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IMMOBILIZED LIPASES AS CATALYSTS FOR FAT HYDROLYSIS

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases of considerable physiological significance and industrial potential that can catalyze numerous reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis (Jaeger et al, 2002). In oleochemical industry they can be used for production of fatty acids (Knežević et al, 1998) or monoacylglycerols (Šiler–Marinković et al, 1995) from triacylglycerides. Lipase–catalyzed hydrolysis is an energy–saving process, much more favorable than the conventional Colgate–Emery process which applies high pressurized steam at high temperature and produces undesirable color impurities. Considering the high catalytic power and exceptional stereo–, regio– and chemoselectivity of lipases, the small number of their commercial applications in the industry is surprising. The reason is that the lipase reaction systems are too complex, usually consisting of two immiscible phases: an aqueous phase with dissolved enzyme and an organic phase with dissolved substrate (Knežević et al, 1998). The application of lipase has been deterred in these reaction systems by the high cost of enzymes, contamination of products with residual protein, slow reaction rate, and lack of an ideal emulsion reactor system to cater the complex interfacial heterogeneous hydrolysis. Most of the above problems could be overcomed using immobilized lipases. In order to design a productive system for palm oil hydrolysis by lipase from *Candida rugosa* we studied four different systems: (a) immobilization in the lecithin/isooctane reversed micelles; (b) immobilization on a polymer support; (c) immobilization in alginate by electrostatic extrusion; and (d) immobilization on Cuprophane membrane in a hollow fiber module. Lipase immobilization efficiencies, activities and stabilities in repeated palm oil hydrolysis batches were assessed and compared.

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MATERIALS AND METHODS

Candida rugosa lipase (890 units mg^{-1}) purchased from Sigma Chemical Co. (St. Louis, MO) was used for experiments without further purification. Refined Malaysian palm oil (Vital Vrbas, Yugoslavia) with a saponification value of 199.5 and a molar mass of 845 g mol^{-1}, was used as the substrate for lipase hydrolysis. Bovine serum albumin (BSA, Sigma, St. Louis, MO) was used as a standard for protein. Isooctane of p.a. grade was purchased from Merck (Darmstadt, Germany) and used as the organic solvent. Deoiled Soya lecithin (Lucas Meyer, Hamburg) was used as a surfactant for preparation of reversed micelles. Copolymer SGE–A2–94 obtained by suspension polymerization of glycidyl methacrylate (Rhom–Darmstadt, Germany) and ethylene glycol dimethacrylate (Rhom–Darmstadt, Germany) was used as a support for lipase immobilization. Sodium alginate and calcium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Olive oil emulsion from Sigma Chemical Co. (St. Louis, MO) was used to assess the lipase activity. All other chemicals were reagent grade. Capillary dialysis modules (model E2) donated from INEX–Hemofarm DD (Vršac, YU) made of Cuprophane with a molecular weight cut–off of 5000 Da were used as hollow fiber membrane for lipase immobilization. The module consisted of approximately 7000 fibers with an internal diameter of 200 mm, a wall thickness of 8 mm, and length of 16 cm. The total membrane area was 1.0 m^2.

Preparation of reversed micellar solution. Reversed micellar solutions were prepared by injecting an appropriate volume of concentrated stock solution of the lipase in phosphate buffer, (100 IU of lipase solution in buffer, pH=7.0) into the mixture of organic solvent and lecithin under vigorous stirring. The value of R (defined as the molar ratio of water to surfactant) was fixed at 15 for all experiments, as we previously defined as optimum (Šiler–Marinković et al. 1995).

Lipase immobilization on SGE–A2–94 polymer support. Macroporous copolymer SGA–A2–94 was synthesized according to the procedure described by Jovanović et al, 1994. Particles with diameters in the range of 150–500 μm were used. Lipase from *C. rugosa*

was immobilized on the SGA–A2–94 copolymer by adsorption (Mojović et al, 1998).

Lipase immobilization in alginate beads. Alginate beads were produced by electrostatic droplet generation, as previously described by Knezevic et al (2002). In order to prevent leakage of the lipase out of alginate beads an additional membrane based on poly–L–lysine was made (Ma et al, 1994). The amount of immobilized enzyme was determined according to the procedure described by Knežević et al (2002).

Immobilization on hollow fiber membrane. The preparation of the lipase solution and immobilization of the lipase was described by Knežević et al. (2004).

Analytical methods. Lipase contents were measured in the initial lipase solutions and in aqueous effluents using the Lowry method with BSA as a standard at 550 nm. Concentrations of free fatty acids produced were measured in the collected oil samples by titration with 0.1 M KOH in ethanol, using phenolphthalein as indicator. The degree of hydrolysis was calculated from the acid value and the saponification value of the oil in the reaction mixture (Mojović et al. 1998). The lipase activities were estimated by a standard olive oil emulsion method. The immobilization efficiency (%) was determined as described previously (Knežević et al 2004; Mojović et al, 1998).

RESULTS AND DISCUSSION

Comparison of different C. rugosa immobilized systems used for palm oil hydrolysis. Figure 1 presents schematics and photographs of the three of four immobilized lipase systems used in this study. The key parameters needed for development of the process with immobilized enzyme are enzyme loading achieved; the initial activity of biocatalyst and the stability in repeated batches. In addition, the kinetic properties of immobilized systems in the concrete reaction may also differ, thus making certain systems more appropriate for specific applications. These parameters are thoroughly investigated and data obtained for immobilized *C. rugosa* lipase are summarized in Table 1. On the basis of experimental results for palm oil hydrolysis the productivities of the immobilized systems were calculated and presented in Figure 2.

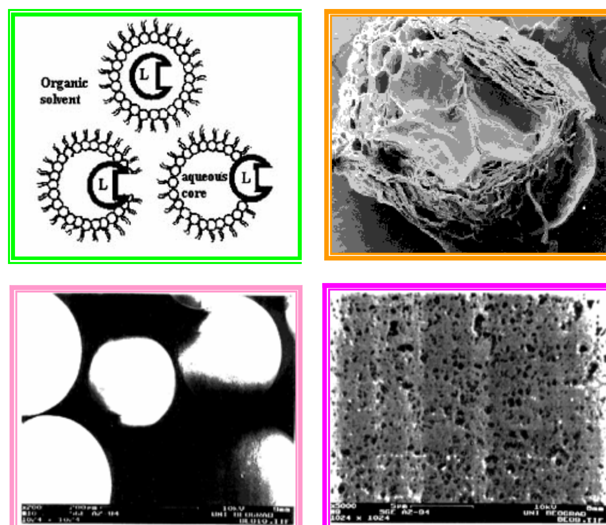

Figure 1. Schematics and photographs of the three immobilized lipase systems. Upper left is a schematic of the reverse micellar system with indicated location of enzyme within the micellar core. Upper right is a scanning micrograph of the alginate bead. Lower left is a scanning micrograph of SGE–A2–94 particles and lower right of the particle surface morphology.

Reversed micellar system greatly increases substrate solubility and homogeneity that results in favorable kinetics of reaction (reaction rate constant $k=1.537\ h^{-1}$). However, the system lacks stability for reuse. Consequently, the overall system productivity in palm oil hydrolysis is low (106.5 moles of fatty acids per kg of lipase).

Polymer SGE–A2–94 exhibited rather high affinity for binding of *C. rugosa* lipase and a loading of 18.1 mg/g was achieved. Optimal conditions for obtaining immobilized catalyst with high activity were 42°C and pH=5.8. However, immobilized lipase was loosing the initial activity in subsequent runs reaching the productivity of 386.7 moles of fatty acids per kg of lipase after six batches.

High immobilization efficiency of 99% was attained with *alginate*. Significant decrease of the activity of the alginate immobilized lipase was observed after the third

Table 1. The comparison of immobilized systems

Immobilized system	Temperature of reaction (°C)	Mass of immobilized lipase	Immobilization efficiency (%)	Reaction rate constant[a] (h^{-1})	Relative activity[b] (%)
Reversed micelle	30 37	–	–	0.642 1.537	100
Polymer SGE–A2–94	37	18.1 mg/g	73	0.876	83
Alginate beads	37	10 mg/g	99	–	75
Membrane hollow fiber	30	155 mg/m^2	40	0.269	42

[a]Reaction rate constants were determined on the basis of kinetic model by Knežević et al (1998). Model didn't fit well experiments for alginate immobilized lipase b Activity was relative to reversed micellar system taken as 100% of activity.

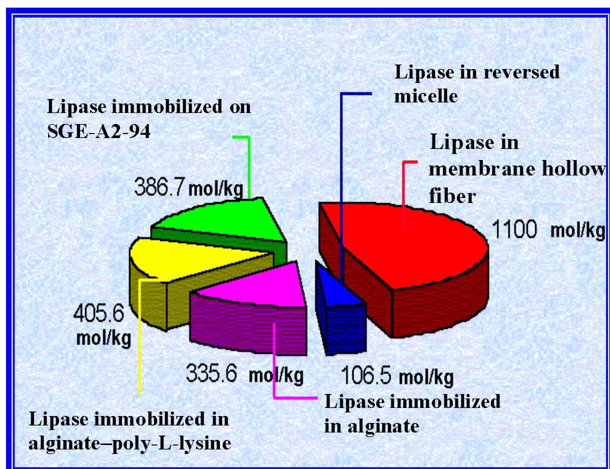

Figure 2. Comparison of the productivity of immobilized systems with Candida rugosa in the hydrolysis of palm oil

run. This was in agreement with the findings of Betigeri et al (2002). A moderate productivity of 335.6 moles after seventh batch was achieved by the lipase immobilized in alginate beads. An increase in productivity of 21 % was accomplished by making a poly–L–lysine membrane around alginate beads which contributed to the enhancement of the stability of the immobilized system (Figure 2).

Despite a pretty low immobilization efficiency of *membrane hollow fiber* (40%, Table 1) which was also reported by Van der Padt, et al. (1990), this system achieved very high productivity of 1100 mol/kg in palm oil hydrolysis. The ten times higher productivity than the one in reversed micelles was due to significantly higher stability of this system and also due to the optimization of the substrate flow regime through the hollow fiber modulus (Knezevic et al., 2004).

CONCLUSIONS

Immobilization of lipase from Candida rugosa in reversed micelles resulted in a high enzyme activity and favorable kinetics, and a low stability in the reaction of palm oil hydrolysis. The productivities of the lipase immobilized on copolymer SGE–A2–94 and in alginate beads were around three times greater than the one of the reversed micellar system, mostly because of enhanced stability. The lipase immobilized in the membrane hollow fiber was by far the most productive and stable system in palm oil hydrolysis, ten times more productive than reversed micellar system.

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