# THE ENZYMATIC HYDROLYSIS OF LIPIDS IN A HYDROPHILIC MEMBRANE BIOREACTOR

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# THE ENZYMATIC HYDROLYSIS OF LIPIDS IN A HYDROPHILIC MEMBRANE BIOREACTOR

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**Proefschrift**

ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
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des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

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# STELLINGEN

1 De enzymatische hydrolyse van triglyceriden door *Candida rugosa* lipase is te beschrijven als 3 reactiestappen, elk met een tweede orde kinetiek in de olie- respectievelijk de water-opgeloste reactiecomponenten. De overall reactie heeft een schijnbare orde van 1,75 in de totale ester molfractie (*dit proefschrift*).

2 De sterke affiniteit van wateropgeloste lipases voor grensvlakken geldt niet alleen voor hydrofobe grensvlakken, hetgeen in overeenstemming is met adsorptie aan het lipide-substraat, maar ook voor bepaalde typen hydrofiele grensvlakken (*dit proefschrift*).

3 Een membraanreactor verdient de voorkeur boven een emulsie-systeem voor omzettingen met een hoge toegevoegde waarde met behulp van kostbare lipases.

4 Door de waterfractie in een emulsie te beschouwen als de substraatconcentratie voor de hydrolyse van triglyceriden, wordt voorbijgegaan aan het effect van glycerol op de reaktiekinetiek en het evenwicht (Han, D., J.S. Rhee, S.B. Lee (1987) *Biotechnol. Bioeng.* 30, 381-388).

5 Incubatie assays zijn niet geschikt voor de bepaling van de invloed van de temperatuur op de initiële omzettingssnelheid (Khor, H.T., N.H. Tan, C.L. Chua (1989) *J. Am. Oil Chem. Soc.* 63, 538-540).

6 Het is onjuist om de oliefractie in een emulsie te beschouwen als analoog voor de substraatconcentratie in het Michaelis Menten kinetiek model.

7 De sensorische analyse van boterzuur, gehydrolyseerd uit tributyrine, is een onaangename doch zeer gevoelige kwalitatieve bepaling van de aanwezigheid van lipases.

8 De op zich terechte constatering dat er voor het doorvoeren van milieumaatregelen grote investeringen nodig zijn houdt niet in dat een milieuverantwoorde economie gebaseerd moet zijn op economische groei (*Brundtland rapport: "Our common Future"*).

9 Selectie van onderzoeksonderwerpen op basis van subjectieve argumenten, zoals de geurwaarneming van visolie, dient zo veel mogelijk vermeden te worden.

10 Uit het feit dat de ligfiets geen, en de fietscomputer wèl wijdverbreide toepassing vindt, blijkt dat bij het doorvoeren van vernieuwingen de verhoging van efficiency vaak van ondergeschikt belang wordt geacht.

Stellingen behorende bij het proefschrift:
"The enzymatic hydrolysis of lipids in a hydrophilic membrane reactor"
W. Pronk
Wageningen
13 maart 1991

*Voor Marielle*

# VOORWOORD

In 1984 werd, onder begeleiding van Piet Kerkhof, een aanvang gemaakt met het in dit proefschrift beschreven onderzoek. Nadat ik in een doctoraalvak aan het onderzoek had gewerkt, kreeg ik de gelegenheid om een promotieonderzoek aan hetzelfde onderwerp uit te voeren. Dit leek me een goede gelegenheid om meer ervaring op te doen op het gebied van biotechnologisch onderzoek. Op dat moment was ik de enige persoon die aan olie / membraanreaktoren werkte en ook al was ik met die vette spullen op het lab niet erg geliefd, toch expandeerde de olie / membraan groep zich sterk. De vergelijking met een olievlek lijkt hier op zijn plaats. Na ongeveer een jaar begonnen Albert van der Padt en Jos Keurentjes een promotie-onderzoek, respectievelijk naar de ester-synthese in een lipase-membraanreaktor en naar de scheiding van vetten en vetzuren met behulp van membranen. Het overleg binnen deze groep heb ik altijd als stimulerend en motiverend ervaren. Nog een jaar later startte Gerard Boswinkel een onderzoek naar specifieke toepassingen van de hydrolyse in een membraanreaktor. Doordat dit onderwerp sterk gerelateerd was aan het hydrolyse onderzoek ontstond er een samenwerking die niet alleen nuttig was, maar ook buitengewoon prettig. Binnen het onderzoek van specifieke toepassingen startte Anja Janssen een onderzoek naar de synthese van suikeresters. Enige tijd later begon Marjan Geluk met een onderzoek naar enkele fundamentele en praktische aspecten van de olie / membraan reaktoren in samenwerking met DSM. Het kringetje werd gesloten toen bleek dat ik na het promotieonderzoek bij laatstgenoemde firma een aanstelling kon krijgen als research-medewerker.

De coördinerende en sturende kracht achter dit alles was Klaas van 't Riet. Voor de begeleiding bij het hydrolyse onderzoek wil ik hem hierbij graag bedanken. Op het gebied van oliën en vetten is de kennis en inbreng van de heren Arisse en van Dalen (Rhenus BV) zeer zinvol geweest. Dr. Büchele (ENKA) adviseerde over het gebruik en de eigenschappen van ENKA membranen.

Tijdens het verloop van het onderzoek hebben studenten op diverse deelgebieden een bijdrage geleverd aan het onderzoek. Ik wil hen in chronologische volgorde noemen: Kees van Helden, Jos Putker, Ellen le Clercq, Albert Knol, Machteld van der Burgt, Epie Postmus, Hanneke van Hazendonk, Raymond Vaessen.

Het onderzoek werd gefinancierd door de Programma Commissie Membraantechnologie (PCM) en de Stichting Technische Wetenschappen (STW), door ENKA BV en door Rhenus (Verenigde Oliefabrieken BV).

# CONTENTS

# OUTLINE OF THE THESIS

The enzymatic hydrolysis of lipids can be an attractive alternative to the conventional chemical-physical hydrolysis process for certain applications. Since two phases are involved in the hydrolysis reaction, a membrane reactor might be an appropriate reactor system. In this thesis two membrane reactors are presented: a hydrophilic membrane reactor with immobilized enzyme and a hybrid emulsion/membrane reactor in which the enzyme is present in a free form.

In chapter 1 the hydrolysis reaction and the properties of lipase enzymes are reviewed. The immobilization methods and reactor systems published in literature are reviewed.

In chapter 2 the concept of the hydrophilic membrane reactor is presented. The use for continuous and batch processes is demonstrated and the kinetics as a function of the fatty acid concentration at zero glycerol concentration is determined experimentally.

In chapter 3 the mass transfer in the membrane reactor is studied. The diffusion of fatty acids in the hollow fiber core is modelled. The diffusion of glycerol in the membrane wall is measured and a model is derived from these measurements. The models are used to predict the mass transfer limitation as a function of the conversion rate.

In chapter 4 the hydrolysis kinetics is studied as a function of the product concentrations. The lipid hydrolysis was considered as three hydrolysis steps and alternatively as a single step. The data of glycerol and fatty acid influence are combined into a model predicting the activity as a function of product concentrations and time.

In chapter 5 the influence of several reactor parameters is described : temperature, pH and different salts in the water phase. The temperature data are modelled for the prediction of temperature effects on activity and productivity.

In chapter 6 the interfacial behaviour of lipase is studied in three different reactor systems: a stirred vessel with controlled interface, an emulsion and a membrane reactor.

In chapter 7 the concept of the hybrid reactor is presented. The influence of emulsion composition on membrane fluxes is investigated and the continuous use of a hybrid reactor for the hydrolysis of lipids is demonstrated.

In chapter 8 the interfacial behaviour of the enzyme is considered in a broader context. Further, the membrane reactors presented in this thesis are compared with other reactors used for hydrolysis reactions.

Chapter 1

# INTRODUCTION

## GENERAL

In this thesis the enzymatic hydrolysis of triglycerides is described. Triglycerides are the main constituents of naturally occurring oils and fats. A triglyceride molecule is an ester of glycerol and three fatty acids. Many different kinds of fatty acids can be distinguished, which differ among each other in chain length and structure. Triglycerides can be hydrolyzed either chemically or enzymatically. The resulting products are fatty acids which are lipophilic, and glycerol, which is hydrophilic. In this reaction therefore two phases are involved: a lipid phase and a water phase. Fatty acids further can be saponified by the addition of metal hydroxides resulting in the formation of soaps, which are strongly surface-active.

Oils and fats have been utilized by man for nutritional and domestic purposes since early times. In the Roman era olive oil was a very valuable commodity and the principal oil used for foods and ointments. In these times oils and fats were already processed to form fatty acids and soaps, to be used as cleaning agents. Animal fats were mixed with water and ashes (which contain caustic metal ions), and after boiling for several days a soap solution was obtained. Cake soap was a luxury product that came into common use not until the 19$^{th}$ century. The fact that enzymes can catalyse fat hydrolysis was proved by Claude Bernard in 1856 (1). Fats were split into fatty acids and glycerol by pancreatic juice which contains lipase. The earliest process for industrial enzymatic hydrolysis of fats was based on the addition of ground castor beans to an oil in water emulsion (2). Presently, a Japanese oil industry (Miyoshi Oil Co.) is known to apply the enzymatic hydrolysis process on a commercial scale. However, up to now the major proportion of fatty acids is produced non-enzymatically in large scale continuous processes at high temperature and pressure.

Lipases are generally considered as a class of esterases. The definition of these enzymes is as follows: Esterases are enzymes which catalyse the hydrolysis or formation of ester bonds. Lipases (EC 3.1.1.3) are defined as esterases which are active at the lipid-water interface only. Consequently, lipases are not able to hydrolyse soluble esters. This effect can be demonstrated with an ester-substrate which

has a limited solubility in water, such as triacetin. At low concentrations, this substrate is completely soluble in water. Hydrolysis does not occur with lipase in the system. With increasing triacetin concentration, oil droplets are formed and in that situation the substrate is hydrolyzed readily (3).

Lipases adsorb strongly to the lipid substrate (4), but also to a variety of other hydrophobic interfaces such as silicones (5), hexane (4) and the air-water interface (6). However, in the active site of the enzyme a lipophilic binding group can not be found. Therefore the enzyme is bound to have a hydrophobic head (supersubstrate binding site) which is separate from the reactive site (7). A hypothetical molecular mechanism of lipid hydrolysis by pancreatic lipase was reported by Brockerhoff (8), based on the known mechanism of chymotrypsin. A hydrophobic leucine is located next to a serine moiety in the active site of the lipase molecule (9). A nucleophilic attack of the hydroxy group of serine is resulting into hydrolysis by a charge relay system in which aspartic acid and histidine are involved. In a recent publication the three-dimensional structure of *Mucor miehei* lipase was revealed using X-ray crystallography (10). The catalytic center indeed was found to consist of a triad of serine, aspartic acid and histidine. The active center has a close resemblence with that of protein hydrolysing enzymes such as trypsin.

Lipases can be isolated from a a great number of origins. A complete survey will not be given here, but only some current or remarkable lipases will be mentioned. Early lipase research concerning purification and characterization has been carried out with pancreatic lipase. This work was reviewed by Desnuelle (11). Apart from the animal source, the microbiological source of lipases has become important for the last 20 years. Yeasts and fungi proved to be very efficient lipase-producers; Lipases with varying properties can be obtained from e.g. *Geotrichium candidum* (12), *Pseudomonas fluorescens* (13), *Rhizopus arrhizus* (14), *Rhizopus delemar* (15), and *Chromobacterium viscosum* (5). Finally, lipases have been isolated from plant sources such as barley and green malt (16).

As already pointed out, different lipases may have different properties. An important aspect of lipase kinetics is the specificity. Three different kinds of specificity can be distinguished:

- **positional specificity**

Positional specificity for the 1- and 3- position of a glyceride is common among lipases. Many microbial lipases (*Mucor Miehei, Aspergillus niger, Rhizopus delemar* etc.) but also pancreatic lipase possess this kind of specificity.

- **fatty acid specificity**

Fatty acid specificity is less common; most lipases have a broad spectrum of hydrolyzable substrates and only minor differences in hydrolysis rate are occurring. There is one exception: The lipase from *Geotrichium candidum* has a marked selectivity for fatty acids with a double bond at the 8-position (12).

- **stereospecificity**

Many lipases selectively hydrolyse only one of the two enantiomers of a chiral ester (17).

Another important aspect of lipase kinetics is the stability of the enzyme. For some applications, such as laundry enzymes, a high stability at elevated temperatures is required. Therefore thermostable lipases have been selected: a lipase from *Humicola lanuginosa* appeared to have a temperature optimum at 60°C (18).

The lipase used in this study originates from the yeast *Candida rugosa* (19). It does not possess specificity for position and a slight specificity for fatty acids. The following fatty acids are hydrolyzed with decreasing rate: oleic, lauric, palmitic, myristic, stearic acid (20).

## APPLICATIONS OF LIPASES

A number of conversions can be carried out by lipase. The reactions that are commercially important can be divided into three different groups:

### Esterification

At low water concentrations, esterification can be brought about. A broad variety of esters can be synthesized. Esterification of glycerol and fatty acids results in the production of mono-, di-, and triglycerides. (21, 22) These products can be used as stabilizers or surfactants in food applications (21). Esterification of glucose and fatty acids gives sugar esters, which are powerful surfactants (23).

### Transesterification

When lipase is incubated with different glycerides at a low water concentration, hydrolysis and resynthesis will result in acyl migration. This reaction is called transesterification or interesterification. When transesterification takes place between glycerides and fatty acids, the word acidolysis or glycerolysis (24) is used. The application of this type of reaction is predominantly in the production of a valuable cocoa butter equivalent from cheap starting materials (25). Chemical transesterification is also applied. With this process however, also exchange of fatty acids at the 2-position occurs and the melting properties are affected adversely.

Enzymatic transesterification is also applied for the production of chemicals, such as the formation of monoacyl glycols by transesterification of glycols and ethylcarboxylates (26).

### Hydrolysis

When the water concentration in the water phase of a two-phase reaction system is high enough, the equilibrium is on the acid side, and hydrolysis can occur.

Hydrolysis of glycerides is applied mainly for the production of bulk fatty acids for the chemical industry. Derivatives of fatty acids include fatty amines, fatty acid methyl esters, fatty alcohols, dicarboxylic acids *etc*. The bipolar character of many of these molecules makes them very suitable for the production

of a variety of soaps and detergents. Other applications include alkyd resins, paints, rubbers, lubricants, or use in the processing of leather, textile and paper (27). Glycerol is considered as a side-product. It can be used by the cosmetic industry for lotions and creams, shampoos etc. Derivatives of glycerol are used in gums, lubricants, etc. (28).

The major part of fatty acids is produced by the Colgate-Emery process. It is based on chemical hydrolysis at high temperature (250°C) and high pressure (50 bar) and is carried out in a counter-current column reactor. Drawbacks of the process are the energy consumption and the breakdown of products. Particularly multiple unsaturated fatty acids are unstable at the conditions in the reactor and may undergo side reactions. The resulting products affect the quality of the lipid phase adversely and cause colouring of the water/glycerol phase (29).

In addition to glycerides, a number of other esters can be hydrolyzed by lipase, such as indoxyl-acetate (30) and copolyesters (31).

Many lipases display a selectivity for stereo-isomers; Pure (S)-propanol was made by the asymmetric hydrolysis of (±)-1,2-diacetoxy-3-chloropropane (17). Pure (S)-propanol is a precursor for the production of β-adrenergic blocking agents. Stereospecific hydrolysis was also reported by Lavayre *et al.* (32).

Lipase-catalyzed hydrolysis is also used for food and household applications; Examples are the flavour development in dairy products and the use of microbial lipases in the formulation of laundry detergents (33).

Studies on kinetical aspects of the enzymatic hydrolysis reaction have mainly been devoted to more or less simplified empirical models. A well-founded study on the kinetics was not found in literature.

## IMMOBILIZATION OF LIPASE

For the commercial production of fatty acids, lipase can be used either in a free form or immobilized. In literature different methods for the immobilization of lipase were described:

A classical immobilization method is solvent-precipitation of lipase on Celite (34). This method was applied for the interesterification reaction. Further, adsorption to different materials such as the hydrophobic matrix spherosyl (35) and polysaccharides (36) has been applied. Precipitation or adsorption methods were also combined with crosslinking of the adsorbed protein in order to increase stability (34, 37). Besides, covalent binding in combination with a spacer was described for the binding of lipase (38). Finally, the principle of entrapment can be used, for example in hydrophilic photo-crosslinkable resins (39).

Membranes are a different group of immobilization matrices. Immobilization onto a hydrophobic microporous membrane (polypropylene) by adsorption was reported for the hydrolysis, esterification and acidolysis of glycerides (40, 41, 24). Adsorption to an acrylic hydrophobic membrane was reported by Taylor et al. (42). The advantage of membranes as immobilization supports is that a membrane can eliminate mass transfer limitations, while acting as the reaction interface.

## REACTORS FOR THE ENZYMATIC HYDROLYSIS OF LIPIDS

In a reactor for the enzymatic hydrolysis, two prerequisites have to be fulfilled: The interface between lipid and water phase has to be created and the enzyme has to adsorb to this interface. The higher the volumetric interfacial area, the higher can be the amount of adsorbed enzyme and thus the volumetric activity of a reactor.

For a free enzyme, the interface can be created by emulsification. This is generally done by mechanical stirring, but alternative methods have been described such as a countercurrent column with perforated plates (43). In an emulsion, the free enzyme will adsorb spontaneously to the interface. A drawback of such a system is that it is difficult to recover the enzyme for reuse. Centrifugation of an emulsion after hydrolysis can result in a partial recovery of the enzyme only (44). An alternative, labour-intensive method to apply free enzyme is inclusion in a membrane sandwich (45).

For an enzyme immobilized to porous or solid beads, the situation is more complex. Although a large interface is created in an emulsion, the beads will be wetted by one of the two phases only, depending on the extent of hydrophilicity of the bead. The phase which is not adhering to the carrier will contact the outer surface of the carrier only occasionally and will be excluded almost completely from the inner surface of porous particles. Therefore immobilization to particles generally results in a very low activity yield (46). This problem was partially solved by immobilization onto the hydrophilic core of an amphiphilic gel with a hydrophobic coat on this hydrophilic core (47). However, frequent regeneration has to be applied in order to remove the glycerol, which accumulates in the core.

For a membrane-immobilized lipase, two different types of reactors can be distinguished: permeation types and diffusion types. In the *permeation-type*, after adsorption of the enzyme, a dispersion of lipid and water is forced through the pores of the membrane. A microporous acrylic membrane was used by Taylor *et al.* (42, 48). The activity-yield of immobilization was reported to be low. A possible explanation is the fact that a high proportion of the lipase is not in contact with both phases. Regions within the membrane where no convection occurs can be responsible for this phenomenon.

In a *diffusion-type* membrane reactor, the enzyme is immobilized at the membrane wall and the two phases flow each on one side of the membrane, without a forced flux through the membrane. Transport through the membrane takes place by diffusion. The interface is created at the surface of the membrane, and an efficient use of enzyme can be obtained since the enzyme is immobilized at this membrane. In principle there are two different types: In the *hydrophobic* membrane reactor the enzyme is adsorbed to a hydrophobic membrane and the water phase flows on the side of the membrane where the enzyme is immobilized. An open, microporous membrane is applied in order to minimise limitations of lipid diffusion through the membrane. A fine pressure control is needed to prevent leakage of lipid into the water phase. In the scale up of this reactor type pressure control is critical (49). On the other hand, an advantage of the immobilization to such a porous membrane is that the available adsorption area per superficial membrane area is very high, so that the membrane can be used very effectively. In the *hydrophilic* membrane reactor the enzyme is adsorbed to a hydrophilic membrane and the lipid phase flows on the side of the membrane where the enzyme is immobilized (figure 1). The use of an ultrafiltration membrane prevents the lipid phase from penetrating through the membrane (50; this thesis). The available adsorption

area is equal to the superficial membrane area since this membrane has no macro-pores. Advantages of this system as compared to the hydrophobic reactor are the low membrane costs of the type of membrane applied and the absence of leakage of the lipid phase. In the work presented here, an attempt is made to gain more insight in the properties of the hydrophilic membrane reactor and to optimise relevant reactor parameters. The enzyme kinetics play an important role in reactor design. Therefore the enzyme kinetics and dependence on pH and temperature were studied intensively in the membrane reactor and in emulsion systems. The comparison of the kinetics of the free and the immobilized enzyme gave information on the influence of immobilization (adsorption) on the activity of lipase in a membrane reactor *vs.* lipase in an emulsion. Apart from the enzyme kinetics, mass transfer is an important aspect of the reactor. As shown in figure 1, lipids and fatty acids diffuse from and to the bulk lipid phase and glycerol and water diffuse through the membrane from and to the inner fiber wall where the enzyme is located. The comparison of conversion rate and diffusion rate indicates which factor is rate limiting.

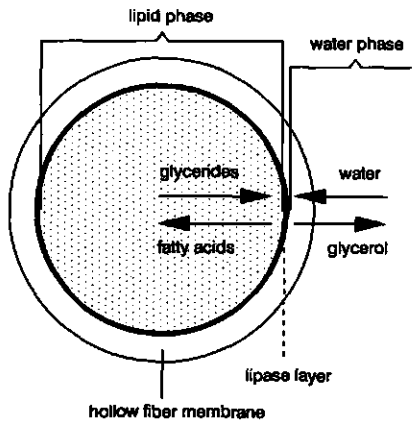


**Figure 1:** *Cross section of a cellulose hollow fiber with lipase immobilized to the inner membrane wall.*

When the basic knowledge on enzyme kinetics and mass transfer has been gathered, more insight can be obtained from models which are based on these data. Such models can be used to optimise operation parameters or to predict productivity. Another application could be the use for cost calculation or feasibility studies for potential users.

Summarizing, this study is meant to provide the basic knowledge of the enzymatic hydrolysis of lipids in a membrane reactor, in order provide more insight in the theory of lipase catalyzed hydrolysis with and without immobilization and in order to provide data necessary for the design of industrial applications.

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# THE HYDROLYSIS OF TRIGLYCERIDES BY IMMOBILIZED LIPASE IN A HYDROPHILIC MEMBRANE REACTOR.

## SUMMARY

In this chapter a method is described to immobilize lipase from *Candida rugosa* on a hollow fiber membrane, and the use of such a system for the hydrolysis of lipids is reported. The membranes were ENKA hydrophilic Cuprophan-type hollow fibers, having a large specific surface area. The immobilized lipase exhibited a high stability: the half-life time was 43 days at a temperature of 30°C. Furthermore, it is proved that kinetic studies can be carried out with this system, operated in a batch or continuous mode. The relation between conversion rate and degree of hydrolysis was determined. On this basis, a dynamic model of the process was developed that describes the relation between reaction conditions and the conversion rate.

## INTRODUCTION

The hydrolysis of oils and fats is an important industrial operation: world-wide, $1.6.10^6$ tons of fatty acids are produced every year by this process. The products (fatty acids and glycerol) are basic materials for a wide range of applications (1). The hydrolysis reaction yields 1 mole of glycerol and 3 moles of fatty acid per mole of triglyceride. Triglycerides, from here on called "lipids", do not dissolve in the water phase, so the reaction has to take place at the interface of the water and lipid phase. The products, fatty acid and glycerol, are dissolved in the lipid and water phase, respectively. As the reaction is reversible, both the hydrolysis rate and the final composition depend on the fatty acid concentration in the oil phase and on the glycerol concentration in the water phase (2).

Conventional processes are based on the hydrolysis at an elevated temperature, in some cases in the presence of a catalyst or at high pressure (2). This results in a high energy input; breakdown of products may also occur, especially in the case of highly unsaturated fatty acids. The by-products dissolved in the

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water phase cause a problem in the isolation of glycerol from this phase.

The reaction can be catalysed by enzymes of the class lipase (EC 3.1.1.3). Enzymatic hydrolysis of lipids is interesting, because it could overcome some of the drawbacks of the high-temperature process. Lipases have been applied already on an industrial scale for the hydrolysis of lipids (3). Lipase in a free form will adsorb onto the lipid-water interface, which explains the high reactivity of the enzyme toward the insoluble substrate (lipid). However, when the enzyme is immobilized on a solid carrier, it is difficult to bring the enzyme into contact with both substrate phases. When the carrier has a hydrophobic character, there will be a limited contact of the water phase with the lipase. For example, an activity yield of 18.3% was obtained after immobilization of lipase in a hydrophobic photo-crosslinkable resin (4). Conversely, a hydrophilic carrier hinders the lipid phase from reaching the lipase (5).

A membrane-based lipase reactor without immobilization has been published by Molinari and Drioli (6). It comprises a stirred vessel and a membrane unit in a closed loop configuration. In the stirred vessel, an emulsion is formed and the hydrolysis reaction takes place. The permeate formed by ultrafiltration of the emulsion in the membrane unit is enriched in the reaction products.

The membrane also can be used for immobilization of lipase and the simultaneous separation of the phases by the membrane. The membrane can be hydrophobic or hydrophilic. In the first case, the lipid phase penetrates the membrane. The lipase must then be immobilized to the "water side" of the membrane. A reactor based on this principle has been studied by Hoq *et al.* (7,8,9) for the hydrolysis of lipids.

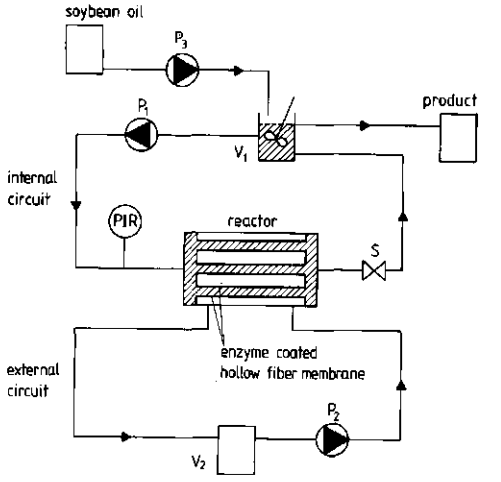
In our reactor, the hydrophilic Cuprophan membrane is used (10) and the lipase is immobilized on the "lipid side" of the membrane. The membrane is not permeable for the lipid phase. Water permeates through the membrane to the enzyme layer, thus supplying reaction water; glycerol diffuses through the membrane back into the water phase; the fatty acids remain in the lipid phase. The volumetric activity is linearly dependent on the membrane-specific surface area. Therefore, use is made of hollow fiber-membrane units with a high surface/volume ratio and a low price per surface area. Since the lipid phase cannot penetrate the membrane (not even at pressures up to 1 bar), the hydraulic pressure caused by circulation of the lipid phase is enough to maintain a good phase separation and no accurate pressure control is necessary. This is an important advantage in comparison with the hydrophobic membrane reactor described by Hoq *et al.* (7,8,9). A disadvantage is the lower activity per membrane surface area. However, this is compensated by the lower price of the membrane per surface area.

In the scale-up stage of this process it will be useful when a prediction can be made on the reactor performance as a function of the membrane surface area, the percentage hydrolysis, and other relevant factors. The purpose of the study reported here is (1) to show that the immobilized enzyme hollow fiber membrane reactor with hydrophilic membranes can be used for lipid hydrolysis, and (2) to develop an engineering model that can be used for scale-up of the process.

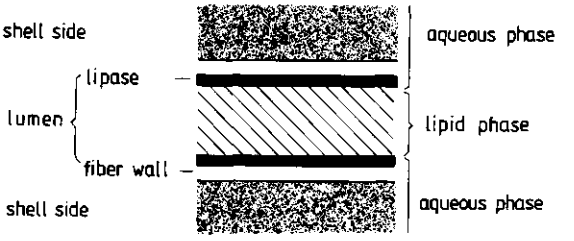
## METHODS AND MATERIALS

### Experimental set-up

The experimental set-up is shown in Figure 1(a). The reactor consisted of a hollow fiber membrane module, made by ENKA-Membrana AG (FRG). It contained 9.170 fibers with an internal diameter of 0.215 mm, a wall thickness of 0.016 mm, and a length of 0.21 m; the total membrane area was 1.3 $m^2$. The fibers were made of cellulose (Cuprophan) symmetrical membranes (cut-off = 5.000).




(a)




(b)

**Figure 1.** *(a) The experimental set-up. (b) Schematic representation of a cross section through a hollow fiber.*

The lipid phase was present in the lumen of the fibers and in vessel $V_1$, called the "internal circuit", and was circulated by pump $P_1$. The volume of the stirred vessel $V_1$ was 50 mL, and of the tubing, 10 mL. The total volume of the internal circuit was 150 mL. The water phase was present in the shell surrounding the fibers and in the water vessel $V_2$, called the "external circuit." The water phase had a total volume of 200 mL and was circulated by pump $P_2$.

The reactor was kept at a constant temperature of 30°C. The pressure of the internal circuit, caused by the hydraulic resistance of the fibers, was registrated continuously by the pressure indicator PIR (WIKA, Type 891.10.500, 1.6 bar) connected to a recorder.

In the batch mode, pump $P_3$ was off. Oil and water both were circulated with a flow rate of 10 mL/min. In the continuous mode, pump $P_3$ added a predetermined flow of lipid to the internal circuit, and an equal flow of product was removed by a level controller in vessel $V_1$. In order to prevent inhibition by high glycerol concentrations, the water phase was replaced each day by distilled water.

## Materials

Lipase powder from *Candida rugosa*, produced by Meito sangyo (type "OF"), was used in this study. It contains 12.7% [w/w] of protein and had an activity of ca 54 U/mg as determined by the lipase assay (see above).

In all experiments the lipid phase was soybean oil of edible quality with a fatty acid content of 0.3%, a non-hydrolyzable fraction of 0.5%, a mean fatty acid molecular weight of 276 D, and a density of 917 $kg.m^{-3}$. It was supplied by Rhenus BV (Rhenen, The Netherlands).

## Immobilization procedure

The internal circuit was filled with a solution of lipase in water, valve S was closed, and the solution was ultrafiltrated at a flow rate of 25 mL/min. Without intermission, this was followed by ultrafiltration of 150 mL of pure water. The water permeated, together with dissolved molecules smaller than 5.000, while the lipase and other macromolecules were retained. They probably formed a gel layer on the membrane, as a result of concentration polarization. Then, valve S was opened and oil was pumped through the inner circuit until the outgoing fluid was free from water droplets. Protein and lipase quantities were determined in the permeate and outgoing water, using the assays described below. Finally, the external circuit was filled with water, to which 0,15% sodium azide was added in order to prevent microbial growth.

The situation at the membrane surface is depicted in Figure 1(b). Water is outside the fibers and permeates through the hydrophilic fiber wall to the enzyme layer, while lipid is inside the fibers. So the enzyme is located at the interface, which is necessary for its activity.

## Analytical methods

The lipase activity was determined in the following assay: 1 mL of tibutyrin with 50 mL of a solution containing 0,15 [w/v] Arabic gum, 2 m*M* maleic acid, pH 6, was sonified in a Branson "B-12" ultrasonic emulsifier. The emulsion was stirred in a vessel thermostated to 30°C. The enzyme solution was added and the pH was kept at 6 by addition of 0.1 NaOH. One unit is defined here as the amount of lipase requiring the addition of 1 µmole of NaOH/min.

Protein measurements were performed according to a modified Lowry procedure (11).

The degree of hydrolysis (synonymous to "fatty acid content") was determined by titration of a lipid sample (ca. 1 g) in 20 mL ethanol, with 0.5 *N* NaOH. The degree of hydrolysis (*Z* [%]) was calculated as follows:

$$Z = \frac{W \cdot v \cdot T}{10 \cdot m} \qquad (1)$$

where: $W$ = the mean molecular weight of the fatty acids (276); $v,T$ = the volume [mL] and molarity of the titrated NaOH solution, respectively; $m$ = the weight of the oil sample [g].

The conversion rate (*CR* [mL.h$^{-1}$.m$^{-2}$]) is defined as the volume of lipid hydrolyzed completely per membrane surface area per hour. In batch experiments the conversion rate was calculated as follows:

$$CR = \frac{dZ(t)}{dt} \cdot \frac{V}{100.A} \qquad (2)$$

where: $V$ = volume of the lipid phase [mL]; $A$ = membrane surface area [m$^2$]; $t$ = time [h]

In continuous experiments, relation (3) was used for the calculation of the conversion rate,

$$CR = \frac{\phi \cdot Z}{100 \cdot A} \qquad (3)$$

where $\phi$ = net flow rate [mL.h$^{-1}$].

The standard conversion rate (*SCR* [mL.h$^{-1}$.m$^{-2}$]) is defined as the conversion rate that should be attained when $Z$ = 0%:

$$SCR = \frac{CR \cdot CR(0)}{CR(Z)} \qquad (4)$$

where $Z$ is the actual degree of hyrolysis and $CR(0)/CR(Z)$ represents the ratio of the conversion rates at a fatty acid content of 0% and Z%.

The glycerol content in the water phase was determined by refractive index measurements. The correlation between refractive index and glycerol content [w/v] is given in (12).

## RESULTS AND DISCUSSION

### Immobilization

One hundred mL of a 20 mg/mL lipase solution (in total $1.1 \cdot 10^5$ U, 0.28 g protein) was ultrafiltrated, as described in the methods and materials section. The quantity of protein and lipase that was immobilized could be determined from the balances by calculating the difference between the quantity that was added and the quantity in wash water and permeate. An uncertain factor was the quantity of protein dispersed in the lipid phase after wash out. It was assumed that no protein was present in this phase. From these calculations it follows that 18 ± 7% of the total protein was immobilized. The lipase activity balance showed

an immobilized quantity of 14 ± 5%.

After immobilization, the reactor was filled with oil (internal circuit) and a 0.15% sodium azide solution (external circuit), and both phases were circulated for a period of 110 h in order to let the system stabilize. After this period samples were taken from the oil circuit at regular times. These were stirred for one week with a twofold volume of water, and the degree of hydrolysis was registrated before and after to detect small quantities of lipase. No lipase activity could be found in any of the samples, which indicates that no active enzyme is washed out during the experiments.

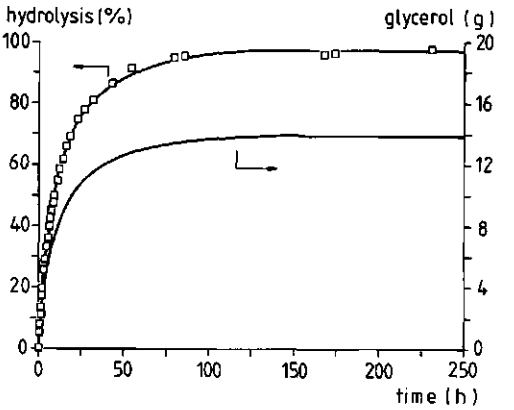



**Figure 2:** *The degree of hydrolysis (□ : measurements; the solid line through these points is the best fit) and the glycerol amount as a function of time in a batch experiment.*

## Selection of the temperature

The reactor was kept at a temperature of 30°C. This temperature resulted from the experiments:

I. At room temperature (20-23°C), complications occurred: In continuous mode, the degree of hydrolysis decreased gradually, with an analogous increase in oil circuit pressure. In batch experiments, the pressure was raised above 1 bar when the fatty acid content went above 70-80%. Besides, the produced lipid phase was not clear and a precipitate could be observed upon storage. From these results we concluded that at room temperature the fatty acids or possibly mono- or diglycerides form insoluble complexes that clog the tiny fibers.

II. At 40°C, no pressure increase with time occurred, but the conversion rate decreased rapidly (17% $day^{-1}$), most probably due to denaturation of the lipase.

III. At 30°C, there still was no pressure increase with time, and the denaturation rate was much lower; 1.6% $day^{-1}$ (see the section on continuous experiments). The pressure in the internal circuit, caused by the hydraulic resistance of flow through the fibers, amounted to 40 mbar.

## Batch experiment: kinetics

The kinetics as a function of the fatty acid content were derived from a batch experiment. The conditions of this experiment are described in the methods and materials section. The following remarks must be made:

1. The batch experiment was started after a continuous experiment of 4 days. In this period the conversion rate of the reactor was stabilized at a value of 59.6% hydrolysis at a net flow rate of 6.9 $mL.h^{-1}$.

2. Oil samples decreased the volume of the inner circuit. This was corrected for.

3. The water phase was replaced every day by distilled water. A correction was made for the samples taken from the water phase.

4. The total volume of the inner circuit was 173 mL.

The results of fatty acid measurements are shown in Figure 2. From these measurements, the theoretical course of the amount of glycerol in the water phase was determined, assuming that one molecule of triglyceride results in one molecule of glycerol. The measured total amount of glycerol in the water phase showed a good correspondence with the theoretical line. Deviations from this line were within the experimental error. From these results, the conclusion can be drawn that the triglycerides are converted into fatty acids without the formation of mono- and diglycerides. This conclusion is confirmed by Khor *et al.* (13), who demonstrated that during the lipase-catalyzed hydrolysis less than 9% [mole/mole] of the total lipid amount consists of diglycerides, while monoglycerides cannot be detected at all.

In order to determine the relation between conversion rate and fatty acid content, the degree of hydrolysis as a function of time was differentiated numerically and plotted against the fatty acid content, as shown in Figure 3. The results show that the conversion rate is strongly dependent on the fatty acid content.

If the data from Figure 3 are plotted and fitted in the power law relation, a good correlation is observed at a reaction order of 1.7. These results are different from the results of Yamane *et al.* (9), who concluded that under certain conditions a first-order description of the kinetics was valid.

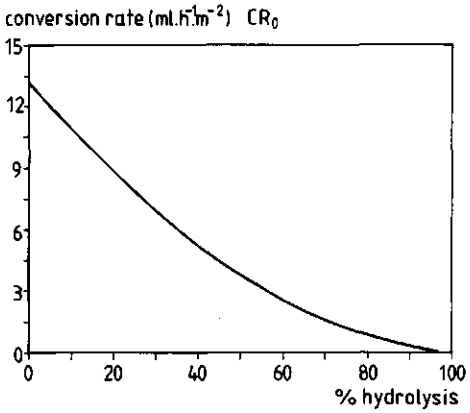



**Figure 3:** *The conversion rate as a function of percentage hydrolysis. Data derived from Figure 2.*

## Continuous experiments

### *Stability*

The stability of the reactor was examined in a long-term continuous experiment. The net flow was adjusted to 10.8 mL/h. The initial fatty acid content was 37.5%. The degree of conversion vs. time is given in Figure 4.

It is evident from Figure 3 that the conversion rate as such is not an unambiguous measure for the activity of the system. Therefore, the activity of the system was expressed in the standard conversion rate (*SCR*) value, which was defined in the Methods and Materials section. The values of *CR(0)* an *CR(Z)* in this expression were calculated from the relation shown in Figure 3. The course of *SCR* against time is given in Figure 4, too.

As discussed for the immobilization experiment, there is no wash out of enzyme. So the inactivation observed should be a result of lipase denaturation only. The decrease in conversion rate has a logarithmic course, corresponding to first-order denaturation. The best fit, shown in Figure 4, leads to an inactivation constant of $6.8.10^{-4}$ $h^{-1}$, corresponding with an inactivation rate of 1.6% per day or a half-life time of 43 days. Hoq *et al.* (7) observed a half-life time of 2 days in the case of lipase immobilized in a hydrophobic membrane reactor at a temperature of 40°C. When 18% glycerol was present in the water phase of this system, the half-life time was increased to 15 days.

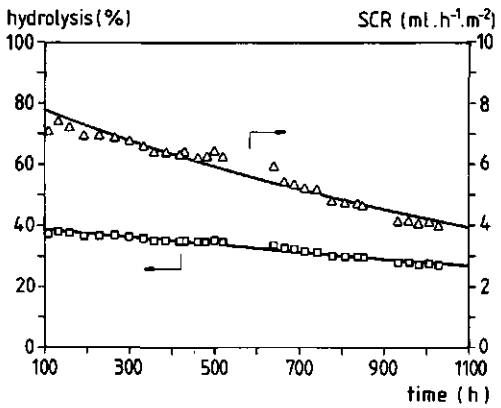



**Figure 4.** *The degree of hydrolysis (□) and the standard conversion rate (△) in a long-term continuous experiment.*

*Flow variations*

In another experiment with the same immobilization procedure, the degree of hydrolysis after an equilibration period of 110 h amounted to 43.7% at a net flow of 12.3 mL/h. Then, the net flow rate was changed several times. The experimental results on these flow variations are presented in Figure 5. In the caption to this figure, the flow rates and corresponding time intervals are indicated. The simulation in this figure is based on the following model.

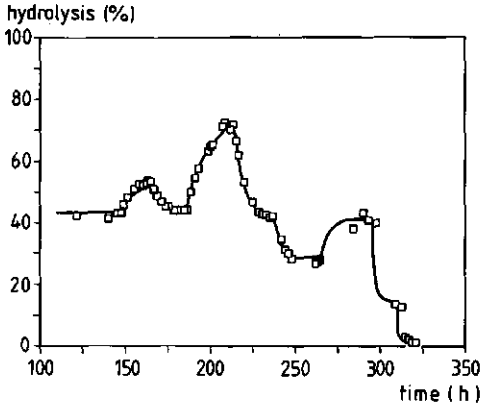



**Figure 5.** *The degree of hydrolysis as a function of varations in net flow rate (ϕ). The continuous line represents the degree of conversion as calculated by the model. Up till t = 147.5 the net lipid flow rate was 12.3 mL.h$^{-1}$. Then, the flow was adjusted to the following values: 7.5 mL.h$^{-1}$ (t = 147.5 h); 12.3 mL.h$^{-1}$ (t = 165 h); 1.7 mL.h$^{-1}$ (t = 186 h); 12.3 mL.h$^{-1}$ (t = 214 h); 25.9 mL.h$^{-1}$ (t = 237.5); 12.3 mL.h$^{-1}$ (t = 265 h); 66.5 mL.h$^{-1}$ (t = 295 h); 500 mL.h$^{-1}$ (t = 310 h).*

## Theoretical model

A theoretical model of the reactor as a whole was developed, based on the following assumptions:

1. The internal circuit can be considered a perfect mixed reactor because the circulation time through the fibers is more than an order of magnitude lower than the characteristic time for the hydrolysis in the reactor.

2. The inactivation of the enzyme is first order with an inactivation constant of $6.8.10^{-4}$ h$^{-1}$.

3. The relation between conversion rate and fatty acid content is as shown in Figure 3.

4. The diffusion of glycerol through the membrane does not influence the conversion rate. This was confirmed as follows: Calculations as well as measurements on glycerol diffusion through the membrane showed that the concentration difference of glycerol across the membrane is less than 17 m*M* (1.6 g.L$^{-1}$) at a conversion rate of 20 mL.h$^{-1}$.m$^{-2}$. In addition, model experiments showed that glycerol has no markable

influence on the kinetics of the reaction below a concentration of 50 $g.L^{-1}$.

5. The diffusion of fatty acids and glycerides in the fiber to and from the interface where the reaction takes place is not rate limiting. This was confirmed by the observation that the circulation rate of the lipid phase through the fibers has no influence on the conversion rate.

The balance over the system can be given by

$$V\frac{dZ(t)}{dt} = P(t,Z)\cdot A\cdot 100 - \phi(t)\cdot Z(t) \qquad (5)$$

where : $V$ = volume of the internal circuit [mL]; $P(t,Z)$ = the fatty acid production [$mL.h^{-1}.m^{-2}$]; $A$ = membrane surface area [$m^2$]

The production kinetics is described by the function $CR(Z)$, which is shown in Figure 3. In order to calculate the fatty acid production, this function must be corrected for inactivation and the initial activity of the system:

$$P(t,Z) = \left(\frac{CR(Z)}{CR(0)}\right)\cdot SCR\cdot e^{-kt} \qquad (6)$$

where: $k$ = the inactivation constant mentioned in assumption 2; $SCR$ = the standard conversion rate of the system at the start of the experiment

$CR(Z)$ and $CR(0)$ are the conversion rates at a percentage hydrolysis of Z and 0%, respectively, as given in Figure 3.

Discretization of relation (5) gives

$$Z(t+dt) = Z(t) + dt\cdot\frac{100\cdot A\cdot P(t,Z) - \phi(t)\cdot Z(t)}{V} \qquad (7)$$

The size of the time step was chosen in such way that further reduction of the time step had no significant influence on the calculated values. Figure 5 shows the simulated curve based on this model. The simulated and measured values show a good correspondence; small deviations occur, probably due to inaccuracy in flow determination or fatty acid measurement.

The model is based on separately determined parameters for the kinetic data, membrane surface area, and process conditions. This means that it can be used for scale-up purposes and for optimization without the need of elaborate experiments on a large scale.

## CONCLUSIONS

A membrane bioreactor for the hydrolysis of lipids was developed, which acts at moderate temperature, so that no transformation of the fatty acids can be expected. It has been observed visually that during hydrolysis experiments the water/glycerol phase is a very clear solution and the lipid phase shows no significant decolorization.

The batch experiment showed that all glycerol formed migrates into the water phase. For the batch experiments it was shown that the amount of glycerol formed corresponds with the amount of fatty acids. This indicates that triglycerides are hydrolyzed to fatty acids without the formation of significant amounts

of mono- and diglycerides. The reactor exhibits a high stability at 30°C.

The theoretical model predicts results of batch as well as continuous experiments, provided that the initial activity is known. The model can be used for scale-up calculations.

## SYMBOLS

| | |
|---|---|
| $\phi$ | net flow rate of lipid [mL.h$^{-1}$] |
| $A$ | membrane surface area [m$^2$] |
| $CR$ | conversion rate, defined as the volume of lipid hydrolyzed per hour [mL.h$^{-1}$.m$^{-2}$] |
| $CR(Z)$ | conversion rate [mL.h$^{-1}$.m$^{-2}$] as a function of $Z$ |
| $k$ | logarithmic inactivation constant [h$^{-1}$] |
| $m$ | weight of the lipid sample |
| $P$ | fatty acid production [mL.h$^{-1}$.m$^{-2}$] |
| $SCR$ | standard conversion rate [mL.h$^{-1}$.m$^{-2}$], defined as the conversion rate corresponding to $Z$ = 0% |
| $t$ | time [h] |
| $T$ | molarity of the titrated NaOH solution |
| $v$ | volume of the titrated NaOH solution [mL] |
| $V$ | volume of the internal circuit [mL] |
| $W$ | mean molecular weight of the fatty acids |
| $Z$ | degree of hydrolysis [%] |

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Chapter 3

# MASS TRANSFER OF GLYCEROL AND LIPIDS IN HYDROPHILIC HOLLOW FIBERS OF A MEMBRANE BIOREACTOR


## SUMMARY

In this chapter, the mass transfer of lipid- and water- dissolved components in the hollow fiber membrane reactor is studied. The diffusion of glycerol across the membrane was studied in model diffusion experiments. The diffusion coefficient of glycerol was calculated to be $2.1 \cdot 10^{-10}$ $m^2.s^{-1}$, while the literature value for infinite dilution amounts $9.4 \cdot 10^{-10}$ $m^2.s^{-1}$. Based on the measured overall mass transfer coefficient, concentration differences and productivities were calculated as a function of the conversionrate of the immobilized lipase. The results show that glycerol diffusion is not rate limiting (< 3.3%) for the range of maximum conversion rates obtained until now in the membrane reactor (up to 0.33 mole.$m^{-2}.h^{-1}$). For the case of complete diffusion limitation of glycerol/water, the observed activity is calculated to be 18.3 mole.$m^{-2}.h^{-1}$.

The diffusion in the lipid phase was studied using a calculated diffusion coefficient and a numerical model integrating laminar flow, diffusion and lipolytic reaction kinetics. The results show that lipid-phase diffusion limitation is low (< 5%) for conversion rates up to 0.34 mole.$m^{-2}.h^{-1}$, while almost complete diffusion limitation (> 99.6%) is reached for conversion rates of the immobilized lipase higher than 1000 mole.$m^{-2}.h^{-1}$. For the case of complete lipid-diffusion limitation the conversion rate is calculated to be 24 mole.$m^{-2}.h^{-1}$. Thus, also with respect to the lipid phase, the inhibition due to diffusion is relatively small for the conversionrates reached until now in the membrane reactor.


## INTRODUCTION

The suitability of a membrane reactor for the enzymatic hydrolysis of lipids was demonstrated in a previous article (this thesis, chapter 2). In case of lipolysis in an oil/water emulsion, the lipase adsorbs at the lipid/water interface where the reaction takes place. In a hollow fiber reactor, numerous cellulosic hollow fibers are embedded in a membrane module, creating a high specific surface area (5.000 up to 10.000 $m^2.m^{-3}$). The lipase enzyme is immobilized on the inner side of the fibers. In a previous article (this thesis, chapter 2), a calculation method was presented for the conversion of the activity measured in a continuous experiment into the Standard Conversion Rate (SCR), which is defined as the fatty acid production rate at zero fatty acid concentration and at zero glycerol concentration. In the membrane reactor,

the SCR amounts from 40 - 100 $ml.m^{-2}.h^{-1}$ (0.13 - 0.33 $mole.m^{-2}.h^{-1}$).

In the case of immobilized biocatalysts the mass transfer of the reacting components may become rate limiting in some cases (1). For the two-phasic hydrolysis reaction mass transfer occurs in the lipid as well as the water phase. In a hydrophilic membrane reactor the components in the water phase (water and glycerol) have to diffuse through the membrane and through the boundary layer outside the fiber. The components in the lipid phase (triglycerides and fatty acids) have to diffuse in a radial direction inside the fibers. The literature data on mass transfer in lipolytic membrane reactors are limited and concern hydrophobic membrane reactors only. Yamane (2) used a kinetic model to describe hydrolysis kinetics based on reaction limitation only (first order kinetics) or on limitation of lipid diffusion through the membrane only. However, kinetics and mass transport were not measured or predicted independently and therefore it was not possible to judge if the reaction was diffusion controlled or kinetics controlled.

In this chapter the diffusion rate of glycerol through the cellulosic membrane will be measured and the mass transfer of lipid flowing through the hollow fibers will be modeled. The combination of the model data on diffusion of water/glycerol and lipids with the measured data on the enzymatic activity indicates which of these factors is rate limiting.

## MATERIALS AND METHODS

### Materials:

All measurements with the cellulose membrane were carried out with a membrane module with a total membrane area of 0.77 $m^2$, an effective fiber length of 0.21 m and a packing density of 42%. It was kindly provided by ENKA AG (Wuppertal, Germany). The membrane was made of regenerated cellulose (Cuprophan™) with a cut-off value of 5.000 D. The hollow fibers had an internal diameter of $2.15 \cdot 10^{-4}$ m and a wall thickness of $8.0 \cdot 10^{-6}$ m. Lipase of *Candida rugosa* was purchased from Meito Sangyo, Japan.

### Glycerol diffusion experiments

Measurements on glycerol diffusion were done in the membrane module, thoroughly rinsed with demineralized water. Shell side and lumen of the fibers were connected to a pump and a flask resulting in two separate circuits, referred to as external and internal circuit respectively. The volumes of the circuits were determined accurately in each experiment and were 400 mL. The vessels in both circuits were stirred vigourously by magnetic stirring devices. Gear pumps with constant flow-characteristics were used (Ismatec MV-type gear pumps) in order to prevent flow and pressure fluctuations. After filling the external circuit with demineralized water, the internal circuit was filled with a glycerol/water mixture with a glycerol concentration of 170 $kg.m^{-3}$. From this time on (t=0) the volumes and glycerol concentrations were measured for both circuits in the flows leaving the module as well as in the flows entering the module. Glycerol was measured by refractive index measurement as reported earlier (this thesis, chapter 2).

## Theory on glycerol diffusion

Due to the concentration difference across the membrane an osmotic water flow will exist from the circuit with the lower glycerol concentration (external circuit) to the circuit with the higher glycerol concentration (internal circuit). On the other hand, the pressure head caused by the flow of water/glycerol through the fibers results in a flow in the opposite direction. The net flow across the membrane from the external into the internal circuit is defined as the membrane flux ($\phi$, [$m^3.s^{-1}$]), which is determined experimentally from volume changes of the circuits. The total glycerol mass transfer from the internal to the external circuit ($J_t$, [$kg.m^{-2}.s^{-1}$]) is the sum of transport due to diffusion and due to convection (flux) as reflected in equation 1.

$$J_t = K(c_{ic} - c_{ec}) - \frac{\phi c_{ec}}{A} \qquad (1)$$

with: $$\phi = \frac{\partial V_{ic}}{\partial t} = -\frac{\partial V_{ec}}{\partial t}$$

in which $K$ [$m.s^{-1}$] represents the total diffusional mass transfer coefficient; inside the fibers, in the membrane wall and the outside the fibers. $c$ is the glycerol concentration [$kg.m^{-3}$]. In equation (1) the assumption is made that the diffusional mass transfer of glycerol does not attribute to the volume change of the circuits.

The measured mass transfer rate of glycerol from the external to the internal circuit through the membrane can be calculated from the measured change in time of the product of glycerol concentration and volume of the internal circuit ($c_{ic}$) as shown in equation 2.

$$J_t = -\frac{\partial\left(\frac{c_{ic} \cdot V_{ic}}{A}\right)}{\partial t} = -\frac{V_{ic}}{A} \cdot \frac{\partial c_{ic}}{\partial t} - \frac{c_{ic}}{A} \cdot \phi \qquad (2)$$

The same equation is valid for the external circuit.
According to Perry (3) the overall diffusion coefficient ($D_o$) of a component in a membrane is a function of the intrinsic diffusion coefficient ($D$), the tortuosity of the membrane ($\beta$) and porosity of the membrane ($\epsilon$) as shown in equation 3. The tortuosity of the membrane represents the ratio between the average diffusion path length through the membrane and the thickness of the membrane.

$$D_o = D \cdot \frac{\epsilon}{\beta^2} \qquad (3)$$

In the case that the diffusional resistance is determined by the membrane wall only, equation 1 can be modified by introducing the membrane thickness $d$ [m] as:

$$K = \frac{D_o}{d} = \frac{D.\epsilon}{d.\beta^2} \qquad (4)$$

From the measurements of volumes of and glycerol concentrations in internal and external circuit as a function of time, the value of $K$ was calculated at several values of $t$ using equations 1 and 2. The same

calculation carried out for the external circuit gave the same results within experimental error. The intrinsic diffusion coefficient ($D$) was calculated from $K$ using equation 4. The membrane porosity ($\epsilon$) was assumed to be 0.5 (4), while the membrane thickness ($d$) was $8.0 \cdot 10^{-6}$ m.

## Modeling of glycerol diffusion

Using the average value of $K$, the glycerol concentration difference across the membrane was calculated as a function of the conversionrate. The conversionrate was transferred into the Standard Conversion Rate (SCR) using the relation for the conversionrate as a function of the glycerol concentration reported earlier (this thesis, chapter 4). The relative activity (ratio of conversionrate and SCR) and the glycerol concentration difference across the membrane ($\Delta c_m$) were calculated as a function of SCR.

# RESULTS AND DISCUSSION

## Glycerol diffusion experiments

In order to investigate if boundary layers limit the mass transfer rate, experiments were carried out with various recirculation rates. An example of the concentration change in time of internal and external circuit (both the average of in- and outcoming concentration) is shown in figure 1 (flow rate ≈ 98 mL/min; initial glycerol concentration in the internal circuit = 217.2 kg.m$^{-3}$). The other experiments showed a comparable profile.

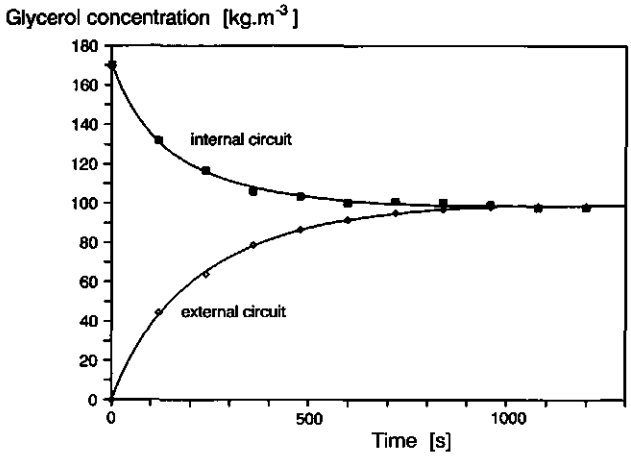


**Figure 1:** *Example of concentration vs. time profile for the diffusion of glycerol through the cellulosic membrane at a recirculation rate of 98 mL/min.*

The initial mass transfer coefficients, calculated from the glycerol concentration change in time as described, are summarized in table I.

| recirculation rate of both circuits [mL.min$^{-1}$] | *K* [m.s$^{-1}$] |
|---|---|
| 11.3 | $3.43 \cdot 10^{-7}$ |
| 50 | $8.96 \cdot 10^{-7}$ |
| 98 | $1.35 \cdot 10^{-6}$ |
| 145 | $1.21 \cdot 10^{-6}$ |
| 193 | $1.15 \cdot 10^{-6}$ |

**Table I:** *Initial mass transfer coefficients for glycerol diffusion through the cellulosic membrane measured for different recirculation rates.*

The mass transfer rate reaches a plateau value above a recirculation flow of 50 mL/min. Above this flow rate apparently the influence of boundary layers can be neglected. In this range the mass transfer coefficient and the overall diffusion coefficient are related according to equation 4. Therefore, further calculations on the diffusion were done for a flow which is above this minimum value (98 mL/min). The membrane tortuosity as reported in literature is in between 2.0 and 4.9 (5). In this report, a value of 3.45 was assumed. Now, from equations 1, 2 and 4, mass transfer coefficients and the diffusion coefficients were calculated for different times after the start of the experiment (table II). The average diffusion coefficient was found to be $2.10 \cdot 10^{-10}$ m$^2$.s$^{-1}$. This value is lower than the literature value for infinite dilution ($9.4 \cdot 10^{-10}$ m$^2$.s$^{-1}$). This can be expected because the measured diffusion coefficient is an effective diffusion coefficient and differences can occur due to wall effects which slow down the mass transfer. Further, a deviation of the tortuosity from the assumed value can influence the calculated value of the intrinsic diffusion coefficient.

| time [s] | *K* [m.s$^{-1}$] | *D* [m$^2$.s$^{-1}$] |
|---|---|---|
| 240 | $1.08 \cdot 10^{-6}$ | $2.06 \cdot 10^{-10}$ |
| 360 | $1.13 \cdot 10^{-6}$ | $2.14 \cdot 10^{-10}$ |
| 480 | $1.10 \cdot 10^{-6}$ | $2.09 \cdot 10^{-10}$ |
| **average:** | $1.10 \cdot 10^{-6}$ | $2.10 \cdot 10^{-10}$ |

**Table II:** *Calculated mass transfer coefficients (K) and diffusion coefficients (D) for different times after the start of a diffusion experiment at a recirculation rate of 98 mL.min$^{-1}$.*

The enzyme kinetics of lipase is influenced by the glycerol concentration. The kinetics of *Candida rugosa* lipase immobilized on the cellulosic membrane was reported before (this thesis, chapter 4); the initial rate as a function of the glycerol concentration as well as the equilibrium composition of lipid and aqueous phase were reported. A high activity per surface area will result in a high concentration difference and thus in a reduced enzyme activity. In order to get insight in these phenomena the concentration difference across the membrane was calculated as a function of enzyme activity immobilized (SCR) using the mass transfer relation with the data calculated above and the relation of activity as a function of the glycerol concentration and SCR reported earlier. Diffusion limitations in the lipid phase are neglected. The result of these calculations is shown in figures 2a and 2b.

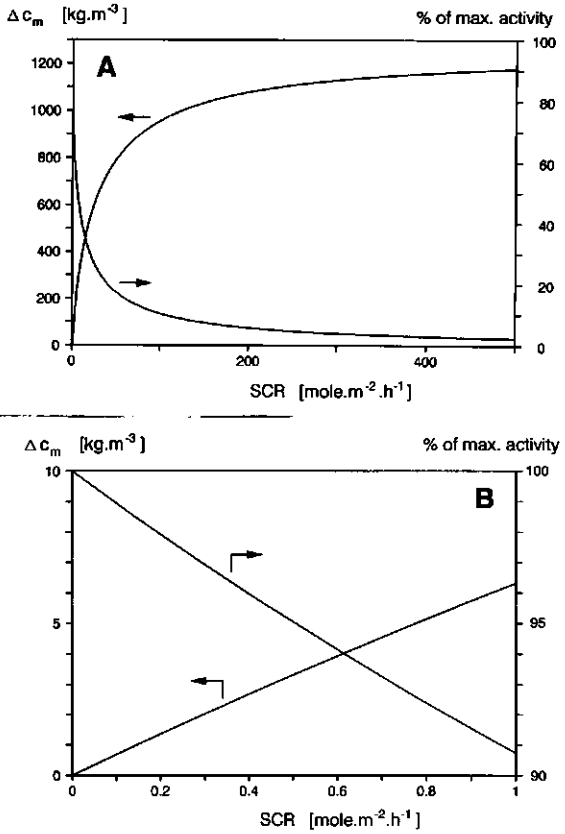


**Figure 2a and 2b:** *Concentration difference of glycerol across the membrane ($\Delta c_m$) and percentage of the maximum activity as a function of the amount of enzyme activity immobilized.* **X-axis:** *SCR (the maximum activity which would be reached when there would not be diffusion limitation in the lipid or water phase);* **left Y-axis:** *glycerol concentration difference across the membrane ($\Delta c_m$);* **right Y-axis:** *calculated activity relative to the maximum activity (activity/SCR). Diffusion limitation in the lipid phase is neglected.*

At the maximum activity currently obtained in the membrane reactor (up to 0.33 mole.m$^{-2}$.h$^{-1}$) the relative activity is 96.7% (figure 2b). Hence it can be concluded that the kinetical experiments that are be reported in this thesis (chapter 2 and 4) were not influenced by diffusion limitation of glycerol. However, when a substantially higher activity is reached, for example by immobilization of a high-activity lipase preparation, diffusion limitation can be expected: For example, 90% activity remains at an SCR of 1.08 mole.m$^{-2}$.h$^{-1}$. For the case of complete diffusion limitation ($SCR \rightarrow \infty$) the conversionrate can be calculated to be 18.3 mole.m$^{-2}$.h$^{-1}$, assuming that the diffusion coefficient does not change with concentration.

## Lipid phase diffusion

Apart from water and glycerol, there is mass transfer of lipid components to and from the interface. Triglycerides diffuse from the bulk to the fiber wall and fatty acids diffuse into the opposite direction. Reynolds numbers of lipid flow through the hollow fibers are in the range of $4.9 \cdot 10^{-4}$ to $4.9 \cdot 10^{-3}$. Therefore the flow in the fibers is fully laminar with a parabolic flow profile and radial mass transfer is totally brought about by diffusion. Further, the Peclet number, calculated from the diffusion coefficient and the average flow velocity, is $1.1 \cdot 10^{6}$ to $1.1 \cdot 10^{7}$ for flow rates used in the reactor ($2.0 \cdot 10^{-4}$ to $2.0 \cdot 10^{-3}$ m.s$^{-1}$). This indicates that axial diffusion is negligibly small in comparison with axial transport by convection.

Another assumption of the model is that no diglycerides and mono-glycerides are formed during conversion. In chapter 4 of this thesis it will be demonstrated that the level of these intermediary products remains low at low glycerol fractions. Besides, the description of kinetics using the power law relation as a function of fatty acids alone was proved to be satisfying (this thesis, chapter 4). Therefore the intermediary products will be neglected in the model.

The diffusion coefficient of components in infinitely diluted solutions can be calculated from molecular data by the relation of Wilke and Chang (6). For low concentrations of fatty acid in triglyceride, the diffusion coefficient is calculated to be $5.3 \cdot 10^{-11}$ m$^2$.s$^{-1}$; for low concentrations of triglycerides in fatty acids it is $2.4 \cdot 10^{-11}$ m$^2$.s$^{-1}$. Taking into account the law of mass conservation the diffusion coefficients of both components have to be identical for a mixture of two components. The diffusion coefficient of fatty acids in triglycerides can be calculated as follows (7):

$$D = (D_{12}^{\circ})^{(M_1)} \cdot (D_{21}^{\circ})^{(M_2)} \cdot \left(1 + \left(\frac{\partial \ln \alpha_1}{\partial \ln M_1}\right)\right) \qquad (5)$$

with $D$ the diffusion coefficient, $\alpha$ the activity coefficient and $M$ the molar fraction. Since triglycerides and fatty acids are similar molecules which do not interact strongly in a physical or chemical way, the lipid phase is expected to behave like a highly ideal mixture. Therefore the term $(\partial \ln \alpha_1)/(\partial \ln M_1)$ is approaching zero and the diffusion coefficient of the mixture will be in between the coefficients of both pure components, depending on the composition. In view of the relatively small difference between the coefficients, the average value ($3.85 \cdot 10^{-11}$ m$^2$.s$^{-1}$) was used for the mixture in all calculations.

Finally, the density of fatty acids or a mixture of fatty acids and triglycerides was assumed to be the same as the density of pure triglycerides. This assumption was confirmed by measurements.

The differential equation of a two-component system for laminar flow in a pipe (hollow fiber) with neglect of axial diffusion is given in equation 6 (8).

$$\frac{D}{r}\cdot\left(\frac{\partial^2 C}{\partial r^2}\cdot r + \frac{\partial C}{\partial r}\right) = \bar{v}\cdot\left(1-\frac{r^2}{R^2}\right)\cdot\frac{\partial C}{\partial l} \qquad (6)$$

with C the triglyceride concentration (kg.m-3). For single-pass conversion three boundary conditions can be formulated:

1) the radial flow profile is symmetrical; this includes that there is no concentration gradient in the central axis:

$$\left\{\frac{\partial C}{\partial r}\right\}_{r=0} = 0 \qquad (7)$$

2) the incoming oil contains triglycerides only:

$$C_{(l=0)} = 1 \qquad (8)$$

3) at the fiber wall the diffusion rate equals the reaction rate; The reaction rate was fitted to the power law relation yielding a reaction order of 1.75 (this thesis, chapter 4). The boundary condition can therefore be given by:

$$D\left\{\frac{\partial C}{\partial r}\right\}_{r=R} = -k\cdot C^{1.75} \qquad (9)$$

with k the reaction constant [$kg^{-0.75}.m^{3.25}.s^{-1}$], which represents the rate at zero fatty acid and glycerol concentrations, and which is equivalent to the standard conversionrate (SCR) which was introduced before (this thesis, chapter 2).

A numerical solution method was followed. The differential equation (eq. 6) was discretisized by the method of Crank Nicolson and the following form was obtained.

$$C_{i,j+1} - \left\{\frac{Ddl}{4\bar{v}\left(1-\frac{r^2}{R^2}\right)}\right\}\cdot\left\{\frac{C_{i+1,j+1}-2C_{i,j+1}+C_{i-1,j+1}}{dr^2} + \frac{C_{i+1,j+1}-C_{i-1,j+1}}{2r\,dr}\right\} =$$

$$C_{i,j} + \left\{\frac{Ddl}{4\bar{v}\left(1-\frac{r^2}{R^2}\right)}\right\}\cdot\left\{\frac{C_{i+1,j}-2C_{i,j}+C_{i-1,j}}{dr^2} + \frac{C_{i+1,j}-C_{i-1,j}}{2r\,dr}\right\} \qquad (10)$$

The subscripts *i* and *j* refer to the discrete radial and axial coordinates respectively.

This equation can be written for all coordinates *(i,j)*. All equations on a fixed axial position together form a tridiagonal matrix. Starting from $j=dl$ the boundary conditions give relations for $i=0$ and $i=R$ and also for all concentrations at coordinate $j=0$. The solution of this set of equations was performed by the Thomas-algorithm (9). In the same manner the next layer can be calculated up to the end of fiber.

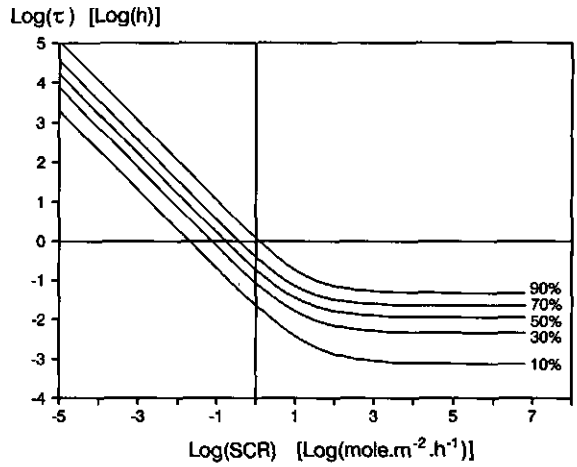


**Figure 3:** *Residence time (τ) needed for a lipid conversions of 10%, 30%, 30%, 70% an 90% (lipid fraction at the end of the fiber = 0.9, 0.7, 0.5, 0.3 and 0.1 kg.m$^{-3}$ respectively) as a function of rate constant k, expressed as SCR (mole.m$^{-2}$.h$^{-1}$). Diffusion limitation in the water phase is neglected in the model.*

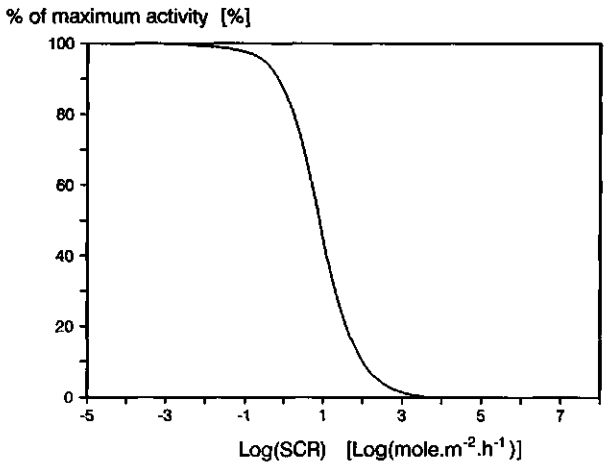


**Figure 4:** *The percentage of the calculated conversionrate over the maximum conversionrate (the amount of activity in the absence of diffusion limitation) plotted as a function of the maximum conversionrate (for a conversion of 50%) as calculated from the values shown in figure 3. Diffusion limitation in the water phase is neglected in the model.*

The number of steps is a critical parameter for the accuracy of the calculation. For each value of the reaction constant, the calculations were carried out for a wide range of step sizes. The results were converging for increasing step numbers and for the final calculations the step number was fixed to a value above which no considerable change in the results occurred. Typically for the fiber length 150 steps and for the fiber width 100 steps were taken. For different values of $k$ the fiber length necessary to attain a certain degree of conversion was calculated using the numerical algorithm. The residence time $\tau$, the time needed to obtain the required conversion, was calculated from the fiber length; the SCR was calculated from $k$. The resulting curves are shown in figure 3. For relatively low values of SCR, $\tau$ is proportional with the SCR, which implies that the reaction is rate-limiting. For relatively high values of SCR, $\tau$ is not influenced by SCR, which means that the conversion is not controlled by the reaction, but by the diffusion. In order to visualize the extent of inhibition due to concentration differences caused by diffusion, the calculated SCR was related to the maximum SCR (without diffusion limitation) and plotted in figure 4 (for a conversion of 50%).

From figure 3 and 4 appears that diffusion already influences the reaction at very low rates. Though, the extent of inhibition due to diffusion limitation remains relatively small for a broad range of reaction rates: while a diffusion inhibition of 1% is calculated for an SCR of 0.005 mole.$m^{-2}.h^{-1}$, the inhibition has only increased to a value of 5% for an SCR of 0.34 mole.$m^{-2}.h^{-1}$. It can be concluded that diffusion has only a minor influence on the activity for the reaction rates obtained until now in the membrane reactor (maximum rates 0.13 to 0.33 mole.$m^{-2}.h^{-1}$). Even when the conversion rate of the reactor could be increased by a factor 10, the inhibition due to diffusion remains will remain limited (30%). Almost complete diffusion limitation (> 99.6%) is reached for immobilized activities higher than 1000 mole.$m^{-2}.h^{-1}$. For extremely high conversionrates, the maximum conversionrate for complete diffusion limitation can be calculated from the intersection of the two asymptotes in figure 3: for a conversion of 90% it was calculated to be 24 mole.$m^{-2}.h^{-1}$. This means that, from the viewpoint of lipid-phase diffusion, the activity can be improved at maximum with a factor of about 100 as opposed to the reactor that is used now.

## CONCLUSIONS

Based on several assumptions and the glycerol diffusion measurements in a hollow fiber membrane module, the diffusion coefficient of glycerol in the cellulosic membrane was calculated to be $4.58 \cdot 10^{-10}$ $m^2.s^{-1}$. The deviation for the literature value for infinite dilution ($9.4 \cdot 10^{-10}$ $m^2.s^{-1}$) can be explained by wall-effects, or deviations from the assumptions made.

Although the model is rather an indication than a high-precision approximation, the results of the model can be used to get insight in the order of magnitude of the mass transport phenomena in the membrane: From the results it was concluded that glycerol diffusion will not be rate limiting for the range of activities obtained in the membrane reactor: for an SCR of 0.33 mole.$m^{-2}.h^{-1}$ (maximum value obtained) the calculated relative activity is 96.7%.

The diffusion in the lipid phase has a low impact on the reaction rate (< 5%) for reaction rates up to 0.34 mole.$m^{-2}.h^{-1}$, and 1% inhibition occurs at a rate of 0.005 mole.$m^{-2}.h^{-1}$. Therefore, with respect to lipid diffusion as well as glycerol diffusion, diffusion will not be rate limiting for the maximum conversionrates reached until now in the membrane reactor (0.13 to 0.33 mole.$m^{-2}.h^{-1}$). Normal conversionrates are an order of magnitude lower than the maximum conversionrate due to the presence of reaction products

and inactivation. Therefore, under reaction condition the influence of diffusion is even lower.

For the theoretical case of complete limitation of water/glycerol diffusion (occurring when $SCR \rightarrow \infty$ in figure 2) the conversionrate is 18.3 mole.m$^{-2}$.h$^{-1}$. Complete limitation of lipid phase diffusion ($SCR \rightarrow \infty$ in figure 3) results in a maximum conversionrate of 24 mole.m$^{-2}$.h$^{-1}$. Therefore, it can be concluded that lipid diffusion and glycerol diffusion are of the same order of magnitude and will both determine the reaction rate at extremely high conversionrates. For both the lipid and the glycerol diffusion limitation, the theoretical maximum observed activity (*ca* 20 mole.m$^{-2}$.h$^{-1}$) is a factor 60 higher than the maximum activity measured until now (0.33 mole.m$^{-2}$.h$^{-1}$).

## ACKNOWLEDGEMENT

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## SYMBOLS

| | | |
|---|---|---|
| $\alpha$ | [-] | activity coefficient |
| $A$ | [m$^2$] | membrane area |
| $c$ | [kg.m$^{-3}$] | glycerol concentration |
| $C$ | [kg.m$^{-3}$] | triglyceride concentration |
| $d$ | [m] | membrane thickness (8.0·10$^{-6}$ m) |
| $D$ | [m$^2$.s$^{-1}$] | intrinsic diffusion coefficient |
| $D_O$ | [m$^2$.s$^{-1}$] | overall diffusion coefficient |
| $J$ | [kg.m$^{-2}$.s$^{-1}$] | net mass transfer rate (water phase) |
| $k$ | [kg$^{-0.75}$.m$^{3.25}$.s$^{-1}$] | conversion rate per membrane area at $c=0$. |
| $K$ | [m.s$^{-1}$] | mass transfer coefficient (water phase) |
| $l$ | [m] | longitudinal coordinate |
| $M$ | [mole.mole$^{-1}$] | concentration (molar fraction) |
| $r$ | [m] | radial coordinate |
| $R$ | [m] | radius of fiber (1.075·10$^{-4}$ m) |
| SCR | [mole.m$^{-2}$.h$^{-1}$] | standard conversion rate: virtual rate at zero glycerol and fatty acid concentration, expressed as mole of fatty acid formed per hour, divided by the membrane area (this thesis, chapter 2) |
| $\bar{v}$ | [m.s$^{-1}$] | average superficial flow rate in fiber |
| $V$ | [m$^3$] | volume of circuit |
| $\beta$ | [-] | tortuosity of the membrane |
| $\Delta c_m$ | [kg.m$^{-3}$] | glycerol concentration difference across the membrane |

| | | |
|---|---|---|
| $\epsilon$ | [-] | porosity of the membrane |
| $\phi$ | [$m^3.s^{-1}$] | flux through the membrane |
| $\tau$ | [h] | residence time of lipid in the fiber |

## Subscripts:

| | |
|---|---|
| av | average |
| ec | external circuit |
| ic | internal circuit |
| *i* | discrete radial coordinate |
| *j* | discrete axial coordinate |
| *t* | time [s] |
| 1 | dissolved component (fatty acid) |
| 2 | solvent phase (triglyceride) |

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Chapter 4

# THE INFLUENCE OF FATTY ACID AND GLYCEROL ON THE KINETICS OF FAT HYDROLYSIS BY CANDIDA RUGOSA LIPASE IN A MEMBRANE REACTOR


## SUMMARY

The kinetics of lipid-hydrolysis by *Candida rugosa* lipase was investigated in a membrane reactor and in an emulsion system. Two models were chosen to describe the kinetics of the enzyme:
(1) The hydrolysis of triglycerides to fatty acids was considered to be a chain reaction with the intermediary products di- and mono-glyceride; each step was assumed to be a reversible second-order reaction. The reaction rate constants were determined from batch experiments. The experimental results could be described with this model.
(2) For process optimization and control, a model based on the power law was developed. For this model, the rate of hydrolysis was measured as a function of fatty acid and glycerol concentrations. Relations for the initial rate and equilibrium ester fraction as a function of the glycerol concentration were determined. Further, an order of 1.75 in the hydrolyzable ester fraction in the lipid phase was found to be valid for a wide range of glycerol concentrations. The model with order 1.75 gave much better results when compared to a similar model of order 1.0. Although simpler, the order 1.0 model can not be used. The power law model was applied in the simulation of a reactor composed of three modules. The fatty acid production rate was calculated for this reactor system as a function of the outcoming glycerol concentration at different conditions.

No substantial differences could be observed between the kinetics of the free enzyme and the membrane-immobilized enzyme.


## INTRODUCTION

In chapter 2 the immobilization of lipase (E.C. 3.1.1.3) in a hydrophilic hollow fiber membrane reactor was described. No wash-out of the enzyme could be observed in continuous experiments, and the stability was measured (half life time: 43 days). In batch experiments, the kinetics as a function of the fatty acid concentration was measured in the presence of a water phase without glycerol. The lipase used in that

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study (from *Candida rugosa*) is at the moment the most suitable enzyme for bulk, non-specific fatty acid production (1). When the enzyme is used for industrial production of fatty acids, the glycerol formed is a valuable side-product. It can be used for a number of applications such as cosmetics and the production of polyether polyols (2). Downstream processing is easier and less expensive for a water phase containing higher glycerol concentrations. To make this side product profitable, a minimum concentration of 0.033 mole.mole$^{-1}$ (15% [w/w]) is required (3). The kinetics of the hydrolysis reaction however will be influenced by the glycerol concentration: the equilibrium will shift to the ester-side and the reaction rate is expected to slow down. Therefore, for an optimization of the glycerol concentration the kinetics as a function of glycerol and fatty acid concentrations should be known.

The enzymatic hydrolysis of glycerides is a reaction in which the two substrates and the two products are present in two different phases; the lipid phase for glycerides and fatty acids, the water phase for water and glycerol. The enzyme (lipase) is active at the lipid/water interface and the enzyme-kinetics is a function of the concentration of substrates and products in the corresponding phases. The formation of free fatty acids from triglycerides takes place in different steps. The complete scheme of the reaction steps is given in figure 1. For the chemical-physical hydrolysis of lipids in a one-phasic system at high temperatures, a fundamental model based on figure 1 was developed and fitted to experimental results by Van der Sluijs (4). However, no complete study on the enzymatic hydrolysis kinetics could be found in literature, either for the *Candida rugosa* lipase or for other lipases.

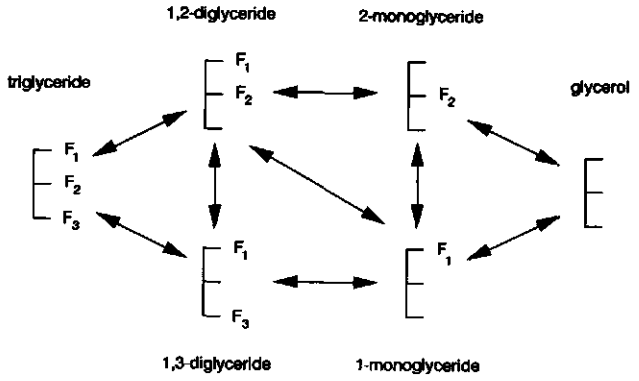


**Figure 1:** *The complete reaction scheme of hydrolysis of triglycerides. In each hydrolysis step a fatty acid molecule is produced and a water molecule is consumed, while in each esterification step a water molecule is produced and a fatty acid molecule is consumed. These products are omitted in the scheme.*

Yamane *et al.* (5) concluded that a first-order model was superior to a zero-order model. A first-order model was also tested by Linfield *et al.* (6,7) and a good fit was obtained for a limited range of substrate concentrations. However, the effect of accumulation of glycerol in the water phase was neglected. Similar results were obtained by Khor *et al.* (8).

The data of progress curves (fatty acid fraction *versus* time) catalyzed by *Pseudomonas fluorescens*

lipase were plotted in a double-reciprocal way by Kosugi *et al.* (9,10). A model based on a linear relation in such a plot was set up, in spite of a low correlation (no statistical data available). The maximum degree of hydrolysis, which was a variable parameter in the model, was found to be depending on the enzyme concentration. This is in contradiction with the thermodynamical theory of equilibria.

Another approach to the study of lipid phase kinetics is to vary the concentration of glycerides in an organic solvent. The ester concentration in the fat/solvent phase can be considered as the substrate concentration. In a hydrophilic membrane reactor with triacetin dissolved in toluene, Guit *et al.* (11) proved that the hydrolysis rate obeyed Michaelis Menten kinetics with respect to the triacetin concentration in the solvent. Michaelis Menten kinetics was also appropriate for interesterification in a one-phasic system (12). In other publications the variation of the substrate concentration in a solvent had an evident influence on kinetics (13,14,15) or no influence could be detected (16).

The adsorption of lipases to lipid interfaces can be described by a Lineweaver-Burk type adsorption isotherm (17); the activity reaches a plateau value when the lipid fraction in an emulsion is increased. From such a curve, an analogue of $K_m$ could be defined as the lipid fraction where the activity is half the maximum rate. This '$K_m$' however is dependent on the enzyme concentration, in contradiction to the $K_m$ in the Michaelis Menten kinetics. Therefore, the concept of Michaelis Menten kinetics is not valid for such a situation. For a study of kinetics the concentrations of substrate and products should refer to the phases they are dissolved in. The dependence on the surface area (or the lipid fraction in an emulsion) is a different subject.

It can be concluded that the literature on the kinetics in dependence of the fatty acid concentration is sparsely and not consistent. For process engineering calculations of the membrane bioreactor the knowledge of the kinetics is essential. It is the objective of this paper to provide these data.

In most publications the influence of glycerol in the water phase, and mono- and di-glycerides in the lipid phase are not included in the description of the kinetics. Since these factors will have an important influence on the kinetics, we tested a model of the kinetics based on the consecutive formation of diglyceride, monoglyceride and fatty acids plus glycerol. The isomers of mono- and diglycerides were not taken into account in view of the complexity of measurement and mathematics of such a model, so that the reaction scheme of figure 2 was obtained.

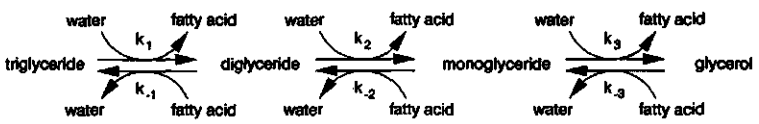


**Figure 2**: *The simplified reactionscheme based on 3 hydrolysis steps, including the water and fatty acid molecules formed or consumed.*

For optimization and control of the enzymatic lipid hydrolysis process, a more empirical description of the process can also be used. Input variables then should be easy to measure and the output should give the fatty acid formation rate and the glycerol formation rate. The most appropriate input variables are the ester fraction, the glycerol fraction and the net flow rates of the phases. In order to obtain such a model, equilibrium and non-equilibrium properties of the reactor were studied.

Kinetics measurements were made in the membrane reactor as well as in the emulsion system. In principle, the membrane reactor is more appropriate for the measurement of two-phase reaction kinetics than an emulsion since the interfacial area remains constant in the membrane reactor and the flow and conditions of the two phases can be controlled independently.

## EXPERIMENTAL

### Materials

The lipid phase was refined soybean oil of edible quality, supplied by Rhenus BV (Rhenen, The Netherlands). The lipase (*Candida rugosa*) was purchased from Meito Sangyo, Japan (type: "OF"). It contained 12.7% [w/w] of protein and was measured to have an activity of 65 ± 4 U/mg for tributyrin as a substrate at 30°C.

### Analytical methods and definitions

The glycerol- and water fractions were calculated from the refractive index of the water phase (18). The ester fraction (E, [-]) was defined as the molar ratio between the esterified fatty acids and the total amount of free- and esterified fatty acids. It was calculated from the acid value and the fatty acid content, measured by titration. The equilibrium ester fraction ($E_{eq}$) was defined as the ester fraction at reaction equilibrium conditions.

Two methods were used for the determination of the ester fraction in the emulsion experiments: (1) inactivation of the enzyme by immediate addition of a 20-fold volume of ethanol (containing phenolphtaleïn) to a sample of the emulsion and subsequent titration of the total emulsion; (2) inactivation of the enzyme by rapid heating up to 80°C of a sample of the emulsion, followed by centrifugation and titration of the lipid phase. Results of the two methods corresponded for larger reaction times. In the initial stage of the batch experiments method (1) was used in view of the faster inactivation.

The composition of the lipid phase in batch experiments was determined by HPLC gelchromatography. A lipid sample (30 ± 3 mg) was dissolved in 1 mL tetrahydrofuran, injected on two serial PLGEL gelpermeation columns (30 cm, Polymer labs.), and analyzed with a refractive index device. The peaks of triglyceride and diglyceride had a partial overlap. The choice of the integration method, either on basis of baseline separated peaks or on basis of tailing peak and rider, was made visually for each chromatogram. The mass concentration [kg.m$^{-3}$] was determined from the integrated data using calibration curves of the pure glycerides. The fractional concentration [mole.kg$^{-1}$] was calculated from these assuming an identical density for all lipid components (917 kg.m$^{-3}$).

## Membrane reactor experiments

The membrane module, produced by ENKA-Membrana AG, W-Germany, contained cellulose hollow fiber membranes (Cuprophan™) with a total membrane area of 1.36 $m^2$ or 1.20 $m^2$. The fibers had an internal diameter of 0.215 mm, a wall thickness of 0.016 mm and a length of 0.21 m; the membrane cut-off was 5.000 D for proteins.

Lipase (*Candida rugosa*) was immobilized to the cellulosic membrane by ultrafiltration of a lipase solution. The procedure described before (chapter 2) was applied here with some slight modifications: A lipase solution containing 0.5 g of crude lipase was ultrafiltrated at a pressure of 0.5 up to 0.6 bar, followed by ultrafiltration of 300 mL of demineralized water.

In the experimental set-up the lipid and water phase were recirculated and a continuous flow of soybean oil could be added to the lipid circuit, as was described in chapter 2. However the following conditions were different: The water phase had a total volume of 1100 mL; The recirculation flow of both phases was 40 mL.$min^{-1}$; The pressure-drop over the fibers of the membrane reactor (internal circuit) as caused by the flow of lipid was 0.1 up to 0.2 bar.

In a continuous experiment, the conversion rate (*CR*, [mole.$h^{-1}$.$m^{-2}$]) represents the amount of fatty acid formed per hour per unit of membrane surface area. *CR* is calculated from the net flow rate ($\phi$, [$m^3$.$h^{-1}$]), the difference in acid fraction in the in- and outgoing oil ($E_s$-$E$), the fatty acid molecular weight ($M_f$, [kg.$mole^{-1}$]) and the membrane surface area ($S$, [$m^2$]) as:

$$CR = \rho \cdot \frac{\phi \cdot (E_s - E)}{S \cdot M_f} \qquad (1a)$$

The conversion rate in continuous experiments with continuous replacement of the water phase and negligible glycerol concentration (index $g=0$, or $CR_{g=0}$) was transformed into the virtual activity at $E=1$ ($CR_{E=1,g=0}$) as described before (chapter 2), in which publication $CR_{E=1,g=0}$ was abbreviated to '*SCR*' (standard conversion rate). Besides this calculation, the conversion rate at $E=1$ was also measured in continuous experiments at controlled glycerol concentrations. In these experiments a high net flow of oil (20 mL.$min^{-1}$) was applied, resulting in an ester fraction above 0.97. The glycerol fraction was measured and adjusted if necessary.

The equilibrium ester fraction at different glycerol concentrations was determined in long term batch experiments and was defined as the ester fraction at the moment that no change in ester fraction could be observed during three days or more at a constant glycerol fraction in the water phase.

For *CR* experiments, the water compartment was filled with 1000 mL of a water/glycerol mixture. The glycerol concentration was determined frequently and adjusted if necessary. 500 mL of soybean oil was pumped in a single pass mode through the fibers and the reactor was filled and recirculated as described before for the batch experiment (chapter 2). The glycerol fraction was kept constant by a continuous replacement of the water phase, while the lipid phase was recirculated only (batch mode). Lipid samples were taken by an auto sampler device. The influence of the sampling on the reactor volume was corrected for. In these experiments, *CR* was calculated as:

$$CR = -\frac{\rho \cdot V}{M_f \cdot S} \cdot \frac{\partial E}{\partial t} \qquad (1b)$$

## Emulsion experiments

A stirred thermostated vessel with baffles was used for the emulsion experiments. The dimensions were as follows: vessel diameter: 100 mm; four-bladed paddle stirrer with blades 10 x 8 mm; four baffles of 10 x 100 mm (width x height). The stirring rate was 8 $s^{-1}$. The volume of the water/glycerol phase (containing 0.015% (w/v) sodium azide) was 600 mL, the lipid volume was 150 mL. In the thermostated vessel the phases were stirred and brought to 30°C during 20 minutes. Then 0.1 g of lipase was added. In a separate experiment it was demonstrated that under these conditions not the interfacial area but the amount of lipase is rate-limiting (chapter 6). Thus changes in interfacial area due to different process conditions do not influence the reaction rate. The reaction was followed for 7 to 15 hours, depending on the reaction rate. The conversion rate at $E=1$ (initial rate) was calculated from the initial slope of the progress curve. The equilibrium ester fractions at different glycerol concentrations were determined in batch experiments after 15 hours. The glycerol concentration of the water phase was measured at the end of the experiments after phase separation. The conversion rate as a function of the ester fraction was determined in batch experiments. The increase of the glycerol fraction in the water phase was less than 0.004 mole.mole$^{-1}$ in all emulsion experiments, due to the high water/oil ratio.

For the emulsion system as well as the membrane reactor, the conversion rate as a function of the ester fraction was calculated from the progress curves ($E$, $t$ profiles) by differentiation of polynomal fits as described in a chapter 2.

## Counter- and co-current experiments with 3 modules

For the test of the empirical power law model, experiments were carried out with three membrane modules placed in series (module A, B, C). In a previous contuous experiment, the Standard Conversion Rate ($CR_{t=0,E=1,g=0}$) of each module was determined. For the series experiment, the lipid and water phase of each module was recirculated and a net flow of lipid and water was applied which was small (< 40 mL.h$^{-1}$) as compared to the recirculation flow (ca. 1800 mL.h$^{-1}$). Therefore, the water- and lipid phases of each single module can be considered as ideally mixed phases and the process consists of three ideally mixed reactors in series with co- or counter-current flow of lipid and water phases. A net flow of lipid substrate (34.2 mL.h$^{-1}$ of soybean oil) was added to module A, and by overflow devices from A → B → C. A net water flow was applied similarly either in a co-current mode (A → B → C), or in a counter-current mode (C → B → A). The net water flow was 6.52 mL.h$^{-1}$ for the co-current experiment and 13.55 mL.h$^{-1}$ for the counter-current experiment.

## THEORY

### Model based on 3 hydrolysis steps

A model based on a reaction-scheme of 3 consecutive hydrolysis steps as shown in figure 2 was used here. Elaboration of this scheme yields the following relations (eq. 2 to 5), where $T$ = triglyceride concentration, $D$ = diglyceride concentration, $M$ = monoglyceride concentration, $F$ = fatty acid concentration, $G$ = glycerol concentration, $W$ = water concentration, all expressed as mole.kg$^{-1}$:

$$\frac{dT}{dt} = -k_1 \cdot T \cdot W + k_{-1} \cdot D \cdot F \qquad (2)$$

$$\frac{dD}{dt} = k_1 \cdot T \cdot W - k_{-1} \cdot D \cdot F - k_2 \cdot D \cdot W + k_{-2} \cdot M \cdot F \qquad (3)$$

$$\frac{dM}{dt} = k_2 \cdot D \cdot W - k_{-2} \cdot M \cdot F - k_3 \cdot M \cdot W + k_{-3} \cdot F \cdot G \qquad (4)$$

$$\frac{dF}{dt} = k_1 \cdot T \cdot W - k_{-1} \cdot D \cdot F + k_2 \cdot D \cdot W - k_{-2} \cdot M \cdot F + k_3 \cdot M \cdot W - k_{-3} \cdot G \cdot F \qquad (5)$$

The equilibrium constant of each step is defined as the ratio between the two rate constants, as:

$$K_n = \frac{k_n}{k_{-n}} \qquad (6)$$

n = 1, 2, 3.

The overall equilibrium constant can be derived from the product of the equilibrium constants of the three different steps, as:

$$K_{ov} = K_1 \cdot K_2 \cdot K_3 = \frac{D \cdot F}{T \cdot W} \cdot \frac{M \cdot F}{D \cdot W} \cdot \frac{G \cdot F}{M \cdot W} = \frac{G \cdot F^3}{T \cdot W^3} \qquad (7)$$

The measured data were normalized to $CR_{g=0}$ to correct for differences between membrane modules and were fitted to the model as presented by the equations 2 - 6 using the non-linear fit algorithm of Marquardt (19). The resulting concentration, time model curves were calculated numerically. The fatty acid mass balance was used to check the results of the calculations.

## RESULTS AND DISCUSSION

### Model based on 3 hydrolysis steps

The results of the measurements of mono- di- and triglycide concentrations in batch hydrolysis experiments are shown in figures 3, 4 and 5 for different glycerol concentrations. The model based on 3 hydrolysis steps (figure 2, equations 2-6) was applied to these data. The rate constants were calculated by fitting the total set of measured lipid phase concentrations (mono- di- triglycerides of figures 3 - 5 and

the fatty acid concentrations not shown) to the model, after correction for the differences in $CR_{t=0,E=1,g=0}$ of the different modules. The resulting rate constants are presented in table A. The model curves of the different experiments are also shown in figures 3, 4 and 5. The results indicate that the model, with second-order kinetics of each individual step, can describe the reaction data. This has not been reported before for the two phasic lipid hydrolysis. When the experiments at different glycerol concentrations were fitted individually to the model, a somewhat higher correlation was observed (data not shown). However, the rate constants then are different for the different glycerol concentrations. Such differences are not very likely kinetically.

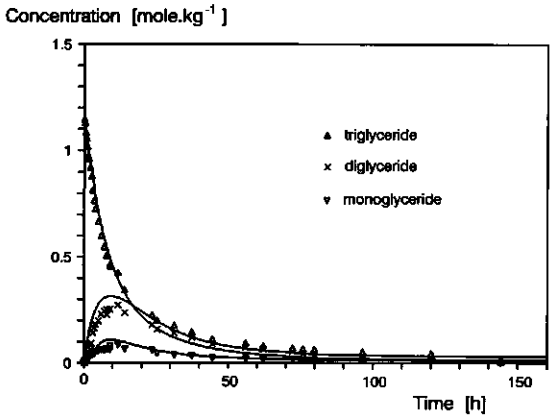


**Figure 3:** *Measurements of batch conversion in the membrane reactor at a glycerol fraction of 0 mole.kg$^{-1}$ in the water phase and curves of the model based on 3 hydrolysis steps.*

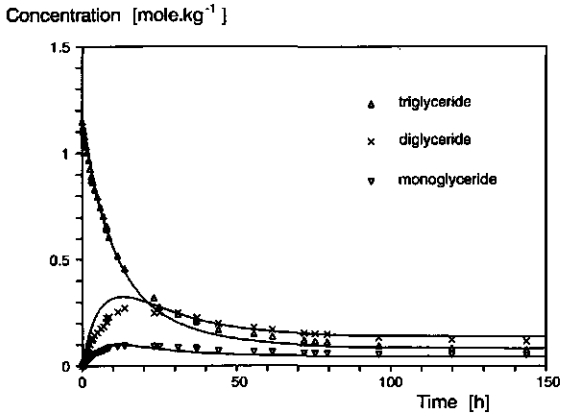


**Figure 4:** *Measurements of batch conversion in the membrane reactor at a glycerol fraction of 3.26 mole.kg$^{-1}$ (30%, w/w) and curves of the model based on 3 hydrolysis steps.*

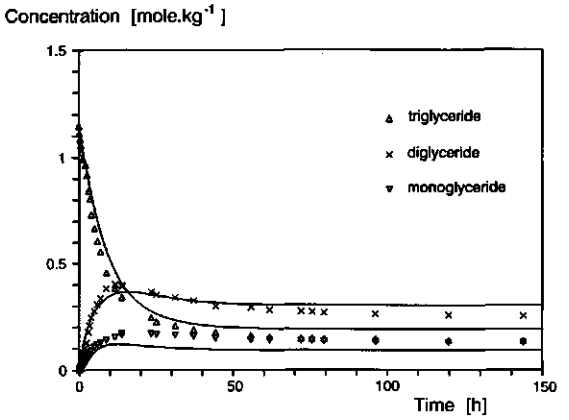


**Figure 5:** *Measurements of batch conversion in the membrane reactor at a glycerol fraction of 5.43 mole.kg$^{-1}$ (50%, w/w) and curves of the model based on 3 hydrolysis steps.*

| step | T → D | D → M | M → G |
|---|---|---|---|
| $k_+$ | 0.061 | 0.122 | 0.250 |
| $k_-$ | 0.488 | 5.12 | 0.055 |
| K | 0.125 | 0.024 | 4.55 |
| $K_{ov} = K_1.K_2.K_3 = 1.37 \cdot 10^{-2}$ | | | |

**Table A:** *Calculated rate constants [kg.hr$^{-1}$.mole$^{-1}$] and equilibrium constants [-]*

## Equilibrium

The equilibrium conditions were determined by measuring the equilibrium ester fractions in several batch experiments with differing glycerol concentrations for an emulsion as well as a membrane system. The results of these experiments (figure 6) indicate that no differences exist between emulsion and membrane system. This can be expected since the equilibrium situation is determined thermodynamically.

The emulsion-data show a linear relationship with a high correlation (correlation coefficient = 0.98) in the range of glycerol fraction from 0 to 0.24 mole.mole$^{-1}$, or 0 to 61% [w/w]. Extrapolation of this line to a glycerol fraction of 1 yields an $E_{eq}$ of 1.75 instead of the theoretical value of 1.0. The relation will therefore deviate from linearity in the range of 0.24 to 1 mole.mole$^{-1}$.

Figure 6 also shows the equilibrium curve obtained from $K_{ov}$ (eq. 7) of the model based on three hydrolysis steps. Also this model prediction can be concluded to be in a good agreement with the measured equilibrium data.

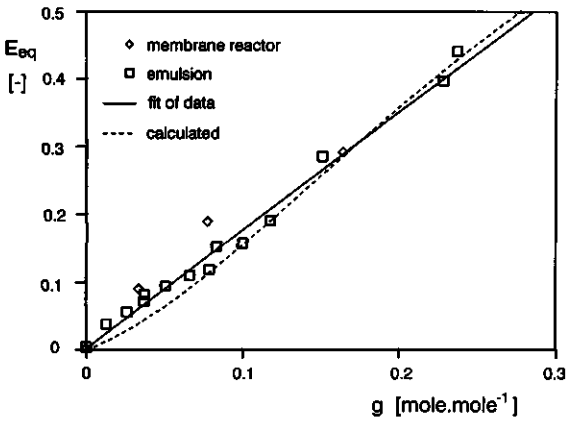


**Figure 6:** *Equilibrium ester fractions in emulsion and membrane reactor as a function of glycerol mole-fraction in the water phase. The straight line is the fit of the measured data, as described by equation 8. The dotted line represents the equilibrium as calculated from the model based on 3 hydrolysis steps (equation 7, data from table A).*

## The conversion rate at E=1 as a function of the glycerol fraction

The initial rate of hydrolysis ($CR_{E=1}$) was measured as a function of the glycerol fraction for membrane reactor and emulsion system. In figure 7, the reciprocal normalized rate at $E=1$ as a function of the reciprocal water content is shown. A linear relationship fits the data. For comparison, in figure 7 also the initial rate is shown as calculated from the model based on 3 hydrolysis steps (dotted line). It predicts the same trends, however with a quantitative deviation up to about 30%.

The mode of plotting in figure 7 corresponds with the Lineweaver-Burk plot used in the description of Michaelis Menten kinetics. Yet, Michaelis Menten kinetics is not valid here because the intercept in figure 7 has a negative value (-6.85), and a statistical analysis excludes the intercept to be positive. For Michaelis Menten kinetics this would lead to the impossibility that the maximum conversion rate (the reciprocal of the intercept) has a negative value. With Michaelis Menten kinetics valid it could be expected that for high water fractions the reaction would become pseudo zero-order in the water-substrate, resulting in a plateau-value of the initial rate. From the results presented here it appears however that the conversion rate increases with increasing water content up to the situation of pure water. The increase of activity at increasing water fractions in glycerol was shown before by Hoq *et al.* (20). It can be concluded that Michaelis Menten kinetics are not valid for the hydrolysis reaction in function of the water concentration.

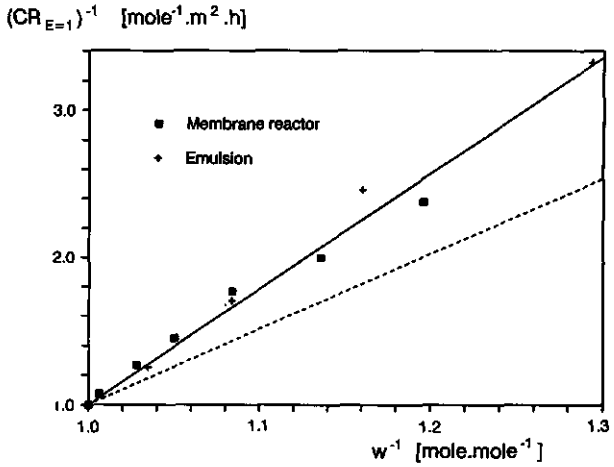


**Figure 7:** *Double reciprocal plot of the initial conversion rate normalized to a conversion rate of 1 at g=0, as a function of the water fraction (1-g) for membrane reactor and emulsion. The dotted line represents the initial rate as calculated from the model based on 3 hydrolysis steps, with the data of table A (normalized to $k_1$ = 1).*

## Power law model

The power law model was based on the following three kinetical descriptions: (a) the influence of glycerol on the equilibrium ester fraction, (b) the influence of glycerol on the conversion rate at $E=1$, and (c) the power law relation.

**(a)** The relation for the equilibrium ester fraction ($E_{eq}$) as a function of the glycerol fraction can be derived from the straight line in figure 6, as:

$$E_{eq} = \alpha_1 \cdot g \qquad (8)$$

with $\alpha_1$ = 1.75 [mole.mole$^{-1}$]

**(b)** The conversion rate at $E=1$ is decreasing with increasing glycerol fraction (figure 7) and can be described as:

$$CR_{E=1} = CR_{E=1,g=0} \cdot \left( \alpha_2 + \frac{\alpha_3}{1-g} \right)^{-1} \qquad (9)$$

where: $w$ = 1 - $g$ ; $\alpha_2$ = -6.85 [-] ; $\alpha_3$ = 7.85 [mole.mole$^{-1}$]

This description is valid in the range of glycerol fractions from 0 to 0.24 mole.mole$^{-1}$.

**(c)** In order to test the suitability of the power law relation the conversion rate was determined as a function of the ester fraction at different glycerol fractions in batch experiments in the membrane reactor and the emulsion system. The conversion rate was normalized by using the ratio of $CR$ and $CR_{E=1}$. The ester fraction was normalized by using the fraction $(E-E_{eq})/(1-E_{eq})$. The results are shown in figures 8 and 9 for the membrane reactor and the emulsion system respectively. Ester fractions within 15% deviation from

the equilibrium ester fraction ($\ln\{(E-E_{eq})/(1-E_{eq})\} < 2$) are not shown because in this range the relative error in conversion rate is unacceptably high (more than 50%). Curve fit assuming a linear relation between $\ln(CR/CR_{E=1})$ and $\ln\{(E-E_{eq})/(1-E_{eq})\}$ for the different experiments at several glycerol fractions in membrane reactor and emulsion system gives an average slope of 1.75 (figure 8 and 9; slope 1.75 is indicated by the dotted line). Based on this value, the power law model reads:

$$\frac{CR}{CR_{E=1}} = \left(\frac{E - E_{eq}}{1 - E_{eq}}\right)^{1.75} \qquad (10)$$

Equation 10 can be combined with equatic 's 8 and 9 to give a description of the kinetics as a function of ester fraction, glycerol fraction and $CR_{E=1,g=0}$ as:

$$CR = CR_{E=1,g=0} \cdot \left(\frac{E - a_1 g}{1 - a_1 g}\right)^{1.75} \cdot \left(a_2 + \frac{a_3}{1-g}\right)^{-1} \qquad (11)$$

For the prediction of the product concentrations in membrane reactor experiments, the time dependence should be introduced in equation 11. On assumption of a first-order inactivation (chapter 2), the conversion rate can be written as a function of time as:

$$CR = CR_{t=0,E=1,g=0} \cdot e^{-k_i \cdot t} \cdot \left(\frac{E - a_1 g}{1 - a_1 g}\right)^{1.75} \cdot \left(a_2 + \frac{a_3}{1-g}\right)^{-1} \qquad (12)$$

$CR_{t=0,E=1,g=0}$ can be determined from the steady state conditions of a previously performed continuous experiment using relations 1-a and 11 or, when the glycerol fraction is zero, using the formula for the *SCR* (chapter 2).

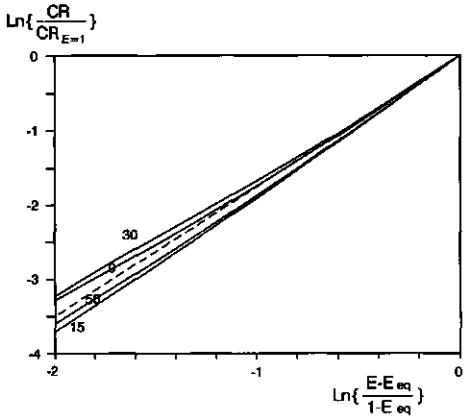


**Figure 8:** *Double logarithmical plot of normalized conversion rate and normalized ester fraction of the batch experiments in the membrane reactor at glycerol concentrations of 0.0 mole.mole$^{-1}$ (0% [w/w]), 0.0334 mole.mole$^{-1}$ (15% [w/w]), 0.0774 mole.mole$^{-1}$ (30% [w/w]) and 0.164 mole.mole$^{-1}$ (50% [w/w]). Each line is derived from a batch experiment performed at the glycerol concentration (% w/w) denoted to that line.*

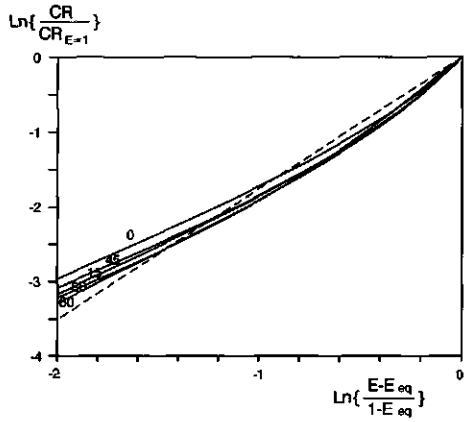


**Figure 9:** *Double logarithmical plot of normalized conversion rate and normalized ester fraction of the batch experiments in the emulsion at glycerol concentrations of 0.0 mole.mole$^{-1}$ (0% [w/w]), 0.0334 mole.mole$^{-1}$ (15% [w/w]), 0.0774 mole.mole$^{-1}$ (30% [w/w]), 0.138 mole.mole$^{-1}$ (45% [w/w]) and 0.227 mole.mole$^{-1}$ (60% [w/w]). Each line is derived from a batch experiment performed at the glycerol concentration (% [w/w]) denoted to that line.*

The model of equation 12 was applied to the measured data in order to visualize the deviation of the model as compared to the measurements. Also the sometimes used and simpler model with an order of 1.0 was applied. Since in the membrane reactor experiments the glycerol fraction remained constant, a description of the ester concentration as a function of time could be obtained by numerical integration of equation 11. $CR_{t=0,E=1,g=0}$ was determined in a previously performed continuous experiment and the constants $a_1$ to $a_3$ were as given above. The model of order 1.75 appeared to give a good description of the measurements, contrary to the model of order 1.0 which has a much lower correlation. An example of the fits is shown in figure 10. In figure 10 also a curve is shown of the first-order model fitted to the initial rate instead of the set of data points. Also with this method the model of order 1.0 is valid only in a very limited range. The literature data are in agreement with this observation: the application of a first-order model by Linfield *et al.* (6) gave a good correlation only in a limited range of substrate fractions.

Mass transfer calculations of lipid diffusion in the lipid phase and glycerol diffusion in the membrane showed out that mass transfer is not rate limiting in the membrane reactor (chapter 3). However, in the membrane reactor the lipase is present in an immobilized form and though the adsorptive immobilization is a very mild method, immobilization could affect the kinetical properties. The results in figure 6-9 however show that there is little or no difference between the membrane reactor and the emulsion system. Therefore it can be concluded that adsorption on the cellulosic membrane does not affect the lipase kinetics.

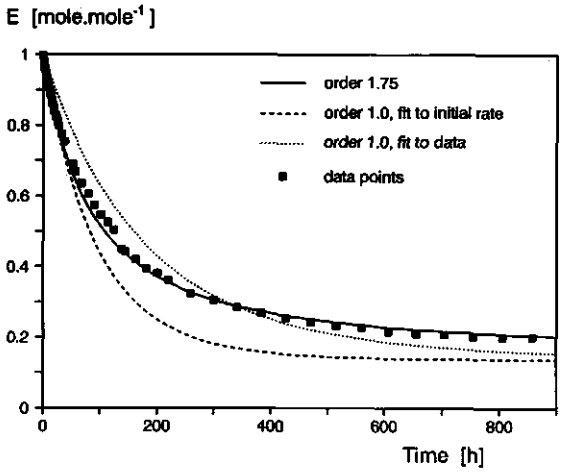


**Figure 10:** *Data of a batch experiment in the membrane reactor at a glycerol fraction of 0.0774 mole.mole$^{-1}$ (30% [w/w]) and the fits of models with order 1.75 and 1.0.*

## Counter- and co-current experiment with 3 modules; test of the power law model

The experimental test was done on three modules placed in series. Beforehand, the activity at $t=0$ ($CR_{t=0,E=1,g=0}$) and the stability (inactivation constant, $k_i$) of each module was determined in continuous experiments. The results are shown in table B. Then, the reactors were first operated in counter-current mode and next in co-current mode. In each situation the concentrations of ester and glycerol were followed until the change of concentration in time was according to the inactivation rate. This situation was reached after 219 h and 504 h, for the co-current and counter-current experiment respectively.

| | Module A | Module B | Module C |
|---|---|---|---|
| $CR_{t=0,E=1,g=0}$ [mole.h$^{-1}$.m$^{-2}$] | 0.1803 | 0.0662 | 0.0794 |
| $k_i$ [h$^{-1}$] | $1.96 \cdot 10^{-3}$ | $1.36 \cdot 10^{-3}$ | $1.81 \cdot 10^{-3}$ |

**Table B:** *Conversion rates and inactivation constants for the three modules used for the application of the empirical power law model.*

In equation 12, the conversion rate was expressed in terms of ester fraction and lipid net flow rate as given in equation 1-a, and an analogous equation was made in terms of glycerol fraction and water net flow rate (equation not shown). Thus, for the 3 modules in series, 6 equations with 6 unknown variables were obtained, which could be solved. The model calculations and measured data of a counter-current and a co-current experiment are shown in table C.

The model predicts the results for the lipid and water phase and the limited deviations are not showing an apparent trend. Possible causes of error in the glycerol fraction are the evaporation of water from the water-phase vessel and the formation of mono- and di-glycerides. HPLC analysis confirmed the occurrence of small amounts of mono- and diglycerides, particularly at higher glycerol fraction.

| | module A | | module B | | module C | |
|---|---|---|---|---|---|---|
| | *model* | *meas.* | *model* | *meas.* | *model* | *meas.* |
| **Co-current** | | | | | | |
| Ester fraction [mole.mole$^{-1}$] | 0.392 | 0.386 | 0.580 | 0.560 | <u>0.750</u> | <u>0.740</u> |
| glycerol fraction [mole.mole$^{-1}$] | 0.0202 | 0.0208 | 0.0416 | 0.0494 | <u>0.0803</u> | <u>0.0791</u> |
| **Counter-current** | | | | | | |
| Ester fraction [mole.mole$^{-1}$] | 0.465 | 0.449 | 0.601 | 0.606 | <u>0.751</u> | <u>0.760</u> |
| glycerol fraction [mole.mole$^{-1}$] | <u>0.0286</u> | <u>0.0326</u> | 0.0207 | 0.0197 | 0.0125 | 0.0117 |

**Table C:** *Measurements and model predictions in counter- and co-current experiment. Underlined are the measured values and the values predicted by the model for the outflow of the reactor cascade.*

## Simulations with the power law model

From the measurements above it appears that in the counter-current mode a high final glycerol fraction can be obtained without a dramatic decrease of the activity. The minimum glycerol concentration for efficient downstream processing (0.0334 [mole.mole$^{-1}$] or 15% [w/w]) can easily be reached. It is self-evident that the efficiency (fatty acid production rate) of the process is lower for higher glycerol concentrations. Using the power law model, the fatty acid production rate of a process can be calculated as a function of the outgoing glycerol fraction for the glycerol range where the model is valid (0 to 0.23 mole.mole$^{-1}$) and a comparison can be made for co-current and counter-current flow mode. In industrial processing the lipid phase is required to contain an ester fraction of no more than about 0.05 mole.mole$^{-1}$. Taking these requirements into account the fatty acid production rate was calculated using the model based on equation 12. This was done so for counter- and co-current flow in a series of three reactors

(each with and $S = 1.2$ m$^2$ and an *SCR* value (= $CR_{t=0,E=1,g=0}$) of 0.066 mole.m$^{-2}$.h$^{-1}$). Figure 11 shows the fatty acid production rate as a function of the outgoing glycerol fraction for different water-phase net-flows, resulting in different glycerol fractions. The slope of productivity *versus* glycerol concentration is higher in the co-current mode than in counter-current, as was expected. The model shows that at a glycerol fraction of 0.0334 mole.mole$^{-1}$ (which is the minimum concentration for glycerol recovery) the efficiency of the counter-current process has decreased by 76%. This concentration can not be reached in the co-current process since the equilibrium ester fraction is higher than 0.05 for this glycerol fraction (equation 8).

The model was also used to calculate the productivity at a final ester fraction of 0.75 for co- and counter-current in function of the glycerol fraction (figure 11). The influence of glycerol on the reaction rate and equilibrium is expected to be less severe for this lower conversion value. Indeed, the activity-decrease in the presence of glycerol is less and the difference between co- and counter-current is smaller.

The model calculations above indicate that the empirical power law model can be used for process optimization of the enzymatic fat hydrolysis.

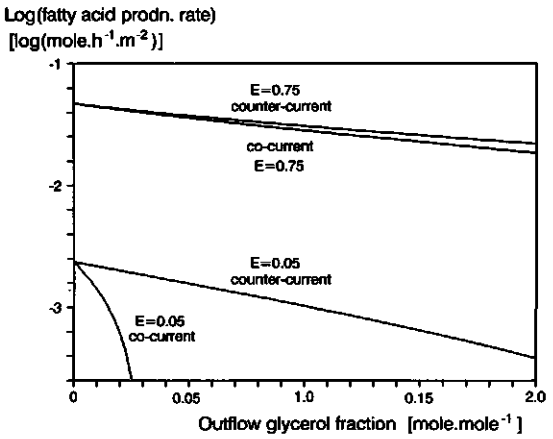


**Figure 11:** *Calculations based on the emperical model: the fatty acid production rate [mmole.h$^{-1}$] as a function of final glycerol fraction for final ester fractions of 0.05 and 0.75 for counter- and co-current operation of 3 membrane reactors.*

## ACKNOWLEDGEMENT

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## SYMBOLS

$a_1$ [mole.mole$^{-1}$], $a_2$ [-], $a_3$ [mole.mole$^{-1}$]: constants in power law model

| | |
|---|---|
| $CR$ | conversion rate [mole.h$^{-1}$.m$^{-2}$] |
| $D$ | diglyceride concentration [mole.kg$^{-1}$] |
| $E$ | ester fraction [-] |
| $E_{eq}$ | equilibrium ester fraction |
| $E_s$ | ester fraction of the inflow oil |
| $F$ | fatty acid concentration [mole.kg$^{-1}$] |
| $g$ | glycerol fraction [mole.mole$^{-1}$] |
| $G$ | glycerol concentration [mole.kg$^{-1}$] |
| $k$ | rate constant [kg.h$^{-1}$.mole$^{-1}$]; subscripts in k refer to the steps in figure 2 |
| $k_i$ | inactivation constant [h$^{-1}$] |
| $K$ | equilibrium constant [-]; subscripts in K refer to the steps in figure 2 |
| $K_{ov}$ | overall equilibrium constant [-] |
| $K_m$ | Michaelis Menten constant |
| $M$ | monoglyceride concentration [mole.kg$^{-1}$] |
| $M_f$ | average molecular weight of the fatty acids in soybean oil (0.278 kg.mole$^{-1}$) |
| $S$ | membrane surface area [m$^2$] |
| $SCR$ | Standard Conversion Rate [mole.m$^{-2}$.h$^{-1}$] |
| $T$ | triglyceride concentration [mole.kg$^{-1}$] |
| $t$ | time [h] |
| $V$ | volume of the lipid phase [m$^3$] |
| $w$ | water fraction [mole.mole$^{-1}$] |
| $W$ | water concentration [mole.kg$^{-1}$] |
| $\phi$ | net flow rate [m$^3$.h$^{-1}$]) |
| $\rho$ | density of soybean oil (917 kg.m$^{-3}$) |

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Chapter 5

# PARAMETERS INFLUENCING HYDROLYSIS KINETICS OF LIPASE IN A HYDROPHILIC MEMBRANE BIOREACTOR


## SUMMARY

The hydrolysis of lipids by *Candida rugosa* lipase was studied in a membrane reactor as a function of several reactor parameters: temperature, pH, $Ca^{2+}$ and $Na^{+}$ in the water phase.

The initial hydrolysis rate of the free enzyme as a function of the temperature was shown to follow Arrhenius kinetics with an activation energy of 12.3 kJ.mole$^{-1}$ in the range of 25°C to 55°C. Above 55°C the inactivation is too fast for an accurate determination of the initial rate. Literature data about the effect of temperature on the hydrolysis rate of *Candida rugosa* are not consistent, possibly due to different measuring methods.

The influence of temperature on the stability was investigated for the membrane reactor and for the free enzyme. A stabilizing effect of immobilization was observed in the membrane reactor as compared to the free enzyme. The immobilized enzyme was a factor four more stable; the activation energy for the inactivation reaction was found to be 201 kJ.mole$^{-1}$ for both free and immobilized enzyme.

Combination of the relations for activity and stability rendered a model which described the activity and productivity as a function of temperature and time. The results of model calculations showed that for long operation times like that used in a membrane reactor, the total productivity can increase with decreasing temperature. The optimal temperature is in the range of 25 - 30°C. However, a lower limit of the operating temperature is the melting point of the lipid substrate used.

The pH optimum for the initial activity of free as well as membrane-immobilized lipase appeared to be 6.5 - 7.0. In the acidic range the immobilized enzyme had a higher activity than the free enzyme. Above



This chapter was submitted for publication by the authors Wouter Pronk, Gerard Boswinkel and Klaas Van 't Riet.

pH 7 the membrane reactor was clogged by the formation of soaps.

In the presence of $CaCl_2$ (10 mM) the stability of the membrane-immobilized lipase increased with more than a factor 10. The effect of NaCl was less favourable: At low concentrations (0.1 M) no influence was observed while at higher concentrations (1 M) the stability decreased with a factor 20.

## INTRODUCTION

The enzymes of the class lipase (EC 3.1.1.3) have been studied intensively for the last few years (1,2). The research efforts are directed towards several types of reactions catalyzed by lipases such as esterification, hydrolysis and interesterification (transesterification). The lipase from the yeast *Candida rugosa* (formerly: *Candida cylindracea*) is an appropriate enzyme for the hydrolysis of lipids due to its availability and price. The enzyme can be used for hydrolysis of all kinds of triglycerides since it does not possess specificity towards position and only a very limited specificity towards the fatty acid moiety (3,4).

In view of the reusability of the enzyme, many different methods of immobilization were applied. Covalent methods were used to immobilize lipase to polysaccharides (5), carboxymethyl-cellulose (6) and polyacrylamide gels (7). Another method applied for immobilization of lipases is entrapment in a photocrosslinkable resin (8). Finally, adsorption was used to immobilize lipase to amphiphilic gels (9) and to different types of membranes (10,11,12). The immobilization of *Candida rugosa* lipase on cellulose membranes for the hydrolysis of lipids was described in chapter 2. The opportunity of this membrane reactor is that the enzyme is immobilized at the interface of the lipid and water phase: the water phase impregnates the hydrophilic membrane, while the lipid phase at the other side of the membrane (where the lipase is immobilized) can not permeate the hydrophilic membrane. Soybean oil was used as a model-substrate and the temperature was kept at 30°C in all these studies.

The effect of operating parameters on the activity of free and immobilized enzyme should be known for an optimal use of the enzyme. The influence of fatty acid and glycerol on the kinetics of the reaction will be described in chapter 4 of this thesis. From these data, the net flows of lipid and water phase can be optimized in a continuous process. Additionally, the influence of pH, temperature and effectors should be analyzed. Investigations of these parameters are reported in this chapter.

The role of pH of the water phase is complicated in the hydrolysis of triglycerides. As for other enzymes, there will be a pH value or pH range for lipase at which the conformation of the enzyme as a catalyst is optimal, while extreme pH conditions will cause an irreversible inactivation of the enzyme. On the other hand, the pH determines in which state the free fatty acids are present. At pH values above the dissociation constant the fatty acids will dissociate and are able to form soaps when cations are present. Ionized fatty acids will more easily dissolve in the water phase; soaps will adsorb to the water/lipid interface or form micelles in the water phase. Due to the complexity of phenomena and the lack of literature data, the influence of pH cannot be predicted, and has to be measured.

The **temperature** of the reactor has a dualistic effect. A higher temperature increases the initial activity of the enzyme, but decreases its stability. Therefore, activity and stability both should be investigated. The results can be used to model the activity of the lipase as a function of temperature and time.

Lipases are generally known to be influenced by **effectors**, but the effect is strongly dependent of the type of lipase and the conditions. The effects of known effectors like $CaCl_2$ and NaCl have to be investigated.

## MATERIALS AND METHODS

### Materials and analysis

The lipase of *Candida rugosa* (type 'OF') was purchased from Meito Sangyo, Japan. The lipid phase was soybean oil of edible quality which was supplied by Rhenus B.V., The Netherlands. Hydrophilic cellulose hollow fiber membranes (1.36 or 1.2 $m^2$), made of Cuprophan™, were kindly provided by ENKA Inc.. The fatty acid content was determined by titration of the free fatty acids as described before (14).

### Effect of temperature and pH on free lipase

The activity of free lipase with tributyrin as a substrate was measured in the following assay: 1 mL of tributyrin with 50 mL of a solution containing 0.1% [w/v] Arabic gum, 2 mM maleic acid, pH 6, was sonified in a Branson "B-12" ultrasonic emulsifier. The emulsion was stirred in a vessel thermostated to a predetermined temperature and the pH was adjusted to the desired value. The enzyme solution (0.1 mg of previously dissolved crude enzyme) was added and the pH was maintained constant by the addition of 0.01 mM of NaOH. One unit is defined as the amount of active lipase requiring the addition of 1 µmole of NaOH per minute. In the experiments with varying pH, the temperature was 30°C; in experiments with varying temperature, the pH was 6.0.

The activity of free lipase with soybean oil as a substrate was measured with an emulsion in a stirred vessel. The vessel had a diameter of 8.9 cm and four baffles of 12 x 1 cm. A 4-bladed paddle-stirrer was used with blades of 1 x 0.8 cm. The stirring speed was set to 8 $s^{-1}$ and the temperature was maintained at 30°C by means of a water jacket. The water phase (600 mL) was buffered in the same way as described for the membrane reactor, the lipid phase was soybean oil of edible quality (150 mL).

The effect of temperature on the stability of free lipase was determined by incubating a lipase solution in water (10 mg/mL) in a water bath. At regular time intervals, samples were taken and the remaining activity was determined at a temperature of 30°C and pH 6 by the tributyrin assay described above. The experiments were carried out *in duplo*.

## Effect of temperature and pH on activity of lipase in the membrane reactor

Immobilization of *Candida rugosa* lipase onto the cellulosic membrane was carried out by ultrafiltration of a lipase solution in the internal circuit, resulting in adsorption of the enzyme to the inner wall of the fibers. The lipid phase was circulated through the lumen of the fibers and the water phase was circulated in the shell of the membrane module. The exact procedure of immobilization and operation of the reactor has been described before (chapter 3). The lipid substrate was soybean oil of edible quality.

The effect of **temperature** was investigated in a membrane reactor operated in a continuous mode. After a stabilization period of at least 110 hours, the temperature of the reactor system was adjusted by regulation of the thermostated case in which the reactor was placed. The activity of the reactor was calculated from the net flow rate and the degree of hydrolysis. The activity was converted into the conversion rate at zero fatty acid content, which was described as 'Standard Conversion Rate' (*SCR*) in chapter 3. The *SCR* was followed in time until the initial value had decreased for at least 30%. The glycerol concentration was kept below 0.5% [w/w] by replacing the water phase frequently. The pH (6 ± 0.2) was corrected if necessary.

The effect of **pH** was measured in a reactor operated in the continuous mode at 30°C. The buffer was 0.01 M sodium phosphate, except for pH 3 and 2.5 where a citrate buffer was used (0.01 M). The pH value was measured and controlled within ± 0.2. The activity of the reactor was measured as described in the section for the temperature experiment, and was followed for at least 60 h. The glycerol concentration was controlled in the way given above. In between the experiments at different pH, the activity of the reactor was measured by a change of the water phase to pH 6 for 60 hrs. The activity in the pH-experiment was corrected for the decrease in activity with time as measured at pH 6.

## The influence of effectors

The influence of NaCl was investigated in the membrane reactor: after immobilization and stabilization of the activity, 0.1 M NaCl was added to the water phase and the effect was registered. Next, 1 M NaCl was added and the activity was measured. The influence of $CaCl_2$ (10 mM) was investigated also after immobilization and stabilization.

## RESULTS AND DISCUSSION

### Effect of temperature on activity

The temperature influence on lipase activity was determined in the assay system with tributyrin as a substrate at pH 6. Above 55°C the addition of NaOH was not linear in the time scale of measurement (*ca* 1 minute) and therefore an accurate measurement of the initial rate was not possible above this temperature. The activation energy of the hydrolysis reaction ($\Delta E_h$) was calculated from the activity plot using the Arrhenius equation:

$$A = A_\infty \cdot e^{\left(-\frac{\Delta E_h}{RT}\right)} \qquad (1)$$

The linearized plot of the initial activity data is shown in figure 1, yielding an activation energy of 12.3 kJ.mole$^{-1}$ and $A_\infty$ = 0.53 mole.h$^{-1}$.mg$^{-1}$. This fit is shown in figure 1 as the straight line. The effect of temperature on activity in the membrane reactor was tested in a continuous system with recirculation of both phases. Due to the large mean residence time in this reactor however, the denaturation overruled the higher activity when the temperature was increased. Therefore no accurate measurements of the activity as a function of temperature could be made in the membrane reactor.

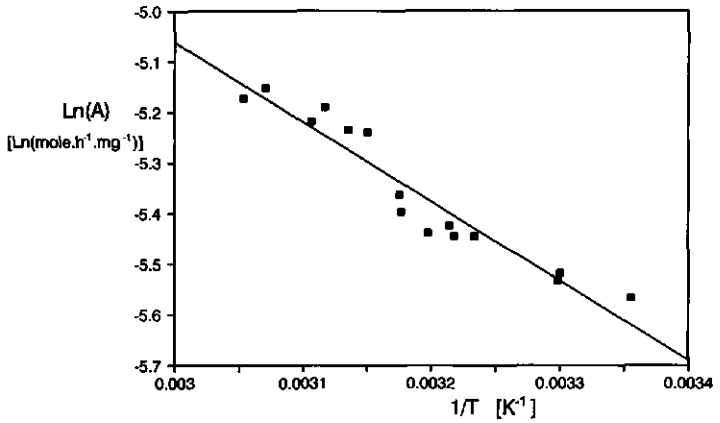


**Figure 1:** *Arrhenius plot of the activity of free lipase with tributyrin as a substrate.*

Activation energies of the hydrolysis reaction catalyzed by *Candida rugosa* lipase were reported to be 9.6 kJ.mole$^{-1}$ for olive oil (13), 38 kJ.mole$^{-1}$ for tributyrin and 36 kJ.mole$^{-1}$ for triolein (7). Thus, the value found here is within the range reported elsewhere.

## Effect of temperature on stability

In the membrane reactor, the influence of temperature on stability was examined in a recirculating flow reactor operated in a continuous mode. In chapter 2, it was shown that no wash-out of enzyme occurs after a stabilization period of 110 h. The activity was calculated as described in the Methods and Materials section. The curves of activity *versus* time were similar to the results shown in chapter 2.

The temperature dependence of the inactivation can be expressed analogous to the activity as:

$$k_i = k_{i\infty} \cdot e^{\left(-\frac{\Delta E_i}{RT}\right)} \qquad (2)$$

The activity of the membrane reactor was followed in time for different temperatures, and the inactivation constants were calculated. The linearized plot of the data is shown in figure 2.

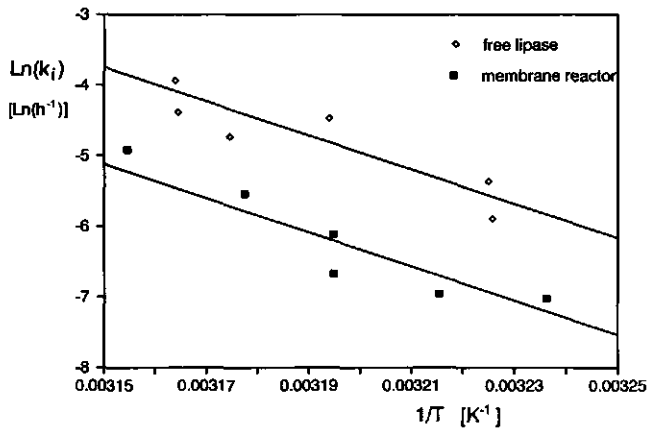


**Figure 2:** *Arrhenius plot of the stability of free and membrane-immobilized lipase.*

The storage stability of free lipase as a function of temperature was measured with the tributyrine assay at different temperatures. The inactivation constants are plotted in figure 2. The activation energy of inactivation ($\Delta E_i$) was calculated to be 201 kJ.mole$^{-1}$, on the assumption that the slope of the lines for membrane reactor and free enzyme are equal. The absolute level of the lines differ such that the immobilized

lipase is a factor four more stable than the free lipase ($k_{i,\infty}$ = 6.5·10$^{30}$ and 2.6·10$^{31}$ h$^{-1}$ for membrane reactor and free lipase, respectively).

Stabilization of enzymes upon immobilization is a general phenomenon for all kinds of systems (14). According to a commonly accepted mechanism for this stabilization, the conformational change involved with inactivation is more difficult to attain for the immobilized enzyme, due to its lower mobility as compared to the free enzyme.

In literature, the inactivation of lipase was reported to be the sum of two first-order inactivation processes (15): An initial inactivation with a high inactivation constant is followed by a stage of slower inactivation. In some of our experiments, indeed a more pronounced activity decrease appears to occur in the initial period for the free lipase. The results shown in this report are measured in the period after this decrease. For the membrane reactor, the initial stage of inactivation is not relevant for the membrane reactor because the experiments are always carried out after a stabilization period of 110 hours.

## Model of activity and productivity as a function of temperature

Both initial activity and stability influence the activity and productivity after a certain time of reaction. The activity of free lipase can be expressed as a function of initial activity, inactivation constant, and time as shown in equation 3.

$$A(t) = A(0) \cdot e^{(-k_i \cdot t)} \tag{3}$$

Combination of equation (3) with equations (1) and (2) gives:

$$A(t) = A_\infty \cdot e^{\left(-\frac{\Delta E_h}{RT}\right)} \cdot e^{\left(-t \cdot k_{i,\infty} \cdot e^{\left(-\frac{\Delta E_i}{RT}\right)}\right)} \tag{4}$$

The total productivity in a given period of time at a fixed temperature ($P(t)$) is the integral of the activity in time:

$$P(t) = \int_0^t A \, dt \tag{5}$$

It is assumed that equation 1 with the activation energy of free lipase is valid also for membrane-immobilized lipase (replacing *SCR* for *A* in equation (1)), when the inactivation constant is replaced by that for the membrane-immobilized lipase. Thus equations (4) and (5) can be used to calculated the activity and productivity in the membrane reactor. In figure 3, the calculated activity is shown as a function of time for five different temperatures.

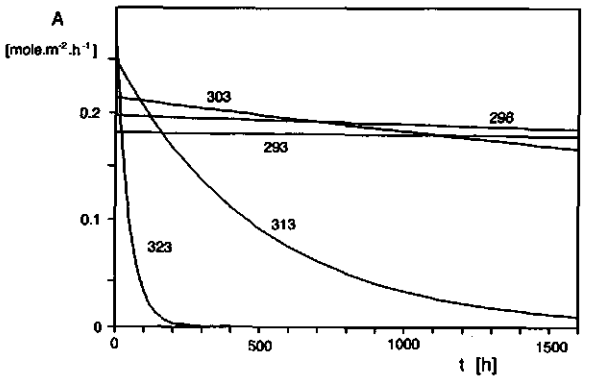


**Figure 3:** *Model calculations of activity (A or SCR) versus time for different temperatures in the membrane reactor. Legends refer to the temperature in K. Parameter values:* $A_\infty$ *= 28.5 mole.m*$^{-2}$*.h*$^{-1}$*;* $k_{i,\infty}$ *= 6.5·10*$^{30}$ h$^{-1}$*;* $\Delta E_i$ *= 201 kJ.mole*$^{-1}$*;* $\Delta E_h$ *= 12.3 kJ.mole*$^{-1}$*.*

The productivity, calculated numerically from equations (4) and (5), is presented in figure 4a and 4b for times up to 1600 h and 100 h respectively for the membrane reactor, and in figure 4c up to 100 h for a hypothetical continuous reactor with free lipase. The plots show that at an increase in temperature the positive effect on the activity is overruled very easily by the negative effect on the stability, so that the total productivity can increase with a decrease of temperature. For a membrane reactor where extended operation stability of the enzyme is an important advantage, the temperature therefore should be kept as low as possible. The melting point of the hydrolysis mixture (fatty acids, mono- and diglycerides) determines the lower limit of the temperature. For soybean oil, this lower limit was found to be around 20°C. For high melting fats, such as tallow, the temperature should be kept well above 40°C. The model can calculate the productivity at that temperature and can be used for cost comparison purposes with alternative processes such as solvent dissolution or the use of thermoresistant lipases. Comparison of figures 4b and 4c indicates that for longer operation times a membrane reactor is favoured to the emulsion reactor: for example, at 323 K the activity of the emulsion system has decreased to zero at t=30 h, while the membrane reactor is still active at t=100 h.

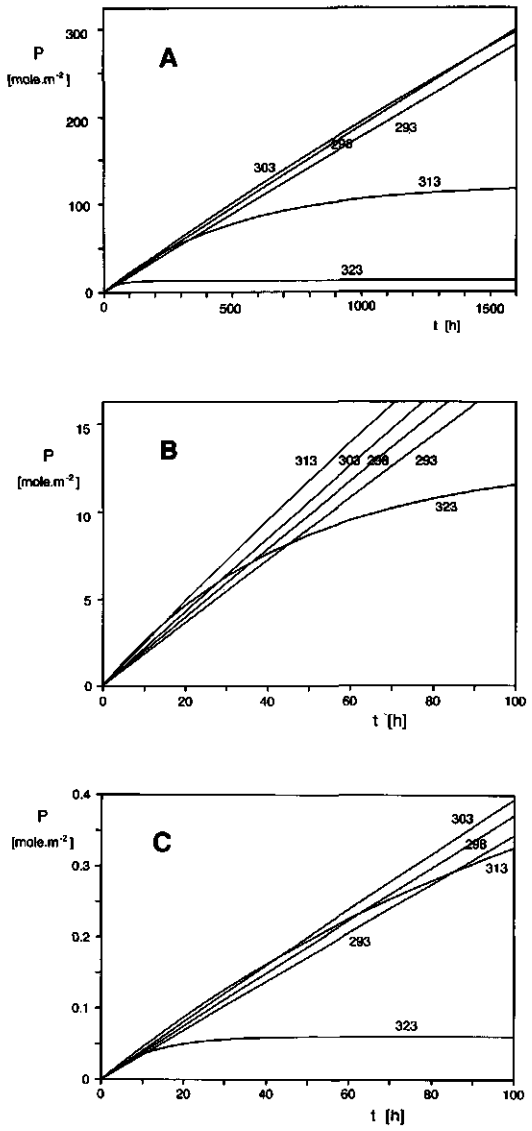


**Figure 4:** *Model calculations of productivity versus time for different temperatures for times up to 1600 h (fig. 4a) and 100 h (4b) in the membrane reactor and for times up to 100 h in a hypothetical continuous emulsion reactor (fig. 4c). Legends refer to the temperature in K. Parameter values: Membrane reactor:* $A_\infty$ *= 28.5 mole.m⁻².h⁻¹;* $k_{i\infty}$ *= 6.5·10³⁰ h⁻¹;* $\Delta E_i$ *= 201 kJ.mole⁻¹;* $\Delta E_h$ *= 12.3 kJ.mole⁻¹. Emulsion reactor: same values except* $A_\infty$ *= 0.53 mole.h⁻¹.mg⁻¹;* $k_{i\infty}$ *= 2.6·10³¹ h⁻¹.*

In practice, the membrane reactor will be operated until the activity decreases below a certain value. From figure 3 it appears that the operation time will decrease upon increasing temperature. In the comparison of two different temperatures, the break-even point of productivity (figure 4) always occurs after the break-even point of activity (figure 3). Thus, when higher temperatures are required, an optimization has to be made between regeneration plus enzyme costs and productivity profits.

The curves further show very clearly that the productivity optimum depends on the time of incubation, and that the temperature profile of activity (figure 3) is different from that of the productivity (figure 4). This can possibly explain the fact that Khor *et al.* (16) found a temperature optimum of 37°C for lipase incubated during a certain period of time. Those authors such as Khor *et al.* (16) that measure the conversion in a given period of time, therewith measure the productivity instead of activity.

## The effect of pH

The effect of the pH on free lipase (substrate: tributyrin) and on immobilized lipase (substrate: soybean oil) is shown in figure 5. For free as well as membrane-immobilized lipase the pH optimum is 6.5-7.0. At pH 3 or lower the stability was very low in comparison with neutral pH values. From figure 5 appears that below pH 7 the relative activity of the immobilized lipase is higher than the activity of the free enzyme. This effect may be the result of either the immobilization or the difference in substrate. The pH profiles presented in literature (19) show that the pH profile of a high molecular-weight substrate (olive oil) has a sharper pH optimum than for tributyrin. Conversely, our results show that the immobilized lipase active on a high molecular weight substrate (soybean oil) has a broader pH optimum. This can only be explained by the fact that the immobilized enzyme has a different (broader) pH optimum towards the acid region than the free lipase. A comparison towards the basic region of the pH profile is not possible with this system, because the formation of soaps in the buffered water phase interferes with the activity measurements.

In several publications the pH optimum of *Candida rugosa* lipase was reported: pH optima were 6.0 for olive oil (13,4), 7.5-8.3 for a palm oil/hexane mixture (16) and 7.0 for triolein (7). The observation of low stability below pH 3 is in agreement with the results published by Sohn *et al.* (15) who showed that lipase has the highest stability at pH 5-7 and by Celebi and Caglar (17) who reported a pH range of optimum stability at pH 5 - 6. It should be noticed that the pH range of optimum stability is approximately corresponding with the pH range of optimum activity.

In the membrane reactor at pH 7 or higher the pressure drop of the flow through the fibers and the viscosity of the lipid phase increased with time and after 1 to 3 days the reactor was clogged up completely. The pH of the water phase decreased very rapidly in these experiments. These phenomena can be explained as follows: The dissociation constant (pK) of short chain fatty acids is 4.8 - 5.0 (18) and the pK of long-chain fatty acids can be estimated to be around 5.5 from extrapolation. Thus at alkaline pH values soaps will be formed which are not soluble in the lipid phase and which have a low solubility in the water phase. Therefore the soaps will crystallize or form insoluble complexes which block the lumen of the fibers.

A decrease in activity at higher pH could further be explained by the fact that soaps form inactive complexes with lipases, as reported by Wang *et al.* (19).

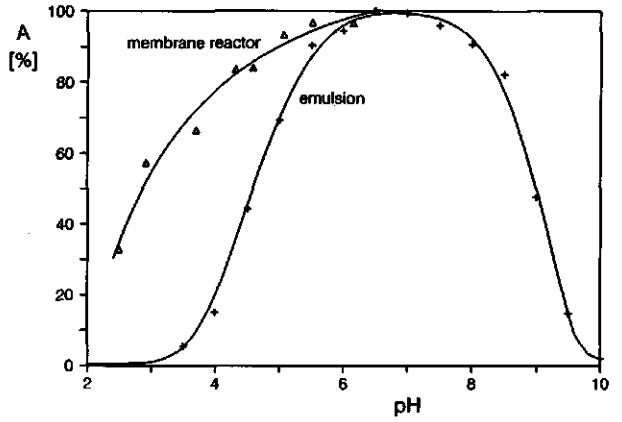


**Figure 5:** *Relative activity of free and membrane-immobilized lipase as a function of pH.*

In the experiments described in chapter 2 of this thesis, the water phase contained no buffers. The pH of the demineralized water varied from 6.5 to 7. During conversion the pH was measured. It decreased to a stable value of 5.5 to 6.0 with increasing residence time. The decrease in pH is probably due to the buffering effect of fatty acids which partly dissolve in the water phase. Also in weakly buffered systems (0.05 M phosphate buffer) the pH changes into the direction of the dissociation constant of the fatty acids (approximately 5.5). At the final pH value of 5.5 - 6 the activity is over 90% of the activity at the pH optimum and the maximum enzyme stability is obtained. Soaps are not formed and blocking of the fibers does not occur under these conditions. Adding to this the fact that the omission of buffer includes a considerable cost reduction in an industrial process, it can be concluded that for hydrolysis by *Candida rugosa* lipase, a non-buffered reactor system seems to be preferable.

## The influence of effectors

In the membrane reactor, 0.1 M NaCl did not influence the activity. However, when 1 M NaCl was added, the activity increased for several hours to about 120% of the initial activity and then decreased rapidly (about 50% activity after two days), which means a 20 fold decrease of the stability. From lipase assays of the lipid phase it was proved that lipase was washed out in the presence of 1 M NaCl. This can

be explained as follows: The lipase is adsorbed to the membrane material by a combination of interactions among which the ionic interaction. When the salt concentration is increased, the ionic interaction is becoming weaker and the enzyme is released from the membrane surface. This causes an emulsification of the enzyme (initially higher activity) followed by a wash-out of the enzyme (rapid activity decrease).

The effect of $CaCl_2$ (10 mM) was studied in a continuously operated reactor at 30°C. Immediately after addition, the activity decreased slightly from a *SCR* value of 0.047 to 0.045 mole.$m^{-2}$.$h^{-1}$. Then the activity was followed during one week and the inactivation constant was calculated to be $6.0 \cdot 10^{-5}$ $h^{-1}$. From this it follows that the half life time in the presence of $CaCl_2$ is 483 days while the half life time without $CaCl_2$ was 43 days. A stabilizing effect of $CaCl_2$ (by a factor 2) was also reported for an esterification reaction by *Chromobacterium viscosum* lipase immobilized in a hydrophobic membrane reactor (10).

At higher pH values, $CaCl_2$ may be expected to form calcium soaps with the released fatty acids and thus block the interface. This may explain the inhibiting influence of $CaCl_2$ reported by Khor *et al.* (16) at pH 7.5. The effect of $CaCl_2$ is strongly dependent on the type of lipase. For example, $CaCl_2$ is known to be a cofactor of pancreatic lipase in the alkaline pH range, rendering the enzyme in a conformation able to adsorb to the lipid/water interface.

## CONCLUSIONS

Arrhenius kinetics appears to be appropriate for the hydrolysis reaction as well as the inactivation of the enzyme. The activation energy of the hydrolysis reaction was found to be 12.3 kJ.$mole^{-1}$ for the free lipase. The activation energy of the inactivation reaction was determined as 201 kJ.$mole^{-1}$. Lipase immobilized in the membrane reactor had a four times higher stability than the free enzyme. The data on temperature dependence of stability and activity were combined in a model predicting the activity and productivity in time as a function of the temperature. Model predictions showed that an increase in temperature has a much stronger effect on stability than on the initial activity. Thus, for long operation times the highest productivity is reached at moderate or low temperatures. For high-melting substrates the operating temperature in a membrane reactor has to be above the melting point and will be higher than the optimal temperature for stability. The calculations show that the model can be used for the simulation and optimization of the enzymatic hydrolysis process.

The composition of the water phase was demonstrated to influence several aspects of the hydrolysis kinetics: The pH optimum for activity was 6.5 - 7.0 for both the free and the membrane-immobilized enzyme. At lower pH values the immobilized lipase had a higher relative activity than the free lipase. At pH values above 7.0 the measurement of the activity in the membrane reactor (immobilized lipase) was impossible due to soap formation and concomittant clogging of the hollow fibers. Furthermore, the pH affected the stability, with severely decreased stability at pH values around and below pH 3. The addition of salts resulted in either a decrease or an increase in stability, depending on the type of salt added and the

concentration: When NaCl (1 M) was added to the water phase of the membrane reactor the stability was found to decrease, probably due to the wash-out of the lipase. A lower concentration (0.1 M) did not affect the stability. Low concentrations of $CaCl_2$ (10 mM) however increased the stability of the immobilized lipase by more than a factor 10.

NaCl, pH and $CaCl_2$ show that lipase stability is a complex process as it concerns the inactivation of the enzyme as such and the influence on the adsorption of the lipase to the oil/water interface.

## ACKNOWLEDGEMENT

This work was supported by the Dutch Programme Committee on Membrane Technology (PCM), the Dutch Foundation of Technical Sciences (STW), and by Rhenus, a division of Verenigde Oliefabrieken BV (Rotterdam, The Netherlands). Membrane modules were kindly provided by ENKA AG, Technical Membranes, Wuppertal (FRG). We gratefully acknowledge Raymond Vaessen and Ellen le Clercq for their contributions to this work.

## SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| $A$ : | activity for the free lipase [mole.h$^{-1}$.mg$^{-1}$] |
| $\Delta E_h$ : | activation energy of the hydrolysis reaction [kJ.mole$^{-1}$] |
| $\Delta E_i$ : | activation energy of the inactivation reaction [kJ.mole$^{-1}$] |
| $k_i$ : | inactivation constant [h$^{-1}$] |
| $P$ : | productivity [mole.m$^{-2}$] |
| $R$ : | gas constant [J.mole$^{-1}$.K$^{-1}$] |
| $SCR$ : | standard conversion rate [mole.m$^{-2}$.h$^{-1}$] |
| $T$ : | absolute temperature [K] |
| $t$ : | time [h] |

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Chapter 6

# THE INTERFACIAL BEHAVIOUR OF LIPASE IN FREE FORM AND IMMOBILIZED IN A HYDROPHILIC MEMBRANE REACTOR.


## SUMMARY

The influence of the enzyme load on lipase kinetics was investigated in a stirred cell with a known interfacial area, an emulsion and a membrane reactor. From the measurements of activity and protein in the stirred cell, it was calculated that at saturation of the interface a multimolecular lipase layer is adsorbed to the lipid/water interface, while only a part of this layer is active. In contrast, the membrane reactor attained the highest activity at a surface load of approximately a monolayer of lipase. For enzyme loads far below saturation, the specific activity of lipase in the membrane reactor and stirred cell are of the same order of magnitude (1.7 and 1.0 mole.$h^{-1}$.$g^{-1}$ respectively). This indicates that the enzyme activity does not differ very much for an enzyme adsorbed to the lipid/water interface or to the membrane. However, the activity seems to be dependent on the physical state of the interface, since in an emulsion system a much higher activity was found (9.0 mole.$h^{-1}$.$g^{-1}$). A decrease in the purity of the lipase preparation offered for immobilization in the membrane reactor did not influence the membrane reactor activity. This is an indication for the hypothesis that lipase adsorbs preferentially to the membrane as compared to other proteins.


## INTRODUCTION

In chapter 2 of this thesis the immobilization of lipase in a hydrophilic membrane reactor for the hydrolysis of lipids was described. Though only physically adsorbed to the membrane wall, the immobilized enzyme was proven to be stable and no wash-out of enzyme could be detected. The hydrophobic glycerides do not mix with the water phase required for hydrolysis and therefore the reaction will take place at the interface of the lipid and water phase. The fact that lipase is working at the interface was confirmed (1): Pancreas lipase was completely adsorbed from an aqueous solution to the interface with a long-chain triglyceride phase, and the enzyme was migrating back into the aqueous phase when the emulsion was broken (1). The interface appeared to be a prerequisite for lipase to be active; triacetin was reported to be hydrolyzed in an emulsion and not in a homogeneous system at concentrations where it is soluble (2);


This chapter was submitted for publication by the authors Wouter Pronk and Klaas Van 't Riet.

blocking of the interface of a micro-emulsion, resulting in loss of activity was reported (3).

In a homogeneous system, usually the reaction rate is proportional to the enzyme concentration. In a two-phasic system as studied here, the reaction rate can be expected to be proportional to the amount of enzyme, until the interface is fully covered with enzyme (4). When more enzyme is added a part of the enzyme cannot reach the interface and can be expected to be inactive.

A drawback of the use of emulsion systems for kinetic studies is that the interfacial area is affected by the formation of hydrolysis products (di-, mono- glycerides and fatty acids) and by the enzyme added. In order to avoid these effects, a stirred cell was used in this paper, in which the interfacial area is constant and though the mass transfer is not hindered (figure 1).

In the membrane reactor, lipase is adsorbed to a hydrophilic membrane wall. The adsorption behaviour and the activity of the immobilized lipase may differ from the free enzyme situation. The objective of this paper is to get a better understanding of the interfacial behaviour of lipase by studying the adsorption of and hydrolysis by lipase as a function of the amount of enzyme in different systems. The results will serve to compare the behaviour of free and membrane-immobilized lipase, and thus to get more insight in the interfacial and catalytic properties of the immobilized enzyme.

## METHODS AND MATERIALS

Lipase of *Candida rugosa* (type "OF") was purchased from Meito Sangyo, Japan. It has a molecular weight of 120.000 (5), and a protein content of 15% (w/w). The specific activity was 66 Units per mg crude enzyme. 1 Unit (U) is defined as 1 μmole of fatty acid released per minute at 30°C with tributyrine as a substrate. The lipid phase was soybean oil of edible quality, provided kindly by Rhenus BV, The Netherlands. The membrane modules containing Cuprophan™ cellulosic hollow fiber membranes with internal diameter 215 μm were provided kindly by ENKA, Germany. Two types of membrane modules were used: a membrane with thickness 16 μm (total membrane area 1.36 $m^2$ or 1.2 $m^2$) and a membrane with thickness 8 μm (total membrane area 0.77 $m^2$).

The fatty acid content in the lipid phase was determined by titration of the free fatty acids. The quantitative lipase activity was performed by a pH-stat method at pH 6 with tributyrine as a substrate. The method was described in chapter 2 in detail. A qualitative lipase assay was used for the detection of low lipase-levels: An emulsion was made by stirring of 10 mL of the sample with 10 mL of soybean oil and 10 mL of water. The fatty acid content of the lipid phase was followed for at least one week.

Protein analysis was done according to a modification of the Lowry method (6). Protein determinations in lipid/water emulsions were done after a specific extraction procedure: the emulsion (10 g) was mixed with hexane (25 mL), hexanol (5 mL) and 0.1 N NaOH (4 mL). After phase separation, the Lowry assay according to (6) was performed on samples of the water phase.

The stirred cell experiments were carried out in the apparatus shown in figure 1. The volume of the lipid phase was 220 mL, the volume of the water phase was 235 mL. The vessel diameter was 0.081 m and the area of the lipid/water interface was 0.00515 $m^2$. The temperature of the water jacket was maintained at 30°C. The stirring speed was set to 0.67 $s^{-1}$ for the upper stirrer (lipid phase), and 1.78 $s^{-1}$ for the lower stirrer (water phase) turning in the opposite direction, providing a stable interface. These conditions provided maximum convection just without dispersion of the phases. Before a hydrolysis experiment, the phases were stirred and thermostated for at least 20 minutes. Then, the dissolved enzyme

was added to the water phase and samples were taken from the lipid phase at regular time intervals. The initial reaction rate was determined by linear regression of fatty acid - time plots up till a fatty acid content of 5% at maximum. For this small concentration range the conversion could be regarded as constant in time.

The interfacial protein and activity in the stirred cell experiments were determined as follows: After stirring and thermostating the vessel with lipid and water phases for 20 min. the enzyme solution was added. The total activity added was determined from the activity measurement of the lipase solution added. The protein content of the water phase was determined with the modified Lowry method (6). The water phase activity was determined 15 minutes after addition of the enzyme. No activity could be detected in the lipid phase. The adsorbed (interfacial) activity was determined in the stirred cell from the difference between the added total activity and the measured water phase activity. It was assumed that no activity-loss or denaturation occurred in the short period of the experiment.

The emulsion experiments were carried out in a stirred thermostated vessel with a diameter of $9 \cdot 10^{-2}$ m with four baffles of $1 \cdot 10^{-2}$ m width. The temperature was maintained at 30°C. 150 mL of soybean oil (edible quality) and 600 mL of demineralized water were added and the emulsion was stirred for at least 15 min. After addition of the enzyme the fatty acid content was determined each 15 sec. Samples were taken and inactivated by heating of the sample up to 80°C.

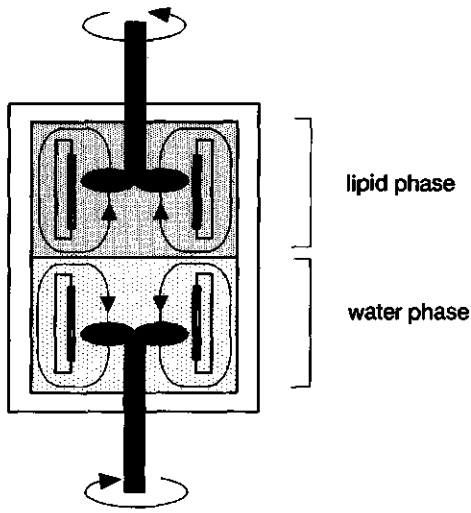


**Figure 1:** *Schematic presentation of a stirred cell with controlled interface (from Dekker et al. (7)). The two stirrers rotate in opposite directions.*

In the membrane reactor experiments, immobilization with the ultrafiltration/adsorption method was carried out according to the procedure described chapter 2: Before immobilization, modules were rinsed with demineralized water for at least 20 h; 0.5 g of crude lipase was added in the ultrafiltration procedure and next the fibers (lipid circuit) were filled with the lipid phase while the shell side (water circuit) was filled with the water phase. The lipid circuit was operated in a continuous mode and the water circuit in a semi-continuous mode for a period of at least 200 h. The product of net flow rate and fatty acid concentration difference of the in- and outflow is the fatty acid production rate. This production rate was converted into the Standard Conversion Rate (SCR) as described in chapter 2. The SCR is defined as the conversion rate at zero fatty acid concentration and at zero glycerol concentration (mole fatty acid.$m^{-2}.h^{-1}$).

The amount of protein immobilized on a membrane module was calculated from a mass balance before and after immobilization. The amount of protein offered for immobilization was determined from Lowry measurements of the enzyme solution used (0.15 g of enzyme in 250 mL). After immobilization, the protein concentration was determined by the Lowry method in the ultrafiltrate and in the enzyme solution which was driven out by the lipid phase. The protein concentration in the emulsion appearing after the water phase and in the outflow lipid phase (collected up till 250 h after immobilization) was determined by the extraction method described above. The net flow of the lipid phase was collected up till 250 h after immobilization and the protein concentration was determined by the extraction method.

The influence of the enzyme purity on the activity in the membrane reactor was investigated by immobilization of an enzyme solution (0.5 g lipase) to which 1.0 g of inert protein was added (bovine serum albumin, BSA).

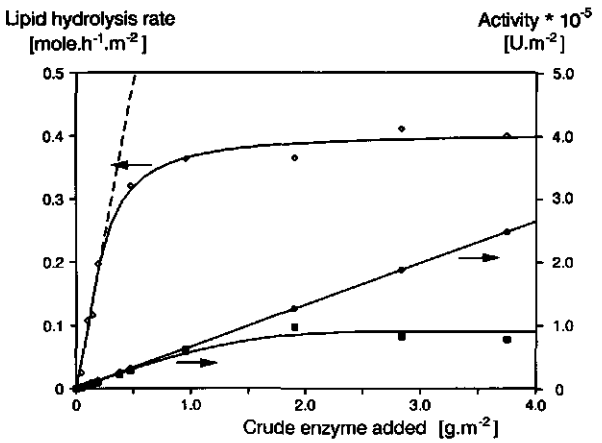


**Figure 2:** *Activity and adsorption in the stirred cell as a function of the enzyme load:*
◇ *: Measured hydrolysis rate;*
—— *: Slope of measured hydrolysis rate at low loads;*
■ *: Measured adsorbed amount of lipase activity;*
○ *: Added amount of lipase activity.*

## RESULTS

For the stirred cell system, the mass transfer rate was estimated from relations for the Sherwood, Reynolds and Schmidt numbers as presented by Dekker *et al.* (7). The mass transfer coefficient in the lipid phase can be calculated to be $6 \cdot 10^{-6}$ and $6 \cdot 10^{-7}$ m.s$^{-1}$ for a completely mobile and a completely rigid interface, respectively. In the stirred cell, the maximum conversion rate divided by the surface area was 0.4 mole.m$^{-2}$.h$^{-1}$, corresponding with $2.8 \cdot 10^{-8}$ m$^3$.m$^{-2}$.s$^{-1}$ (= m.s$^{-1}$) (figure 2). Even at the lowest mass transfer coefficient and the highest conversion rate, the concentration difference in the boundary layer is below 5% of the maximum concentration difference. Therefore, mass transfer limitation is neglected.

For different amounts of enzyme the initial rate of lipid hydrolysis and the amount of lipase-activity adsorbed to the interface were determined in the stirred cell. The results are shown in figure 2. The slope of the linear range of the curve at low surface loads, as indicated by the dotted line, amounts 1.0 mole.h$^{-1}$.g$^{-1}$.

The activity of lipase in an emulsion of soybean oil and water as a function of the enzyme load is shown in figure 3. The slope of the linear range of the curve at low loads, as indicated by the dotted line, shows the maximum specific enzyme activity. This activity was calculated to be 9.0 mole.h$^{-1}$.g$^{-1}$.

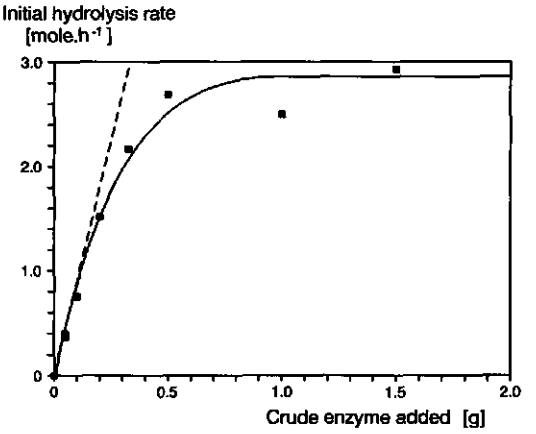


**Figure 3:** *Activity in the emulsion as a function of the enzyme load:*
■ *: Measured hydrolysis rate;*
—— *: Slope of measured hydrolysis rate at low loads.*

The activity of the membrane reactor was followed in time for different enzyme loads as described in the experimental section. At higher enzyme load, some wash-out of enzyme was detected in the period after immobilization (200 h) using the qualitative lipase assay, and also the observed activity decreased considerably in this period. After 200 h the activity-decrease stabilized to the value corresponding to the rate of enzyme denaturation as reported in chapter 2. Moreover, no activity could be detected anymore in the lipid phase using the qualitative lipase assay. The SCR value was therefore defined at 200 h after immobilization. The resulting SCR value for different loads is shown in figure 4. The slope of the linear range of the curve at low surface loads is indicated by the dotted line and amounts 1.7 mole.h$^{-1}$.g$^{-1}$.

From the protein balance before and after immobilization the amount of lipase immobilized in the

membrane reactor at saturation conditions was calculated to be 4.4 mg protein.m$^{-2}$ (Table I).

In order to answer the question if only lipase or the total protein fraction adsorbs to the membrane, the purity of the lipase preparation was reduced artificially by the addition of BSA. After immobilization of the solution in the normal procedure with 0.42 g.m$^{-2}$ of crude enzyme + 0.83 g.m$^{-2}$ of BSA, the activity at t=200 h appeared to be 0.045 mole.h$^{-1}$.m$^{-2}$.

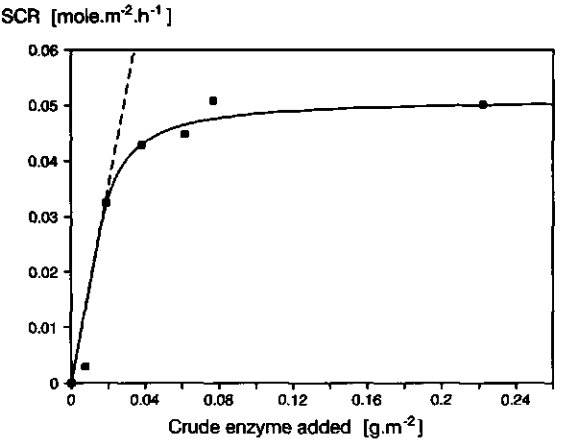


**Figure 4:** *The hydrolysis rate in a membrane reactor as a function of the amount of lipase added in the immobilization procedure.*
■*: Measured hydrolysis rate;*
——— *: Slope of measured hydrolysis rate at low loads.*

## EVALUATION OF THE EXPERIMENTAL DATA

The lipase content of the preparation used was calculated by comparison of the activity as measured in a standard assay with the activity of pure lipase (5). This calculation yields a lipase content of 4.7% (w/w); this implies that the protein fraction contains 31% (w/w) of pure enzyme.

In the stirred cell the hydrolysis rate increases linearly with the load, and no lipase activity is found in the bulk water phase for enzyme loads below 0.2 g.m$^{-2}$. This brings on that below a surface load of 0.2 g.m$^{-2}$ all lipase added adsorbs. The specific activity of the lipase in this range amounts 1.0 mole.h$^{-1}$.{g crude enzyme}$^{-1}$, which is the real activity, since mass transport was proven to be not limiting. For high loads, the surface area activity approaches a plateau value.

The amount of enzyme preparation *active* at the interface (not to be confused with the amount *adsorbed*) at the level of saturation can be calculated as 0.4 g crude enzyme.m$^{-2}$ (19 mg.m$^{-2}$ on a pure lipase basis) from a division of the maximum lipid hydrolysis rate (0.4 mole.h$^{-1}$.m$^{-2}$) and the specific activity in the linear range (1.0 mole.h$^{-1}$.g$^{-1}$).

| | |
|---|---|
| permeate: | 8.75 mg protein |
| water phase driven out and water/oil emulsion | 7.59 mg protein |
| extractions from lipid net flow: | |
| first period | 0.14 mg protein |
| second period | 0.09 mg protein + |
| Total amount non-immobilized: | 16.57 mg protein |
| Total amount added | 22.50 mg protein - |
| Immobilized amount: | 5.93 mg protein |
| **Surface load:** | **4.4 mg protein.m$^{-2}$** |

**Table I:** *Protein balance before and after immobilization in the membrane reactor with a membrane area of 1.36 m$^2$.*

A similar calculation is carried out for the maximum adsorbed activity ($9.0 \cdot 10^4$ U.m$^{-2}$), divided by the slope in the linear range at low surface loads ($6.6 \cdot 10^4$ U.{g crude enzyme}$^{-1}$). This calculation gives the amount of enzyme *adsorbed* to the interface at saturation of the interface: 1.4 g crude enzyme.m$^{-2}$, that is 66 mg.m$^{-2}$ on a pure lipase basis. A number of assumptions underlie these calculations, yet they indicate that at saturation of the interface only a part of the adsorbed enzyme is active. This phenomenon is also illustrated in figure 5 which shows the specific activity of the adsorbed enzyme in the stirred cell, as obtained from a division of the hydrolysis rate with the adsorbed activity. Below an adsorbed lipase amount of about $1.1 \cdot 10^4$ U.m$^{-2}$, corresponding to 8 mg pure lipase.m$^{-2}$, the specific activity is maximum. Above this surface load, the specific activity is decreasing. A monolayer of pure lipase can be calculated to have a surface load of 5.9 mg.m$^{-2}$, on the assumption of globular conformation of the molecules (molecular weight 120.000). Consequently, the lipase load at the maximum level of activity (8 mg pure enzyme.m$^{-2}$) corresponds with 1 - 2 layers. The adsorbed amount of enzyme at saturation of the interface (1.4 g crude enzyme.m$^{-2}$ = 66 mg pure enzyme.m$^{-2}$) consists of about 11 lipase layers. The specific activity at saturation, which is lower than the maximum specific activity at low loads, corresponds to only a part of this layer (0.4 g.m$^{-2}$ = 3.2 'monolayers'). This observation is in good agreement with Ekiz *et al.* (8): In a study with tributyrin as a substrate, lipase was shown to have a lower specific activity at concentrations above which a monolayer is formed at the interface. Van der Padt *et al.* showed that the lipase load at saturation of the interface in an emulsion of decanoic acid and glycerol/water is 170 mg crude enzyme.m$^{-2}$ (9), which is also more than a monolayer. These observations could be explained by the hypothesis that the lipase molecules in the interfacial enzyme layer are in a dynamic equilibrium between the lipid interface and the boundary layer. Since the enzyme can only be active at the interface, the observed specific activity

of the adsorbed enzyme will be decreased.

In the membrane reactor, the slope of the curve of activity *versus* load at low loads was found to be 1.7 mole.$h^{-1}$.$g^{-1}$ (dotted line in figure 4). This slope is of the same order of magnitude as found in the stirred cell experiments (1.0 mole.$h^{-1}$.$g^{-1}$), but is much smaller than the slope found for the emulsion experiments (9.0 mole.$h^{-1}$.$g^{-1}$). It can be concluded that the activity in the membrane reactor is in between the activities found in the stirred cell and the emulsion. The emulsion shows a higher activity. For esterification, van der Padt *et al.* found values of 0.13 mole.$h^{-1}$.$g^{-1}$ for the membrane reactor and 0.09 mole.$h^{-1}$.$g^{-1}$ for the emulsion system (9). Since esterification is a slower process the activities are lower. In contrary to the hydrolyzing system, the membrane and emulsion system give approximately the same value.

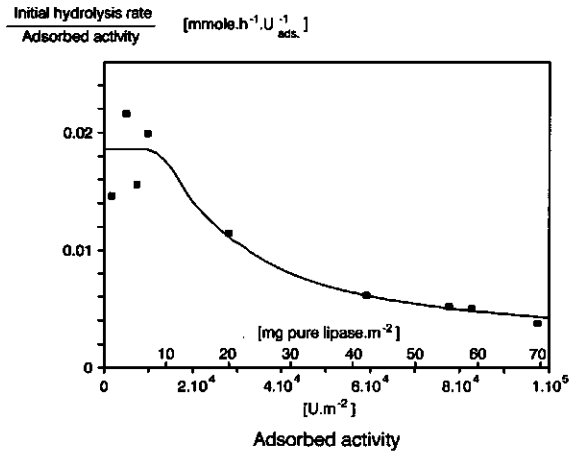


**Figure 5:** *The activity of the adsorbed lipase as a function of the amount of enzyme adsorbed in the stirred cell, as calculated from the data in figure 2.*

The amount of enzyme active at the membrane at saturation of the interface was calculated by division of the maximum level of the lipid hydrolysis rate and the initial slope of figure 4. The amount of crude enzyme active at the membrane thus calculated is 0.029 g.$m^{-2}$ (corresponding to 4.35 mg protein.$m^{-2}$ or to 1.4 mg pure lipase.$m^{-2}$). Though lower, this value is of the same order of magnitude as the optimum load found for the esterification membrane reactor (0.075 g crude enzyme.$m^{-2}$). The maximum load in the esterification was about twice this value (9).

In case of saturation of the interface, the amount of protein immobilized on the membrane was measured and calculated as 4.4 mg.$m^{-2}$ (Table I). This is about the same value as the load of activity calculated from the activity curve (4.35 mg protein.$m^{-2}$). A monolayer of protein is, depending on the molecular weight, 3 - 6 mg.$m^{-2}$, while a monolayer of lipase is 6 mg.$m^{-2}$. Thus it seems that about one monolayer is adsorbed. Conversely, in the stirred cell three to four layers of lipase are active and even more layers may be adsorbed. The reason for the difference between the membrane reactor and the stirred cell might be that in a membrane reactor molecules that desorb are flown out of the system, while in the stirred cell there exists a dynamic equilibrium between adsorbed enzyme and the bulk water phase.

The bulk water phase in the stirred cell is not removed from the system, which enables the formation of a multi-molecular layer. Thus it might be that in the membrane reactor only the strongly bonded monolayer is stable.

The activity of the membrane reactor in the immobilization experiments where BSA is added to the lipase, was measured as 0.045 mole.$h^{-1}.m^{-2}$ at saturation conditions. This value is approximately equal to the activity for a lipase preparation without BSA addition (0.05 mole.$h^{-1}.m^{-2}$). This observation indicates that the lipase adsorption is not influenced by the presence of other proteins. This can possibly be explained by the fact that lipase adsorbs to the membrane interface in preference to other proteins. This explanation is in agreement with the highly selective adsorption of lipase in the stirred cell experiments, and with the adsorption of lipase to other interfaces, as reported in literature: The adsorption of lipase to a hydrophobic (polypropylene) membrane was reported to be independent of the purity of the lipase preparation (10). Besides, adsorption to hydrophobic interfaces such as siliconized glass beads and the air-water interface was reported (11,12,13). Lipases by definition act at an oil-water interface. This property may explain its ability to adsorb also to other hydrophobic interfaces.

## CONCLUSIONS

The specific activity of the adsorbed protein in the stirred cell shows the following pattern: the specific activity is maximum below an adsorbed lipase amount of about $1.1 \cdot 10^4$ U.$m^{-2}$, corresponding to 8 mg pure lipase.$m^{-2}$. This load corresponds with 1 - 2 layers of pure lipase. Above this surface load, the specific activity is decreasing. The adsorbed amount of enzyme at saturation of the interface corresponds with about 11 lipase layers, but only a part of this layer can be active.

For the membrane reactor, the amount of protein immobilized to the membrane was calculated as 4.4 mg.$m^{-2}$ (Table I). This load corresponds with a monolayer of a protein-mixture or with a monolayer of lipase. When the lipase/protein ratio of the enzyme preparation is decreased by the addition of BSA, the activity in the membrane reactor at saturation conditions is unaffected. This indicates that lipase adsorbs preferentially to the membrane in comparison to other proteins.

For all systems studied, at relatively low surface loads, the activity is proportional with the lipase load. This slope was found to be 1.7 mole.$h^{-1}.g^{-1}$ for the membrane reactor, 1.0 mole.$h^{-1}.g^{-1}$ for the stirred cell, and 9.0 mole.$h^{-1}.g^{-1}$ for the emulsion.

## ACKNOWLEDGEMENT

This work was supported by the Dutch Programme Committee on Membrane Technology (PCM), the Dutch Foundation for Technical Sciences (STW) and by Rhenus BV, a division of Verenige Oliefabrieken BV (Rotterdam, The Netherlands). Membranes were kindly provided by ENKA AG (Wuppertal, W-Germany).

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Chapter 7

# A HYBRID MEMBRANE-EMULSION REACTOR FOR THE ENZYMATIC HYDROLYSIS OF LIPIDS


## ABSTRACT

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. The opportunity 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].


## INTRODUCTION

With the rapid developments in biotechnology, the enzymatic hydrolysis of lipids has been investigated intensively for the last decade. Yet, 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. Though, 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 in a free or 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 of one of the two phases unless immobilization is carried out on a surface which is permeable for just one of the phases, resulting in the enzyme to be immobilized at the interface


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of the lipid- and water phase. A membrane can provide this condition (chapter 2; 1). In case of the low-price lipase used in the hydrolysis process, the membrane cost will be a considerable part of total cost (2) and thus an effective utilization of membrane area is essential. Instead of immobilization matrix, a membrane can also be utilized as a separation medium. In the latter case, the membrane-use prevents an enzyme present in free form in the emulsion reactor from being washed out. This principle was used by Tanigaki *et al.* (3). 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.* (3) is very labour-intensive. An advantage of an emulsion reactor is the fact 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 (4). Thus, in principle a high volumetric activity can be reached at high enzyme loads and with vigorous stirring. Yet, the emulsion reactor as such 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 (5). This method, based on centrifugal emulsion separation with re-use of the water phase, yielded an enzyme-recovery of just about 90%.

In this paper, a reactor is presented in which lipase is active in a free form in an emulsion and which though can be operated in a continuous mode. The water phase and the lipid phase are continuously removed from the system using 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 tributyrine as a substrate at pH 6 (this thesis, chapter 2). 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 a period of one week during which several samples were taken and the degree of hydrolysis was determined. The activity decreases with the substrate concentration as shown in chapter 2 of this thesis. The results are expressed as standard conversion rate (SCR, mole.h$^{-1}$), defined as the activity of the reactor at 100% triglyceride substrate (chapter 2). In contrast to the membrane-immobilized system, the conversion rate in the hybrid reactor was not expressed relatively to the membrane area, since 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:

- Polysulfone hollow fiber membrane H1P10-43 (Amicon, Denvers, USA) with a total membrane area of 0.08 $m^2$ and a cut-off value of 10.000.
- Poly-Vinylidene-Di-Fluoride (PVDF) flat-sheet ultrafiltration membrane (cut off: 10.000) from Rhone-Poulenc (Courbevoie, France), membrane area $6.4 \cdot 10^{-3}$ $m^2$.
- Polypropylene (PP) flat-sheet microfiltration membrane from ENKA (Wuppertal, FRG) with an average pore size of 0.1 μm, and a membrane area of $6.4 \cdot 10^{-3}$ $m^2$.
- Polypropylene (PP) hollow fiber microfiltration membrane from Organon Tecnika (Boxtel, The Netherlands), 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, USA).

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 in the first place visually. If the membrane seemed appropriate, also a quantitative water analysis was carried out.

The permeate flux of the hydrophilic and hybrophobic membrane 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 en 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 6.5 $L.h^{-1}$ at minimum.

The stirred vessel had a total volume of 1.9 L (diameter 19 cm and height 11.3 cm) and contained four baffles (1 x 17 cm). A two-bladed paddle-stirrer with holes as described by Dekker *et al.* (6) was used. The stirrer was designed in such a way to provide optimum mixing (convection) in the total volume of the vessel, at a minimal stirring rate and shear rate. 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 and 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 described above. The temperature was maintained at 30 C by placing the whole experimental set-up in a thermostated case. By adjustment of 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. If the addition of $CaCl_2$ was mentioned, the concentration was 10 mM.

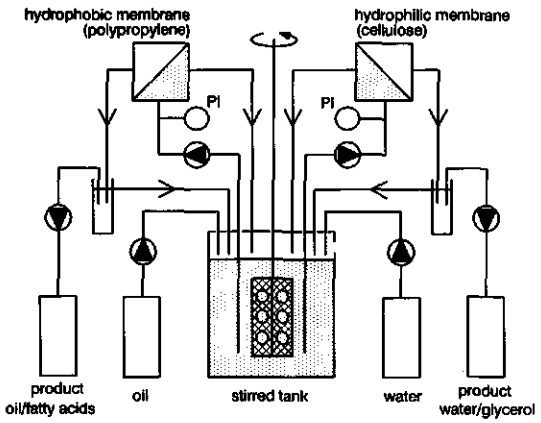


**Figure 1:** *Schematic presentation of the hybrid reactor. PI=pressure indicator.*

## RESULTS AND DISCUSSION

### Selection of the hydrophobic membrane

Keurentjes *et al.* (7) have shown that adsorption can easily occur on a hydrophobic membrane surface. Due to this adsorption the membrane can become more hydrophilic and then the membrane can loose after some time its selective permeability for the lipid phase. The polysulfone microfiltration membrane showed these properties. It had good separation properties initially, but 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 in time was almost equal for the three membranes. Based on the observations above, the PP membrane was judged as the most appropriate one. 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 was selected therefore.

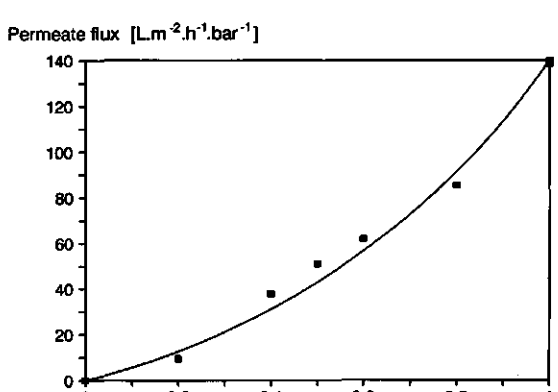


**Figure 2:** *Permeate flux of the polypropylene hollow fiber membrane as a function of the oil fraction [v/v] of an oil/water emulsion.*

## 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 can possibly be explained from the fact that a smaller fraction of the dispersed oil comes into contact with the membrane. 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 in the order of 0.2% [w/w] (8). 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 ultrafiltrated over a cellulosic membrane (Cuprophan™) for immobilization purposes (chapter 2 of this thesis). In these experiments the water phase appeared to permeate without leakage of

lipase or lipid for pressures up to 1.5 bar. Therefore, this membrane type was expected to be appropriate for use in the hybrid reactor.

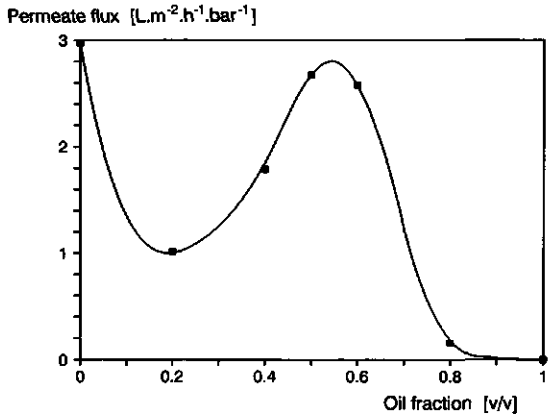


**Figure 3:** *Permeate flux of the cellulose hollow fiber membrane as a function of the oil fraction [v/v] of an oil/water emulsion.*

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 results, 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. The occurrence of this flux optimum however could not be explained.

## Properties of lipase in the stirred vessel

In the stirred vessel the influence of the amount of enzyme on the conversion rate in an emulsion with an oil fraction 0.5 [v/v] was examined. The curve has a similar pattern as shown before for esterification (9): Up to loads of 0.5 g of crude enzyme the specific activity was constant (9 $mole.h^{-1}.g^{-1}$) and the total activity increases linearly with the load of enzyme. At higher loads (0.5 g to 5 g) the specific activity decreases while the total activity yet increases. The total activity of the reactor levels off to a maximum of 14.5 $mole.h^{-1}$ at enzyme loads above 5 g.

The inactivation of lipase in a stirred emulsion at the same temperature and stirring rate as in the hybrid reactor is shown in figure 4. The decrease of activity appears to obey first order kinetics; the rate 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 $6.8 \cdot 10^{-4}$ $h^{-1}$ (chapter 2), which implies that the immobilized lipase is a factor 7 more stable than the lipase in emulsion.

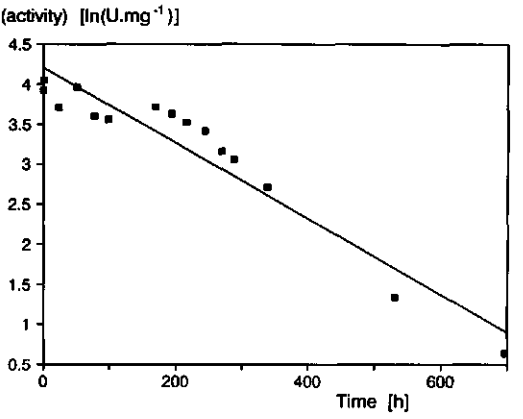


**Figure 4:** *Inactivation of lipase in an emulsion. The activity was determined in samples of the total emulsion using the tributyrin activity assay.*

## Hybrid reactor experiments

The conditions in the hybrid reactor experiments were based on the experiments 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 had not only an optimum at an oil fraction of 0, but also 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] (4). An oil fraction of 0.5 [v/v] therefore provides the optimum conditions for the process. From a viewpoint of effective enzyme use, a low enzyme load, resulting in a high specific enzyme activity, is preferable. From a viewpoint of an optimum reactor activity however, a high enzyme load should be applied. A potential drawback of high loads is strong 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. In this period, the flux of the polypropylene membrane stabilized to a value of 0.56 $L.m^{-2}.h^{-1}.bar^{-1}$. The flux of the cellulosic 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 $L.m^{-2}.h^{-1}.bar^{-1}$. These flux decreases are probably caused by severe fouling of the membrane. For the hydrophilic membrane, the flux through the membrane was reversed by reversal of the flow direction of the pump every 48 hours. Then, after switching the pump in the original direction, the flux restored to a value of 0.065 $L.m^{-2}.h^{-1}.bar^{-1}$.

Lipase activity analysis was done in the permeate of both membranes using the qualitative assay. No lipase could be detected in both permeates 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 cellulosic membrane

was a clear water solution, without any oil layer on top, which indicates the absence of any oil permeation. The permeation of lipid through the cellulosic membrane is unlikely in view of the small pores 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 (chapter 2 and chapter 5 of this thesis, respectively).

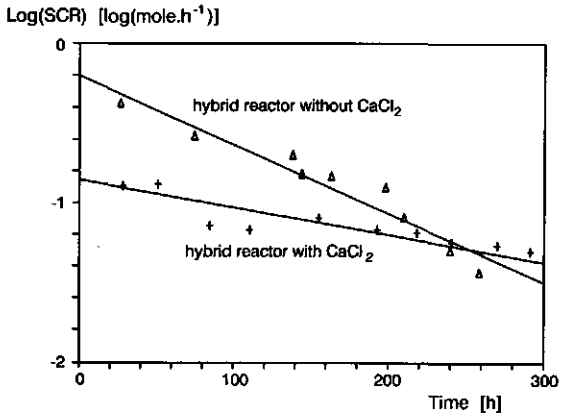


**Figure 5:** *Activity of the hybrid reactor, expressed as SCR (Standard Conversion Rate) as a function of time for lipase in the presence and absence of $CaCl_2$ (10 mM). The activity was determined from the product of the lipid permeate flow and the fatty acid fraction in the permeate. All experiments were carried out at 30 C with 0.5 gram of lipase.*

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 and on 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 (10), who showed that inactivation in a stirred vessel is mainly caused by the inactivation at the air-water interface. The inactivation constants (without $CaCl_2$) in the hybrid reactor and in the stirred vessel are $1 \cdot 10^{-2}$ $h^{-1}$ and $4.7 \cdot 10^{-3}$ $h^{-1}$, respectively, which means that the stability in the stirred vessel is a factor 20 higher than in the hybrid reactor. The main difference between the two systems is the fact that the emulsion is pumped through the membrane units in the hybrid system. If this would be the reason of the higher inactivation, shear forces might possibly play a role in the inactivation mechanism in a membrane unit. An alternative

explanation can be the loss of activity due to the formation of a fouling layer on the membrane.

The SCR at t=0 is 0.64 mole.$h^{-1}$ in the absence of $CaCl_2$. This corresponds with 0.76 mole.$m^{-2}$.$h^{-1}$ since the total membrane area is 0.84 $m^2$. In the cellulosic membrane reactor with immobilized lipase the activity after stabilization is 0.03 mole.$m^{-2}$.$h^{-1}$ (chapter 2). Therefore, it can be concluded 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, in principle the enzyme amount relative to the membrane area can be increased, resulting in a higher activity relative to the membrane area.

The stability of the system is considerably less than in the cellulosic membrane reactor with immobilized lipase. The addition of $CaCl_2$ to the water phase can only partially overcome this difference; Though, also the membrane-immobilized lipase is stabilized by this addition.

## CONCLUSIONS

It can be concluded that the objectives of this study have been attained: The operation of a continuous hybrid hydrolysis reactor was 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. This however does not imply that for commercial application the hybrid system always will be preferred to a system with membrane-immobilized lipase. Advantages of the membrane reactor are the higher stability and the lower 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.

## ACKNOWLEDGEMENT

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## ABBREVIATIONS

| | |
|---|---|
| SCR | Standard Conversion Rate |
| PI | pressure indicator |
| PP | polypropylene |
| PVDF | polyvinylenedifluoride |

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Chapter 8

# DISCUSSION AND CONCLUSIONS

In this thesis a method is presented to immobilize lipase to a hydrophilic membrane. In the membrane reactor the water and lipid phases remain separated in two different circuits, which enables continuous operation. The immobilized enzyme is more stable than the free enzyme and is not released from the membrane during continuous operation. Besides the hydrophilic membrane reactor with immobilized enzyme, a hybrid reactor is studied in which the enzyme is present in a free form in an oil/water emulsion. Phase separation is carried out using membrane loops. The principle of the hybrid reactor has been demonstrated. Though, optimization of the system has to be carried out with respect to membrane fouling and optimization of the enzyme load. In this chapter the two hydrolytic systems are compared with other lipase hydrolysis reactors. Further, some fundamental aspects of the adsorption of the enzyme are discussed.

## Comparison of reactor systems for the enzymatic hydrolysis of lipids

In chapter 7, it was demonstrated that a hybrid reactor (HR) composed of a stirred vessel, a hydrophilic and a hydrophobic membrane loop, can be used for the hydrolysis of lipids. In the HR the membrane serves to retain the enzyme in the reactor system while the reaction products are removed. Since the membrane area is not directly related to the activity, as in the membrane reactor (MR), it provides the opportunity to save on membrane cost by increasing the ratio of enzyme and membrane area. The results from the presented experiments indicate that the initial membrane-surface related activity of the HR (0.76 mole.$m^{-2}$.$h^{-1}$) indeed is higher than the initial activity in the MR (0.04 - 0.33 mole.$m^{-2}$.$h^{-1}$).

In the MR, the volumetric activity is restrained by the volumetric membrane area, which is a function of the hollow fiber diameter and packing density. In the hollow fiber modules used in this study the volumetric activity (as related to the reactor compartment) was 1650 mole.$m^{-3}$.$h^{-1}$. In the HR, the volumetric activity is restrained by the volumetric interfacial area of the emulsion. This area, and therewith the volumetric activity, can be influenced by the stirring conditions. With the conditions described in chapter 7 the volumetric activity is 380 mole.$m^{-3}$.$h^{-1}$ (as related to the reactor compartment), which is a factor 5 lower than the value obtained in the MR. The lower value in the HR is presumably the result of a lower

volumetric interfacial area. The interfacial area can be influenced by the stirring conditions. The comparison of the (initial rate) values in HR and MR indicates that under the experimental conditions the volumetric interfacial area of the hollow fiber membrane module is higher than that of the emulsion in the HR.

The activity in a reactor system further can be related to the amount of enzyme used. For the HR this calculation gives an activity of 1.28 mole.$h^{-1}$.{g crude enzyme}$^{-1}$. In the MR, the enzyme-related activity was shown to be a function of the surface load (chapter 6). For low enzyme loads, the activity was 1.7 mole.$h^{-1}$.{g crude enzyme}$^{-1}$, which is in good agreement with the HR activity. For high enzyme loads, only a part of the enzyme offered was immobilized. At the maximum level of activity (0.05 mole.$m^{-2}$.$h^{-1}$), the enzyme load was 0.1 g.$m^{-2}$, which yields an enzyme-related activity of 0.5 mole.$h^{-1}$.{g crude enzyme}$^{-1}$.

While the enzyme-related activity is equal for the HR and the MR, the stability in the MR is an order of magnitude higher than in the HR (MR inactivation constants: $6.8 \cdot 10^{-4}$ $h^{-1}$ and $6.0 \cdot 10^{-4}$ $h^{-1}$ in the presence and absence of $CaCl_2$, respectively; HR inactivation constants: $1 \cdot 10^{-2}$ $h^{-1}$ and $4 \cdot 10^{-3}$ $h^{-1}$). Stabilization is a general phenomenon observed for immobilized enzymes, and for lipases.

A hydrophobic membrane reactor was studied by a Japanese group (T. Yamane, M.M. Hoq, Nagoya University, Japan). The opportunity of this system is the fact that the membrane has an open, microporous structure, which permits the adsorption of a high amount of enzyme in comparison to the superficial surface area: enzyme loads of 2.27 g crude enzyme.$m^{-2}$ (0.34 g protein.$m^{-2}$) were reported (1). From an activity, enzyme-load curve the specific activity was calculated as 1.46 mole.$h^{-1}$.{g crude enzyme}$^{-1}$, which value is in agreement with the value found for the hydrophilic membrane reactor which was reported in chapter 6 (1.7 mole.$h^{-1}$.{g crude enzyme}$^{-1}$). This indicates that both in the hydrophobic and the hydrophilic MR the adsorbed enzyme has the same activity which presumably is its optimum activity. The maximum hydrolysis activity at zero product concentrations, related to the superficial surface area, was calculated to be around 3 mole.$m^{-2}$.$h^{-1}$ (1), while in the hydrophilic reactor a maximum activity of 0.33 mole.$m^{-2}$.$h^{-1}$ was obtained. This ten fold higher activity can be attributed to the higher enzyme load in the hydrophobic MR. The stability of the hydrophobic MR ($t_{1/2}$ = 2 days at 40°C) is lower than of the hydrophilic MR ($t_{1/2}$ = 16 days at 40°C), possibly due to wash-out of the enzyme.

A potential drawback of the hydrophobic MR is the leakage of lipid through the membrane into the water phase in large-scale processes. The pressure difference across the membrane was reported to be very critical for the lipid-leak and could not be controlled satisfactory (2). Another drawback of the hydrophobic membrane is its high costs relative to the cellulosic hollow fibers used in the hydrophilic MR, and the lower enzyme stability (half life time: 2 days at 40°C).

A different system combining emulsions and separation steps was published by Tanigaki *et al.* (3): Lipid hydrolysis was performed in a stirred system. Next, centrifugation of the total reaction mixture was carried out resulting in three different fractions: a lipid phase, a water phase and an interfacial layer. The lipase in the water phase was concentrated by ultrafiltration using a polyacrylonitrile membrane. In the subsequent batch cycle, the interfacial layer and the concentrated enzyme were added to the lipid and water phase. Repeated batch operation showed that in this way the enzyme is recovered quantitatively. An advantage of such a system as compared to the HR is the higher stability reported. The absence of a hydrophobic membrane implies lower investment costs, and possibly lower enzyme loss due to fouling. A drawback of such a system is that it is very labour-intensive. The long-term stability of the enzyme was not measured, but the stability can be expected to be less than in the MR.

Bühler *et al.* (4) published a continuous system based on reaction in an emulsion and separation

by centrifugation. In each cycle 90% of the lipid and 90% of the water phase were removed by centrifugation. The remaining interfacial layer was recycled. Obviously, a relatively high proportion of the enzyme (10%) was washed out in the water phase, probably due to the equilibrium of lipase between interface and water phase. This invoked the need for continuous enzyme addition: in order to obtain a stable activity, 0.62 g of crude enzyme (*Candida rugosa*) had to be added per kg of fatty acid produced. As compared to the reactor system of Tanigaki *et al.* (3), the advantage of continuous operation did not outweigh the very high enzyme loss.

The comparison above shows that a great number of variables must be considered for the selection of an optimum reactor system for a certain application. An advantage of the MR is the high enzyme stability, which can be a crucial benefit for valuable enzymes in high-added value applications. For low-added value applications the membrane costs of the hydrophilic MR may be prohibitive. In the hydrophobic MR, the membrane area needed is a factor 10 lower. However, this effect will be compensated at least partially by the lower enzyme stability and the higher costs per membrane area. The HR system is providing high potentials since a relatively low membrane area is needed in comparison with the amount of lipase active in the emulsion. Further development of the HR is needed in order to obviate the effects of fouling.

## Adsorption and interfacial behaviour

In chapter 6, the interfacial behaviour of lipase was studied. The results indicate that at saturation conditions in a stirred cell a multilayer of enzyme adsorbs. Only a part of this multilayer can be active. A possible explanation for this phenomenon is the formation of a multi-enzyme complex on the interface. Only the molecules that are in contact with the interface can be active, and thus the average activity of the adsorbed enzyme is lower than that of a monolayer.

In an emulsion system, the enzyme was shown to have a 9 times higher specific activity than in the stirred emulsion at loads below saturation (chapter 6). This can not be explained by the multi-enzyme theory, but rather indicates an influence of shear rate on the interfacial boundary layer. Though, mass transport was shown not to be rate limiting. Further fundamental research in this area is needed to elucidate these phenomena.

The adsorption of lipase to the hydrophilic membrane was studied in chapter 6. At saturation of the membrane interface, a protein balance showed that 4.4 mg protein.$m^{-2}$ was adsorbed. The SCR was found to be 0.05 mole.$m^{-2}$.$h^{-1}$, and the specific activity can be calculated as 11.4 mole.$h^{-1}$.{g protein}$^{-1}$. The slope of the activity, enzyme-load curve is 1.7 mole.$h^{-1}$.{g crude enzyme}$^{-1}$ for loads below saturation. This value represents also the specific activity of the enzyme, since at low loads no wash-out of enzyme was detected. Assuming the specific activity calculated from the protein balance and from the initial slope are equal, the adsorbed enzyme can be calculated to be 15% of the crude enzyme, which value corresponds with the protein content of the free enzyme. This calculation indicates that not only the pure lipase, but the total protein fraction of the preparation is adsorbed to the membrane. Conversely, the BSA experiments indicate that lipase adsorbs in preference to other proteins to the membrane: the activity at saturation was not influenced if the adsorption was carried out with a preparation to which BSA was added. Also a purified lipase preparation did not result in a higher SCR (unpublished results). The reason for this phenomenon may be the inaccuracy in the protein determination or the fact that lipase adsorbs to only

a part (30%) of the interface very specifically, while the remaining part of the interface is covered with other proteins. It can be concluded that the mechanism of lipase adsorption on the membrane can not be understood completely on basis of the experiments shown and that specific research in this area is needed.

The fact that lipase adsorbs to hydrophobic interfaces may be explained from its catalytic activity at lipid interfaces. However, the fact that lipase also adsorbs to the hydrophilic cellulose interface is not so self-evident. The measurements of activity and enzyme load indicate that not a multi-enzyme gel-layer is adsorbed, but that the enzyme adsorbs in a protein monolayer. Also the fact that the enzyme is released from the membrane in the presence of high salt concentrations (chapter 5) is an indication for adsorption. Adsorption to the hydrophilic cellulose surface though is not very likely. The exact interactions which play a role could possibly be found if a more detailed structure of the cellulosic membrane would be known. Possibly, during the manufacturing process the more hydrophobic groups or impurities in the material are directed to the membrane surface, resulting in an interfacial layer with a relatively high content of functional or hydrophobic groups. The theory that the manufacturing process somehow influences the membrane characteristics is supported by the fact that the enzyme adsorption is influenced by the membrane thickness: adsorption to 16 μm membranes was much better than adsorption on thinner membranes (unpublished results). The characteristics of the membrane are also influenced by aging of the membrane: the longer the operation time, the higher the SCR obtained. The process of aging is accelerated by treatment at higher temperatures (ENKA, personal communication), as rinsing with hot water. The effect of membrane age may be the reason for different SCR values reported in this thesis. In the experiments of the influence of enzyme load (chapter 6) similar membranes were used with a comparable age and a similar pretreatment method was applied.

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# SUMMARY

The production of fatty acids from lipids (fats and oils) currently takes place in a physical chemical process at a high temperature and pressure. Fatty acids are applied in numerous products such as soaps, detergents and chemicals for pharmaceutical, household and industrial applications. For certain applications the conventional fatty acid production process is not suitable because of side reactions or because of the impurity of the end product. For that kind of applications an enzymatic process, taking place under atmospheric pressure and moderate temperatures, can be appropriate. Another advantage of the enzymatic process is the relatively low energy input.

A potential drawback of the enzymatic hydrolysis is however the high enzyme cost as compared to the added value of the end product. This invokes the necessity to re-use the enzyme (lipase). Re-use can be carried out readily when the enzyme is immobilized. Usually enzymes are immobilized to an inert carrier. This method however is not appropriate for lipases since the hydrolysis reaction takes place at the interface of a lipid and a water phase; the spontaneous adsorption of the free enzyme to the interface will not take place when it is bonded to a carrier. A membrane however provides the opportunity to immobilize the enzyme just at the interface of lipid and water. For the hydrophilic membrane used in this thesis, the lipase was immobilized on the lipid-side of hollow fiber membranes (chapter 2). The water phase can diffuse in the membrane, while the membrane is impermeable for the lipid phase. This results in an interface at the lipid-side of the membrane where the enzyme is present. The aim of this study was to further develop the knowledge on the membrane reactor and to optimize the system. This was done by investigating fundamental aspects such as reactor parameters, hydrolysis kinetics and mass transfer, as well as practical aspects such as operation mode and system design.

The occurrence of possible mass transfer limitations was studied in chapter 3. The diffusion of glycerol/water in the membrane wall as well as the diffusion of lipid components in the core of the hollow fiber membrane was studied using mass transfer models. The rate of both diffusion phenomena appeared to be an order of magnitude higher than the conversion rates reached. Therefore, diffusion was concluded not to limit the conversion rate in the membrane reactor.

In chapter 2 the concept of the hydrophilic membrane bioreactor was described and the results of batch hydrolysis experiments were presented. From these results a power law model for the activity as a function of the fatty acid concentration was derived. This model was further worked out in chapter 4; the value of the power was determined more accurately and the influence of the glycerol concentration was incorporated in the model. The kinetics of the free and immobilized enzyme showed no substantial differences. In chapter 4 also a more fundamental model was proposed based on the three consecutive hydrolysis steps. This model could predict the concentrations of fatty acids, mono-, di- and triglycerides in batch experiments at different glycerol concentrations.

The stability was studied in chapter 2 in continuous-flow experiments. The half life time of lipase in the membrane reactor was determined as 43 days. In chapter 5 it was shown that the stability of the system

can be increased by more than a factor 10 by the addition of $CaCl_2$ to the water phase. Under comparable conditions, the stability of the membrane-immobilized lipase was shown to be a factor 4 more stable than the free enzyme. For both systems, Arrhenius kinetics were valid for the enzyme inactivation. Also the hydrolysis reaction complies with Arrhenius kinetics, as proven for the free enzyme. Models for the prediction of the effect of temperature on activity and productivity showed that on a long term the lowest possible temperature is favoured. Further, in chapter 5 the influences of pH and other additions to the water phase were investigated.

The immobilization of lipase on the membrane was carried out by ultrafiltration of a lipase solution. In a period of 100 h after immobilization, wash-out of enzyme took place (chapter 2 and 6). Thereafter, no free enzyme could be detected any more in the reactor. Ultrafiltration results in a gel layer; There are strong indications that finally a monolayer of lipase remains on the membrane. This would imply that only the lipase-membrane interactions and not the inter-lipase interactions are strong enough to withstand the shear force.

The interfacial behaviour of the free enzyme was studied in a stirred cell with a controlled interface. Under saturation conditions, the free enzyme adsorbed to the lipid/water interface in a multi-molecular layer (corresponding to *ca.* 11 lipase layers). Only a part of this layer appeared to be active (corresponding to *ca.* 3 lipase layers). The activity related to the amount of protein below saturation corresponded with the activity related to the amount of protein immobilized in the membrane reactor. Therefore, it can be concluded that immobilization to the membrane does not result in a loss of activity.

An alternative hydrolysis reactor is presented in chapter 7. It consists of an emulsion and two circuits, in which ultrafiltration is carried out with a hydrophilic and a hydrophobic membrane. Thus the water/glycerol product flow is removed by the hydrophilic membrane and the fatty acid/glyceride product flow is removed by the hydrophobic membrane. In contrary to the hydrophilic membrane reactor, the reactive area in this hybrid reactor is not restricted by the membrane area. This implies that in the hybrid reactor the ratio of conversionrate and membrane area can be higher than in the hydrophilic membrane reactor. Indeed, the initial activity of the hybrid reactor, as related to the membrane area, was proved to be higher than the activity of the hydrophilic membrane reactor. A further development of the hybrid reactor system is needed in order to prevent membrane fouling and enzyme inactivation and to optimize the fluxes of the membranes.

It can be concluded that the hybrid has high potentials, but that further research is needed. The hydrophilic membrane reactor can be concluded to be a stable system yielding pure products glycerol/water and fatty acid/lipid. By adjusting the net flow rates of the phases, the final glycerol and fatty acid concentrations in the product flows can be regulated. The effects of temperature and product concentrations on the production rate in time can be predicted using the models presented. Thus the results can serve to evaluate the performance of this type of reactor for industrial lipid hydrolysis. The selection of an optimum hydrolysis reactor system for a certain application will require a similar evaluation for all alternative systems.

# SAMENVATTING

De produktie van vetzuren uit oliën en vetten vindt momenteel voornamelijk plaats in een fysisch-chemisch proces bij hoge temperatuur en druk. Vetzuren worden toegepast in talrijke produkten zoals zepen, schoonmaakmiddelen en chemicaliën voor farmaceutische, industriële en huishoudelijke toepassingen. Voor bepaalde toepassingen is de conventionele vetsplitsing echter niet optimaal vanwege het optreden van zijreakties of de onzuiverheid van het eindprodukt. Voor dit soort toepassingen kan een enzymatisch proces, wat plaatsvindt bij gematigde druk en temperatuur, beter geschikt zijn. Een ander voordeel van het enzymatisch proces is de relatief lage energiebehoefte.

Een mogelijk nadeel van de enzymatische hydrolyse zijn de hoge kosten van het enzym (lipase) in vergelijking met de toegevoegde waarde van het eindprodukt. Hierdoor wordt hergebruik van het enzym noodzakelijk. Dit kan bereikt worden door immobilisatie van het enzym. Normaal worden enzymen geïmmobiliseerd aan vaste dragers. Deze methode is echter niet geschikt voor lipases aangezien de hydrolyse-reaktie plaatsvindt aan het grensvlak van de olie- en de waterfase; de spontane adsorptie aan het grensvlak die optreedt in geval van vrij enzym is niet mogelijk indien het enzym gebonden is aan een drager. Een membraan kan de gelegenheid bieden om het enzym juist op het grensvlak van olie- en waterfase te immobiliseren. In geval van het hydrofiele membrane wat in dit proefschrift gebruikt is, wordt het lipase geïmmobiliseerd aan de olie-kant van een holle-vezel membraan. Het membraan houdt de oliefase tegen, maar de waterfase kan door het membraan diffunderen. Hierdoor ontstaat er dus een grensvlak aan de olie-kant van het membraan op de plaats waar het enzym vast zit. Het doel van het hier beschreven onderzoek was de verdere ontwikkeling van kennis op het gebied van lipase-membraan-reaktoren en de optimalisatie van deze systemen. Hiertoe werden zowel fundamentele aspecten (stof-transport, kinetiek, reaktor parameters) als meer praktische aspecten onderzocht.

Het stoftransport wordt in hoofdstuk 3 beschreven: de diffusie van glycerol/water in het membraan zowel als de diffusie van de olie-componenten in de vezel-kern werden bestudeerd met behulp van stoftransportmodellen. De snelheid waarbij beperkingen op gaan treden bleek voor beide een orde groter dan de tot nog toe bereikte omzetsnelheden. Hieruit werd geconcludeerd dat de omzetting niet beperkt wordt door stoftransport.

In hoofdstuk 2 wordt het concept van de hydrofiele membraan bioreaktor beschreven en worden de resultaten van batchgewijze hydrolyse getoond. Uit deze batch experimenten werd een kinetiek model op basis van de machtswet als functie van de vetzuurconcentratie afgeleid. Dit model werd verder uit-gewerkt in hoofdstuk 4: de waarde van de macht werd nauwkeuriger bepaald en de invloed van de glycerolconcentratie werd in het model verwerkt. Er konden geen wezenlijke verschillen ontdekt worden tussen de kinetiek van het vrij enzym en die van het membraan-gebonden lipase. In hoofdstuk 4 werd tevens een kinetiek model beschreven dat gebaseerd is op de drie hydrolyse stappen van triglyceride via diglyceride en monoglyceride naar vetzuren. Met behulp van een dergelijk model kunnen de concentraties van de genoemde produkten in batchgewijze experimenten tamelijk nauwkeurig voorspeld worden.

De stabiliteit van het systeem wordt in hoofdstuk 2 onderzocht in kontinue experimenten. In de hydrofiele membraanreactor werd een halfwaardetijd van 43 dagen bepaald. In hoofdstuk 5 wordt beschreven dat de stabiliteit van enzym een factor 10 hoger is in aanwezigheid van $CaCl_2$. Onder vergelijkbare omstandigheden is het membraangebonden enzym een factor 4 stabieler dan het vrije enzym. In beide systemen is Arrhenius kinetiek van toepassing op de enzym inaktiveringsreaktie. De hydrolyse reaktie voldoet ook aan Arrhenius kinetiek in geval van vrij enzym. Er werd een model opgesteld waarmee de invloed van de temperatuur op de aktiviteit en produktiviteit voorspeld kan worden. Uit simulaties bleek dat op de lange termijn de hoogste produktiviteit bereikt wordt bij de laagst mogelijke temperatuur. Voorts werden in hoofdstuk 4 het effect van de pH en van toevoegingen aan de waterfase beschreven.

De immobilisatie van lipase werd uitgevoerd door ultrafiltratie van een oplossing van het enzym in water. Tot 100 uur na deze procedure werd afspoeling van het enzym waargenomen (hoofdstuk 2 en 6). Na deze periode kon in de reaktor geen vrij enzym meer aangetoond worden. Door ultrafiltratie wordt een enzym-gellaag gevormd op het membraan. Er zijn aanwijzingen gevonden dat uiteindelijk een monolaag lipase op het membraan achterblijft. Dit zou betekenen dat alleen de lipase-membraan interactie sterk genoeg is om weerstand te bieden tegen de afschuifkrachten.

Het grensvlakgedrag van het vrije enzym werd onderzocht in een geroerde cel met een beheersbaar oppervlak. Onder verzadigingscondities adsorbeert het enzym in een meer-moleculaire laag (overeenkomend met *ca.* 11 lipase lagen). Slechts een gedeelte van deze laag (overeenkomend met *ca.* 3 lipase lagen) bleek aktief te zijn. De aktiviteit per hoeveelheid geadsorbeerd eiwit (bij beladingen lager dan verzadiging) kwam overeen de aktiviteit per hoeveelheid geïmmobiliseerd eiwit in de membraanreaktor. Hieruit kan geconcludeerd worden dat de immobilisatie van het enzym aan het membraan geen invloed heeft op de aktiviteit.

In hoofdstuk 7 werd een nieuw type hydrolyse reactor besproken. Het systeem bestond uit een emulsie en twee circuits waarin ultrafiltratie met een hydrofoob en een hydrofiel membraan werd uitgevoerd. De waterfase (water/glycerol) werd verwijderd door het hydrofiele membraan en de oliefase (vet/vetzuur) werd verwijderd door het hydrofobe membraan. In deze zogenaamde hybride reaktor wordt het reaktieve oppervlak niet beperkt door het membraanoppervlak, hetgeen wèl het geval is in de hydrofiele membraanreaktor. Hierdoor kan in principe een kleiner membraanoppervlak gebruikt worden. Uit de experimenten bleek inderdaad dat de initiële aktiviteit per oppervlakte eenheid membraan hoger was dan in de hydrofiele membraanreaktor. Voor een verdere ontwikkeling van de hybride reaktor is voortgezet onderzoek nodig: de membraanvervuiling en enzym-inaktivering moet verminderd worden en de membraanfluxen moeten worden geoptimaliseerd.

Geconcludeerd kan worden dat de hybride reaktor goede mogelijkheden biedt, maar dat voortgezet onderzoek nodig is. Verder kan geconcludeerd worden dat de hydrofiele membraanreaktor een stabiel systeem is waarmee zuivere produktfasen (vet/vetzuur en glycerol/water) geproduceerd kunnen worden. Door het instellen van de netto stroomsnelheden kunnen de produktconcentraties in beide fasen gereguleerd worden. Het effect van temperatuur en produktconcentraties op de produktiesnelheid als functie van de tijd kan voorspeld worden met behulp van kinetiek modellen. De verkregen resultaten kunnen gebruikt worden om de toepasbaarheid van dit type reaktor voor industriële toepassingen af te wegen. De keuze van een optimaal reaktor systeem voor de hydrolyse van oliën en vetten voor een bepaalde toepassing vereist een soortgelijke optimalisatie van alle alternatieve systemen.

# CURRICULUM VITAE

De auteur werd geboren op 23 juli 1961 te Naarden. Hij behaalde zijn VWO diploma in 1979 aan het Willem de Zwijger Lyceum te Bussum. Van 1979 tot 1985 studeerde hij aan de Landbouwuniversiteit Wageningen in de studierichting Moleculaire Wetenschappen, oriëntatie Biotechnologie. In de doctoraalfase deed hij onderzoek bij de vakgroep Biochemie en bij de vakgroep Erfelijkheidsleer. Tevens startte hij bij de sectie Proceskunde van de vakgroep Levensmiddelentechnologie het onderzoek naar de hydrolyse van triglycerides in een membraanreaktor. Dit was aanleiding tot een promotieonderzoek, waarmee in 1985 werd gestart. Het onderzoek werd gefinancierd door de Programma Commissie Membraantechnologie en door Rhenus BV.

Vanaf 1989 is de auteur werkzaam bij de sectie Milieu en Veiligheids Research van DSM Research te Geleen.