Lipase Immobilization in a Hollow Fibre Membrane Reactor: Kinetics Characterization and Application for Palm Oil Hydrolysis*

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Dedicated to the 80th birthday of Professor Elemír Kossaczký

Feasibility of lipase immobilization in a hydrophilic hollow fibre membrane reactor for oil hydrolysis has been demonstrated. A simple immobilization technique was applied resulting in about 155 mg m^{-2} of immobilized lipase at approximately 40 mass % yield. The immobilized lipase in the membrane reactor had favourable kinetic properties. The reaction in the membrane reactor was well approximated by one-substrate first-order reversible kinetics, previously established for a microemulsion reactor, implying the same reaction mechanism in both systems. Estimation of kinetic parameters revealed that the immobilized lipase retained activity equivalent to approximately 44 % of that of the free lipase but the final conversions were higher with the immobilized lipase. At the enzyme load employed in this study, the loss of activity due to enzyme desorption had only a small effect on the reactor stability and oil hydrolysis. The membrane reactor operated for up to 137 h with no significant loss in productivity. These results imply that the proposed immobilization technique and hollow fibre reactor system with immobilized lipase provide a promising solution for applications of lipase for oil hydrolysis at industrial scale.

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 [1]. The broad substrate specificity makes lipases usable in a wide field of applications and their market is still growing [2]. One of the most promising fields of lipases application is the production of fatty acids and glycerol *via* hydrolysis of oils and fats for the food and pharmaceutical industries [3, 4]. However, lipase reaction systems are complex, usually consisting of two immiscible phases: an aqueous phase with dissolved enzyme and an organic phase with dissolved substrate [5]. Applications of lipase have been deterred in these reaction systems by high enzyme cost, contamination of products by residual protein, low reaction rates, and lack of an ideal emulsion reactor system to carry out the complex interfacial heterogeneous hydrolysis. On the other hand, the biphasic membrane reactors with lipases immobilized in the membranes can be used to enhance the productivity of enzymatic process by improving substrate/lipase contact, by providing a simple and reversible means of enzyme immobilization and by effecting the removal of inhibitory reaction products [6]. In addition, enzyme membrane reactors offer advantages with respect to conventional enzyme reactors for the membrane ability to operate simultaneously as an enzyme carrier and a phase separation barrier combining a chemical reaction with a selective mass transfer through the membrane. Therefore, the use of biphasic membrane reactors with immobilized lipase is highly attractive, especially when hollow fibre reactor configuration is used, since it provides the highest surface to volume ratio without the need for membrane support.

Several authors studied lipase immobilization and fat and oil hydrolysis in hollow fibre reactors. *Hoq et al.* [7] presented a hydrophobic hollow fibre membrane system for fat hydrolysis with lipase immobilized at the water-glycerol side of the membrane. This system was later used also for oil hydrolysis and ester syn-

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thesis by several groups of authors [8—10]. On the other hand, *Pronk et al.* [11] presented a hydrophilic hollow fibre membrane system for hydrolysis of soybean oil with lipase immobilized at the oil side of the membrane. This system was also subsequently used in many studies on lipase catalysis [12—14]. Both membrane systems have advantages and it is not yet determined, which membrane system is preferable for industrial application. An advantage of the hydrophilic membrane system could be the reduced enzyme desorption because of its insolubility in the organic phase. In addition, catalytic activity per membrane area is of the same order of magnitude regardless of the membrane type or thickness. However, if estimated per amount of immobilized enzyme, catalytic activity is much higher for hydrophilic membrane systems [15].

In most studies performed in hollow fibre reactor systems with immobilized lipase, reactor operating regimes and reaction kinetics were not thoroughly investigated and the Michaelis—Menten approach was commonly used [11, 13, 14]. However, the reaction kinetics is more complex than the commonly assumed one-substrate one-product irreversible reaction. In addition, diffusion limitations must be eliminated and the intrinsic kinetics of enzymatic catalysis in this system must be measured.

Recently, palm oil hydrolysis by lipase from *Candida rugosa* in a biphasic oil/aqueous hollow fibre membrane reactor was studied [16]. The authors investigated the reactor operating regime with respect to flow patterns in reactor zones, lipase desorption, and hydrolysis rates and determined the optimal reactor operating regime for palm oil hydrolysis. In this work, under the optimal flow conditions, the kinetics of palm oil hydrolysis by immobilized lipase in the membrane reactor was investigated and compared to the results obtained for free lipase in the microemulsion reaction system. The objective of this work was to define a kinetic model describing the long-run oil hydrolysis by immobilized lipase in the membrane reactor important for commercial applications. In addition, hydrolytic activity of the immobilized and free enzymes was compared and stability of the immobilized enzyme was tested in several hydrolysis batches.

THEORETICAL

In order to interpret and analyze the obtained experimental results, kinetic model derived for the palm oil hydrolysis in a microemulsion system was applied [3]. This model implies that the oil hydrolysis is a one-substrate two-product first-order reversible reaction and that the water concentration does not influence the reaction rate. In such case, the mechanism of oil hydrolysis may be represented as follows

$$\mathrm{S} + \mathrm{H_2O} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{P_1} + \mathrm{P_2} \qquad (A)$$

where S is the ester bond which can be attacked by lipase, P_1 and P_2 are products (free fatty acids and glycerol residues), and k_1 and k_2 are the rate constants for ester bond decomposition and formation, respectively. The product concentration, C_p, is given by the expression

$$C_\mathrm{p} = C_{\mathrm{p}1} = C_{\mathrm{p}2} = C_0 - C \qquad (1)$$

where C_0 is the initial substrate concentration and C is the substrate concentration at any reaction time t.

The rate equation based on mechanism (A) is expressed as follows

$$r = -\frac{\mathrm{d}C}{\mathrm{d}t} = k_1 C - k_2 C_\mathrm{p}^2 \qquad (2)$$

Eqn (2) may be transformed using eqn (1) as follows

$$-\frac{\mathrm{d}C}{\mathrm{d}t} = k_1 C - k_2 \left(C_0 - C\right)^2 \qquad (3)$$

At an equilibrium state, $\mathrm{d}C/\mathrm{d}t = 0$ and $C = C_\mathrm{e}$

$$k_1 C_\mathrm{e} - k_2 \left(C_0 - C_\mathrm{e}\right)^2 = 0 \qquad (4)$$

Substitution of k_2 obtained from eqn (4) into eqn (3) gives

$$-\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{-k_1 \left[C_\mathrm{e} C^2 - C\left(C_0^2 - C_\mathrm{e}^2\right) + C_\mathrm{e} C_0^2\right]}{\left(C_0 - C_\mathrm{e}\right)^2} \qquad (5)$$

where C_e is the equilibrium substrate concentration.

Integration of eqn (5), subject to the boundary condition that $C = C_0$ when $t = 0$, gives

$$\ln \frac{C_0 \left(C - C_\mathrm{e}\right)}{\left(C_0^2 - C C_\mathrm{e}\right)} = k_1 t \frac{\left(X_\mathrm{e} - 2\right)}{X_\mathrm{e}} \qquad (6)$$

where $X_\mathrm{e} = (C_0 - C_\mathrm{e})/C_0$ is the equilibrium degree of hydrolysis and t is the time.

The substrate concentration, C, can be then expressed as a function of the initial, C_0, and equilibrium, C_e, substrate concentrations

$$C = \frac{C_0 \left(C_\mathrm{e} + C_0 M\right)}{C_0 + C_\mathrm{e} M} \qquad (7)$$

where M represents a parameter defined as

$$M = \mathrm{e}^{\frac{k_1 t (X_\mathrm{e} - 2)}{X_\mathrm{e}}} \qquad (8)$$

It was assumed that the reaction catalyzed by immobilized lipase in the membrane reactor follows the same kinetics as that determined for free lipase in the microemulsion system [3]. This assumption is true, if the immobilization method would not influence the structure of the lipase. In addition, plug flow of the substrate mixture in the lumen zone of the reactor

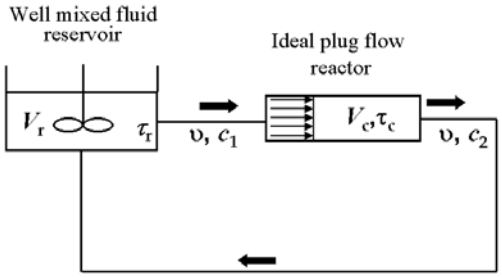

Fig. 1. Scheme of the two-compartment membrane reactor system.

and its ideal mixing in the oil reservoir was expected. Previous results indicated that the assumption of ideal plug flow is probably appropriate for modelling of reactions taking place in the lumen zone of hollow fibre modules, taking into account that only active lumen volume should be considered (≈ 18 % of the lumen volume was almost stagnant) [16]. Experimental system could thus be represented by a two-compartment model consisting of a plug flow reactor connected in series with an ideally mixed vessel (Fig. 1). Then, the substrate concentration at the membrane reactor outlet, C_2, can be expressed as a function of the inlet, C_1, and equilibrium, C_e, substrate concentrations

$$C_2 = \frac{C_1 (C_e + C_1 M)}{C_1 + C_e M} \qquad (9)$$

where M is a constant defined as

$$M = e^{\frac{k_1 \tau (X_e - 2)}{X_e}} \qquad (10)$$

and τ is the mean reactor residence time.

Mass balance for the oil reservoir can be written as

$$\upsilon_{C_2} - \upsilon_{C_1} = V_r \frac{dC_1}{dt} \qquad (11)$$

where υ is the oil phase volumetric flow rate and V_r is the reservoir volume.

Substituting eqn (*9*) into eqn (*11*) results in a first-order differential equation, which can be integrated and solved to get the time required to reduce the substrate concentration from its initial value, C_0, to a concentration C according to

$$t = \frac{\tau}{M-1} \left[(1+M) \ln \frac{C - C_e}{C_0 - C} - M \ln \frac{C}{C_0} \right] \qquad (12)$$

In the proposed kinetic models, adjustable parameters are the rate constant, k_1, and the equilibrium degree of hydrolysis, X_e, which were determined by least-squares fits to the experimental data (Figs. 2 and 3).

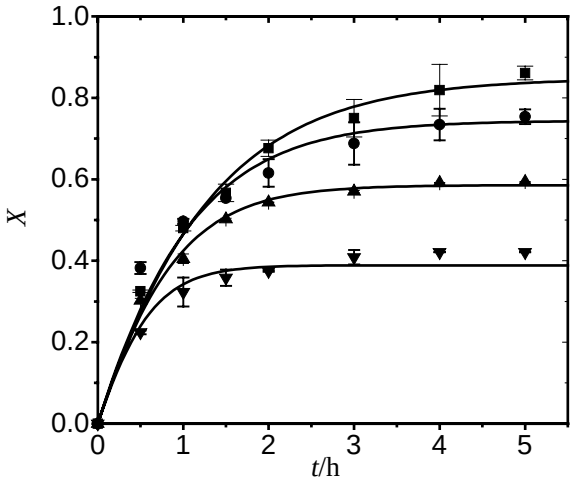

Fig. 2. Experimental (points) and fitted data (solid lines) of palm oil hydrolysis in a free enzyme microemulsion system obtained for the initial substrate concentration of 0.059 mol dm^{-3} (■), 0.090 mol dm^{-3} (●), 0.163 mol dm^{-3} (▲), and 0.326 mol dm^{-3} (▼).

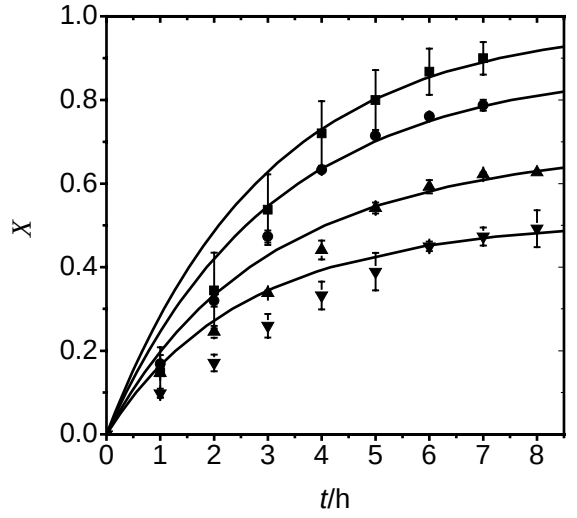

Fig. 3. Experimental (points) and fitted data (solid lines) of palm oil hydrolysis in a membrane reactor obtained for the initial substrate concentration of 0.059 mol dm^{-3} (■), 0.090 mol dm^{-3} (●), 0.163 mol dm^{-3} (▲), and 0.326 mol dm^{-3} (▼).

EXPERIMENTAL

Candida rugosa lipase (EC 3.1.1.3) from Sigma Chemical Co. (St. Louis, MO) with a nominal specific activity of 890 units mg^{-1} was used for experiments without further purification. Refined Malaysian palm oil (importer 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) from Sigma (St. Louis, MO) was used as the standard for protein analysis. Isooctane of anal. grade was purchased from Merck (Darm-

stadt, Germany) and used as the organic solvent. All other chemicals were reagent grade.

Processing equipment consisted of a hollow fibre reactor, oil and buffer reservoirs, two pumps for recirculation of the oil and water phases, and an external water jacket. Capillary dialysis modules (model E2) purchased from INEX-Hemofarm (Vrsac, Yugoslavia) were used as hollow fibre reactors. The reactor module consisted of approximately 7000 fibres with an internal diameter of 200 μm, a wall thickness of 8 μm, and a length of 16 cm. The total membrane area was 1.0 m^2. The hydrophilic fibres were made of Cuprophane with a narrow relative molecular mass cut-off (M_r, CO) of 5000. The detailed specifications of the membrane, experimental set-up for lipase-catalyzed hydrolysis of palm oil, operating protocol, and analytical procedures were essentially the same as those described previously [16]. In each batch experiment 140 cm^3 of the oil phase (palm oil solution in isooctane) was recirculated through the lumen side and 200 cm^3 of the aqueous phase (0.5 M-phosphate buffer of pH 7) was recirculated through the shell side of the reactor. Both phases were recirculated cocurrently upwards through the vertically oriented reactor by peristaltic pumps. The reactor had an external jacket recirculated by hot water to maintain isothermal conditions. During the hydrolysis batches, temperature of the oil and buffer reservoirs and on the exit of the fibre zone was maintained constant at (30 $\pm$ 1) ℃. All hydrolysis experiments were conducted at pH of 7.0 $\pm$ 0.05. Reactor operating conditions were as follows: the reactor active volume of 64 cm^3 and the flow rate of 16 cm^3 min^{-1}, thus giving a reactor residence time of 0.067 h.

Preparation of the lipase solution and immobilization procedure was identical to that employed previously [16]. The reactor lumen was recirculated with an aqueous lipase solution and subsequently filled with the organic solvent, which resulted in lipase immobilization on the inner side of fibres. The amount of immobilized lipase was determined as the difference between the protein content of the lipase solutions before and after the immobilization procedure [16]. Lipase content in the lipase solutions was estimated using the Lowry method with BSA as a standard at 550 nm [17].

Concentration of free fatty acids produced was measured in the emulsion system as well as in the membrane system. Concentrations of free fatty acids produced were measured in collected oil samples by titration with 0.1 M-KOH in ethanol, using phenolphthalein as the indicator. All data represent the averages of triplicate samples with the corresponding error of $\pm$ 5 %. The degree of hydrolysis was calculated from the acid value and the saponification value of the oil in the reaction mixture as described elsewhere [18].

Palm oil hydrolysis by immobilized lipase was investigated in the reactor system described above. Four oil concentrations in the range of 0.059—0.326 mol dm^{-3} based on the ester bond concentration were examined in kinetic experiments. The ester bond concentration was taken as having the value threefold higher than the triglyceride concentration, assuming that lipase from *Candida rugosa* could nonspecifically release 3 mol fatty acids per 1 mol of triglyceride. Since the optimum operating conditions were reported as the flow rate in the reactor lumen side of 16 cm^3 min^{-1} and the flow rate in the reactor shell side of 9 cm^3 min^{-1}, these conditions were employed in all hydrolysis experiments in this study. Aliquots of the oil phase (3 cm^3) were taken at time intervals and dissolved in 6 cm^3 of ethanol—diethyl ether mixture (φ_r = 1 : 1) for free fatty acids analysis. Titration of the aliquots of the aqueous phase with 0.1 M-KOH confirmed that the concentration of fatty acids in this phase was negligible. After each hydrolysis batch, the oil and the aqueous phases were withdrawn and substituted with equal volumes of fresh solutions.

In studies of stability of immobilized lipase, 20 successive batches of palm oil hydrolysis were performed in the same membrane reactor. Each batch lasted for 7—8 h after which the substrate was substituted with the fresh one.

The enzyme reaction in the microemulsion system was assayed as described previously [3]. 100 cm^3 flasks were filled with 12 cm^3 of 160 mmol dm^{-3} soya lecithin solution in isooctane with different concentrations of substrate ranging from 0.059 mol dm^{-3} to 0.326 mol dm^{-3} (based on the ester bond concentration). Hydrolysis experiments were initiated by adding 500 mm^3 lipase solution in 0.5 M-phosphate buffer of pH = 7 (containing 13 mg of free lipase) to the substrate reaction mixture, followed by vortexing until the solution became homogeneous. Reaction was carried out at 30 ℃ using a shaking water bath at 130 strokes per min.

RESULTS AND DISCUSSION

In the experimental reactor, the lipase from *Candida rugosa* was immobilized on hollow fibre membrane. Immobilization technique used in this work was based on noncovalent interactions of water-soluble lipase and hydrophilic hollow fibres. Although lipases do not exhibit high affinity for hydrophilic supports in aqueous solutions, nonpolar environment could improve ionic and other lipase-support interactions [19]. The amount of crude lipase immobilized onto the hollow fibres was (155 $\pm$ 5) mg m^{-2} membrane with approximately 40 mass % yield. Reproducibility of the immobilization method was verified in four independent immobilization experiments with new hollow fibre modules and the average standard deviation of immobilized lipase amount was 6.7 %.

For design and operation of enzyme reactor systems, proper rate equations and kinetic parameters

Table 1. Optimal Parameters of Kinetic Model for Microemulsion and Membrane System and Standard Deviation of Model Fitted Data

Initial concentration C_0/(mol dm^{-3})	Microemulsion system, eqn (*7*)			Membrane system, eqn (*12*)		
	X_e	k_1/h^{-1}	STD/%	X_e	k_1/h^{-1}	STD/%
0.059	0.836	0.648	2.655	0.990	0.339	11.019
0.090	0.741	0.761	0.902	0.910	0.293	8.696
0.163	0.585	0.650	0.601	0.703	0.240	10.639
0.326	0.389	0.508	2.269	0.518	0.260	15.822

are necessary. Kinetic model originally developed for oil hydrolysis in a free-enzyme microemulsion system was used to describe the oil hydrolysis in a membrane reactor system.

Under previously determined optimal flow conditions, the kinetics of palm oil hydrolysis in the membrane reactor was investigated and compared with that of the free enzyme in the microemulsion system. The amount of free lipase corresponded to the amount of the immobilized lipase. Figs. 2 and 3 show a reaction kinetic profile at different initial substrate concentrations (0.059—0.326 mol dm^{-3}) in microemulsion and membrane reactor systems, respectively. Results show that the degree of hydrolysis generally decreased as the initial substrate concentration was increased. The highest degree of hydrolysis of approximately 99 % was achieved for the lowest initial substrate concentration in the membrane reactor. Nevertheless, the time necessary to achieve the chemical equilibrium was generally longer for the immobilized lipase compared with that of the free enzyme. It appears that the enzyme hydrolysis of palm oil in microemulsion system was fast at the initial stage and then slowed down. On the other hand, in the membrane reactor system, the continual removal of glycerol from the membrane by the aqueous phase reduced the back reaction rate and allowed for higher conversion.

Figs. 2 and 3 show the averaged experimental data for microemulsion and membrane systems, respectively. Solid lines represent the best fit of experimental data by the proposed model for different substrate concentrations. Table 1 lists the kinetic parameters obtained from both microemulsion and membrane system experiments. It appears that the reaction rate with immobilized lipase was lower than that observed for free lipase. However, the maximum degree of hydrolysis estimated at four chosen substrate concentrations was always higher compared to the conversion obtained in the presence of free lipase. Goodness of fit for the palm oil hydrolysis in the membrane reactor is shown in Fig. 3. It was shown that the model somewhat overestimated the reaction rate at the beginning of hydrolysis in the membrane reactor (average standard deviation was 11.54 %). Based on the average values of the rate constants at 30 °C (0.64 h^{-1} and 0.28 h^{-1} for the free and the immobilized enzyme, respectively) it was determined that the immobilized

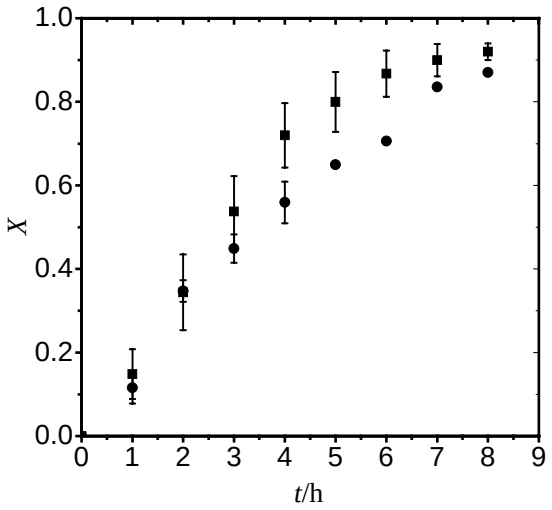

Fig. 4. Palm oil hydrolysis by immobilized lipase in a membrane reactor. Comparison of the first four (■) and the last three (●) experiments of 20 successive runs.

enzyme retained around 44 % of the free enzyme activity.

Main objective of this study was to develop an immobilized membrane system capable of operating for extended period of time with minimum loss of enzyme activity. Stability of the immobilized lipase was assessed in 20 successive batches of palm oil hydrolysis in the same membrane system. Results of the first four and the last three experiments performed in this experimental series at the same experimental conditions are presented in Fig. 4. Observed specific rates in the first four hydrolysis runs were only slightly higher than those obtained after 20 cycles (137 h) despite the considerable loss of the immobilized enzyme [16, 20]. The overall decrease in activity of the immobilized lipase was approximately 15 %.

Factors that contribute to poor reactor performance include enzyme desorption and enzyme inactivation. In this study, the amount of the immobilized lipase ranged from (154.8 ± 5.2) to (62.9 ± 2) mg m^{-2} due to enzyme desorption [16, 20]. Observed loss of enzyme activity due to desorption and enzyme inactivation had only a small effect on the reactor stability and the oil hydrolysis rate (Fig. 4). These results suggest that lipase was initially immobilized in multiple

layers such that the large portion of enzyme was not accessible to the substrate. This portion was also easily desorbed. These results agree well with findings of *Van der Padt et al.* [12] who found that the optimum amount of immobilized lipase was about 75 mg of crude lipase per m^2, the value only two- or threefold higher than the calculated value for the monolayer of pure lipase. In this study, approximately (62.9 ± 2) mg m^{-2} of immobilized active lipase stable for 137 h of operation was prepared.

SYMBOLS

C	substrate concentration	mol dm^{-3}
C_1	substrate concentration at the membrane reactor inlet	mol dm^{-3}
C_2	substrate concentration at the membrane reactor outlet	mol dm^{-3}
C_e	equilibrium substrate concentration	mol dm^{-3}
C_0	initial substrate concentration	mol dm^{-3}
C_p	product concentration	mol dm^{-3}
k_1	rate constant for ester bond decomposition	h^{-1}
k_2	rate constant for ester bond formation	h^{-1}
M	dimensionless parameter ($M = e^{\frac{k_1 t(X_e-2)}{X_e}}$)	
r	reaction rate	mol dm^{-3} h^{-1}
t	reaction time	h
V_c	contactor volume	cm^3
V_r	reservoir volume	cm^3
X	degree of hydrolysis ($X = (C_0 - C)/C_0$)	
X_e	equilibrium degree of hydrolysis ($X_e = (C_0 - C_e)/C_0$)	

Greek Letters

τ	mean reactor residence time	h
υ	oil phase volumetric flow rate	cm^3 min^{-1}

REFERENCES

1. Pandey, A., Benjamin, S., Soccol, C. R., Nigam, P., Krieger, N., and Soccol, V. T., *Biotechnol. Appl. Biochem. 29*, 119 (1999).
2. Jeager, K. E. and Eggert, T., *Curr. Opin. Biotechnol. 13*, 390 (2002).
3. Knezevic, Z., Siler-Marinkovic, S., and Mojovic, L., *Appl. Microbiol. Biotechnol. 49*, 267 (1998).
4. Knezevic, Z., Mojovic, L., and Adnadjevic, B., *Enzyme Microb. Technol. 22*, 275 (1998).
5. Han, D. and Rhee, J. S., *Biotechnol. Bioeng. 28*, 1250 (1987).
6. Giorno, L. and Drioli, E., *TIBTECH 18*, 339 (2000).
7. Hoq, M. M., Koike, M., Yamane, T., and Shimizu, S., *Agric. Biol. Chem. 49*, 3171 (1985).
8. Malcata, F. X., Hill, C. G., Jr., and Amundson, C. H., *Biotechnol. Bioeng. 39*, 984 (1992).
9. Malcata, F. X., Hill, C. G., Jr., and Amundson, C. H., *Biotechnol. Bioeng. 38*, 853 (1991).
10. Mercon, F., Erbes, V. L., Sant'Anna, G. L., Jr., and Nobrega, R., *Braz. J. Chem. Eng. 14*, 1 (1997).
11. Pronk, W., Kerkhof, P. J. A. M., van Helden, C., and van't Riet, K., *Biotechnol. Bioeng. 32*, 512 (1988).
12. Van der Padt, A., Edema, M. J., Sewalt, J. J. W., and van't Riet, K., *J. Am. Oil Chem. Soc. 67*, 347 (1990).
13. Guit, R. P. M., Kloosterman, M., Meindersma, G. W., Mayer, M., and Meijer, E. M., *Biotechnol. Bioeng. 38*, 727 (1991).
14. Giorno, L., Molinari, R., Natoli, M., and Drioli, E., *J. Membr. Sci. 125*, 177 (1997).
15. Bouwer, S. T., Cuperus, F. P., and Derksen, J. T. P., *Enzyme Microb. Technol. 21*, 291 (1997).
16. Knezevic, Z., Kukic, G., Vukovic, M., Bugarski, B., and Obradovic, B., *Process Biochem. 39*, 1377 (2004).
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem. 193*, 265 (1951).
18. Mojovic, L., Knezevic, Z., Popadic, R., and Jovanovic, S., *Appl. Microbiol. Biotechnol. 50*, 676 (1998).
19. Andrade, J. D. and Hlady, V., *Adv. Polym. Sci. 79*, 1 (1986).
20. Knezevic, Z., Vukovic, M., Bugarski, B., and Obradovic, B., in *Proceedings of the 1st International Congress on Bioreactor Technology in Cell, Tissue Culture and Biomedical Applications*, p. 95. Tampere, Finland, 2003.