Enzyme Hydrolysis of Babassu Oil in a Membrane Bioreactor

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ABSTRACT: This work deals with the enzymatic hydrolysis of babassu oil by immobilized lipase in a membrane bioreactor using unmixed aqueous and lipid streams. The experimental work was carried out in a flat plate membrane module with two different membranes: hydrophobic (nylon) and hydrophilic [mixed cellulose esters (MCE)], with different nominal pore sizes ranging from 0.10 to 0.65 μm. *Candida cylindracea* lipase was adsorbed on the membrane surface area, and the reactor was operated in batch mode. The initial enzymatic rate increased from 80 to 150 μmol H^+/min when the organic phase velocity increased from 1.0×10^{-3} to 3.0×10^{-3} m/s, indicating that mass transfer in that phase was the process-limiting step. Calcium ions had a marked effect on immobilized lipase activity, increasing around twofold the lipolytic activity. Long-term experimental runs showed that the immobilized lipase remained stable for at least 8 d. The values for immobilized protein and maximal productivities observed for 0.45 μm membranes were: 1.01 g/m^2 and 193 μmol $H^+/m^2 \cdot s$ for MCE membrane and 0.78 g/m^2 and 220 μmol $H^+/m^2 \cdot s$ for nylon membrane. The productivities obtained are among the highest values reported in the technical literature.

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KEY WORDS: Babassu oil, immobilized lipase, membrane bioreactor, oil hydrolysis.

Membrane processes correspond to a group of technologies that are replacing conventional and well-established steps in the chemical industry. The growing use of membrane technology is basically due to its simplicity and lower energy cost. Biotechnology is an area where membrane technology shows very efficient applications, such as protein separation, liquid clarification, product concentration, cold sterilization and organic liquid dehydration. Membrane bioreactors can combine reaction and product or cell separation in the same equipment. This characteristic is particularly suitable for enzymatic oil hydrolysis, a process that deals with two immiscible phases. Fatty acid production is mainly carried out using a conventional process (Colgate-Emery), which operates under temperature and pressure conditions that cause secondary reactions, demanding further purification steps (1). Enzymatic technology may be regarded as an alternative process, showing the following advantages: (i) the operation is conducted under mild temperature and pressure conditions and (ii) enzyme specificity reduces secondary reactions. Lipases (EC 3.1.1.3.) promote the hydrolysis of oils and fats, acting in the water/lipid interface. Thus, the surface area available for reaction is a key process efficiency factor.

The first reported work on an immobilized lipase membrane bioreactor was presented by Hoq *et al.* (2). These authors used a flat plate membrane reactor with a hydrophobic nylon membrane, on the surface of which lipase was immobilized by adsorption. According to these authors, the main advantages of their system were that it did not require emulsion formation or organic solvent, the enzyme immobilization was simple, and the product could be obtained in a single phase.

To improve the membrane area/reactor volume ratio, Pronk *et al.* (3) used a hollow-fiber membrane reactor with a hydrophilic membrane of cellulose acetate. As the hydraulic pressure in the oil phase was enough to maintain phase separation, an accurate pressure control was not necessary. This fact is an advantage with respect to hydrophobic membrane reactors.

Cuperus *et al.* (4) studied lipase stability in a hollow-fiber membrane bioreactor. These authors stressed that low enzyme stability adversely affected the cost of industrial scale bioprocesses. They stated in their work that the use of a hydrophilic membrane prolongs enzyme stability. Membrane hydrophilicity probably affects the water activity in the vicinity of the enzyme's active sites.

Use of the calcium ion as a lipase cofactor usually improves free lipase stability and activity (5–7). However, the influence of this ion as a cofactor in immobilized enzyme membrane bioreactors has not yet been reported.

The aim of the present work was to evaluate the stability of babassu oil hydrolysis by immobilized lipase in a membrane bioreactor. (The oil from the nuts of the babassu palm is rich in C_{12}, C_{14}, C_{16}, and C_{18}.) The following aspects were investigated: the influence of membrane characteristics and mass transfer in the bioreactor on immobilized enzyme activity, the use of calcium as an immobilized enzyme cofactor, and the reuse of the immobilized biocatalyst.

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EXPERIMENTAL PROCEDURES

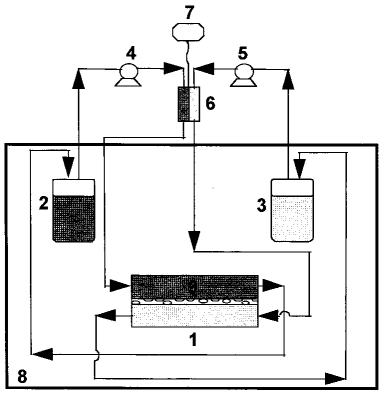
Materials. Babassu oil (refined) was purchased from Dureino (Teresina, Brazil). Meito Sangyo (Aichi, Japan) kindly provided the microbial lipase from *Candida cylindracea*, which is commercially available under the trade name OF. The microfiltration membranes were supplied by Osmonics Inc. (Minnetonka, MN): hydrophobic nylon membranes, with nominal pore sizes of 0.22, 0.45 and 0.65 μm, and hydrophilic mixed cellulose ester (MCE) membranes, with nominal pore sizes of 0.10, 0.22, 0.45, and 0.65 μm.

Membrane bioreactor. The reactor used was a flat plate module composed of two symmetrical rectangular plates (15 × 8 cm) and the membrane, forming two 12-mL vol compartments with a membrane area of 120 cm^2.

Membrane characterization. Pore size distribution and gaseous permeability were determined in a porometer manufactured by Coulter Electronics Limited (Luton, United Kingdom), the Coulter Porometer II model. The results of pore size distribution were used to calculate the membrane's surface porosity.

Lipase immobilization. A protein solution was microfiltered and recirculated through the membrane, allowing enzyme adsorption on the membrane surface. The protein solution (50 mL) was composed of 1 g/L of a commercial lipase preparation dissolved in a buffer solution (citric acid 0.1 mol/L and sodium phosphate dibasic 0.2 mol/L, pH 7.0). The operation conditions for enzyme immobilization were: solution flow rate of 6.8 mL/min, temperature of 23°C, and recirculation time of 30 min.

Reaction system. The experimental setup is illustrated in Scheme 1. The organic and aqueous phases were kept in different reservoirs and recirculated through the reactor by peristaltic pumps. Identical volumes (60 mL) of both organic and aqueous phases were recirculated at fixed flow rates. The reactor's influent organic phase consisted of babassu oil, and the influent aqueous phase was a buffer solution (citric acid 0.1 mol/L and sodium phosphate dibasic 0.2 mol/L, pH 7.0). The reactor and the reservoirs were kept at a constant temperature (40°C). The pressure difference between the liquid phases, at the reactor inlet, was adjusted and controlled to avoid mixing.



SCHEME 1

Analytical methods. The quantity of immobilized protein was obtained from the difference between protein contents measured before and after the enzyme immobilization procedure. Protein was determined according to the classical method by Lowry *et al.* (8). Owing to their solubility, the produced fatty acids remained in the organic phase and were determined by titration with a solution of 0.01 mol/L NaOH up to the end point value of pH 9.6. Lipolytic activity was determined from the initial slope of the reaction progress curve and was expressed in terms of International Units (I.U.), where one I.U. is defined as the amount of enzyme that releases one μmol acid/min under standard conditions. The glycerol produced remained in the aqueous phase, and its concentration was determined by gas chromatography, using Chrompack CP 9000A equipment (Middelburg, The Netherlands), with a WCOT fused-silica capillary column (internal diameter of 0.53 mm and length of 25 m), a flame-ionization detector, and H_2 as the carrier gas at a flow rate of 240 mL/min. Column temperature program: initial temperature of 80°C for 1 min; increase temperature at a rate of 10°C/min; final temperature of 240°C for 5 min.

RESULTS AND DISCUSSION

Membrane characterization. Table 1 presents the results of membrane characterization. Only the 0.45-μm nylon membrane presented a measured pore size diameter equal to the nominal pore size. As observed, relative gaseous permeability increased with the nominal membrane pore size and decreased with membrane porosity for both materials. The larger pores present in low-porosity membranes were shown to be more efficient for gas permeation than the smaller pores in high-porosity membranes.

Immobilization results. The amount of protein immobilized on the membrane surface area was determined for all tested membranes. No significant differences were observed, and the results were confined to a narrow range, between 0.78 and 1.02 g/m^2 for membranes of different materials and characteristics.

Mass transfer in the membrane bioreactor. To study the effect of mass transfer on immobilized enzyme activity, we performed experiments under different flow conditions, varying the aqueous and organic phase velocities. In a first series of experiments the aqueous phase velocity was kept constant (5.44×10^{-3} m/s), and the organic phase velocity was varied. Then, the aqueous phase velocity was varied while the or-

TABLE 1
Morphological Parameters of the Tested Membranes[a]

Nominal pore size (μm)	MCE membranes			Nylon membranes		
	d (μm)	ε (%)	*P*	*d* (μm)	ε (%)	*P*
0.10	0.19	11.34	0.26	—	—	—
0.22	0.32	4.25	0.47	0.33	3.22	0.39
0.45	0.57	1.92	1.01	0.44	1.90	1.16
0.65	0.93	0.80	1.83	1.25	0.39	1.67

[a]Abbreviations: *d*, measured pore size diameter; ε, porosity; *P*, relative gaseous permeability ($cm^3/cm^2 \cdot s \cdot cm$ Hg); MCE, mixed cellulose esters.

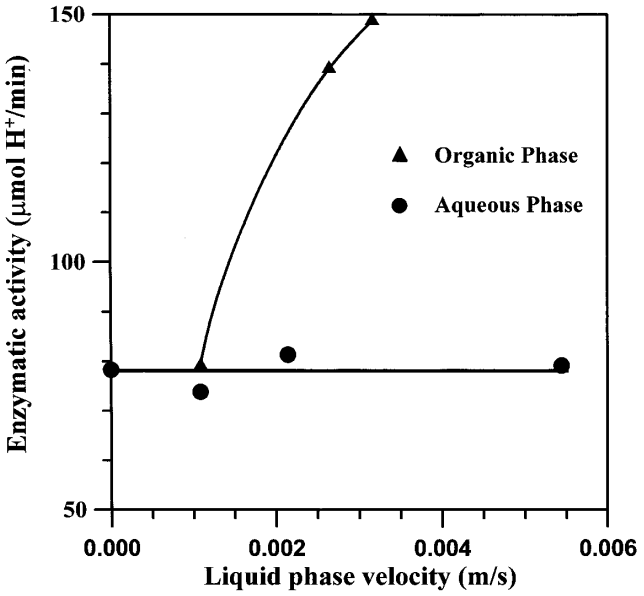


FIG. 1. Enzymatic activity as a function of the liquid phase velocities. (▲) Organic phase velocity variation, keeping aqueous phase velocity constant at 5.44×10^{-3} m/s; (●) aqueous phase velocity variation keeping organic phase velocity constant at 1.08×10^{-3} m/s.

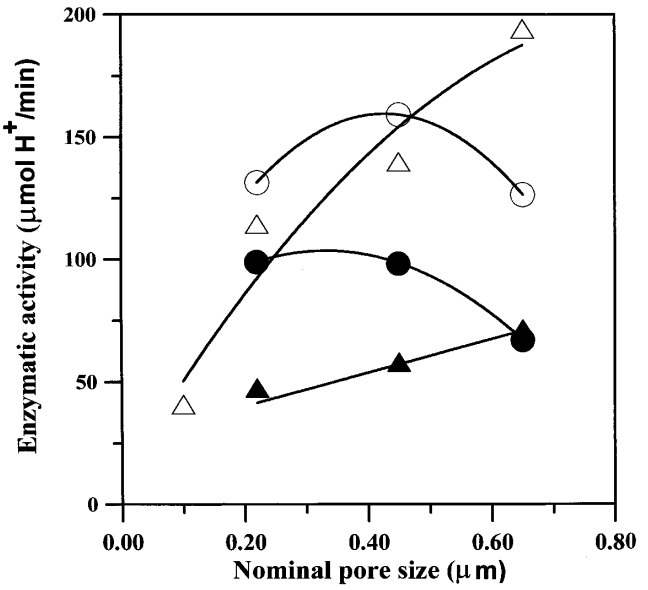


FIG. 2. Activity of the immobilized enzyme in the membrane [△,▲ mixed cellulose esters (MCE), and ○,● nylon] as a function of the nominal pore sizes for two different operation modes: △,○ organic phase in contact with the enzyme adsorbed membrane side; ▲,● aqueous phase in contact with the enzyme adsorbed membrane side.

ganic phase velocity was kept constant (1.08×10^{-3} m/s). The MCE membrane with a nominal pore size of 0.45 μm was used in all experiments.

Figure 1 summarizes these experimental results. The organic phase velocity strongly affected the immobilized enzyme activity, indicating that an improvement in hydrodynamic conditions brings about an appreciable increase in immobilized enzyme activity. This behavior is probable due to enzyme activity inhibition by fatty acids inside the membrane pores. As the organic phase velocity increases, the diffusion of fatty acids from the pores to the bulk organic phase occurs more rapidly because convection removes fatty acids from the bulk phase, promoting a greater concentration driving force for diffusion. Unfortunately, owing to experimental limitations, it was not possible to reach operational speeds high enough for the mass transfer in the organic phase to cease to be a limiting step to the process.

A similar series of events would occur for the removal of glycerol when the aqueous phase velocity increases. In this case no significant effect on the enzymatic reaction rate was observed, as shown in Figure 1. This behavior is a strong evidence that the presence of glycerol does not affect the enzymatic activity.

Lipase inhibition by glycerol is a controversial matter. Hoq *et al.* (2), when studying enzymatic oil hydrolysis in a flat plate membrane bioreactor, verified that glycerol concentrations up to 23% (w/w) in the aqueous phase improved enzyme activity. On the other hand, Holmberg *et al.* (9) observed a reduction in the hydrolysis rate with raised glycerol concentrations. In a previous study (10), when bioreactor hydrodynamics was the focus, it was observed that glycerol accumulation inside the pores did not affect the immobilized enzyme activity.

Hydrolysis results for MCE and nylon membranes. Figure 2 illustrates the variation of enzymatic activity with the nominal membrane pore size for MCE (hydrophilic) and nylon (hydrophobic) membranes for two modes of reactor operation. One operated with the organic phase in contact with the side of the membrane that contained the immobilized enzyme, while the other operated with the aqueous phase in contact with the that side. During these experiments the aqueous and organic phases velocities were 5.44×10^{-3} and 2.64×10^{-3} m/s, respectively.

For the MCE membrane, the activity of the immobilized enzyme was lower for 0.1- or 0.22-μm nominal pore size membranes. This was an unexpected result because the reduction in membrane porosity (associated with a rise in nominal pore size) led to a lower available interfacial area for the reaction. However, this behavior could be explained by the difficulty of penetration of the liquid phases into the small pores, hindering the interface formation. Thus, as the pore size is increased, the penetration of the liquid phases into its interior is facilitated and, consequently, the reaction seems to be favored in membranes that have higher pores sizes.

Another effect that promoted the increase of the enzymatic activity with the nominal membrane pore size for MCE membranes could be related to its hydrophilicity. As pore size increases, there is a higher penetration of the aqueous phase into the pore, pushing the lipid/aqueous interface toward the lipid stream side. This effect also promotes an increase in fatty acid concentration driving force for diffusion from pore to bulk organic phase, preventing its accumulation inside the pores and, consequently, enzyme inhibition.

The results obtained with nylon membranes (Fig. 2) showed maximal activity values for both reactor operation modes when the membrane nominal pore diameter was 0.45 µm. This behavior may be the result of two different and opposite effects. First, as observed for MCE membranes, enzyme activity tends to increase because penetration of the liquid phases is favored in large-pore membranes. On the other hand, as a result of the hydrophobic characteristics of nylon membranes, the organic phase penetrates easily into the membrane pores. This effect increases the distance between the interface and the bulk organic phase, decreases the concentration driving force for fatty acid diffusion, and increases its concentration in the vicinity of the oil/water interface. The accumulation of fatty acids at the interface contributes to enzyme inhibitions. A balance between these effects seems to be favorable for the 0.45-µm nominal pore size membrane, which showed the best performance.

The results shown in Figure 2 also indicate that, for a given nominal pore size, the immobilized enzyme activity is higher when the organic phase is in contact with the side of the membrane on which the enzyme is immobilized. A similar result was obtained by Rucka *et al.* (11), who studied the influence of membrane characteristics on immobilized lipase activity. However, no relationship was determined between the membrane hydrophobicity and enzymatic activity and, unfortunately, no explanation for such behavior was found in the literature.

Effect of calcium ion on immobilized enzymatic activity. The influence of the Ca^{2+} ion on immobilized enzyme activity was investigated in four different experiments: (i) a concentration of 0.02 mol/L of Ca^{2+} was maintained in the enzymatic solution utilized in the immobilization step; (ii) a concentration of 0.02 mol/L of Ca^{2+} was maintained in the circulating aqueous phase during the enzymatic reaction; (iii) a concentration of 0.02 mol/L of Ca^{2+} was used in both the immobilization procedure and reaction; (iv) Ca^{2+} was not used at all. The MCE membrane with a nominal pore size of 0.45 µm was used in all experiments. During these experiments the aqueous and organic phases velocities were 5.44×10^{-3} and 2.64×10^{-3} m/s, respectively.

The immobilization results showed that no significant differences in the amount of adsorbed protein were observed, revealing that the presence of calcium in the enzymatic solution (immobilization step) did not affect the amount of immobilized protein. This also indicates that any lipase molecular conformation change caused by calcium did not affect the enzyme-membrane-binding site.

The effect of calcium ion on immobilized enzyme activity is illustrated in Figure 3. The results show that Ca^{2+} has a positive effect on immobilized enzyme activity, mainly when it is added during both the immobilization and the reaction steps. The addition of Ca^{2+} during one of these steps had a slightly positive effect, indicating, as previously mentioned, that any change in protein conformation by Ca^{2+} binding to the lipase does not affect protein-membrane binding. Noble *et al.* (7) determined the three-dimensional structure of the active site of *Pseudomonas aeruginosa* lipase and identified

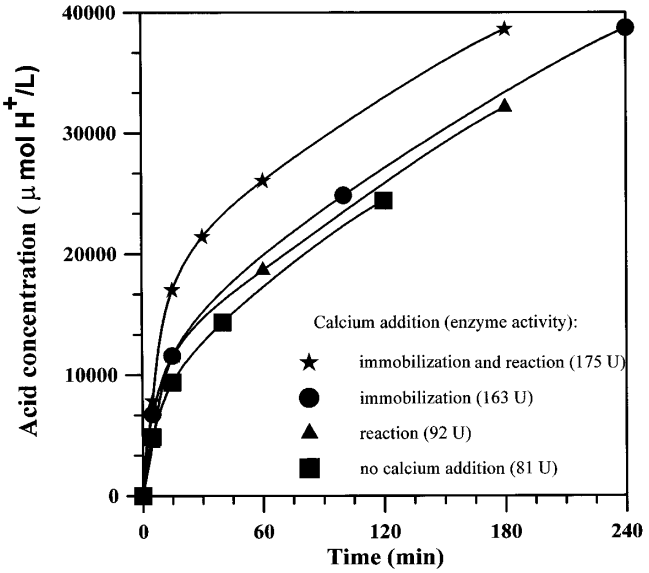


FIG. 3. Effect of calcium ion on immobilized enzymatic activity. MCE membrane, 0.45 µm nominal pore size. For abbreviation see Figure 2.

the binding site for Ca^{2+}, indicating that this ion has a major role on the stabilization of the enzyme's active site structure. However, the influence of this ion as a cofactor in immobilized enzyme membrane bioreactors containing *C. cylindracea* lipase has not yet been reported.

Stability of the immobilized biocatalyst. An important practical aspect for bioprocess viability is the stability of the immobilized biocatalyst. To evaluate this parameter, some experiments were performed. In the first set of experiments, the possibility of immobilized lipase reuse was investigated. The

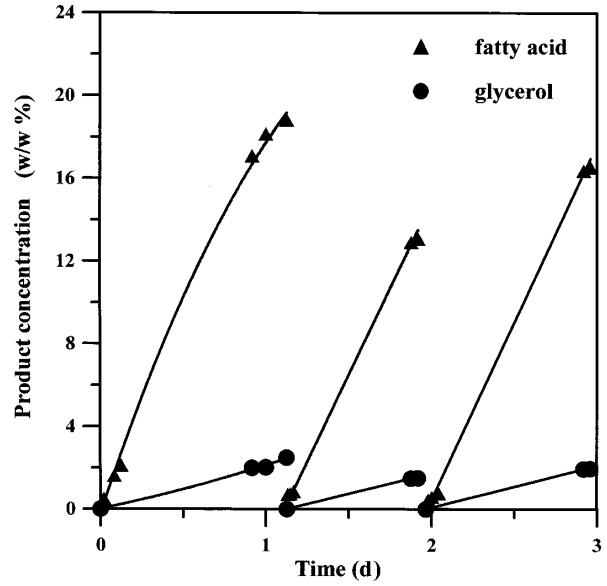


FIG. 4. Reaction progress curves for the three sequential experiments with the same immobilized enzyme membrane and replacement of both liquid phases each 24 h. (▲) Fatty acid concentration in organic phase (hydrolysis degree); (●) glycerol concentration in aqueous phase.

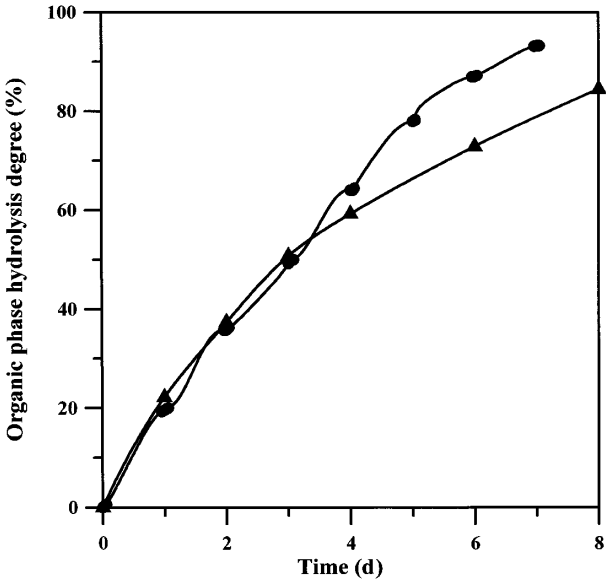


FIG. 5. Reaction progress curves in long-term experiments for two different bioreactor operating modes. (▲) Without membrane replacement during the experiments; (●) with membrane replacement each 24 h. The MCE membrane with nominal pore size of 0.45 μm was used in all experiments. For abbreviation see Figure 2.

MCE membrane with nominal pore size of 0.45 μm was used in all experiments. The experimental conditions were the same as those in the previous section (0.02 mol/L of Ca^{2+} used in the immobilization and reaction steps). After 24 h of reaction, both liquid phases were replaced by new amounts of the original phases. The results are shown in Figure 4. The slope of the curves, mainly the first one, indicated that the production rate of fatty acids decreased with time, probably owing to enzyme activity inhibition. The results in Figure 4 also reveal similar reaction progress curves for three sequential experiments, suggesting that the inhibition caused by fatty acid production is reversible. The similarity among these reaction progress curves also indicates that no lipase desorption occurs during reaction operation, confirming that enzyme adsorption is fairly stable. The absence of protein in both liquid phases during reaction procedures indicates a very low degree of enzyme desorption. Similar results were obtained in other studies with lipase immobilized in membrane reactors (2,10).

To evaluate lipase inhibition by glycerol, we performed a second set of experiments without aqueous phase substitution. Thus, the glycerol produced was not removed from the system, favoring the accumulation of this product. Results (data not shown) indicate that even with glycerol accumulation, the hydrolysis rate was practically identical to that obtained in the experiments conducted with aqueous phase replacement (Fig. 4). This result confirms that fatty acids are the main inhibitors of immobilized enzyme activity.

Long-term experiments were performed to evaluate the drop in immobilized lipase activity in high acid concentration environments. In the first experiment, there was no membrane replacement during the reaction, whereas the membrane was replaced every 24 h in the second experiment. The results shown in Figure 5 indicate that in the second experiment, higher hydrolysis rates were reached after 3 d of reactor operation. A smaller reduction in enzyme activity was observed (even in high acidic environments) when the membrane was periodically replaced.

Process evaluation. A survey of published data on oil and fat hydrolysis catalyzed by lipase in membrane bioreactors is shown in Table 2. Despite the diversity of enzymes, substrates, membranes and reactors, the maximal productivity values obtained in the present work for the 0.45 μm membranes lie well within the highest range of operation.

ACKNOWLEDGMENTS

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TABLE 2
Performance of Membrane Bioreactors for Enzymatic Hydrolysis of Fats and Oils[a]

Authors (ref.)	Substrate	Enzyme-producing microorganism	Reactor (membrane)	Amount of immobilized protein (g/m^2)	Maximal productivity (μmol $H^+/m^2 \cdot s$)
Hoq *et al.* (2)	Olive oil	*Candida cylindracea*	Flat plate (polypropylene)	0.14–3.4	20
Pronk *et al.* (3)	Soybean oil	*C. rugosa*	Hollow fiber (cellulose acetate)	0.65	12
Bower *et al.* (12)	Triolein	*Rhizopus javanicus*	Hollow fiber	—	23
Taylor *et al.* (13)	Tallow	*Thermomyces lanuginosus*	Flat plate (acrylic)	—	23
Rucka *et al.* (14)	Sunflower oil	*Rhizopus* sp.	Flat plate (polytetrafluoroethylene)	—	20
Guit *et al.* (15)	Triacetin	*C. cylindracea*	Hollow fiber (polyacrylonitrile)	1.0–1.5	12
Cuperus *et al.* (4)	Sunflower oil	*C. rugosa*	Hollow fiber (hydrophilic)	—	200
Merçon *et al.* (10)	Babassu oil	*Mucor miehei*	Hollow fiber (polyetherimide)	0.5–1.97	44
This work	Babassu oil	*C. cylindracea*	Flat plate (MCE)	1.01	193
This work	Babassu oil	*C. cylindracea*	Flat plate (nylon)	0.78	220

[a]For abbreviation see Table 1.

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