A Hybrid Membrane-Emulsion Reactor for the Enzymatic Hydrolysis of Lipids

Wouter Pronk[1], **Machteld van der Burgt, Gerard Boswinkel** and **Klaas van 't Riet***
Department of Food Science, Food and Bioprocess Engineering Group, Wageningen Agricultural University, NL 6700 EV Wageningen, The Netherlands

A hybrid reactor, consisting of a stirred vessel, a hydrophilic membrane loop and a hydrophobic membrane loop, is presented for the continuous enzymatic hydrolysis of soybean oil in an emulsion. The permeates of the hydrophilic and the hydrophobic membrane consist of a single water phase and a single lipid phase, respectively. No lipase activity could be detected in the permeates of both membranes, which implies that all enzyme is retained in the system. An important advantage of this system is that it combines the high surface area in an emulsion with the containment of lipase in a membrane reactor. It is further shown that the stability of the system can be improved considerably by the addition of $CaCl_2$ to the water phase. Under comparable conditions the enzyme stability in the hybrid reactor is lower than the stability in a stirred vessel. The composition of the emulsion appears to influence the flux of the membranes. The flux of the hydrophobic membrane increases with an increasing oil fraction of the emulsion while the flux of the hydrophilic membrane has an optimum for two different oil fractions—0 and 0.55 (v/v).

KEY WORDS: Emulsion, enzyme, fatty acid production, hybrid membrane-emulsion reactor, hydrolysis, lipase, lipid, membrane, separation, soybean oil.

As part of the rapid development of biotechnology, enzymatic hydrolysis of lipids has been investigated intensively for the last decade. The enzymatic process has not replaced the conventional physical chemical process (Colgate-Emery), due to the low cost margins of the bulk process and the relatively low energy prices. However, application of enzymatic hydrolysis may be expected in the future for special applications or when the cost of energy rises.

In principle, the enzyme lipase can be used free or in an immobilized form. The reactants form two different phases, and the free enzyme adsorbs to the interface where it is active. Immobilization will cause mass transfer problems in one of the two phases unless immobilization is carried out on a surface that is permeable to just one of the phases, resulting in the enzyme becoming immobilized at the interface of the lipid and water phases. A membrane can provide this condition (1,2). In the case of a low-price lipase used in the hydrolysis process, the membrane cost will be a considerable part of the total cost (3), and thus an effective utilization of membrane area is essential. Instead of an immobilization matrix, a membrane can also be used as a separation medium. In that case, the use of a membrane prevents an enzyme present in free form in the emulsion reactor from being washed out. This principle was used by Tanigaki *et al.* (4). After a batch-hydrolysis, phase separation was carried out, and the enzyme was separated from the water phase by a polyacrylonitrile ultrafiltration membrane. The enzyme could be recovered completely. However, repeated-batch operation as applied by Tanigaki *et al.* (4) is labor-intensive. An advantage of an emulsion reactor is that the reactive area is not limited by the membrane area. The reactive area in an emulsion is the interfacial area, which can be influenced by the stirring rate (5). Thus, in principle, a high volumetric activity can be reached at high enzyme loads and with vigorous stirring. Yet the emulsion reactor is unattractive, because in the conventional process the enzyme cannot be recovered. A different emulsion reactor with enzyme recovery was published by Bühler and Wandrey (6). This method, based on centrifugal emulsion separation with re-use of the water phase, provided a recovery of 90% of the enzyme.

In this paper, a continuous reactor is presented in which the lipase is active in a free form in an emulsion. The water phase and the lipid phase are continuously removed from the system through a hydrophilic and a hydrophobic membrane, respectively. Thus, the so-called hybrid reactor is composed of three different sub-systems—a stirred emulsion vessel, a circuit containing a hydrophilic membrane, and a circuit containing a hydrophobic membrane. This system provides the opportunity to combine the high volumetric surface area of an emulsion with the enzyme retention of a membrane reactor. The objective of this study is to select appropriate membranes and to demonstrate that the system can be operated continuously, while providing pure lipid and water phases without enzyme-leakage.

METHODS AND MATERIALS

The fatty acid content of the lipid phase was determined by titration of a weighed lipid sample. The water content in the lipid permeate was determined by Karl Fisher titration. Lipase of *Candida rugosa* (type "OF") was purchased from Meito Sangyo (Japan).

Two different activity-assays were used. The quantitative assay was a pH-stat method with tributyrin as a substrate at pH 6 (1). A qualitative method to detect low activity levels was based on addition of a sample (10 mL) to an emulsion (10 mL water and 10 mL oil). The emulsion was stirred and incubated at 30°C for one week, during which several samples were taken and the degree of hydrolysis was determined. The activity decreases with substrate concentration as shown by Pronk *et al.* (1). The results are expressed as standard conversion rate (SCR, $mole.h^{-1}$), defined as the activity of the reactor at 100% triglyceride substrate (1). In contrast to the membrane-immobilized system, the conversion rate in the hybrid

[1] Present address: DSM Research, BO-MVR, NL 6160 MD Geleen, The Netherlands.

*To whom correspondence should be addressed at Wageningen Agricultural University, P.O. Box 8129, NL 6700 EV, Wageningen, The Netherlands.

reactor was not expressed relative to the membrane area, because the activity is not proportional to the membrane area.

The hydrophilic membrane was a cellulosic hollow fiber membrane, purchased from Organon Tecnika (Boxtel, The Netherlands) with a cut-off value of 5,000 (for blood proteins) and a total membrane area of 0.77 m^2. The fibers had a diameter of 0.2 mm and the thickness of the membrane was 8 μm. This type of membrane is currently used in artificial kidneys.

Four different hydrophobic membranes were used: i) Polysulfone hollow fiber membrane H1P10-43 (Amicon, Denver, CO) with a total membrane area of 0.08 m^2 and a cut-off value of 10,000; ii) PVDF flat-sheet ultrafiltration membrane (cut off, 10,000) from Rhone-Poulenc (Courbevoie, France), membrane area $6.4 \cdot 10^{-3}$ m^2; iii) Polypropylene (PP) flat-sheet microfiltration membrane from ENKA (Wuppertal, Germany) with an average pore size of 0.1 μm, and a membrane area of $6.4 \cdot 10^{-3}$ m^2; and iv) polypropylene (PP) hollow fiber microfiltration membrane from Organon Tecnika, with a pore size of 0.2 μm and a membrane area of 0.07 m^2. The flat sheet membranes were used in a Megaflow module (type TM-100) from New Brunswick Scientific (Edison, NJ).

The selection of the hydrophobic membrane was carried out by circulation of an emulsion without lipase [oil fraction 0.8 (v/v)] through the membrane module at a pressure head of 0.5 bar, so the mean pressure difference across the membrane was 0.25 bar. The occurrence of water in the permeate was judged visually. If the membrane seemed appropriate, a quantitative water analysis also was carried out.

The permeate flux of the hydrophilic and hydrophobic membranes as a function of the oil fraction and the lipase content was determined by weighing. The emulsion was recycled through the fibers at a pressure head of 0.4 and 0.8 bar for the PP and cellulosic membrane, respectively. So, the mean pressure difference across the membrane was 0.2 and 0.4 bar, respectively. The pumping rate in these experiments was minimally 6.5 $L.h^{-1}$.

The stirred vessel had a total volume of 1.9 L (diameter, 19 cm; height, 11.3 cm) and contained four baffles (1 × 7 cm). A two-bladed paddle-stirrer with holes as described by Dekker *et al.* (7) was used. The stirrer was designed to provide optimum mixing (convection) in the total volume of the vessel, at minimal stirring and shear rates. The vessel was filled to a total volume of 1.7 L. The stirring rate was 450 rpm, unless specified otherwise. There was no air entrainment in the vessel at this stirring rate.

The influence of the enzyme load was determined in an emulsion with an oil fraction of 0.5 (v/v), stirred at 450 rpm at a temperature of 30°C. The stability was determined in an emulsion with an oil fraction of 0.5 (v/v) to which 0.5 g of enzyme was added. The stirring speed was 450 rpm and the temperature was kept at 30°C.

Hybrid reactor experiments. A schematic presentation of the hybrid reactor system is shown in Figure 1. The hydrophilic membrane and the stirred vessel, with an effective volume 1.7 L, were described above. The hydrophobic membrane was the hollow fiber PP membrane, as described above. The temperature was maintained at 30°C by placing the system in a thermostatted case. By adjusting the circulation rate, the permeation rate of both membranes was maintained higher than the net flow rate of oil and water into and out of the stirred tank (30 $mL.h^{-1}$). The overflow vessels caused the surplus permeate to recycle into the stirred vessel. In order to prevent microbial growth, sodium azide [0.03% (w/w)] was added to the water phase. In the stability experiment, the amount of enzyme was 0.5 g. $CaCl_2$ was 10 mM when used.

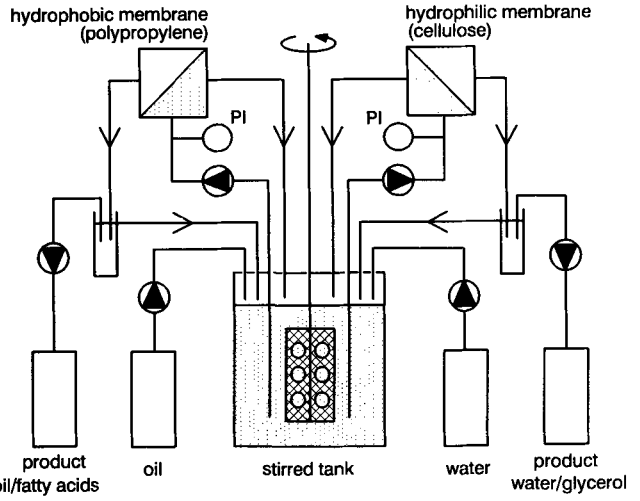

FIG. 1. Schematic presentation of the hybrid reactor. PI=pressure indicator.

RESULTS AND DISCUSSION

Selection of the hydrophobic membrane. Keurentjes *et al.* (8) have shown that adsorption can easily occur on a hydrophobic membrane surface. Due to this adsorption the membrane can become more hydrophilic, and the membrane can then eventually lose its selective permeability for the lipid phase. The polysulfone microfiltration membrane showed these properties. Although it had good separation properties initially, after one hour of operation water drops could be detected by eye in the permeate.

The PVDF and the flat sheet PP membrane could be operated during several days without visible water leakage. At an oil fraction of 0.8 (v/v), the initial flux of the PVDF membrane was 15 $L.m^{-2}.h^{-1}.bar^{-1}$, while the initial flux of the flat sheet PP membrane was 50 $L.m^{-2}.h^{-1}.bar^{-1}$, and of the hollow fiber PP membrane 90 $L.m^{-2}.h^{-1}.bar^{-1}$.The relative flux decrease of the three membranes in time was almost equal. Based on the observations above, the PP membrane was judged the most appropriate. A drawback of the flat sheet configuration of the PP membrane was that at higher pressures (around 1 bar) the membrane was leaking due to mechanical problems with the membrane in the module. The hollow fiber configuration appeared to be more pressure-resistant; the hollow fiber PP membrane could be operated up to pressures of 1.5 bar without damage or visual permeation of water and therefore was selected.

Characterization of the polypropylene membrane. The measurement of the flux of the hollow fiber PP membrane as a function of the oil fraction of the emulsion is shown in Figure 2. The flux decrease at lower oil fractions possibly can be explained from the fact that a smaller fraction of the dispersed oil comes into contact with the membrane.

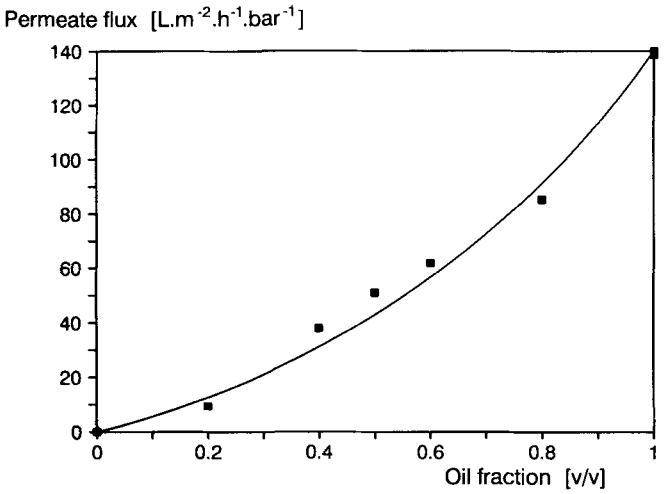

FIG. 2. Permeate flux of the polypropylene hollow fiber membrane as a function of the oil fraction (v/v) of an oil/water emulsion.

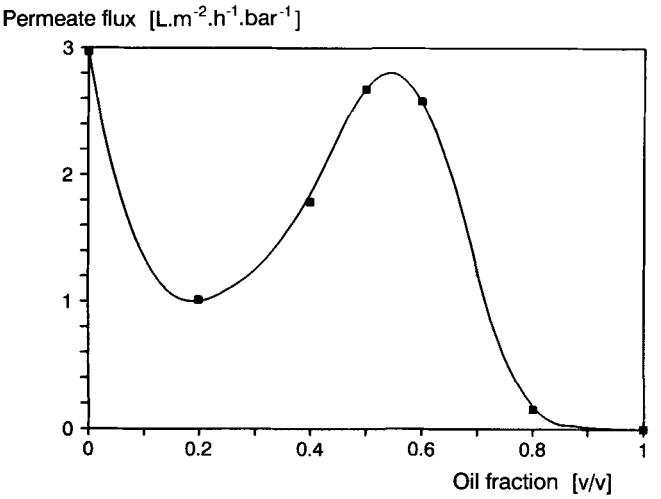

FIG. 3. Permeate flux of the cellulose hollow fiber membrane as a function of the oil fraction (v/v) of an oil/water emulsion.

At an oil fraction of 0.5 (v/v) the permeate contained 0.15% (w/w) of water (Karl Fisher titration). The solubility of water in a triglyceride is about 0.2% (w/w) (9). These results indicate that only the dissolved water is permeating.

The addition of lipase (0.05 $g.L^{-1}$) to an emulsion [oil fraction = 0.8 (v/v)] was tested with the flat sheet polypropylene membrane. It resulted in a substantial flux decrease; two hours after addition of the enzyme the flux had stabilized to a value of 5 $L.m^{-2}.h^{-1}.bar^{-1}$, while the flux without enzyme was 90 $L.m^{-2}.h^{-1}.bar^{-1}$. The water content in the permeate was higher than in the permeate of the emulsion without enzyme [0.7% instead of 0.15% (w/w)], and the permeate was opalescent. This implies that a small amount of emulsified water is permeating when lipase is present. This can be explained by the formation of fine emulsion drops which are able to penetrate the pores of the membrane, due to surface-active components present in the enzyme preparation. No membrane failure was observed during long-term experiments.

Characterization of the hydrophilic membrane. In previous experiments with a lipase-immobilized membrane reactor, emulsions containing lipid, water and lipase were ultrafiltered over a cellulosic membrane (Cuprophan™) for immobilization purposes (1). In these experiments the water phase appeared to permeate without leakage of lipase or lipid for pressures up to 1.5 bar (W. Pronk and K. van 't Riet, unpublished results). Therefore, this membrane type was expected to be appropriate for use in the hybrid reactor.

For the characterization of the cellulosic membrane, the permeate flux was recorded as a function of the oil fraction of an oil/water emulsion. The average values of experiments in triplicate are shown in Figure 3. The maximum deviation of the individual measurements from the values shown was 0.2 $L.m^{-2}.h^{-1}.bar^{-1}$. Therefore, the flux optimum at oil fractions of 0.5–0.6 (v/v) is definitely not within the range of experimental error. This flux optimum could possibly be related to the formation of a bicontinuous system. The occurrence of such a bicontinuous system could be tested by conductivity measurements.

Properties of lipase in the stirred vessel. The influence of the amount of enzyme in the stirred vessel on the conversion rate was examined in an emulsion with an oil fraction 0.5 (v/v). The curve has a similar pattern, as shown before for esterification (10). Loads of up to 0.5 g of crude enzyme gave a constant specific activity, 9 $mole.h^{-1}.g^{-1}$, and the total activity increased linearly with the load of enzyme. At higher loads (0.5–5 g) the specific activity decreased while the total activity increased. The total activity of the reactor levelled off to a maximum of 14.5 $mole.h^{-1}$ at enzyme loads above 5 g.

Figure 4 shows the inactivation of lipase in a stirred emulsion at the same temperature and stirring rate as in the hybrid reactor. The decrease of activity appeared to be first order; the inactivation constant was determined to be $4.7 \cdot 10^{-3}$ h^{-1}. The first-order inactivation constant for the same lipase immobilized in a membrane reactor was found to be $6.8 \cdot 10^{-4}$ h^{-1} (1), which implies that the immobilized lipase is about seven times more stable than the lipase in emulsion.

Hybrid reactor experiments. The conditions in the hybrid reactor experiments were based on the experiments

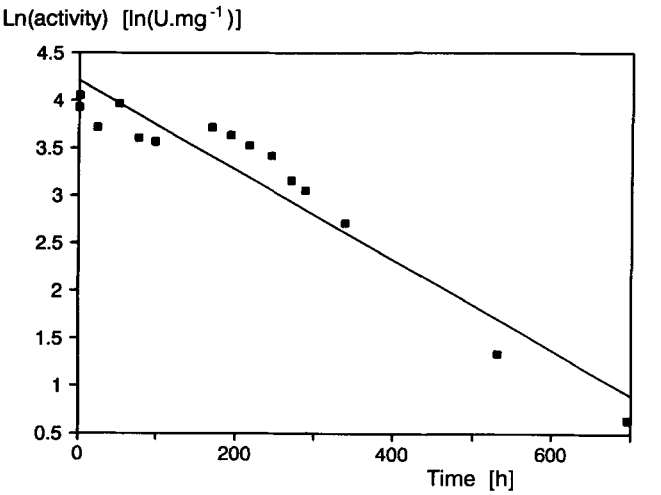

FIG. 4. Inactivation of lipase in an emulsion. The activity was determined in samples of the total emulsion by the tributyrin activity assay.

with the sub-systems. The optimum oil fraction was determined from the flux experiments. The flux of the hydrophobic membrane increased with increasing oil fraction, while the hydrophilic membrane showed two optima at oil fractions of 0 and at 0.5-0.6 (v/v). Furthermore, the volumetric interfacial area in the emulsion has an optimum at an oil fraction of 0.5 (v/v) (5). An oil fraction of 0.5 (v/v) therefore provided the optimum conditions for the process. From a viewpoint of effective enzyme use, a low enzyme load that would result in a high specific enzyme activity is preferable. For optimum reactor activity, however, a high enzyme load should be applied. A potential drawback of high loads is fouling of the membranes. Therefore, a relatively low enzyme load (0.5 g, the maximum value of constant specific activity) was chosen. The stirring speed (450 rpm) was just below the value where air became sucked in, in order to prevent severe denaturation at the air-water interface.

The system was operated in a continuous mode for a period of 18 days. The flux of the polypropylene membrane stabilized at a value of 0.56 $L.m^{-2}.h^{-1}.bar^{-1}$. The flux of the cellulose membrane decreased within several hours to a value of 0.065 $L.m^{-2}.h^{-1}.bar^{-1}$ and then, within several days, decreased further to about 0.025 $L.m^{-2}.h^{-1}.bar^{-1}$. This decrease in flux is probably caused by severe fouling of the membrane. The flux through the hydrophilic membrane was reversed by reversing flow direction of the pump every 48 hr. Then, after switching the pump in the original direction, the flux was restored to a value of 0.065 $L.m^{-2}.h^{-1}.bar^{-1}$.

Lipase activity was analyzed in the permeate of both membranes by using the qualitative assay. No lipase could be detected in either permeate during the whole period. This shows that the active enzyme is contained completely within the system.

Both membranes were tested for their separation capacity. The permeate of the cellulose membrane was a clear water solution, without any oil layer on top, which indicates the absence of any oil permeation. Permeation of lipid through the cellulose membrane is unlikely in view of the small pores present in combination with the high interfacial tension of oil and water. The water content of the permeate of the PP membrane varied between 0.5 and 1.5% (w/w). These results are in agreement with the value of 0.7% (w/w) found with the experiments of the PP membrane and the water/lipid/lipase emulsion described above.

The hydrolysis activity of the system, expressed as SCR, is shown in Figure 5 as a function of time for experiments in the presence and absence of $CaCl_2$ (10 mM). The addition of $CaCl_2$ has a positive effect on the stability. The inactivation constant in the presence of $CaCl_2$ is $4 \cdot 10^{-3}$ h^{-1}, and in the absence of $CaCl_2$, $1 \cdot 10^{-2}$ h^{-1}. A stabilizing effect of $CaCl_2$ was also observed for membrane-immobilized lipase. Inactivation constants of $6.0 \cdot 10^{-5}$ and $6.8 \cdot 10^{-4}$ h^{-1} were found in the presence and absence of $CaCl_2$, respectively [(1); also, W. Pronk, G. Boswinkel and K. van 't Riet, unpublished data].

During the experiment in presence of $CaCl_2$ at t=200 h the stirring rate was decreased from 450 rpm to 180 rpm. No influence on the fatty acid production rate or the stability could be detected. The fact that the stability is unaffected indicates that the stirrer shear forces play a minor role in the inactivation mechanism. This hypothesis is in agreement with a publication by Lee and Choo (11),

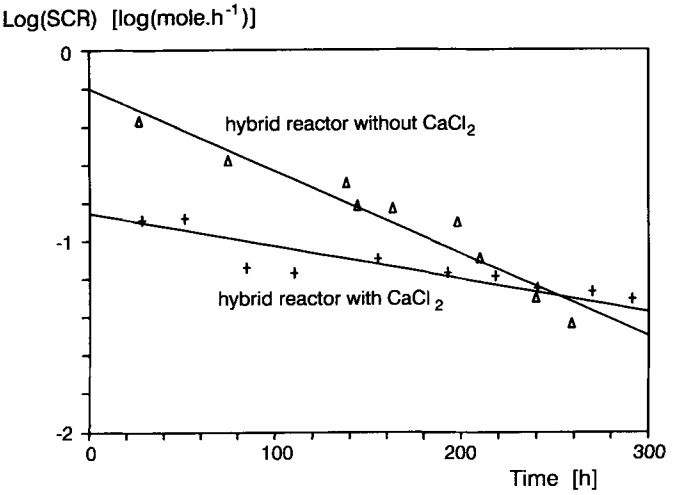

FIG. 5. Activity, expressed as SCR (standard conversion rate), as a function of time for lipase in the hybrid reactor in the presence and absence of $CaCl_2$ (10 mM). The activity was determined from the product of the net lipid flow and the fatty acid fraction in the outgoing flow. All experiments were carried out at 30°C with 0.5 gram of lipase.

who showed that inactivation in a stirred vessel is caused mainly by inactivation at the air-water interface. The inactivation constants (without $CaCl_2$) in the hybrid reactor and in the stirred vessel were $1 \cdot 10^{-2}$ h^{-1} and $4.7 \cdot 10^{-3}$ h^{-1}, respectively, which means that the stability in the stirred vessel is a factor two higher than in the hybrid reactor. The main difference between the two systems was that the emulsion was pumped through the membrane units in the hybrid system. If this were the reason for the greater inactivation, shear forces possibly might play a role in the inactivation mechanism in a membrane unit. An alternate explanation is that the loss of activity was due to the formation of a fouling layer on the membrane.

The SCR at t=0 was 0.64 $mole.h^{-1}$ in the absence of $CaCl_2$. This corresponded with 0.76 $mole.m^{-2}.h^{-1}$ because the total membrane area was 0.84 m^2. In the cellulose membrane reactor with immobilized lipase the activity after stabilization was 0.03 $mole.m^{-2}.h^{-1}$ (1). Therefore, we conclude that the objective of more effective membrane use in comparison to the hydrophilic membrane reactor has been attained in the hybrid reactor. Since the membrane area was not limiting in the experiments shown, the amount of enzyme relative to the membrane area could be increased, resulting in a higher activity relative to the membrane area.

The stability of the system is considerably less than in the cellulose membrane reactor with immobilized lipase. The addition of $CaCl_2$ to the water phase can only partially overcome this difference and the membrane-immobilized lipase is stabilized by this addition as well. The phenomena of fouling and inactivation are now under investigation in separate experiments.

Operation of a continuous hybrid hydrolysis reactor is feasible. The surface-related activity is an order of magnitude higher in the hybrid system than in the membrane reactor, and complete phase separation and enzyme retention have been attained. However, this does not imply that the hybrid system always will be preferred to a

system with a membrane-immobilized lipase for commercial application. Advantages of the membrane reactor are its higher stability and the lesser complexity of the system. The ultimate choice between the two systems will depend on a detailed economical evaluation of both systems, to be carried out after optimization of both systems.

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