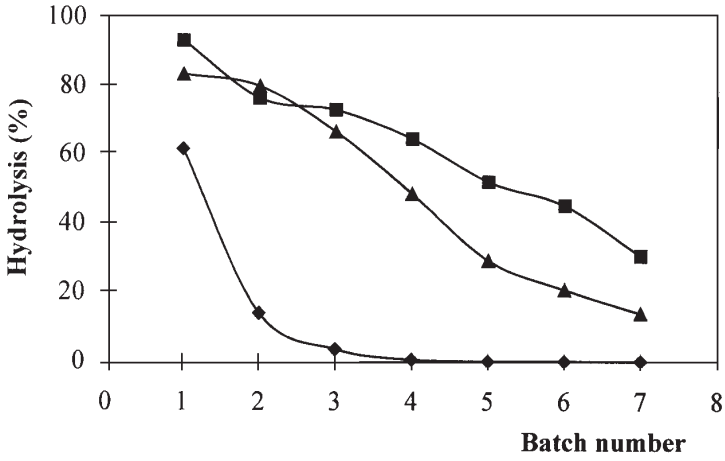

Fig. 1. The degree of hydrolysis of repeatedly used immobilizates on Amberlite IRC-50 (■), Al_2O_3 (▲) and Dowex (◆).

might have an effect on the immobilization, such as time and temperature of immobilization, activation of the immobilizates by drying and the amount of the precipitating agent, were investigated. Of these factors only drying of the immobilizates brought about an activation of the immobilized enzyme, resulting in a 3.3-fold increase in the lipolytic activity of the immobilizate with Amberlite IRC-50 (from 600 to 2000 U/g) and in a 2.4-fold increase of the immobilizate with Al_2O_3 (from 181 to 430 U/g). Regarding the specific activities, the activity of the former immobilizate increased from 40.3 to 355 U/mg immobilized lipase, whereas that of the latter increased from 9.2 to 26 U/mg immobilized lipase. The immobilizates prepared in this fashion were applied to study the factors affecting the hydrolysis of olive oil. The immobilization of the lipase on Amberlite IRC was carried out at 25 °C as well as at 4 °C. Both immobilizates exhibited the same activity. Consequently, the immobilization process was performed at ambient temperature as no energy input was required.

Hydrolysis of olive oil by immobilized lipase

The effectiveness of the immobilized biocatalysts on Amberlite IRC-50 (355 U/mg immobilized lipase) and Al_2O_3 (26.3 U/mg immobilized lipase) was evaluated on the basis of their capability of successfully hydrolysing olive oil under optimal conditions.

Effect of surfactant concentration

Initially, before proceeding with the hydrolysis of olive oil using the immobilizates, it was checked whether the presence of a surfactant in the reaction mixture was indispensable and if so in what amount. The surfactant used was the oleic acid monoester of polyoxyethylene sorbitan (Tween 80). In the reaction system without and with 2 g/L Tween 80, the degree of hydrolysis of olive oil was 97.7 % in both cases. In the system without emulsifier, the emulsion was formed by vigorously mixing the water and oil phases, while in the second case emulsion formation was due to the presence of the emulsifying agent. Although, both emulsions were not very stable, the emulsion with the surfactant was somewhat steadier. A higher content of Tween 80 resulted in the formation of a rather stable emulsion but the degree of hydrolysis decreased to 53.9 % when 10 g/L emulsifier were used. It is obvious that higher concentration of surface-active substance in the interfacial area hindered the access of the enzyme to the substrate, thereby inhibiting the hydrolysis. Hence, in further experiments 2 g/L Tween 80 was employed. It should be mentioned that hydrolysis without the emulsifying agent has the advantage in that the separation of the products is easier. However, the preservation of such an emulsion requires very intensive stirring.

Effect of enzyme concentration on the rate of hydrolysis

From the economical point of view, the substrate concentration should be as high as possible and the amount of soluble or immobilized enzyme as low as necessary to achieve a degree of hydrolysis of over 95 %.

To determine the optimum concentration of lipase for running a 24 h hydrolysis of olive oil, the enzyme concentration was varied from 10 to 800 U/g substrate. The relationship between the lipase concentration and the degree of hydrolysis is shown in Fig. 2. For the soluble enzyme, the hydrolysis was almost completed (97.7 %) when 200 lipase units per gram olive oil were employed. With half of this activity (100 U/g), less than 70 % hydrolysis was obtained, whereas double the amount (400 U/g) allowed for the same percentage of hydrolysis as 200 U/g. Ruckenstein and Wang,[23] working with *Candida rugosa* lipase, came to similar results.

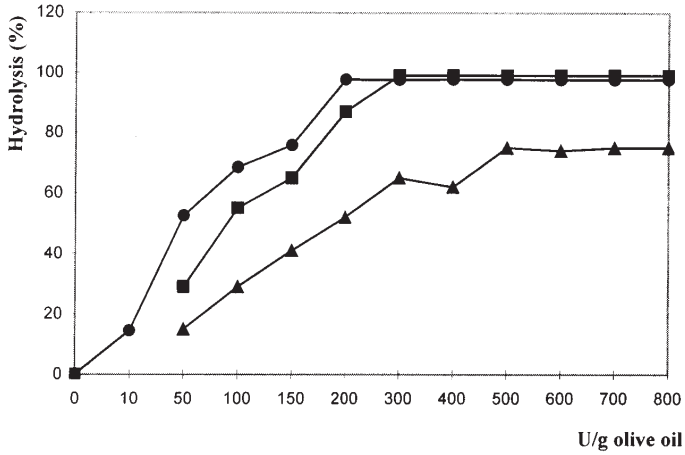

Fig. 2. Effect of lipase concentration on the hydrolysis of olive oil at pH 7 and 30 °C by soluble enzyme (●), immobilizates on Amberlite IRC-50 (■) and on Al_2O_3 (▲).

The course of the hydrolysis with all three forms of the enzyme was linearly proportional to the enzyme concentration up to 150 U/g. As the enzyme concentration was increased, the rate of reaction became asymptotic. When the immobilizates with Amberlite were employed, the highest degree of hydrolysis (99.1 %) was achieved at an enzyme concentration of 300 U/g. If it is calculated how much free and enzyme immobilized on Amberlite is required to achieve the same degree of hydrolysis (97.7 %), it becomes obvious that the free enzyme must be used in three times higher quantities than the immobilized enzyme.

The degree of hydrolysis with Al_2O_3 immobilized lipase was considerably lower reaching a maximum value of 78.5 % above 500 U/g, while with 300 U/g, only 64 % hydrolysis was attained. This could result from the occurrence of multilayer adsorption that might block or inhibit the access of the substrate to the active site. Perhaps, only the enzyme molecules on the external layer of the immobilized preparation were responsible for the demonstrated activity.[24] Another reason for the lower activity could be deactivation of the enzyme during its precipita-

tion with acetone in the immobilization process. In favour of these explanations is the fact that 300 lipase units were provided by 11.5 mg lipase immobilized on Al_2O_3 compared to 0.85 mg lipase immobilized on Amberlite.

Effect of pH and buffer on lipase performance

Not only does the pH value have an effect on the conformation of the enzyme, but it also determines in which state the free fatty acids are in. At pH values above the dissociation constant, the free fatty acids form soaps when cations are present. Ionized fatty acids dissolve more easily in the aqueous phase than unionized ones; soaps adsorb to the water/lipid interface or form micelles in the water phase. Due to the complexity of the phenomena and the lack of literature data, the influence of pH cannot be predicted, and has to be measured.

The effect of pH and the tested buffers on the hydrolytic activity of the soluble and immobilized lipase are depicted in Fig. 3. The pH effect was studied in the region from 4 to 9 using three different buffers: acetate (pH 4, 5, 6), phosphate (pH 6, 7, 8) and TRIS–HCl (pH 7, 8, 9) buffer. At the same pH value, regardless the buffer used, the degree of hydrolysis was almost the same and therefore the results are presented by one curve only. The soluble lipase is effective in the pH range from 4 to 8, producing over 80 % free fatty acids with a maximum activity between pH 6–7, when 97.7 % hydrolysis was registered. In TRIS–HCl buffer at pH 9, the degree of hydrolysis dropped sharply to about 11 %. Both immobilizates exhibited broad pH profiles and

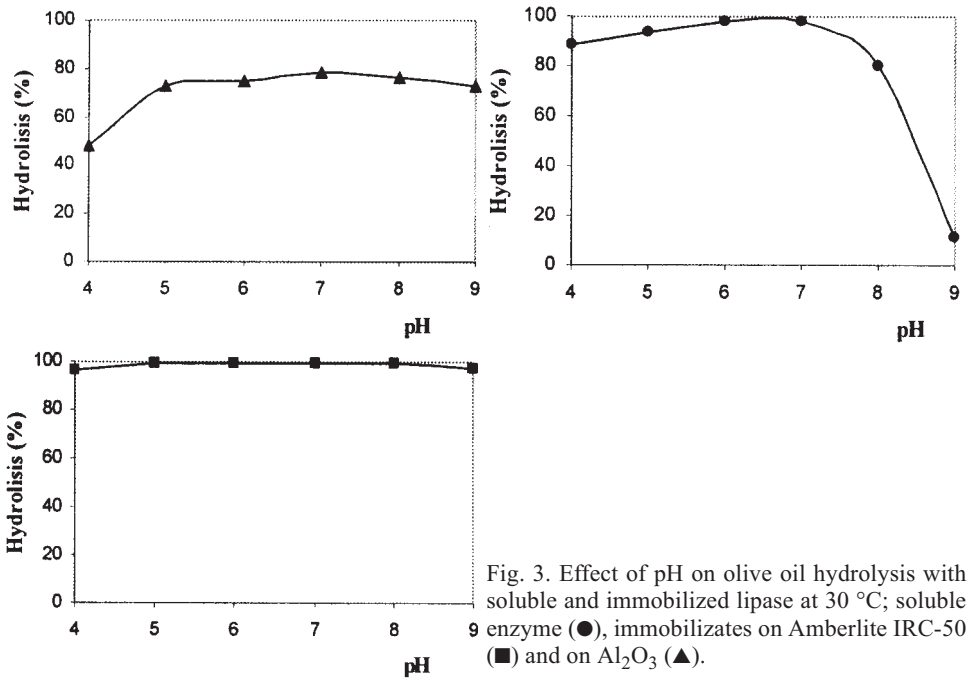

Fig. 3. Effect of pH on olive oil hydrolysis with soluble and immobilized lipase at 30 °C; soluble enzyme (●), immobilizates on Amberlite IRC-50 (■) and on Al_2O_3 (▲).

were less sensitive to pH after immobilization. The Al_2O_3-immobilized lipase hydrolysed to 78.8 % at all pH values except at 4 when it dropped to under 50 %. Additionaly, it is advisable to avoid the lower pH values (4–5) since the isoelectric point of *Candida rugosa* lipase is at about 4.5[25] and the pH of the aqueous phase should be maintaned at least above 5.5 to prevent serious desorption of the enzyme. The Amberlite immobilizate demonstrated excellent stability and activity over the whole investigated range of pH in all three buffers resulting in almost complete hydrolysis of the olive oil (99.1 %). However, the phosphate buffer of pH 7 was chosen for practical reasons since the activation of the Amberlite was performed under these conditions.

A broad pH profile (4 to 8) for *Candida rugosa* lipase immobilized on cuprophane membrane was observed by Pronk *et al.*[26] Lipase immobilized on PBA Eupergite 250L exhibited a narrower profile with the pH otpimum slightly shifted towards the alkaline region, from 8 to 8.5.[3] A rather sharp pH optimum *Candida rugosa* lipase adsorbed on polypropylene (pH 6.8) was reported by Montero *et al.*[16]

Effect of temperature on lipase activity and stability

The temperature dependence of the hydrolytic reaction with soluble and immobilized lipase, shown in Fig. 4, was studied in the interval from 20 to 50 °C. Except at 20 °C, the soluble enzyme yielded over 90 % hydrolysis with 30 °C being the optimum temperature, when 97.7 % of the oil was hydrolyzed. The Al_2O_3-immobilized lipase behaved similarly, only the yield was under 80 % over the whole

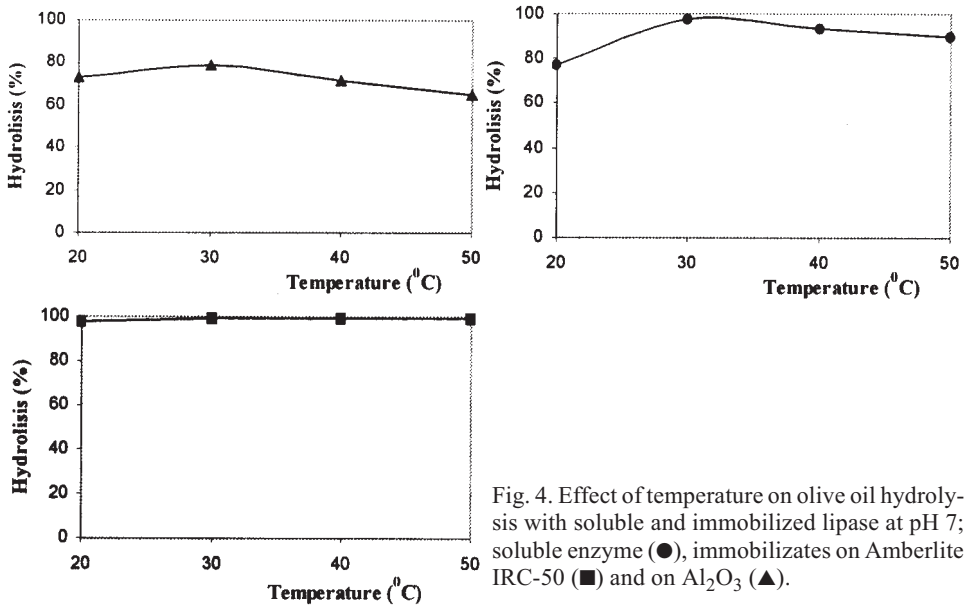

Fig. 4. Effect of temperature on olive oil hydrolysis with soluble and immobilized lipase at pH 7; soluble enzyme (●), immobilizates on Amberlite IRC-50 (■) and on Al_2O_3 (▲).

temperature range. Increasing temperature had no influence on the Amberlite-immobilized lipase, as the enzyme demonstrated almost the same activity (99.1 % degree of hydrolysis) at all studied temperatures. Usually the immobilization procedure results in higher temperature stability and activity of the immobilized enzyme. The optimum temperature shifted from 35 to 45 or 50 °C, which is highly desirable because the higher optimum temperature leads to lower risk of microbial contamination.[1,24] On the other hand, the insensitivity of the Amberlite-immobilizate to temperature, offers another advantage, since working at a lower temperature requires less energy input.

To measure the thermal stability of the enzyme, the temperature data were linearized in an Arrhenius plot (Fig. 5). The calculated activation energies of the soluble enzyme and Al_2O_3-immobilizate were 42.2 kJ/mol and 7.4 kJ/mol, respectively. Increasing the temperature from 30 to 50 °C led to a certain deactivation of both the soluble and Al_2O_3-immobilized enzyme. From Fig. 5, the corresponding energies of deactivation were 3.4 kJ/mol and 8.0 kJ/mol. These values of the energy of deactivation are lower than those cited in the literature. Warmuth *et al.*[3] reported 93.3 kJ/mol for *Candida rugosa* lipase immobilized on various supports. It is obvious that the deactivation energy for Amberlite immobilizates cannot be calculated from Fig. 5. The temperature stability, as well as the pH stability makes this immobilizate attractive to work with.

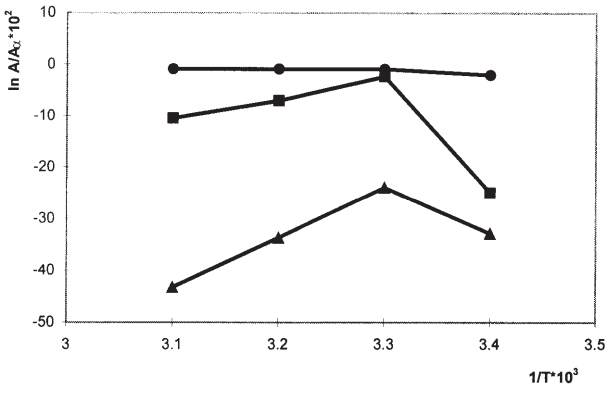

Fig. 5. Arrhenius plot for soluble (●) and immobilized lipase on Amberlite IRC-50 (■) and on Al_2O_3 (▲).

Reusability and stability of the immobilizates

The reusability of the immobilized enzyme is the most appropriate way to evaluate the immobilization procedure. The results in Table II prove that the Amberlite IRC-50 was a good support material for lipase immobilization and that the immobilizate was active enough to perform 99 to 95 % hydrolysis in the first 3 batches, each of which ran for 24 hour. In the sixth batch, the degree of hydrolysis dropped to 83 %.

The immobilized preparation with Al_2O_3 was not only less active than the one with Amberlite IRC-50, but also less stable. The degree of hydrolysis declined sharply to 37.5 % for the sixth batch, which corresponds to the half-life of the immobilizate. For Amberlite immobilizates, however, the half-life was projected to 16 batches, which was an operational time of 384 h. The half-life was determined by extrapolating the data given in Table II (Figure not shown).

TABLE II. Repeated usage of immobilizates on Amberlite IRC-50 and Al_2O_3 for olive oil hydrolysis under optimized conditions

Number of batches	1	2	3	4	5	6
Degree of hydrolysis (%) with immobilizates on:						
Amberlite IRC-50	99.1	98.4	94.7	91.8	86.0	83.1
Al_2O_3	78.7	62.4	56.7	53.5	47.0	35.7

The operational stability is a very important feature for an immobilized enzyme. Not less important is the storage stability. The storage stability of our immobilizates was controlled by day-storage of both at 4 °C for 9 months. During this period the Al_2O_3-immobilizates retained only one quarter of its original activity. The Amberlite IRC-50 immobilizate was again superior, it retained 75 % of its original activity.

CONCLUSION

Among numerous support materials and several techniques, Amberlite IRC-50 and Al_2O_3 were selected for the immobilization of lipase by ionic binding and the combined adsorption method. These two immobilizates had activities of 2000 U/g support (355 U/mg immobilized lipase) and 430 U/g support (26 U/mg immobilized lipase), respectively. They were employed for the batch hydrolysis of olive oil. Under optimized process parameters, the immobilizates on Amberlite IRC-50 brought about up to 99 % hydrolysis of oil while the ones with Al_2O_3 did not exceed 79 %. The immobilized preparation with Amberlite IRC-50 was stable and active over the whole range of pH (4 to 9) and temperature (20 to 50 °C), as well as in repeated usage, having a half-life of 16 batches, which corresponds to an operational time of 384 h. This immobilizate distinguished itself not only with acceptable operational stability but also with good storage stability. Hence, it is rather appropriate for application in hydrolytic processes.

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ИЗВОД

ЛИПАЗА ИМОБИЛИЗИРАНА РАЗЛИЧИТИМ ТЕХНИКАМА НА РАЗНИМ НОСАЧИМА ПРИМЕЊЕНА ЗА ХИУДРОЛИЗУ УЉА

ВИЛМА МИНОВСКА, ЕЛЕОНОРА ВИНКЕЛХАУЗЕН И СЛОБОДАНКА КУЗМАНОВА

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Липаза квасца *Candida rugosa* имобилизирана на Amberlite IRC-50 и Al_2O_3, примењена је за шаржну хидролизу маслиновог уља. Ова два носача су изабрана тестирајући 16 материјала различите природе који припадају неорганским носачима (песак, силика гел, инфузоријска земља, Al_2O_3) неорганским солима ($CaCO_3$, $CaSO_4$), јоноизмењивачким смолама (Amberlite IRC-50 и IR-4B, Dowex 2X8), природним смолама (колофоније), природним биополимерима (натријум-алгинат), синтетским полимерима (полипропилен, полиетилен) и зеолитима. Имобилизација липазе на овим носачима извршена је путем једноставне адсорпције, адсорпцијом и умрежавањем сорбираног ензима, адсорпцијом на јоноизмењивачким смолама, затим комбинованом адсорпцијом и таложењем, само таложењем и заробљавањем у алгинатном гелу. Погодност носача и техника за имобилизацију комерцијалне липазе процењиване су одређивањем активности имобилизираног ензима, задржавањем протеина на носачима, ефикасношћу самог поступка имобилизације, као и вишекратном употребом имобилизата. Способност имобилизата за хидролизу маслиновог уља, била је мера њихове активности. Највећи број имобилизата је показао или слабу активност или стварао проблеме приликом хидролитичке реакције. Само имобилизати добивени јонском адсорпцијом на Amberlite IRC-50 (2000 U/g носача) и са комбинованом адсорпцијом на Al_2O_3 (430 U/g носача) били су активнији и показали задовољавајуће понашање приликом њихове вишекратне употребе у хидролитичкој реакцији. Хидролизу уља овим имобилизатима пратили смо испитивањем утицаја неколико параметара на сам ток процеса и то: концентрације емулгатора, концентрације ензима, pH медијума и температуре. Имобилизат на Amberlite IRC-50 био је стабилан и активан у целом испитиваном подручју pH (од 4 до 9) и температуре (од 20 до 50 °C) и довео до 99 % хидролизе уља. Код вишекратне употребе, овај се имобилизат показао активним и стабилним са временом полуживота од 16 шаржи, што одговара операционом времену од 384 часова. Његова је стабилност складиштења била такође добра, јер је после девет месеци чувања препарат изгубио само 25 % од почетне активности. Имобилизат на Al_2O_3 био је нестабилинији и мање активан од имобилизата на амберлиту. Степен хидролизе, код оптималних услова, није прешао 79 %, а након четврте шарже опао је до 53.5 %.

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