Enzymatic acylglycerol synthesis in membrane reactor systems

Promotor: dr. ir. K. van 't Riet
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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
op vrijdag 11 juni 1993,
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Voorwoord

Al voor, maar zeker ook tijdens mijn onderzoek hebben veel mensen mij een stukje verder geholpen. Een aantal wil ik graag met name bedanken.

Lieve ouders, al vanaf mijn prille jeugd hebben jullie je ingezet om mij te leren omgaan met mijn leesblindheid. Jullie hebben ervoor gezorgd dat ik heb leren lezen, schrijven en rekenen, jullie hebben mij begeleid met de schoolkeuzes. Graag wil ik jullie hiervoor bedanken, zonder die eerste begeleiding was dit werk nooit tot stand gekomen.

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Stellingen

1 Bij de beschrijving van omesteringsreacties met behulp van de zogenaamde omesteringsconversiegraad wordt de eventuele hydrolyse niet meegewogen waardoor het rendement van de reactie wordt overschat.

S.W. Cho, J.S. Rhee, Biotechnol. Bioeng. 41 (1993) 204-210

2 Het probleem bij omesteringsreacties is niet het vinden van de geschikte biocatalysator maar de reactiecondities dusdanig te kiezen dat nauwelijks mono- en diesters zullen ontstaan.

S.W. Cho, J.S. Rhee, Biotechnol. Bioeng. 41 (1993) 204-210
Hoofdstuk 7, dit proefschrift

3 Het meten van de substraatconcentratie op tijdstip t van een enzymatisch gecatalyseerde reactie als functie van de hoeveelheid enzym levert in veel gevallen minder informatie op dan het meten van de concentratie in het verloop van de tijd bij een bepaalde hoeveelheid enzym.

S. Yamaguchi, T. Mase, J. Fermentation Bioeng. 72 (1991) 162-167

4 Bij het ontwerp van het micel-membraanreaktorsysteem voor de hydrolyse van olijfolie zoals beschreven door Prazeres en coauteurs wordt ten onrechte voorbijgegaan aan de noodzaak van continue glycerolverwijdering uit de micelfase.

D.M.F. Prazeres, F.A.P. Garcia, J.M.S. Cabral, Biotechnol. Bioeng. 41 (1993) 761-770

5 Onverwachte metingen vormen de basis voor nieuwe publikaties.

Hoofdstuk 2 en 3, dit proefschrift

6 Het verschil tussen een samenlevingscontract en huwelijksvoorwaarden is 150 gulden.

7 De overheidsmededeling dat het licht is overdag en dus veiliger, is een variatie op de volkswijsheid dat het 's nachts onveilig is op straat.

Verkeersafleidingsbord: Licht overdag, veiliger overdag.

8 Te hard rijden spaart weliswaar tijd op de weg, maar geen tijd aan de pomp.

9 Voor het bestaan van moleculen bestaat geen harder bewijs dan voor het bestaan van God.

10 *Padtstelling:*
De beschikbare tijd is een kwestie van prioriteit.

11 In vele proefschriften wordt in het voorwoord melding gemaakt van het feit dat je nooit in je ééntje kunt promoveren, dit wordt echter zelden in praktijk gebracht.

Stellingen behorende bij het proefschrift "Enzymatic acylglycerol synthesis in membrane reactor systems".

A. van der Padt
Wageningen, 11 juni 1993.

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1 Biomodification of fats and oils

Introduction

Back in 1973, at the American Oil Chemists' Society spring meeting, the potentials and applications of agricultural oils for novel uses were discussed (Tallent *et al.* 1974). At that time only chemical modifications were presented for production of, for instance, fatty amides and glycolipids. Nowadays, besides the chemical route, these compounds are also synthesized on lab scale using enzymes or microorganisms as a catalyst (Schmid 1987). From 1974 onwards it has become clear that the production of basic chemicals from agricultural oils can be achieved by the biomodification and biotransformation of fats and oils. Biomodification can also be applied for food applications. However, both the biocatalysts and the bioreactors involved in these, mainly enzymatic, conversions are expensive and not yet optimized (Yamane 1988).

There are three levels of fat and oil biomodification. Firstly, the genetic engineering and/or cloning of the oil producing crops that may result in oils possessing required properties. Secondly, the selection and fermentation of microorganisms which produce oils or fatty acids, the so-called single cell oil. Finally, the enzymatically catalyzed modification of animal or vegetable fats and oils. This thesis deals about oil and fat modifications using enzymes.

Applications of fats and oils in the chemical industry

Approximately 20% of the world production of oils and fats is utilized in non-food applications, such as detergents, lubricants, paints and plastics (Kaufman and Ruebusch 1990). When applied as a surface active compound, this surface activity of

a compound is usually expressed in a hydrophilic lipophilic balance value (HLB), the higher this value, the better the surface stabilizing capacity is (Cahn and Lynn 1983, Griffin 1979, Young *et al* 1986).
Emulsifying properties depend on the tail length of a fatty acid, a shorter tail will lead to an increased HLB value (Cahn and Lynn 1983). In order to obtain different fatty acid tail lengths, different oil sources can be used. Not only the tail length of the fatty acid is of influence, but the head group also determines the surface stabilising capacity. Some examples are saponified fatty acids, monoglycerides, fatty amines and amides. The latter ones can be used as a lubricant also (Magne *et al.* 1974).
Free fatty acids have a low HLB value of approximately 1 and can be used as antifoamer. Sometimes, an oil can be used directly as antifoamer. For example, rice bran oil contains over 60% w/w free fatty acid and can therefore be used directly (El-Zanati *et al.* 1990). In other cases, the fatty acids have to be liberated prior to use.

Fatty acids or fatty amides can be used with polymer production as an additive to alter the physical characteristics of the polymer (Molnar 1974, Ong *et al* 1989). Fatty acids can also be utilized as a starter material for the production of polymers. Unsaturated fatty acids can be polymerized directly by radical initiated double bond polymerization. Fatty acids can be dimerized chemically and be used for the production of polyamides. Another route to alter the polymer characteristics is coupling of fatty acids or its derivatives to active side groups of a polymer by an addition reaction (Oberkobusch 1990). Some examples of these derivatives are fatty alcohols and fatty amines.
Oils or fatty acids can also be used as the starter material to produce the monomeric esters, to be used for the synthesis of polymers (Kine and Novak 1979).

In the past, paints and varnishes have been based on animal or plant oils (Young *et al* 1986). Nowadays, oil is incorporated chemically as a component of polymer resins. These resins on their turn are dissolved in a petrochemical solvent. For environmental reasons, the use of petrochemicals in paints and varnishes becomes restricted. Again, natural oils come into account as environmentally more friendly solvents. Those oils should be highly unsaturated for fast drying paints, conjugated for high water resistant paints and non-drying for plasticising paint (Young *et al* 1986).

Applications of fats and oils in the food and cosmetic industry

The bulk application of fats and oils is the preparation of edible oil, mayonnaise, chocolate and so on. In these cases the oils or fats are used directly after the conventional oils and fats processing. Sometimes, however, triglycerides are modified in order to improve the quality. This can be done to obtain a change in melting range and taste, an improved shelf life or a more healthful product (Young *et al* 1986).
The melting range of a triglyceride can be manipulated by changing the fatty acid composition of the glyceride (Trommelen 1990). This can be realized either by transesterification of the oil or by the search for another oil source. Not only the melting range changes upon a change in the fatty acid composition, also the health effect of the incorporated fatty acids has to be taken into account (Katan 1990).

Another group of fatty acid esters can be added as flavors. Often this is the ester of a fatty acid and a linear alcohol, such as methyl esters up to myristic esters or terpenes (Heath 1978, Rogers and Fischetti 1980). Terpenes have one or more chiral centres and often only one of the enantiomers has the desired taste or odour (Heath 1978). Therefore, these compounds are preferred to be optically pure.

Fatty acids, monoacylglycerols and sugar esters are used as emulsifiers in food and cosmetics (Tsen 1974, Young *et al* 1986). Emulsifying properties have been discussed in the previous paragraph.

Enzymatic modifications of fats and oils

Possible reactions
When an oil is hydrolyzed, fatty acids are liberated and the triglycerides change into diglycerides, then into monoglycerides and they will end up in glycerol (figure 1). The reverse reaction is the esterification of a fatty acid and glycerol, in this case mono- and diglycerides or even triglycerides can be produced. Both reactions can be catalyzed by the enzyme lipase (Mukherjee 1990). Not only water can act as the nucleophile during the hydrolysis, also an alcohol can act as nucleophile (Yamane 1988). In that case the

reaction is called transesterification (figure 2). A special case of the transesterification is the glycerolysis, now glycerol acts as the nucleophile and a mixture of partial glycerides will be obtained (McNeill *et al.* 1990).

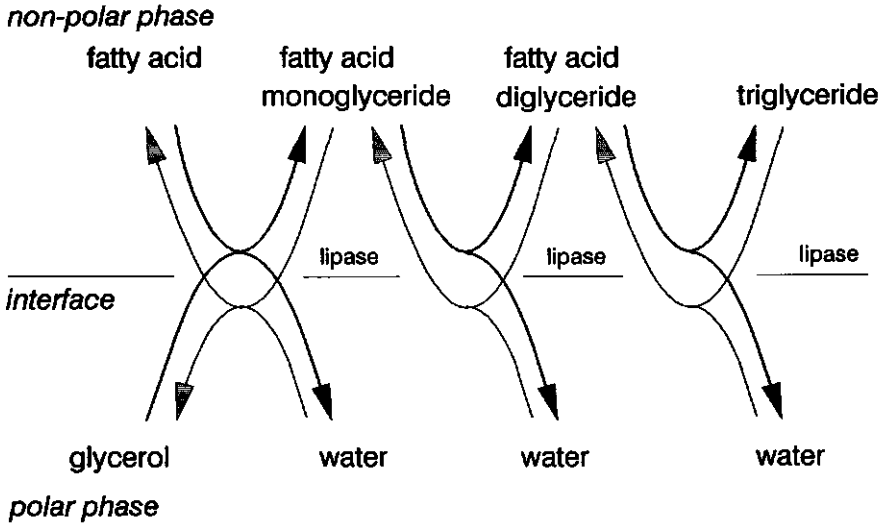

Figure 1: The hydrolysis versus esterification reaction.

Also for esterification, other alcohols can be used. An example is the production of flavor esters, for example an ethyl or butyl ester (Gillies *et al.* 1987, Leitgeb and Knez 1990). Another type of alcohol is the group of carbohydrates. The mono- and diester of carbohydrates and fatty acid can be used as surfactant. These sugar esters can be produced enzymatically in dimethylformamide as solvent (Ampon *et al.* 1991, Riva *et al.* 1988), or in a water fatty acid emulsion (Janssen *et al.* 1990, Seino 1984). In case of application as emulsifier in food, the non-solvent route has to be preferred.

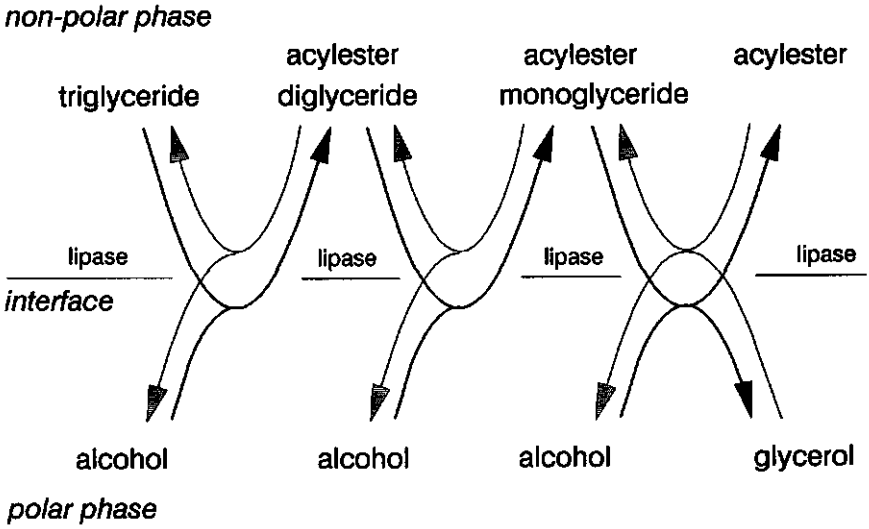

Figure 2: The transesterification reaction. The alcohol involved is glycerol in case of the glycerolysis reaction.

The fatty acid composition of an oil or fat can be changed following two different routes (Macrae 1989). One is called the interesterification process, this occurs when two esters are mixed in the presence of a catalyst (Chang *et al.* 1990). The fatty acids will be exchanged and a random fatty acid composition will be obtained. Another way to change the fatty acid composition is the addition of fatty acids. The esterified fatty acids will be partly liberated while the added fatty acids will be partly incorporated (Luck 1989). This is called the acidolyses.

The reactions as described above can be classified as the traditional lipase catalyzed reactions. Recently, the search for alternative production routes has started for the synthesis of lubricants, monomers for plastic production and even for the polymer itself. Examples are acylamides (Montet *et al.* 1989), hydroxamic acids (Servat *et al.*

1990) and acyllycines (Montet *et al.* 1990) to be used as surface active compounds. All these reactions are catalyzed by an immobilized lipase in a solvent. A relation is found between the yield and type of solvent. The production of monomers such as acrylate monoester has been described (Hajjar *et al.* 1990, Tor *et al.* 1990). Even more, also the production of alkyds and the production of optically active polymers is reported (Geresh and Gilboa 1990, Wallace and Morrow 1989). The degree of polymerization is solvent dependent.

Biocatalyst

When enzymes are involved in the biomodification, a lipase (triacylglycerol hydrolase) is usually chosen (Yamane 1988). Not only lipase can be used in order to catalyze the hydrolysis and esterification reaction, also the use of other enzymes such as phospholipase, subtilisin, trypsin and chymotripsin has been reported in literature (Ampon *et al.* 1991, Riva *et al.* 1988, Yamane 1988, Zaks and Klibanov 1988). This work is restricted to the use of lipase of *Candida rugosa* as catalyst.

Selectivity is an important aspect of lipases. Roughly, four classes of selectivity can be distinguished (Jensen *et al.* 1990). Firstly, the lipid class selectivity. An enzyme can be specific to the type of ester, for example, a difference in selectivity for tri-, di- or monoglycerides, cholesterol esters, methyl esters etcetera. Secondly, regio- or nonregioselectivity, which concerns selectivity for the position of the ester bond in a molecule. *Mucor miehei* lipase only reacts with the 1,3 acylgroups of an oil or fat (regioselective), while *Candida rugosa* lipase can hydrolyse all ester bonds of a triglyceride. Thirdly, fatty acid selectivity, which concerns the selectivity for fatty acid chain length or for the presence of a double bond (*Geotrichum candidum* lipase). Finally, stereo selectivity, some lipases only catalyze the hydrolysis or esterification of one of the two stereo isomers. Of course, often a combination of these selectivities is found (Jensen *et al.* 1990).

Lipase activity

A proposed mechanism of the lipase catalyzed hydrolysis is that the ester, or a fatty acid in the case of esterification, forms a non-covalent enzyme/ester complex with the enzyme. For esterification, this complex transforms into an acyl enzyme complex and

water is released. This is followed by an attack of a nucleophile, an alcohol, to form another covalent complex, which then yields a new ester and the free enzyme (Brockerhoff 1973).

Commonly, lipases are only active at interfaces, which can either be the oil-water interface or the surface of an immobilization carrier. The fact that lipase needs an interface to be active has already been recognized by Sarda and Desnuelle (1958). This implies that Michaelis-Menten kinetics alone cannot describe lipase activity (Ekiz *et al.* 1988, Kierkels *et al.* 1990, Mukataka *et al.* 1985). It can be concluded that the reaction rate in a two phase system is determined by the specific area, the enzyme load, the maximum activity per reaction area and the activity of the enzyme per gram of enzyme.

Lipase stability

In a non-polar environment, the enzyme stability is, besides temperature, also related to the properties of the organic solvent present in the system (Laane *et al.* 1987, Zaks and Klibanov 1988). Also an effect of the concentration can be expected in case of a water miscible solvent (Mozhaev *et al.* 1989). For instance, esterification of fatty acid and glycerol as performed by *Candida rugosa* lipase only occurs at moderate glycerol concentrations (Touraine and Drapron 1988, Tsujisaka *et al.* 1977). At high glycerol contents, the enzyme is inactivated almost instantaneously. This might explain that some authors have concluded that this type of lipase is not capable synthesizing glycerides at all (Hoq *et al.* 1984, McNeill *et al.* 1990). Inactivation is also found by Mozhaev and coworkers (1989). They found that only 50% of the initial α - chymotrypsin activity remained in a 73% by volume glycerol solution.

To improve the stability of the biocatalyst in organic solvents, the biocatalyst can be immobilized (Eigtved *et al.* 1988, Inada *et al.* 1986 and 1990, Mozhaev *et al.* 1990). It has been shown that not only the stability is improved, but also a change in selectivity can be induced by immobilization (Jensen 1990). Another advantage of immobilization is the possibility for reuse of the catalyst.

Equilibrium

In a system which contains an oil or fat and excess of water, the reaction will be in favour of the fatty acid production. Almost 100% fatty acid liberation can be obtained when the glycerol concentration of the water phase remains below 10% (w/w) (Bühler and Wandrey 1988, Pronk *et al.* 1988). Upon an increase of the glycerol concentration, partial glycerides will remain (Pronk 1992).

The water content is a very important parameter for the final equilibrium situation. In case of esterification, water produced during the process will encourage the hydrolysis reaction. Another water source during continuous synthesis is the substrate itself, as the oil at the inlet contains water. This affects the esterification and transesterification. The water accumulates and will enhance the hydrolysis resulting in an oil which contains partial glycerides (Goldberg *et al* 1988, Luck 1989).

Esterification can be achieved when glycerol is brought into contact with a fatty acid in the presence of lipase (Hoq *et al.* 1984, Mukerjee 1990, Touraine and Drapron 1988, Tsujisaka *et al.* 1977). A mixture of mono-, di- and triacylglycerols is found. The higher the glycerol concentration the higher the esterified fatty acid fraction is (Ergan and Trani 1991, Touraine and Drapron 1988). The production can be forced to complete substrate conversion if either the acylglycerol or water is removed (Semenov 1989). An example is found (McNeill *et al.* 1990) where oil is dispersed in a 96.4% glycerol solution as polar phase and all incorporated fatty acids of the starter oil were converted in monoacylglycerols. This occurred because the monoesters did precipitate. Ergan and Trani (1991) showed a procedure to incorporate over 90% of the fatty acids into triacylglycerols, with initial stoichiometric amounts of fatty acid and glycerol. After complete esterification of the glycerol to mono-, di- and triacylglycerols a one-phase system was attained, then the water produced was removed, thus enhancing the esterification of mono- and diglycerides into triglycerides.

As stated, an increase in the water concentration leads to a reduced ester concentration. The ester concentration can be enhanced by using an organic solvent. With esterification, the equilibrium constant can be calculated using thermodynamic activities. The equilibrium constant is not dependent on the presence or type of solvent, however, the activity coefficients do change. This can significantly influence the

equilibrium mole fraction of ester (Halling 1990). The activity coefficients can be calculated and the influence of the solvent upon the productivity can thus be estimated (Janssen *et al.* 1993). Concentrations cannot be used because this is valid for diluted systems, often esterification occurs in non-diluted systems.

Bioreactors

Bioreactors can be divided into one-liquid-phase systems and two-liquid-phase systems. For one-liquid-phase systems all the substrates and products are soluble in a single phase. Examples are esterification, transesterification, interesterification and acidolysis (Chang *et al.* 1990, Leitgeb and Knez 1990, Luck 1989, McNeill *et al.* 1990). Mostly, this single phase is a non-polar one.
In a one-liquid-phase system the lipase must be immobilized on a carrier material to create an interface. As mentioned above, during continuous synthesis, the biocatalyst carrier can adsorb water from the feed stock. This results in a reduced productivity, because the equilibrium will tend toward the hydrolysis reaction (Luck 1989, Macrae 1989). An immobilisation carrier is needed that does not adsorb water. A suitable material will be a hydrophobic matrix. However, enzymes will usually spread on these materials and will thus be inactivated. Therefore, the development of hydrophobic carriers on which the immobilized enzyme stays active is of great importance (Macrae 1989). An example is the development of poly(propyle) or poly(amide) resins (Goldberg *et al* 1988). Another method to dissolve the enzyme in an organic phase is to modify the lipase with poly(ethylene) glycol (Inada *et al.* 1990).

A two-liquid-phase system is required for the hydrolysis of oils and fats and the esterification of various water soluble alcohols (for instance glycerol) and long chain fatty acids (Mukerjee 1990, Pronk *et al.* 1988, Tsujisaka *et al.* 1977). The reaction medium consists of two phases if the fatty acids involved have a minimum tail length of 4 carbon groups. This reaction can be carried out in an emulsion system or a membrane system (Mukerjee 1990). In both systems the oil and water phase are

brought into contact, either by mixing or by a membrane (figure 3). The reaction rate in such a two phase system is determined by the specific area, the enzyme load, the maximum activity per reaction area and the activity of the enzyme per gram of enzyme.

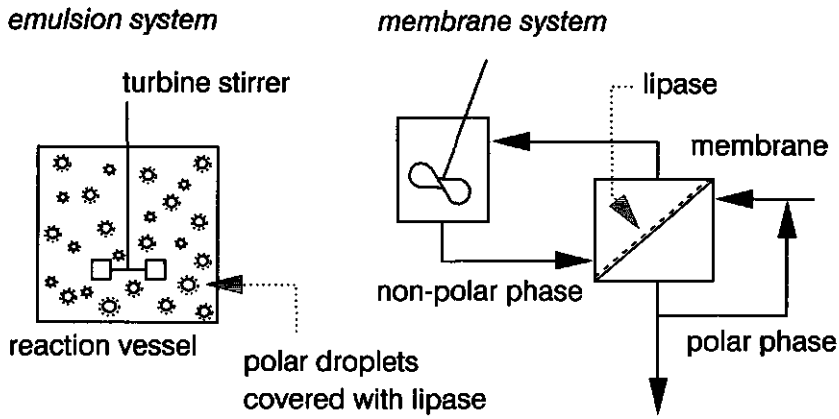

Figure 3: An emulsion system and a membrane system.

As discussed, the production of an excess of free fatty acids, monoglycerides or triglycerides needs continuous product removal. In case of hydrolysis, the water phase has to be controlled in such way that the glycerol concentration remains lower than 10 %. This can easily be done in a membrane system in which the glycerol-water phase and the oil phase are separated (Pronk *et al.* 1988).

McNeill and coworkers (1990) have shown an emulsion reactor for the production of monoesters. In this reactor, the monoesters produced precipitate and a solid monoester enzyme phase is leftover. However, the enzyme is also present in the precipitate and no efficient methods are available to separate the enzyme and monoglyceride, with maintained enzyme activity.

Ergan and Trani (1991) have shown that an excess of triacylglycerols can be produced only when water is removed continuously. In that case all the glycerol is incorporated

and finally a one-phase system is obtained. When starting with a mixture of mono-, di- and triesters and fatty acids, esterification of those partial glycerides and fatty acid can be done in a one-phase reactor such as a packed bed (Goldberg *et al.* 1988).

One of the advantages of the membrane system is that the synthesis can be performed continuously (Hoq *et al.* 1984). The membrane bioreactor can be performed with containment of the enzyme and in-line phase separation. Bühler and Wandrey (1988) presented an emulsion reactor where the phases are separated by centrifugation. The water and oil phase are partly removed, while the interfacial middle layer, which contains the enzyme is returned to the reaction vessel. Due to the high shear rates in the centrifuge, the enzyme stability is not sufficient to make this system commercially attractive. Pronk and coworkers (1991), have shown that the two phases from an emulsion reactor can be separated with a sequence of a hydrophilic and a hydrophobic membrane. In this process, again the stability of the enzyme is the limiting factor.

Aim of this work

As described above, a few questions do arise when a reactor has to be designed for the synthesis of acylglycerols. First of all, the enzyme kinetics in a two-phase system should be known. Secondly, an optimum glycerol concentration can be expected at which a stable enzyme is obtained while esterification proceeds. In other words, does a relation between glycerol concentration and enzyme stability exist? Thirdly, a predictive model for the equilibrium ester concentrations should be available. When these three questions are answered by mechanistic models, these models can lead to an optimal reactor concept for triacylglycerol synthesis or monoester production. The aim of this thesis is twofold: to gain more fundamental knowledge about kinetics and equilibrium of lipase catalyzed esterification and to determine the engineering phenomena that are relevant for the operation of acylglycerol production systems.

Outline of this thesis

This thesis discusses the enzymatic esterification of glycerol and decanoic acid. Reactor concepts are presented for monoacylglycerol and triacylglycerol synthesis.

The reaction rate in a two phase system is determined by the specific area, the enzyme load, its maximum activity per reaction area and the activity of the enzyme per gram of enzyme. The activity of lipase of *Candida rugosa* in a two phase system is discussed for an emulsion system and a membrane system in chapter 2 of this thesis.
The activity and stability of *Candida rugosa* lipase in a glycerol-water mixture is described in chapter 3.
The acylglycerol equilibrium data are discussed in chapter 4. This chapter shows that neither an excess of monoacylglycerols nor an excess of triacylglycerols can be obtained in a two phase system.
An excess triglycerides can be produced only when water is removed continuously and conditions for which all the glycerol is incorporated in the end. In that case, a one-phase system is obtained. This can be done by starting with the creation of a mixture of mono-, di- and triacylglycerols in a two-phase membrane reactor. Afterwards, further esterification of those partial glycerides and fatty acid can be carried out in a one-phase reactor with continuous water removal. One way to avoid water accumulation in a one-phase reactor is the immobilisation of the enzyme onto a membrane which is used for the continuous drying of the oil phase. A more detailed description is given in chapter 5 of this thesis.
Chapter 6 shows a membrane reactor with an in-line adsorption column. This three phase system is developed for the synthesis of a surplus of monoesters. The monoglycerides adsorb preferentially onto the silica column. The reaction proceeds since the monoesters are removed continuously from the reaction medium. The column can be desorbed off-line and the enzyme can be reused.
A general discussion of the potentials and the limitations of the systems presented in this thesis is given in chapter 7. Furthermore, the choices made during this study are discussed reviewing the results.

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2 Membrane bioreactor.

Abstract

Lipases can catalyse the esterification reaction in a two phase system. The *Candida rugosa* lipase catalysed esterification of decanoic acid with glycerol is described in this work for an emulsion system and for a hydrophilic membrane bioreactor. The enzymatic activity is studied in relation to the interface area between the two phases, the enzyme load and the reactor volume. The initial rate per unit interface area, the interfacial activity, is roughly equal for both systems indicating that the cellulose membrane does not hinder the esterification. Because the interfacial activities are equal, the volumetric activity of a membrane system is only specific area related, so a hollow fibre membrane device is preferable. The activity is also a function of the enzyme load. The optimum load in a hydrophilic membrane reactor is one to three times the amount of a monolayer, while in an emulsion system several times this amount. This could indicate that in the emulsion system the adsorption is in a dynamic state while at the membrane surface the adsorption reached its equilibrium state.

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Introduction

This paper deals with lipase catalysed esterification. The general esterification reaction is that of an acid group (often a fatty acid) with an alcohol group with the formation of the ester and a molecule water. Lipases are able to catalyse esterification reactions of fatty acids with alcohols, producing fats and oils and even fine chemicals (chiral compounds, epoxy esters). Sometimes the alcohol and the fatty acid are mutually soluble; usually, however, a two phase system is obtained. This article will be restricted to the two phase system. In case of a two phase system the reaction occurs at the interface in the presence of the enzyme (figure 1). The industrial interest in esterification and hydrolysis is growing very fast. Therefore, research on the process engineering of esterifying systems should point out whether or not these systems are economically viable.

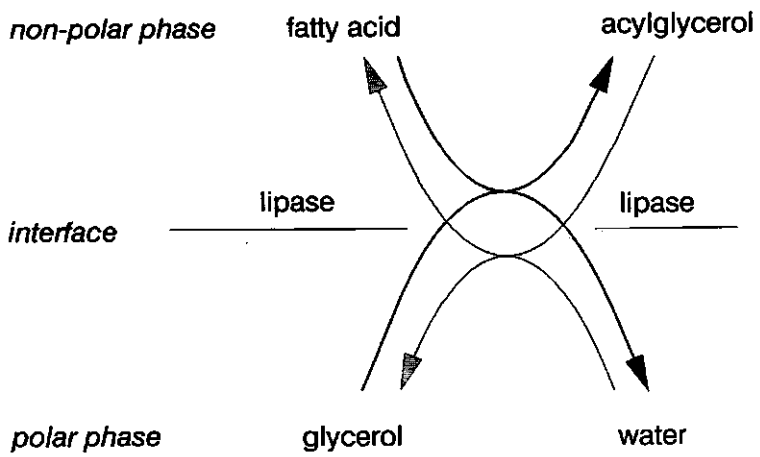

Figure 1: The fatty acid triacylglycerol equilibrium.

In the literature, enzymatic hydrolysis, interesterification and esterification are described. Several publications describe the hydrolyses of oils and fats for the production of bulk fatty acids or special fatty acids (e.g. ricinoleic acid) in an emulsion system (O'Connor *et al.* 1988) or in a membrane system (Pronk *et al.* 1988, Hoq *et al.* 1985a). The fat and oil interesterification experiments are mostly concerned with an immobilised lipase on lab scale (Macrae *et al.* 1983) or on pilot plant scale (Posorske *et al.* 1988). Work is also done on the synthesis of oils and fats by esterification starting with the basic compounds using an enzyme as catalyst (Bell *et al.* 1978, Miller *et al.* 1988, Tsujisaka *et al.* 1977, Okumura *et al.* 1979, Linfield *et al.* 1984, Hoq *et al.* 1984, 1985b). These lipase catalysed reactions at moderate temperature and low water activity are different from the method as presented by Zaks and Klibanov (1985); their experiments showed, that enzymes can also be active in organic media with an extremely low water content (approximately 0.02%), and sometimes also at high temperatures (up to 100 °C).

The literature (table 1) describes three lab scale esterifying systems for two phase reaction media:

1) The use of a solvent to change the two phase system in a one phase system: A solvent is chosen in which both the fatty acid and the alcohol are dissolved e.g. acetone, hexane or methyl isobutyl ketone (MIBK). The obtained enzyme activity is not only solvent dependent, but also a function of the nature of the substrate and its concentration as well as of the enzyme source. For example Bell *et al.* (1978) showed acylglycerol synthesis in acetone catalysed by *Rhizopus arrhizus* lipase, while Miller and coworkers (1988) found almost no activity for the synthesis of propyl myristate in acetone catalysed by *Mucor miehei* lipase. Since most lipases are known to be active at an interface only, the enzyme must be immobilised, creating a solvent / immobilisation carrier interface. Bell *et al.* (1978) used the mycelium of the lipase producing *Rhizopus arrhizus* itself, while Miller *et al.* (1988) used a commercially available immobilised lipase.

Table 1: The esterifying systems in literature.

System	Fatty acid	Alcohol	Lipase	Solvent	Reference
One phase	palmitic -	octanol	*Rhizopus arrhizus*	diisopropyl ether	Bell *et al.* (1978)
	C_4 - C_{10}	octanol	*Mucor miehei*	hexane MIBK acetone among others	Miller *et al.* (1988)
	oleic -	glycerol	*Rhizopus arrhizus*	acetone	Bell *et al.* (1978)
Emulsion	oleic - among others	glycerol among others	*Aspergillus niger* *Rhizopus delamar* *Geotrichum candidum* *Penicillium cyclopium*		Tsujisaka *et al.* (1977) and Okumura *et al.* (1979)
	oleic -	glycerol	*Aspergillus niger* *Pseudomonas sp.* *Mucor pusiilus* *Geotrichem candidum* *Rhizopus delamar*		Linfield *et al.* (1984)
Membrane	oleic -	glycerol	*Candida rugosa* *Mucor miehei* among others		Hoq *et al.* (1984) (1985b)

2) Two phase emulsion system: The alcohol phase, the fatty acid phase and the lipase, either on small particles or 'free', are mixed and thus an emulsion is obtained. The emulsion interface is created by energy input and the emulsifying qualities of the lipase itself. Sometimes an emulsifier is added (Tsujisaka *et al.* 1977). The lipase adsorbs to the interface.

3) The two phase membrane system: The two phases are kept separated by a membrane, this membrane is used as the immobilisation surface. One of the substrates diffuses through the membrane towards the interface between the two phases where the enzyme is immobilised.

The lipases are too costly to be used for a single batch conversion only. Therefore, reuse of the enzyme determines the success of an enzymatic synthesis in case of an industrial process. Two methods for reuse of the enzyme in two phase emulsion systems are published. Firstly, for the hydrolysis of an oil, it was shown that after centrifugation of the emulsion, the recovered interfacial layer contained about 80% of the initial activity (Bühler and Wandrey 1988). Secondly, by immobilisation of the biocatalyst by entrapment in a gel as immobilisation carrier or adsorption or covalent binding on grains the lipase could be recovered and used for several batches (Omar *et al.* 1988). Another method for reuse of the enzyme is immobilisation at the interface between the oil and alcohol phase in a membrane bioreactor. An additional advantage of the membrane reactor is the in situ separation of the fatty acid phase and the alcohol phase.

Hoq *et al.* (1984, 1985b) presented a hydrophobic membrane system for hydrolysis as well as for ester synthesis. The lipase is immobilised at the water-glycerol side of the membrane. A complication appeared to be that lipase does only partly adsorb at the membrane surface at high glycerol contents. In that case an enzyme-glycerol-water solution is circulated at one side of the membrane and high quantities of enzyme are required. Pronk and coworkers (1988) developed an enzymatic membrane system for the hydrolysis of soybean oil catalysed by lipase of *Candida rugosa*. Here the lipase is immobilised at the oil side of the membrane and because the membrane is impermeable for the enzyme, the enzyme cannot dissolve in the glycerol-water phase.

The hydrophilic membrane that is used can withstand transmembrane pressures larger then 10^5 N.m^{-2}, without leakage of the oil phase into the glycerol-water phase, which has distinct operation advantages.

It is not yet possible to determine which membrane system should be preferred for use in a large scale process. More data are needed, particularly about the activity of an esterifying hydrophilic membrane system. The objective of this study is to show the relationship between the enzymatic activity of a membrane bioreactor and a similar emulsion system.

Theory

Immobilisation of a biocatalyst can lead to an activity change not only caused by the immobilisation itself, but also caused by diffusional limitations in the immobilised biocatalyst system. The measured apparent activity of the immobilised system should be compared with the activity of the free enzyme system (the working party on immobilized biocatalysts within the European federation of biotechnology 1983). For a batch reactor the activity equals to the initial substrate removal rate r_i (mole.m^{-3}.s^{-1}):

$$r_i = \left(-\frac{dC_s}{dt} \right)_{t=0} \qquad (1)$$

Where C_S is the substrate concentration (mole.m^{-3}) and t is the time (s). Dealing with an enzymatic heterogeneous equilibrium reaction, three standard activities can be calculated: the volumetric initial rate, the interfacial initial rate and the enzymatic initial rate.

The volumetric initial rate $r_{i,V}$ (mole.m^{-3}.s^{-1}) is based on the total reactor volume, and is a measure given for the batch system volumetric efficiency. By nature of its definition, it is given by:

$$r_{i,V} = r_i \tag{2}$$

The enzymatic initial rate $r_{i,E}$ (mole.s^{-1}.kg^{-1}) is based on the enzyme present and indicates the enzyme activity:

$$r_{i,E} = \frac{r_i}{C_E} \tag{3}$$

where C_E : is the enzyme concentration (kg.m^{-3})

The interfacial initial rate $r_{i,A}$ (mole.s^{-1}.m^{-2}) is based on the interfacial area, and is a measure for the activity on the interface:

$$r_{i,A} = \frac{r_i}{A} \tag{4}$$

where A : is the specific area (m^2.m^{-3})

These three activities should point out whether or not the membrane reactor can compete with an emulsion system. The activity shows the initial rate of the reaction indicating that the higher the activity the sooner the equilibrium state is reached. The equilibrium value is thermodynamically determined so an emulsion experiment ends up with the same concentration as the membrane system.

Experimental

Materials

The 97% pure fatty acid, decanoic acid, was produced by Unichema Chemie (FRG) for this project. Glycerol 99$^+$% was obtained from Janssen (Belgium), hexane from Rathburg (UK), all other chemicals were gained from Merck (FRG). The *Candida*

rugosa lipase (formerly named *Candida cylindracea*) was the enzyme Lipase-OF 360 (Meito Sangyo, Japan). The commercial available Andante membrane (Organon, The Netherlands) was a hollow fibre device containing cellulose (Cuprophane, Enka, FRG) fibres with a diameter of 0.2 mm and a wall thickness of 8 μm, the total area was 0.77 m^2, the reactor volume was 150 mL.

Methods

The emulsion system was run in a thermostated (25 °C) reactor of standard geometry (inner diameter = 0.1 m) with four baffles and a four-bladed turbine (diameter = 0.04 m). The reactor was filled with 500 mL decanoic acid in hexadecane (1:1 w/w) and 220 mL glycerol-water with a known water content. The two phases were mixed (960 rpm) for 30 mins. After that the crude enzyme (2 grams) diluted in 30 mL glycerol-water was added. The initial rate was measured after 20 mins to allow the emulsion to stabilise.

The oil and alcohol circuits of the membrane system were filled. The membrane system had an oil phase volume of 140 ± 10 mL decanoic acid in hexadecane (1:1 w/w) circulating through the inner fibre side (2 L/h). At the shell side, 100 mL glycerol-water phase was recycled (3 L/h). To immobilise the crude lipase, 1 gram was diluted in water, followed by centrifugation (300 s, 30,000 rpm) to remove the cell debris. During the centrifugation step no activity loss occurred. The clear liquid was dispersed in the oil phase, and over a three hours period, ultrafiltrated from the inner fibre side towards the shell side, thus immobilising the lipase on the inner fibre side (Pronk *et al.* 1988). When the system reached equilibrium, the oil phase was refreshed, thereby removing the surplus of free enzyme. Then the initial rate could be measured. The initial rate was measured for a number of glycerol concentrations at 25 °C. The glycerol concentration was determined using a Pleuger refractometer.

Fat phase analysis was done using a Carlo Erba gas chromatograph with a 5 metre CP-sil-5CB (Chrompack, Holland) capillary column and a cold on-column injection system.

Conditions:	injection	80 °C, cooling 10 sec.	
	oven temperature:	80 °C, 1 min isotherm	
		2 °C.min^{-1} up to 320 °C	
	carrier:	He flow:	2.6 mL.min^{-1}.
	detection:	F.I.D.:	370°C
		H_2 flow:	19 mL.min^{-1}.
		air flow:	107 mL.min^{-1}.

The droplet size was measured under the microscope. For each emulsion four samples were taken and placed in a Hemacytometer (improved Neubauer). The Sauter mean diameter d_{32} (m) was calculated for four emulsions with:

$$d_{32} = \frac{\sum(n_i \cdot d_i^3)}{\sum(n_i \cdot d_i^2)} \tag{5}$$

where n_i is the number of droplets with diameter d_i (m). The specific area A (m^2.m^{-3}) is given by:

$$A = \frac{6 \cdot \Phi}{d_{32}} \tag{6}$$

where Φ is the volume fraction of disperse phase (m^3 of disperse phase/m^3 of reactor volume).

Results and discussion:

The esterification was measured in the emulsion system as well as in the membrane system. The initial rate and the equilibrium values were determined at different glycerol concentrations.

An example of a typical run in both systems is shown in figure 2. In the membrane system the fat phase is replaced when equilibrium is reached. The enzyme stays active, even after three batches. Each subsequent batch ends up with a higher free fatty acid equilibrium concentration, due to the reduced glycerol concentration caused by the water produced.

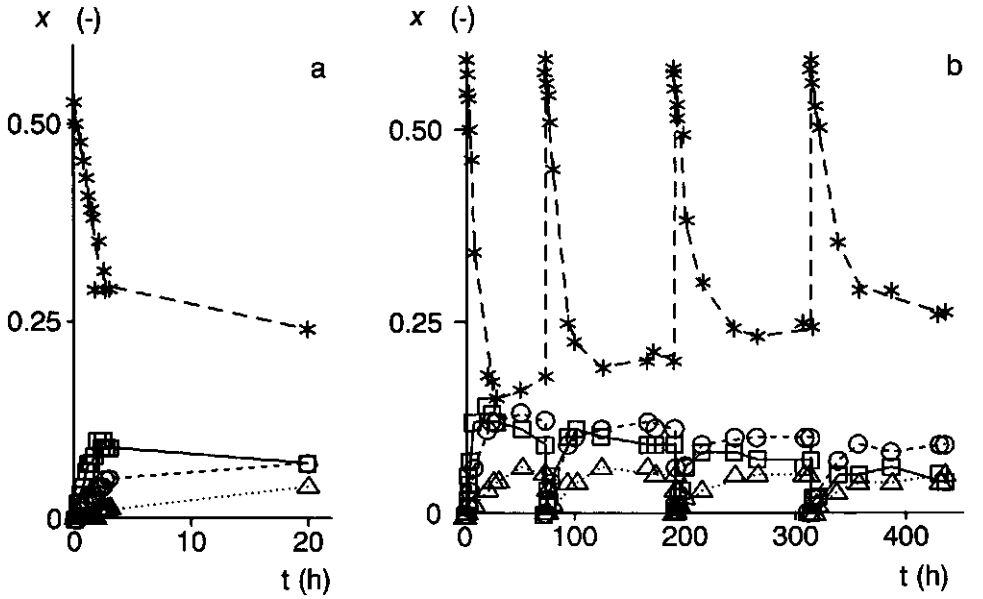

Figure 2: The ester synthesis in an emulsion system (a) and four batches of ester synthesis in a membrane system (b); capric acid (∗), mono- (□), di- (○) and tricaprinate (△).

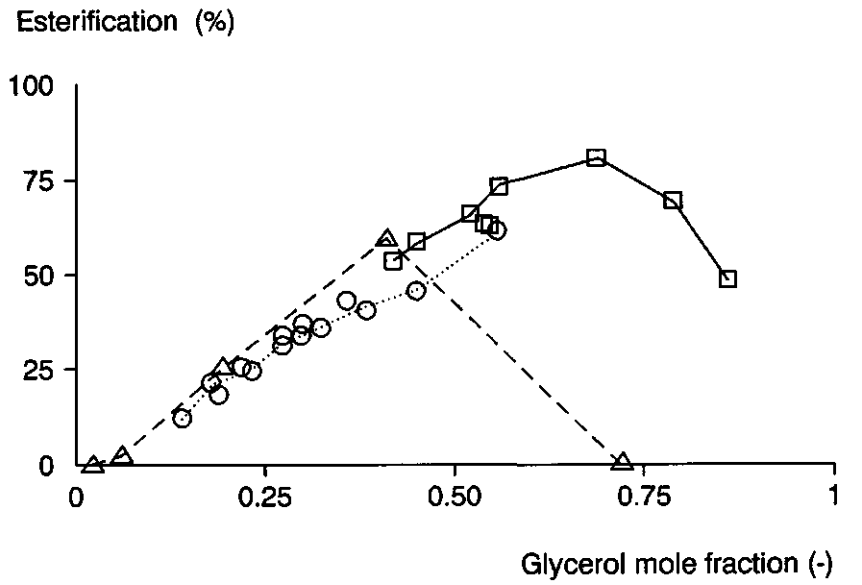

Figure 3: Measured fatty acid equilibrium concentrations at various glycerol concentrations for the emulsion system (○), the membrane system (□) and literature values (△; Tsujisaka et al. 1977).

The equilibrium concentrations for a number of emulsion and membrane batches are shown in figure 3, as well as the values given by Tsujisaka *et al.* (1977) for a glycerol / oleic acid emulsion system. The equilibrium values of Tsujisaka for oleic acid are nearly the same as our values for capric acid. Apparently the equilibrium concentration is mainly dependent on the water concentration for these two cases. At a glycerol concentration higher than 0.65 mole.mole^{-1} the measured equilibrium values decrease with increasing glycerol concentrations. Most probably this is an apparent decrease because the thermodynamical equilibrium might not be reached because of the inactivation of lipase by glycerol. During the reaction the enzyme is inactivated and the inactivation is complete before the equilibrium value can be reached. The inactivation of enzymes in low water activity solvents is correlated with

the log P value (Laane *et al.* 1987). Log P is defined as the logarithm of the partition coefficient in a standard octanol water two- phase system. For glycerol, the calculated log P is negative, resulting in complete inactivation in pure glycerol. The glycerol influence can be reduced by using a solvent, such as THF or 2-pentanone (Bell *et al.* 1978, Zaks and Klibanov 1985) but in that case an one-phase system is obtained.

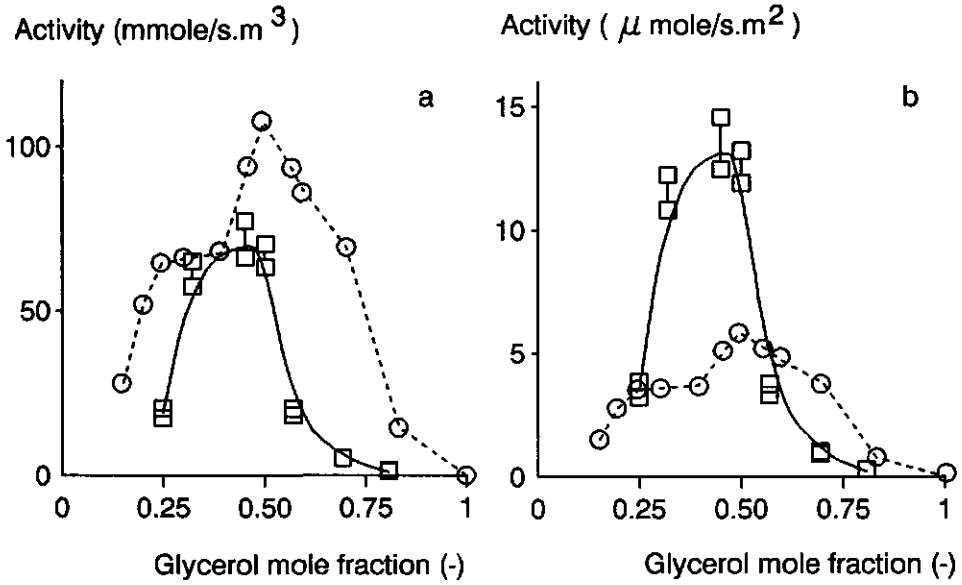

Figure 4: The volumetric activity (a) and the interfacial activity (b) versus the glycerol concentration for both the emulsion system (○) and the membrane reactor (□).

The initial rate is measured for several membrane and emulsion experiments at a variety of the initial glycerol concentrations. The volumetric initial rate is presented in figure 4a. The decrease of activity is indeed observed at high glycerol concentrations. If this is due to inactivation of the enzyme instead of a kinetic effect

of the glycerol, then the membrane system has lost its activity. This is tested for a high glycerol membrane system without activity by diluting the glycerol phase at the start of a following batch. Indeed no activity is measured.

To calculate the interfacial initial rate, the specific area A ($m^2.m^{-3}$) of the emulsion must be known. The Sauter mean diameter d_{32} for this alcohol in oil emulsion is measured as 0.10 ± 0.02 mm, resulting in a specific area A = 18.10^3 $m^2.m^{-3}$. The specific area of the membrane is calculated as the ratio of the membrane surface area and the reactor volume resulting in A = 5.10^3 $m^2.m^{-3}$. The interfacial initial rates are given in figure 4b. The rates of both systems are of the same order, pointing out that, within the range of experimental accuracy of the specific area measurement, the cellulose membrane does not affect the interfacial activity. Optimum esterification takes place where both the equilibrium value as well as the initial rate are high. At glycerol concentrations between 0.3 to 0.5 $mole.mole^{-1}$ glycerol the interfacial initial rate in the membrane system exceeds the emulsion system, while the emulsion system is twice as active at higher glycerol concentrations. The optimum in the membrane activity could be due to the difference in enzyme load at the actual interface, as further discussed below. The very low activity at high glycerol concentrations could be caused by inactivation. As stated above, the inactivation is a function of the glycerol concentration. Assuming that this inactivation is not instantaneous and taking into account that the membrane system is measured after approximately 70 hours using the second oil batch data (figure 2) instead of the 0.4 hours in the emulsion system, the decrease of the activity in the membrane system is indeed likely to be due to the inactivation.

Dealing with an interface reaction, the activity is not only a function of the enzyme activity but also of the enzyme coverage of the interface. To determine the enzyme activity, the enzyme load must be optimized to make sure all the enzyme participates in the reaction, so that the enzymatic activity is not underestimated. Two regions are expected, firstly an increasing activity with the enzyme load, in the case that there is still unoccupied interface left, so the interfacial activity is proportional to the enzyme load. Secondly, once the interface is fully occupied, with one or a number of active enzyme layers, a constant activity is obtained, even when the enzyme load is increased. Now the activity is proportional to the specific area. The change from

enzyme limitation to interface limitation should mark the point where the optimum number of enzyme layers is formed. Assuming that a monolayer is the optimum enzyme occupancy the load could be calculated based on the Stokes radius. O'Connor and coworkers (1988) estimate that in this case an interface can contain 1.6 mg pure pancreatic lipase.m^{-2}. This corresponds to 31 mg crude *Candida rugosa* (5 % pure lipase).

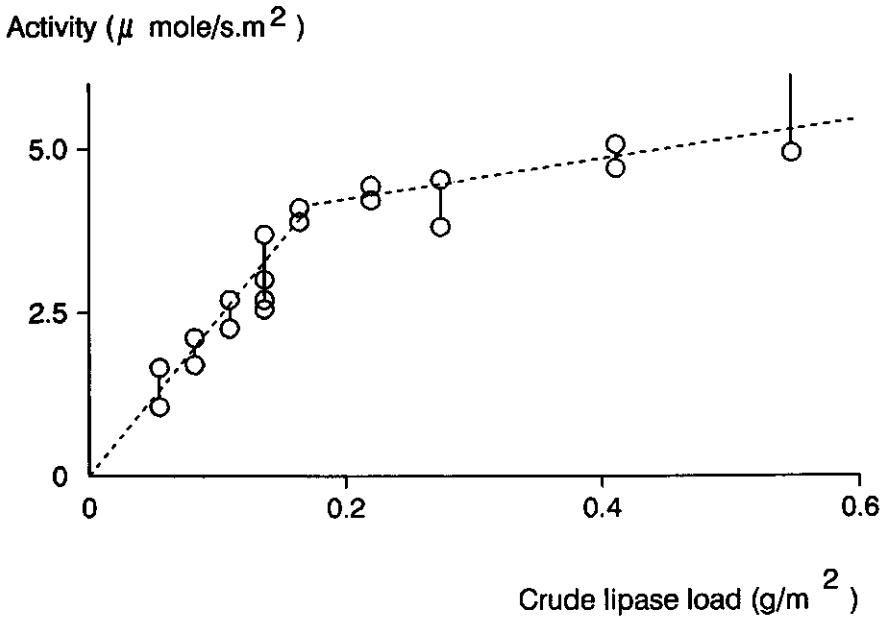

Figure 5: The interfacial activity versus the enzyme load in the emulsion system.

The occupation of the interface in the emulsion system is studied measuring the interfacial initial rate at different enzyme loads (figure 5). Indeed two regions can be distinguished, firstly up to 170 mg crude lipase.m^{-2}, the rate is enzyme limited. Secondly, for loads higher than 170 mg crude lipase.m^{-2}, the rate is interface limited. The slight increase in rate could be due to the increase in the interfacial area as a result of the emulsifying qualities of the lipase, while the interfacial area was assumed

constant for the calculations.
When assumed that only lipase adsorbs, the optimum load in the emulsion system of 170 mg crude lipase.m^2, equals five times the value calculated above for a monolayer coverage. This relative high load could indicate three different mechanisms. A possibility is that an active multilayer is formed. This assumption is not very likely, because the enzymatic activity in the membrane system is higher than in the emulsion system, even though the optimum enzyme load is lower (see below). Another possibility is, that the excess of lipase is necessary because lipase only partly adsorbs at the fat / water-glycerol interface (Ekiz *et al.* 1988). Then a certain bulk concentration is needed to obtain an optimum enzyme layer at the emulsion interface. Moreover, due to the continuous dispersion and coalescence process that takes place in the emulsion, the surface is continuously created and destroyed. In this case the adsorption is still in a dynamic state, and takes place at the continuous created and thus unoccupied interface. Now both the dispersion rate and the adsorption rate are of great importance. The adsorption rate is enhanced when a surplus of lipase is present in the bulk. Because the enzyme load is calculated as the quotient of the enzyme added and the calculated specific area, the actual enzyme load should be corrected for the enzyme present in the bulk, which results in a higher enzymatic activity than the calculated apparent enzymatic activity. Unfortunately the enzyme concentration in the bulk cannot be measured, and moreover this surplus is necessary to obtain the optimum enzymatic activity, therefore, the optimum load is defined as the quotient of the crude lipase added and the interfacial area. Up to the optimum load, the enzymatic activity is constant, and in this region the enzymatic activity $r_{i,E}$ = 25 mmole.s^{-1}.kg^{-1} is characteristic for this system.

This experiment is repeated using a membrane reactor, and the results are given in figure 6. Again, two regions are found, but now the optimum load is reached for 75 mg crude lipase.m^{-2}, which is only two to three times the calculated value needed for a monolayer of pure lipase. At this load the enzymatic activity is 35 mmole.s^{-1}.kg^{-1}, which is 1.4 times the activity measured in the emulsion system. This could indicate two independent mechanisms. Firstly, the membrane may stabilise the enzyme so it keeps its activity during the first batch, while the free lipase in the emulsion inactivates rapidly in 0.57 mole.mole^{-1} glycerol. Secondly, during the immobilisation the enzyme will adsorb preferentially at the membrane surface (Brent *et al.* 1983).

This can be explained by a mechanism by which at the start, all proteins near the membrane surface adsorb, but all proteins with a lower adsorption energy are replaced in time by lipase with a higher adsorption energy, so a relative high interfacial activity is obtained after immobilisation. This is stressed by the non linearity of the enzyme limited region (figure 6). The optimum enzymatic activities are presented in table 2.

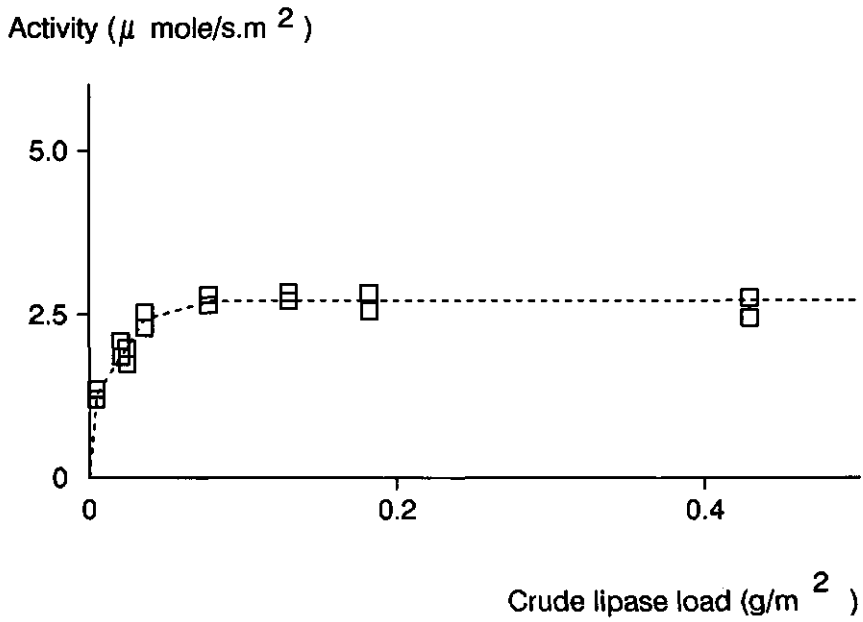

Figure 6: The interfacial activity in a membrane system versus the enzyme load.

In the literature only a hydrophobic membrane device is described for the enzymatic ester synthesis (Hoq *et al.* 1984, 1985b). The activities of this continuous system are represented in table 2. There are three differences between the systems which influence the activity:

Table 2. *The activities for the emulsion system and membrane system as well as Hoq's system (1985b) with the temperature corrected activity between brackets*

	Emulsion system this work	Membrane system this work	Membrane system (Hoq 1985b)
Membrane:		hydrophilic	hydrophobic
Temperature:	25 °C	25 °C batch	40 °C continuous
Fatty acid:	capric acid	capric acid	oleic acid
Glycerol:	0.57 mole.mole^{-1}	0.57 mole.mole^{-1}	0.87 mole.mole^{-1}
Enzyme:	*Candida rugosa*	*Candida rugosa*	*Mucor miehei*
mmole.s^{-1}.m^{-3}	97.	19.	28. (11.)
μmole.s^{-1}.m^{-2}	4.1	3.5	11.3 (4.5)
mmole.s^{-1}.kg^{-1}	25.	35.	0.8 (0.3)

1) A higher temperature gives a higher activity (Q_{10} = 2, Lehninger, 2nd edition). The corrected values are placed in brackets.

2) Different lipase type. It is shown in literature (Okumura *et al.* 1979, Hoq *et al.* 1985b) that other kinds of lipase give other activities. Hoq and coworkers (1985b) found no activity at all using *Candida rugosa* in the hydrophobic membrane reactor (Hoq *et al.* 1985b). This could be due to the high glycerol concentration of 0.87 mole.mole^{-1} resulting in a rapid inactivation so no esterification is measured (see figure 4).

3) Membrane material. The advantage of an asymmetric hydrophobic membrane, which has a rough surface, is the high surface area on microscopical scale per square metre visible membrane, so apparently a high interfacial activity is gained. Therefore, it is not surprising that the interfacial activity of Hoq's system is higher.

However, a disadvantage is the fact that the fat / alcohol interface is at the alcohol phase side of the membrane. The enzyme is therefore immobilised on the alcohol side and so there will be a distribution of enzyme adsorbed and enzyme solubilised in the alcohol phase so a surplus of the enzyme should be added (Hoq *et al.* 1985b). Using a hydrophilic membrane, with the enzyme in the oil phase, the enzyme cannot dissolve in the alcohol phase, because it cannot pass the membrane. This phenomenon explains the forty times higher enzymatic activity for the hydrophilic system.

Both membrane systems have advantages. The hydrophilic system has a higher enzymatic activity, so this system could be used dealing with expensive enzymes taking the low interfacial activity for granted. On the other hand, a hydrophobic unit could be used for bulk processes, using cheap lipases and high production flows. For industrial processing, the hydrophilic membrane system has the advantage that the membrane can withstand transmembrane pressures without leakage of the oil phase. For the development of an industrial process, the three activities have to be studied related to the operational costs.

Conclusions

The *Candida rugosa* lipase catalysed esterification of decanoic acid with glycerol is described for an emulsion system and for a hydrophilic membrane bioreactor. The initial rate per unit interface area, the interfacial activity, is roughly equal for both systems indicating that the cellulose membrane does not hinder the esterification. Because the interfacial activities are equal, the volumetric activity of a membrane system is only specific area related, so a hollow fibre membrane device is preferable.

The activity is also a function of the enzyme load. The optimum load in a hydrophilic membrane reactor is one to three times the amount of a monolayer, while in an emulsion system several times this amount. This could indicate that in the emulsion system the adsorption is in a dynamic state while at the membrane surface the adsorption reached its equilibrium state.

Acknowledgement

This work is funded by the Dutch Ministry of Economical Affairs and the Unilever Research Laboratory Vlaardingen. The authors wish to thank N.K.H Slater, J. Kloosterman, B. Barmentlo and W. Bel for their cooperation in this project.

Symbols

A	specific area	($m^2.m^{-3}$)
Activity	Activity	($mole.s^{-1}.m^{-3}$)
C_E	enzyme concentration	($kg.m^{-3}$)
C_S	fatty acid concentration fat phase	($mole.m^{-3}$)
d_{32}	Sauter mean diameter	(m)
d_i	diameter i	(m)
n_i	number of droplets with diameter d_i	(-)
r_i	initial rate	($mole.s^{-1}.m^{-3}$)
$r_{i,A}$	interfacial based initial rate	($mole.s^{-1}.m^{-2}$)
$r_{i,E}$	enzyme based initial rate	($mole.s^{-1}.kg^{-1}$)
$r_{i,V}$	volume based initial rate	($mole.s^{-1}.m^{-3}$)
t	time	(s)
x	mole fraction	(-)
Φ	volume fraction dispersed phase	($m^3.m^{-3}$)

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3 Enzyme stability

Abstract

For the enzymatic synthesis of esters, the enzyme stability in alcohol-water mixtures is of great importance. When the activity of an enzyme is monitored with time in such a solution, often two inactivation regions can be distinguished. This paper presents a model to describe both inactivation regions for *Candida rugosa* lipase. The inactivation can be described with a two-step model, assuming the native enzyme reversibly altering its conformation to a form having a lower activity. The reversibility is experimentally verified. Both forms do inactivate at the same irreversible rate to a completely inactive form. Inactivation rates are related to the glycerol concentration. During inactivation, aggregate formation is found. The activity of immobilized enzyme is reduced to the same level of activity as is found for free lipase. Based on the inactivation model the initial activity of an esterifying system is calculated. It is shown that the esterification rate approximates the hydrolysis rate.

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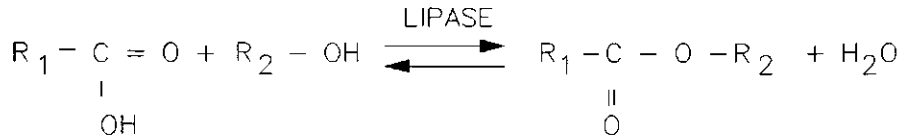
Introduction

In the last decade lipases have been used for the production of specific esters:



An example of an esterification reaction is the synthesis of glycerides (acylglycerols), the mono-, di- and triesters of fatty acids and glycerol. Esterification can be achieved in case a high fatty acid or glycerol concentration is used. At equilibrium a mixture of fatty acid, glycerol, esters and water is obtained. The reaction will only lead to a complete fatty acid conversion if one of the products is continuously removed during the reaction (Eggers *et al.* 1984, Ergan *et al.* 1990, Martinek and Semenov 1981a and Martinek *et al.* 1981b). The conversion rate is a function of the substrate concentration as well as the enzyme concentration and enzyme activity. The enzyme inactivation rate is related to the process conditions such as temperature, type of enzyme, presence of an organic solvent and whether or not the enzyme is immobilized.

When the reaction is performed at ambient temperatures and mild process conditions, the inactivation can often be neglected (Lee and Choo 1989). However, in case of esterification, a high alcohol concentration combined with a low water content is preferred. These conditions may lead to inactivation (Guagliardi *et al.* 1989, Lazar *et al.* 1988, Mozhaev *et al.* 1989 and 1990b and Touraine and Drapron 1988), which can either be due to the high alcohol concentration or to the lowered water activity. One of the proposed mechanisms of this type of inactivation is the removal of the essential water which results in a conformation change of the enzyme resulting in inactivation (Mozhaev *et al.* 1990a and Zaks and Klibanov 1985). In water-alcohol mixtures, not only the water activity changes upon varying the alcohol concentration, but also the dielectric properties of the solution. The dielectric quality of the microenvironment

of the enzyme determines its stability and can lead to protein precipitation in solvents having a reduced dielectric constant and hence impaired activity (Klibanov 1983a, Lehninger 1975). Linfield and coworkers (1984) have shown that lipase precipitates in a glycerol-water solution while losing its catalytic activity. This inactivation is reversible, because at the addition of water, the enzyme redissolves and regains its activity. It is the first aim of this study to determine the mechanism behind the inactivation of *Candida rugosa* in glycerol-water mixtures and whether this inactivation is reversible or irreversible.

The esterification can be performed either by using free enzymes in an emulsion system (Linfield *et al.* 1984, Okumura *et al.* 1979, Touraine and Drapron 1987 and 1988, Tsujisaka *et al.* 1977 and chapter 2) or by using immobilized enzymes (Ergan *et al.* 1990, Hoq *et al.* 1984 and 1985 Schuchs and Mukherjee 1989 and chapter 2). Reaction times in emulsion systems usually are short as compared to the reaction times required in immobilized enzyme systems (chapter 2). In most cases the inactivation in an emulsion system can be neglected. However, in an immobilized enzyme system inactivation is of importance. This 'long-term' inactivation is studied in this paper to gain insight in the inactivation rate of an immobilized enzyme system running for days or even weeks. In the literature models are presented to describe first order inactivation (Bailey and Ollis 1977) and the multiple step inactivation (Henley and Sadana 1986). The second aim of this article is to determine suitable models for the inactivation of *Candida rugosa* lipase.

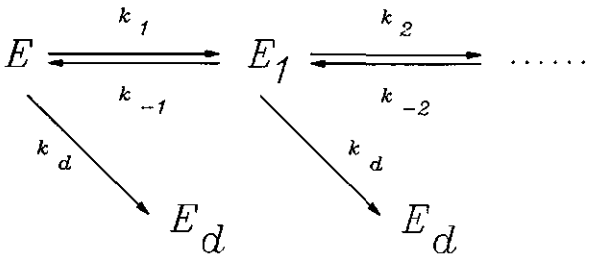
Immobilization can influence the enzyme activity in two different ways. Firstly, during the immobilization itself, the enzyme conformation can alter and thus the activity is reduced or increased as a result of multiple interactions with the support material (Mozhaev *et al.* 1990a and Klibanov 1983b). Secondly, this interaction can stabilize the activity of the enzyme. The measured inactivation of the free enzyme should therefore be compared with the inactivation of an immobilized enzyme system. It is the third aim of this study to determine the difference between *Candida rugosa* lipase inactivation in a free enzyme system versus a membrane immobilized system.

When the inactivation rate is known, the activity of an immobilized system can be corrected for the inactivation and the initial activity can be calculated. Once the inactivation is described properly, the necessary tools for process development and optimization are available.

Theory

In systems in which organic solvents are present, enzymes do not necessarily obey first-order inactivation (Guagliardi *et al.* 1989, Lazar *et al.* 1988 and Mozhaev and coworkers 1989 and 1990b). Two inactivation regions can often be distinguished. Initially a rapid decrease in activity is found, here called the 'short term' inactivation, followed by a slower inactivation, the 'long term' inactivation. This can be the result of at least two inactivation mechanisms, both acting at the same time. The 'short term' inactivation can be a fast reversible inactivation as a result of a minor conformation change of the enzyme. Another explanation is the formation of aggregates in a water-miscible neutral organic solvent as a result of the reduced dielectric constant of the system (Klibanov 1983a, Lehninger 1975). For the 'long term' inactivation mechanism, generally an irreversible inactivation is assumed.

Henley and Sadana (1986) have presented a model for multiple step inactivation. The native enzyme E alters reversibly, to partially active forms E_1, E_2 etcetera, each of which may be inactivated irreversibly to a completely inactive state E_d:



(1)

where k_1, k_{-1} and k_d are first-order reaction rate constants (s^{-1}). Assuming only three forms of the enzyme exist: E, E_1 and E_d the enzyme activity $E_{tot}(t)$ (unit.g^{-1}) as a function of time t (s) can be described with (Henley and Sadana 1986):

$$E_{tot}(t) = E_{tot}(0) \cdot \left(A \cdot e^{-\lambda_1 \cdot t} + (1 - A) \cdot e^{-\lambda_2 \cdot t} \right) \quad (2)$$

where λ_1 and λ_2 : are time constants (s^{-1})

A : is the apparent equilibrium constant (-)

The parameters A, λ_1 and λ_2 can be calculated fitting experimental data. This results in parameter values representing the apparent total activity as a function of time. For our system the relation between the parameters of equation 2 and the kinetic constants of equation 1 can be calculated as shown in appendix 1.

Materials

The *Candida rugosa* lipase used in this study is the enzyme preparation Lipase OF 360,000 obtained from Meito Sangyo (Japan). During two experiments the lipase preparations of Biocatalysts (UK) and Sigma (USA) are used. Glycerol 99+% is obtained from Janssen (Belgium), all other chemicals are purchased from Merck (FRG). Demineralized water is used throughout the study. For the membrane bioreactor a cellulose hollow fibre unit is chosen (Andante unit Organon, The Netherlands). The membrane unit contains 6000 fibres with an internal diameter of 0.2 mm and a dry wall thickness of 8 μm. The total membrane surface area is 0.77 m^2. The decanoic acid obtained from Unichema is 95 % pure.

Experimental

Activity measurement

The lipase activity is determined at 30°C by adding a sample (0.1 to 1 mL) of the enzyme-glycerol solution to a homogenized tributyrin assay solution. The assay solution contains 2% (v/v) tributyrin, 0.1 % (w/v) Arabic gum and 2 mM maleic acid (pH = 6). The pH is maintained constant by pH-stat titration with a 0.01 M NaOH solution. The activity is measured in units, where one unit is the amount of enzyme that releases one μmole of butyric acid in one minute under these conditions (1 unit = 16.7 nKat). The activity of the Meito Sangyo lipase equals 65 ± 5 units.mg^{-1} crude preparation.

Glycerol-water-enzyme system

The inactivation of lipase is monitored in a reaction vessel of standard geometry (0.4 L). The influence of the glycerol concentration is studied at 25°C. Approximately 0.5 g lipase is dissolved in water and centrifuged to remove debris (5 min; 30,000 rpm). The supernatant is dissolved in a glycerol-water solution. The solution is stirred at 250 rpm with a four-bladed turbine stirrer in a baffled vessel. The glycerol concentration is measured using a Pleuger refractometer and adjusted if necessary. The enzyme activity is measured with time with the activity assay. Two experiments are done using the Biocatalyst and Sigma preparation, respectively.

Another set of measurements is done in a beaker with a magnetic stirrer and without baffles. Finally, three experiments are done without stirring at all.

Fitting the datum points

The inactivation rate constant k_d equals λ_1 (see appendix 1). Consider:

$$\lambda_2 \gg \lambda_1 \wedge t > \frac{-1}{\lambda_2} \cdot \ln\left(\frac{0.01}{1-A}\right) \qquad (3)$$

then equation 2 can be rewritten to:

$$E_{tot}(t) = A \cdot E_{tot}(0) \cdot e^{-\lambda_1 \cdot t} \quad (4)$$

The time constant λ_1 is the negative slope of the $\ln(E_{tot}(t)/E_{tot}(0))$ versus time plot and $\ln(A)$ is the intercept. The value of 0.01 in equation 3 is arbitrarily chosen. This means that a difference between equation 2 and 4 of 1 % is allowed.

When both λ_1 and A are known equation 2 can be transformed to:

$$\frac{E_{tot}(t)}{E_{tot}(0) \cdot (1 - A)} - \frac{A}{(1 - A)} \cdot e^{-\lambda_1 \cdot t} = e^{-\lambda_2 \cdot t} \quad (5)$$

The left hand side of equation 5 is the relative activity corrected for the influences of λ_1 and A on the activity. The negative slope of the ln(corrected relative activity) versus time plot results in the time constant λ_2.

Fits are made as follows: First of all, for $t > t_1$, the value for λ_1 and A are calculated from the $\ln(E_{tot}(t)/E_{tot}(0))$ versus time plot using linear regression (equation 4). Although linear regression is not the appropriate tool to fit a non linear equation 4, it can be used to fit the parameters of the linearized equation. However, it is not possible to calculate the confidence interval of these parameters.

Secondly, for $t < t_1$ the value for λ_2 is calculated as the negative slope of the ln(corrected relative activity) versus time plot using linear regression (equation 5).

Finally, $-\ln(0.01 / (1-A)) / \lambda_2$ can be calculated (equation 3), this value ought to be equal to t_1, if this is true, the values of A, λ_1 and λ_2 are found, otherwise t_1 has to be adjusted and the calculation procedure starts again.

Water activity influence

Suspended in 19.3 g hexadecane, 51 mg crude lipase preparation is stored under different water activity conditions above saturated salt solutions in a vacuum desiccator. Drying experiments at our laboratory proved, that within one week the samples are equilibrated. This is also described by Valivety and coworkers (1992). After one week of storage, the activity of the enzyme is determined. The salts used are $LiCl_2$ (a_w = 0.11), K_2CO_3 (a_w = 0.43), NaBr (a_w = 0.58), NaCl (a_w = 0.75) and $BaCl_2$ (a_w = 0.90). The water content of the samples is measured by Karl Fisher titration.

Water activity

The water activity a_w in water-glycerol mixtures is calculated from the glycerol mole fraction using the parameters presented by Norrish (1966):

$$a_w = (1 - x_g) \cdot e^{-\frac{2167}{R \cdot T} \cdot x_g^2} \qquad (6)$$

where:			
	R	: the gas constant	($J.K^{-1}.mole^{-1}$)
	T	: temperature	(K)
	x_g	: the mole glycerol fraction	($mole.mole^{-1}$)

Ultracentrifuge experiments

Whether during solubilization in glycerol the molecular weight of lipase changes, is studied in an ultracentrifuge. For this purpose, lipase is purified as follows: Centrifugation of a 25 $g.l^{-1}$ crude lipase solution during 5 min at 30,000 rpm, a dialysis step followed by Sephadex G100 gel filtration at pH = 7. Eluate fractions with a high hydrolysis activity have been pooled. The specific activity of the purified enzyme equals 874 $units.mg^{-1}$ protein, this is a two fold increase of activity. The obtained protein is not completely pure; one other minor band close to the major band could be seen on the SDS-gel (Laemmli 1970).

The mole mass of the purified enzyme (centrifugation 5 min 30,000 rpm, dialysis step followed by Sephadex G100 gel filtration at pH = 7) is measured in a MSE Centricon analytical ultracentrifuge (45,000 rpm, 20°C, λ = 301 nm, 15 mins interval). Approximately 50 mg of the purified enzyme is dissolved in 250 mL 0.7 mole.mole^{-1} glycerol, subsequently diluted in water to a 0.25 mole.mole^{-1} glycerol solution and a second spectrum is measured.

Membrane bioreactor system

The membrane bioreactor consists of the cellulose hollow fibre membrane module, with an internal oil circuit (circa 80 mL 1:1 w/w decanoic acid in hexadecane) and an external glycerol-water circuit. The biocatalyst is immobilized at the inner fibre side (chapter 2). During immobilization, no glycerol is present in the system. At the start of each experiment the water phase is replaced by a glycerol-water solution. The glycerol concentration is kept constant making use of a feed and bleed system. Two systems are run, a batch oil phase system at a glycerol concentration of 0.57 mole fraction (a_w = 0.32) and a continuously-refreshed oil-phase system having a glycerol phase of 0.32 mole fraction (a_w = 0.62). During the batch experiment, the oil phase is replaced several times and after each replacement the initial esterification rate is calculated. The activity in the continuous reactor is calculated using the mass balance. The conversion takes place at 25°C. The activity is normalized by division by the initial esterification rate. The concentration of decanoic acid is measured by diluting the sample in ethanol followed by titration.

Results and discussion

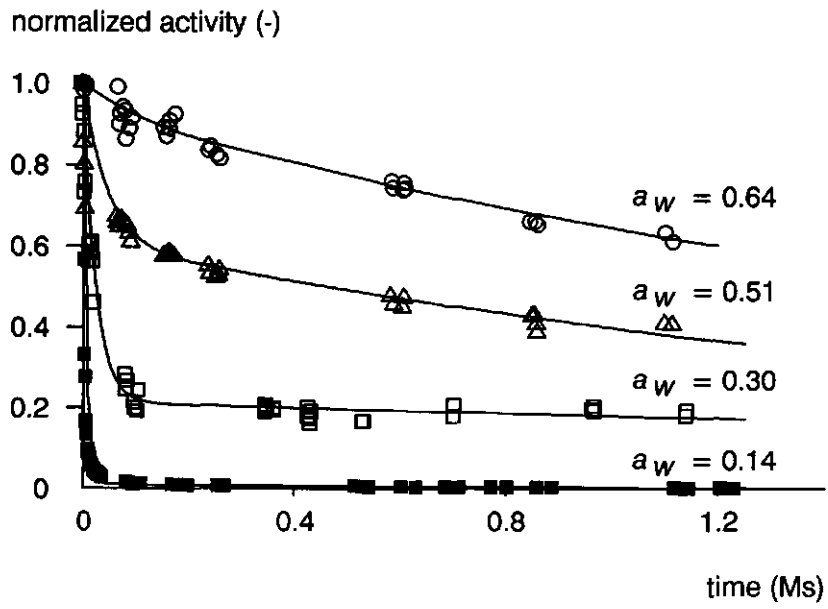


Figure 1. *The normalized Candida rugosa lipase activity measured in water-glycerol mixtures with different water-activities in a turbine stirred tank. Drawn lines are best fits with equation 2.*

The inactivation model and mechanism

The activity of crude *Candida rugosa* lipase preparation is measured in glycerol-water mixtures at different glycerol concentrations (figure 1). The inactivation data can be fitted with equation 2 and the model parameters A λ_1 and λ_2 can be found as a function of the water activity of the system. The best fit is given in figure 1 as the drawn lines. The calculated parameter values are shown in figure 2. The apparent equilibrium constant A is water activity related (figure 2a). At low water activities, A almost equals zero, while at high water activities the apparent equilibrium constant approximates one. This means that at low water activity conditions, the enzyme is almost completely

in its relatively inactive form E_1, while at high water activity conditions the more active form E is more abundant. Thus, both at low (<0.3) and high (>0.8) water activity conditions $\{A \rightarrow 0 \vee A \rightarrow 1\}$, the inactivation model changes from a model with two time constants λ_1 and λ_2 into a model with one time constant λ_1 or λ_2, respectively. Figure 2b shows that the time constants λ_1 and λ_2 are also water activity related, the time constants increase with decreasing water activity.

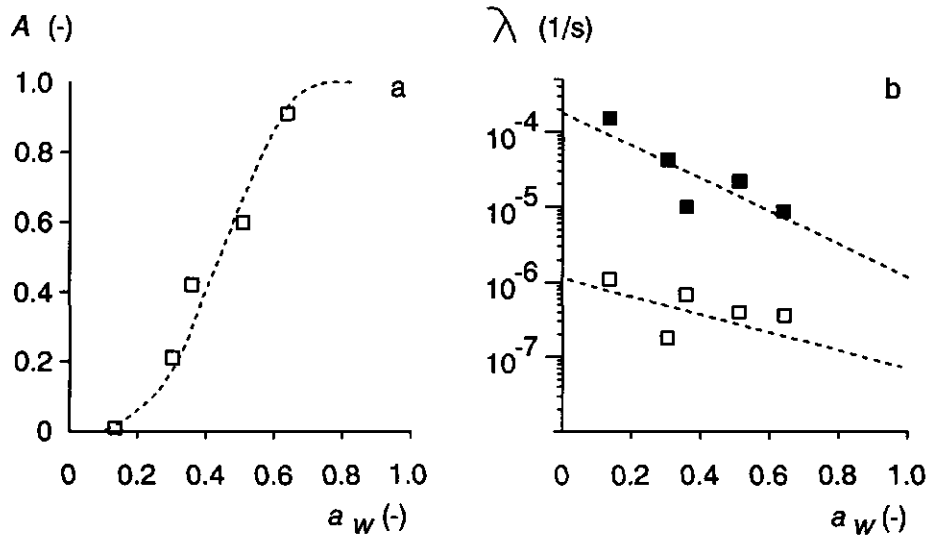


Figure 2. *a) The apparent equilibrium constant A (□) as a function of the water activity. b) The first order rate constants λ_1 (□) and λ_2 (■) as a function of the water activity.*

The two-step model is based on a reversible reaction scheme. Other authors such as Ulbrich *et al.* (1985) explain their results with a model based on the existence of two isozymes with irreversible inactivation. This basic assumption is verified by a step wise increase of the water activity during one experiment. The results show indeed an

increase in activity (figure 3). This experiment proves that a model as proposed by Ulbrich *et al.* (1985) is not valid for this system. Contrary, reactivation takes place, indicating that the inactivation is partly reversible.

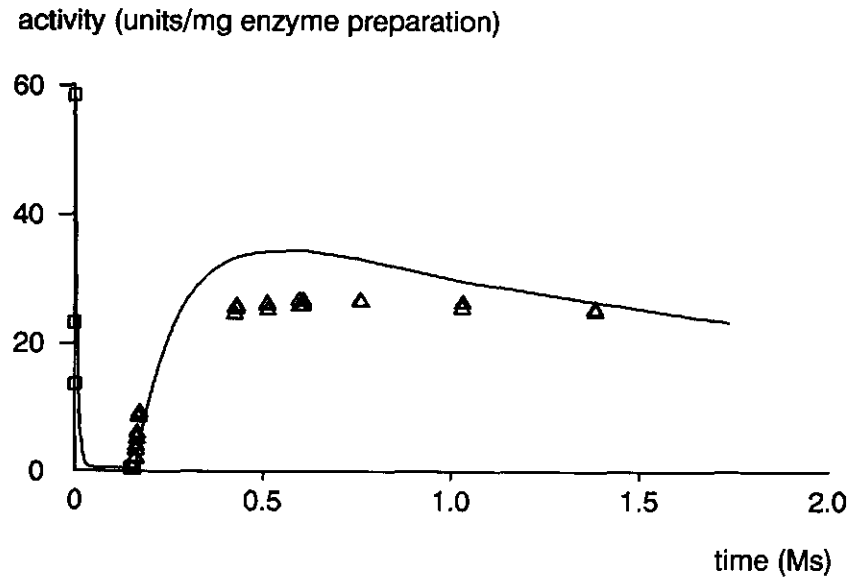


Figure 3. *The measured activity of a lipase solution which is changed from a water activity of 0.14 to 0.64 after 0.16 Ms (2 days) and the activities based on equation A1 with* $\alpha = 0$.

Equation 2 has been derived from the mechanistic model as given in equation 1, assuming initially only E is present and no reactivation takes place. Appendix 1 gives the relation between the fit parameters of equation 2 and the kinetic parameters of equation 1. One parameter has to be assumed to get three equations with three unknowns. The ratio between the activity of E and E_1, α, is the best choice while only the apparent equilibrium constant E is a function of α. Figure 3 shows the curve fit with the A, λ_1 and λ_2 from the curves in figures 2a and 2b and with an α value

of 0. This means that the enzyme in the altered conformation E_1 has no activity at all. The curve fit shows that the parameters, derived from the inactivation experiments, are able to explain a large part of the reactivation experiment. This indicates that the mechanism as proposed in equation 1 might be present.

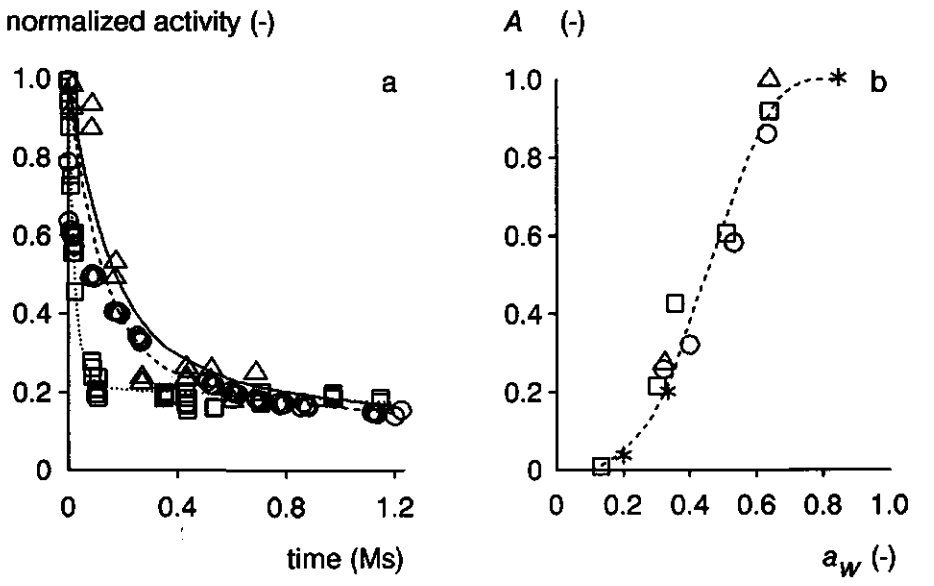


Figure 4. *a) The measured and fitted activities in a glycerol-water mixture at a water activity of 0.30.*
b) The apparent equilibrium constant A as a function of the water activity. Symbols for both graphs: a turbine stirred free enzyme system (□), a magnetic stirred free enzyme system (○), a non-stirred free enzyme system (✳) and a membrane reactor system (△).

The rate of inactivation can be dependent on the process conditions. Figure 4a shows the activity of *Candida rugosa* lipase measured in two different stirred tanks and in a membrane reactor. This graph demonstrates that the 'short-term' inactivation depends

on the process conditions, however, after 200 hours the activities are more or less equal. The apparent equilibrium constants A for a number of such experiments and of lipase which is incubated without stirring are given in figure 4b as a function of the water activity. This figure shows that the apparent equilibrium constant A is related to the water activity, independent of the process conditions used. Therefore, A is a key parameter for the calculation of the activity of an enzyme system.

Equation 2 can be regarded as the summation of two exponential decay functions. With $\lambda_2 \gg \lambda_1$ (see figure 2b), the first term represents the slow 'long-term' activity decrease. The apparent equilibrium constant is the apparent relative active amount subjected to the slow inactivation, *i.e.* representative for the activity level at long term periods (equation 4). Figure 4a and the simulations show that the 'short-term' decrease rate is system dependent. The apparent equilibrium constant is not system dependent, explaining that at large time values the activity becomes the same for the different process conditions. Because data are not measured of λ_1 for all systems, it is not possible to say whether at very long time periods the activity levels again will deviate because of a difference in the slow inactivation rate. In the range measured, this is not the case yet.

One of the questions which has to be answered is whether the lipase inactivation is the same for lipases from different sources. Figure 5 shows the inactivation of *Candida rugosa* (formerly *Candida cylindracea*) of Biocatalysts (U.K.), Meito Sangyo (Japan) and Sigma (USA). It can be seen that all three enzyme preparations show a two-step inactivation. However, the apparent equilibrium activity differs for the different types. Therefore, it is necessary to study the inactivation of every type and source before the initial activity can be calculated from the measured apparent equilibrium activity.

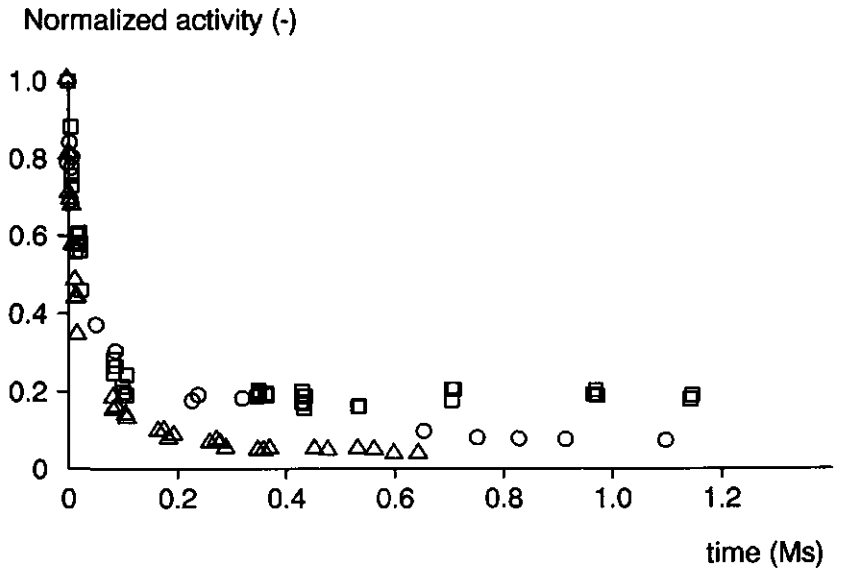


Figure 5 *The inactivation of Candida rugosa (formerly Candida cylindracea) of Biocatalysts (△), Meito Sangyo (□) and Sigma (○).*

Up to this point, the inactivation of lipase is expressed as a function of the water activity of the enzyme system. Whether the inactivation is caused by the reduced water activity or by the high glycerol concentration cannot be discriminated with the above set of experiments. The water activity influence is tested by measuring the activity of batches of crude lipase preparation which have been stored under water activity conditions ranging from 0.1 up to 0.8. Even after one week, the measured activities are equal to the initial activity. This could indicate that during the inactivation experiments in glycerol-water mixtures, the inactivation is not caused by the reduced water activity, but by the glycerol itself. At first sight, the inactivation caused by glycerol is in contrast with the finding in literature that glycerol up to 40 % w/w (a_w=0.87) stabilizes lipase (Brady *et al.* 1988). However, the experiments presented in this work, are carried out in glycerol solution of 70 up to 95 % w/w ($0.11 < a_w < 0.63$). Apparently, the high

glycerol concentration changes the behaviour of glycerol from a stabilizing factor to a solvent which inactivates the catalyst. This is in agreement with the supposition that organic compounds having a low log P value do inactivate microorganisms (Laane *et al.* 1987) and enzymes (Reslow *et al.* 1987). The log P value is defined as the logarithm of the partition coefficient of the compound in a standard octanol-water two-phase system. Biocatalysts are unstable in solvents having a log P $\leq$ 2; in case of glycerol log P = -2.5 (Rekker and de Kort 1979). Hence, inactivation caused by high concentrations of glycerol can be expected.

In the literature, several mechanisms are suggested for the inactivation of enzymes in non-aqueous environments (Mozhaev *et al.* 1990a and Zaks and Klibanov 1985). One of the proposed mechanism is the formation of aggregates due to the change of the dielectric quality of the microenvironment (Klibanov 1983a, Lehninger 1975). To prove the presence of aggregates, the molecular weight of the lipase preparation is monitored using an analytical ultracentrifuge. For this purpose lipase is purified. Purified lipase, dissolved in water, shows the usual pattern of a monodisperse protein. The enzyme is sedimented with a uniform velocity (figure 6a). Assuming that lipase is a globular protein, the molecular weight is calculated to be between 50 and 60 kD. When a sample of purified lipase is solved in 0.7 mole.mole^{-1} glycerol solution, inactivation is obtained. The partly inactivated purified lipase is diluted to a 0.25 mole.mole^{-1} glycerol solution and a second ultracentrifuge run is made. Analogue to the crude lipase, we expect an increase of activity (figure 3). During this reactivation, a polydisperse enzyme solution is obtained (figure 6b). Larger protein molecules do exist that are pulled down with a higher velocity than the smaller ones, leading to a protein distribution over the length of the cuvette. This experiment clearly shows that a molecular weight distribution of lipase can be found in a glycerol-water solution due to the formation of aggregates. Whether this is the only mechanism contributing to the inactivation of crude lipase can not be concluded from this experiment. For instance, other proteins in the crude preparation could influence the process. However, that aggregation takes place is clearly proved by this experiment. The qualitative as well as the quantitative description of aggregation phenomena can be described by a type of equation such as equation 2. This also supports the possible mechanism of aggregation.

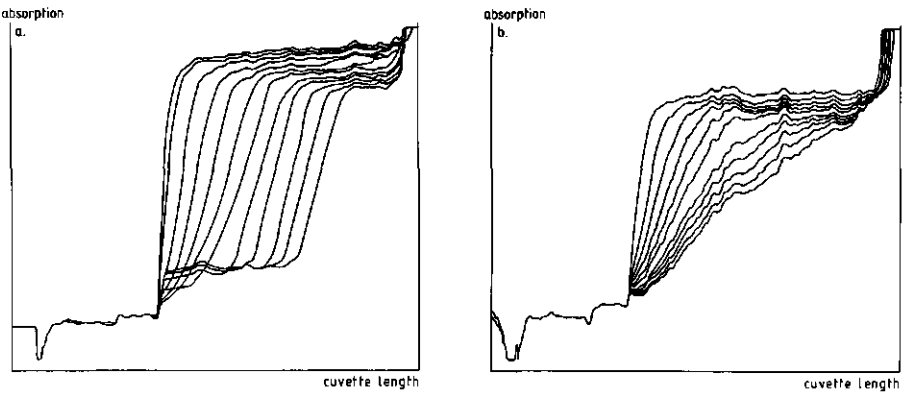


Figure 6. The measured absorption of lipase (a) and pretreated lipase (b) during ultracentrifugation versus the cuvette length, each next observation is done after 900 s.

Consequences for lipase experiments and interpretation in a membrane reactor

An immobilized enzyme system, such as a membrane reactor (figure 4a, $\triangle$), often shows an initial activity decrease. However, when during the start up of an immobilized enzyme system a low water activity is present, inactivation already takes place during the immobilization itself. In this case, the first measured activity is in between the initial activity and the apparent equilibrium activity. After a while (equation 3), the fast reversible inactivation has reached its equilibrium and a first order inactivation can be measured (equation 4). However, it has to be noted that in such cases the initial activity is not equal to the activity at $t = 0$.

When both the apparent equilibrium constant A and the irreversible inactivation constant λ_1 are known, the initial esterification rate can be calculated. As is shown in this paper, A and λ_1 are the same for the free enzyme system and the membrane system, therefore, if the esterification rate of the membrane system is measured at a time t larger than - ln(0.01 / (1-A)) / λ_2 (equation 3) the initial rate can be calculated. In this case t should exceed 0.23 Ms (60 hours). Analogue to equation 4 the initial esterification rate r (mole.s^{-1}.m^{-2}) can be calculated as:

$$r(0) = \frac{r(t)}{A} \cdot e^{\lambda_1 \cdot t} \tag{7}$$

The esterification rate of the membrane bioreactor has been reported in previous work as a function of the glycerol concentration (chapter 2, figure 4b). The data are shown in figure 7, as a function of the water activity. The esterification rates have been measured after allowing the reversible inactivation to take place (0.3 Ms, 96 hours). The initial esterification rate $r(0)$ can be calculated using the values of A and λ_1 of figure 2, and the resulting initial rates are given in figure 7. This plot shows that upon a decrease in water activity the initial esterification rate rises, while the measured equilibrium activity decreases. These calculations show that the measured decrease is due to inactivation. The error range of the calculated initial activity at low water-activities is large due to the fact that both the measured apparent equilibrium activity and constant are low, hence the quotient will have a large error.

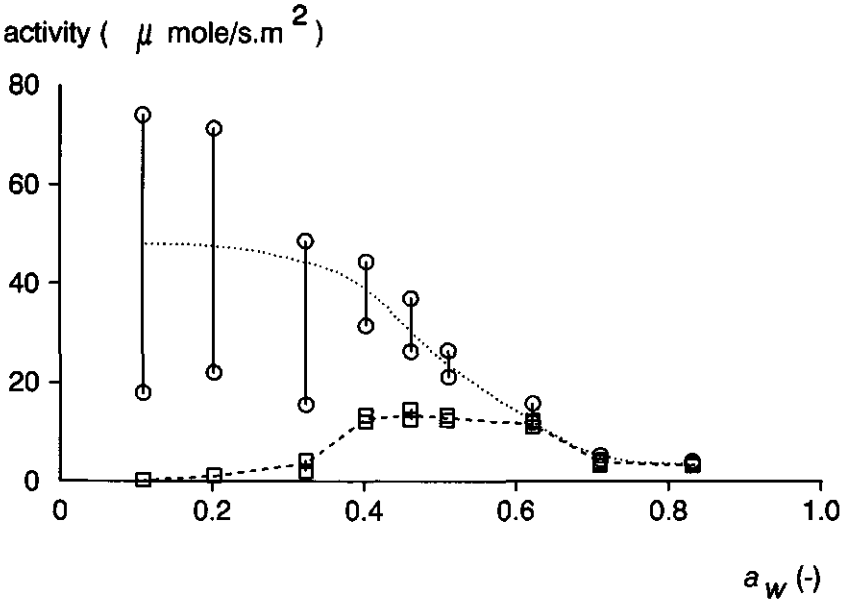


Figure 7. The measured apparent equilibrium activity (□) (Chapter 2) and the calculated initial activity (○) versus the water activity for a membrane reactor.

The calculated initial esterification rate ($15 < E_{tot}(0) < 75$ μmole.s^{-1}.m^{-2}) approximates the activity measured during the hydrolysis of soybean oil (Pronk *et al.* 1991), they have found an activity of 9 to 90 μmole.s^{-1}.m^{-2}. Although an other substrate is used, this calculation indicates that the maximum esterification rate is of the same order of magnitude as the hydrolysis rate. However, in esterifying systems the acting enzyme activity is reduced due to inactivation.

Conclusion

It can be concluded that the inactivation of lipase in glycerol includes both a reversible and a irreversible process. The inactivation is described by a two-step inactivation model. This two-step inactivation occurs for all three types of enzymes tested in this study. The reversible inactivation rate is not only water activity related but differs also for different enzyme systems such as a free enzyme systems and immobilized enzyme systems. The apparent equilibrium activity, defined as the fraction that shows the 'long-term' inactivation, is only a function of the water activity.

The inactivation of lipase is caused by the glycerol concentration and not by the water activity of the system. One of the inactivation mechanisms could be the formation of aggregates.

When the apparent equilibrium constant is measured for the lipase preparation, the initial activity can be calculated. It is shown that the calculated maximum initial esterification rate approximates the maximum hydrolysis rate.

Acknowledgement

This work is partly funded by the Dutch Ministry of Economical Affairs and the Unilever Research Laboratory Vlaardingen.

Symbols

A	apparent equilibrium constant	(-)
a_w	water activity	(-)
$E(t)$	native enzyme activity at time t	(unit.g^{-1})
E_d	completely inactivated enzyme	(unit.g^{-1})

$E_{tot}(t)$	the enzyme activity at time t	(unit.g^{-1})
$E_1(t)$	intermediate enzyme activity at time t	(unit.g^{-1})
K	equilibrium constant	(-)
k_d	first-order inactivation rate constant	(s^{-1})
k_1	first-order reaction rate constant	(s^{-1})
k_{-1}	first-order reaction rate constant	(s^{-1})
R	the gas constant	(J.K^{-1}.mole^{-1})
r	esterification rate	(mole.s^{-1}.m^{-2})
T	temperature	(K)
t	time	(s)
x_g	the mole glycerol fraction	(mole.mole^{-1})
α	the activity ratio	(-)
λ_1	time constant	(s^{-1})
λ_2	time constant	(s^{-1})

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Appendix 1.

For a two step inactivation equation 1 can be written as:

$$\frac{d}{dt}\begin{pmatrix} E(t) \\ E_1(t) \end{pmatrix} = \begin{pmatrix} -(k_1 + k_d) & k_{-1} \\ k_1 & -(k_{-1} + k_d) \end{pmatrix} \cdot \begin{pmatrix} E(t) \\ E_1(t) \end{pmatrix} \tag{A1}$$

The general solution of this equation is:

$$E_{tot}(t) = E_{tot}(0) \cdot \left(A \cdot e^{-\lambda_1 \cdot t} + (1 - A) \cdot e^{-\lambda_2 \cdot t} \right) \tag{A2}$$

When the ratio between the activity of E and E_1 is given by α it follows that:

$$E_{tot}(t) = E(t) + \alpha \cdot E_1(t) \tag{A3}$$

It is assumed, that initially no enzyme with an altered configuration is present:

$$E(0) = E_{tot}(0) \tag{A4}$$

With equations A3 and A4 the relation between the kinetic parameters k_d, k_1, k_{-1} and α with λ_1, λ_2 and A is given by:

$$A = \frac{1 + \alpha \cdot K}{1 + K} \tag{A5}$$

$$\lambda_1 = k_d \tag{A6}$$

$$\lambda_2 = k_1 + k_{-1} + k_d \tag{A7}$$

where K (-) is the equilibrium constant defined as:

$$K = \frac{k_1}{k_{-1}} \qquad \text{(A8)}$$

Fitting experimental results of the total activity gives the empirical parameters A, λ_1 and λ_2. The kinetic parameters cannot be calculated because these are four parameters and only three independent equations are available.

4 Thermodynamic equilibrium

Abstract

Because of the instability of various fatty acids, enzymatic synthesis of esters seems to have advantages over the chemical production thereof. However, a mixture of products and substrates is obtained at equilibrium. In this paper, enzymatic acylglycerol synthesis is discussed with regard to equilibrium concentrations in a non-ideal two-phase system. The equilibrium constants for the different esterification steps are calculated using the UNIFAC group contribution method. The activity based equilibrium constants approximate one. Once the equilibrium constants are known, equilibrium concentrations can be predicted under different conditions using the program TREP (Two-phase Reaction Equilibrium Prediction). It is shown that in a two-phase system with decanoic acid and hexadecane as solvent a mixture of mono-, di- and triester is always obtained without a considerable surplus of any of these. Calculations show that, even with a high glycerol to fatty acid ratio, no pure monoesters can be obtained. Pure triester synthesis can be achieved only under conditions of low water activity.

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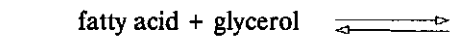
Introduction

Esters of glycerol and fatty acids are used in the food and pharmaceutical industry. Monoglycerides (monoacylglycerols) are utilised as emulsifiers while triglycerides (triacylglycerols) can be added to alter the melting range of a product. Ester synthesis can be catalyzed either by an inorganic catalyst or an enzyme. Enzymatic catalysis has a few advantages over chemical production. The major advantage is that high temperatures can be avoided, thus preventing polymerization of unsaturated fatty acids (Oberkobusch 1990). Another advantage is the selectivity of some lipases for the position of the ester bond or the selectivity for the tail length of the fatty acid (Jensen *et al.* 1990). This selectivity is an extra tool which can be used for the production of tailor made esters. In this paper a non-selective enzyme is used. Ester production often ends up with a mixture of substrates and products (Ergan and Trani 1990, chapter 2). For application of the esters in pharmaceuticals or food, the products must be as pure as possible, therefore insight into the equilibrium state is of great importance to optimize ester production.

The production rate is a function of the concentration and type of catalyst, temperature and substrate and product concentrations. However, the final equilibrium concentrations are catalyst independent and only a function of the thermodynamic properties of the system. Not only do substrate and product concentration play a role, also temperature, pressure and the reaction medium composition determine the final equilibrium situation. In the case of acylglycerol production, three equilibrium reactions are involved:



At equilibrium, a mixture of fatty acids, mono-, di-, triglyceride, water and glycerol is obtained. An excess of triglycerides can be obtained only when the water produced is removed continuously (Ergan *et al.* 1990). Alternatively, an excess of fatty acids can be formed when glycerol is removed (Pronk *et al.* 1988).

The aim of this study is the calculation of the equilibrium constants for the three equilibria in a two-phase system consisting of non-ideal solutions. In this case, activities must be used to calculate the equilibrium constant (Eggers *et al.* 1989). When the equilibrium concentrations are measured, activities can be calculated using the UNIFAC group contribution method, then the activity based equilibrium constant can be calculated. If the equilibrium constants are known and the initial concentrations are given, then it is possible to calculate the equilibrium concentrations using the program TREP (Two-phase Reaction Equilibrium Prediction). It will be shown, that the conditions which lead to pure triester or pure monoester production can be calculated.

Theory

Consider a general condensation reaction:

$$\mathrm{A} + \mathrm{B} \rightleftharpoons \mathrm{C} + \mathrm{water} \tag{4}$$

In which A, B and C are arbitrary chosen components. During reaction, the free energy change ΔG (J.mole^{-1}) of this system is:

$$\Delta G = \Delta G^0 + R \cdot T \cdot \ln\left(\frac{a_C \cdot a_{water}}{a_A \cdot a_B}\right) \tag{5}$$

where	ΔG^0	: standard free energy change for the reaction	(J.mole^{-1})
	a	: activity	(-)
	R	: gas constant	(J.K^{-1}.mole^{-1})
	T	: temperature	(K)

At equilibrium the free energy change ΔG equals zero, in this situation the standard free energy can be calculated as:

$$\Delta G^0 = -R \cdot T \cdot \ln K \tag{6}$$

In which K (-) is the equilibrium constant is given by:

$$K = \frac{a_C \cdot a_{water}}{a_A \cdot a_B} \tag{7}$$

The equilibrium constant K is a constant for a given pressure and temperature. When the equilibrium constant K is known, the standard free energy change for the reaction ΔG^0 can be calculated at this temperature. When an equilibrium situation is reached, the mole fraction of a component i can be measured and the activity can be calculated as:

$$a_i = \gamma_i \cdot x_i \tag{8}$$

where	x_i	: mole fraction	(-)
	γ_i	: activity coefficient	(-)

However, for the system studied here, the activity coefficient γ_i is not known for the different components in the mixture A, B, C and water. Of course, one can measure the activity coefficient at all the different mixture compositions, however, this a very

time consuming job. Alternatively, one can estimate the activity coefficient using *e.g.* the UNIFAC groups contribution method (Fredenslund *et al.* 1977). The equilibrium constant can be calculated as:

$$K = \frac{a_C \cdot a_{water}}{a_A \cdot a_B} = \frac{\gamma_C \cdot \gamma_{water}}{\gamma_A \cdot \gamma_B} \cdot \frac{x_C \cdot x_{water}}{x_A \cdot x_B} \qquad (9)$$

In the case of a two-phase system, it does not matter in which phase the mole fractions are measured to calculate the activities since at equilibrium the activities of both phases are equal. Once K is known, equilibrium concentrations can be calculated when the initial amounts are given. This is done using a computer program.

This approach differs from the methods presented by Eggers and coworkers (1989), Semenov and coworkers (1989) and Halling (1990). These papers describe a way to calculate extractive catalysis in dilute systems. In dilute systems, the partition coefficient is constant. In our case, the partition coefficient is strongly glycerol concentration related. Halling (1990) uses group contribution correlations to predict an equilibrium concentration shift as a function of different organic solvents. This study does not include the influence of solvents upon the ester production. However, the approach as presented in this paper can be used for this purpose. This is done in another study (Janssen *et al* 1993).

Materials

The *Candida rugosa* lipase used in this study is the enzyme preparation Lipase OF 360,000 obtained from Meito Sangyo (Japan). Glycerol 99+ % is obtained from Janssen (Belgium). Decanoic acid is obtained from Unichema and is 95 % pure. All other chemicals are purchased from Merck (FRG). Demineralized water is used throughout the study. For the membrane bioreactor a cellulose hollow fibre unit is chosen (Andante unit Organon, The Netherlands). The membrane unit contains approximately 6000 fibres with an internal diameter of 0.2 mm and a dry wall thickness of 8 μm. The total membrane surface area in such a unit is 0.77 m^2.

Experimental

Emulsion system

Esterification in an emulsion system is performed in a thermostated reaction vessel (25°C) of standard geometry (inner diameter = 0.1 m). The vessel contains four baffles and the mixture is stirred by a four-bladed turbine (diameter = 0.04 m). The reactor is filled with 500 mL decanoic acid in hexadecane solution (1:1 w/w or 2:1 w/w), 250 mL glycerol-water mixture with a known water content and 2 grams of the crude enzyme preparation (chapter 2). After 8 hours, again 2 grams of enzyme is added. Both after 8 hours and after 24 hours, the concentrations of the fat phase and water phase are measured. When the concentrations did not change, these are considered to be the equilibrium concentrations.

Membrane bioreactor system

The membrane bioreactor consists of the cellulose hollow fibre membrane module, with an internal oil circuit (approximately 80 mL 1:1 w/w decanoic acid in hexadecane) and an external glycerol-water circuit (approximately 150 mL). The biocatalyst is immobilized at the inner fibre side (chapter 2). At the start of each experiment the oil phase is replaced. The conversion takes place at 25°C. When no further change in concentrations with time can be measured, the system is assumed to have reached equilibrium if the enzyme is still active. Enzyme activity is tested by replacing the oil phase and measuring renewed ester formation.

Synthesis with water removal

Partial glycerides, hexadecane and decanoic acid, as obtained from the emulsion experiments (total 5 gram), and 1 gram of enzyme preparation are stored without agitation above saturated salt solutions with different water activities. After three weeks of storage, equilibrium is reached and the concentrations are determined. The salts used are $LiCl_2$ (a_w = 0.11), K_2CO_3 (a_w = 0.43), NaBr (a_w = 0.58), NaCl (a_w = 0.75) and $BaCl_2$ (a_w = 0.90) (Young 1967, Goderis 1986).

Fat phase analysis

The composition of the non-polar phase is determined by capillary gas chromatography. From emulsion systems, a sample of approximately 1 mL of the

emulsion is taken and is separated into the polar and non-polar phases by centrifugation (30000 rpm, 5 min). The non-polar phase is diluted (400 times) in hexane, 1 μL is injected 'cold on column' on a 5 m CP-Sil-5-CB column (Chrompack, The Netherlands) placed in a Carlo Erba gas chromatograph. The oven temperature is 80°C at the moment of injection. After one minute the temperature is increased by 20°C.min^{-1} up to 320°C. The F.I.D. detector has been set to 370°C, helium is chosen as carrier gas (4 mL/min). All concentrations are calculated as mole fractions relative to the sum of the amount of solvent, acid and ester.

Water phase analysis

In the water phase the initial glycerol to water ratio is measured using a Pleuger refractometer. Both concentrations are calculated as mole fractions.

Activity coefficient calculations

The activity coefficients are calculated using the UNIFAC model (Fredenslund *et al.* 1977) using the parameter set for the prediction of liquid-liquid equilibria (Magnussen *et al.* 1981). This table is primarily developped for the prediction of liquid-liquid equilibra, including predictions in aqueous-organic two-phase systems at temperatures between 10 and 40 °C. When the water and glycerol activities of water/glycerol mixtures are calculated by using this UNIFAC group contribution parameter set and the formulas of Norrish (1966), a maximum difference of 0.012 in activity is found. This indicates that the prediction of simple water rich solutions is sufficient. The activity coefficients are calculated with reference to an ideal solution in the sense of Raoults' law.

Calculation of the equilibrium constant

For the calculation of the activity coefficients, the mole fraction of all components in each phase has to be known. As only the concentrations of the esters, decanoic acid and the solvent hexadecane is measured in the non-polar phase, the water and glycerol concentration in this phase have to be calculated. Assuming both phases to be at phase equilibrium, the activity of all the components should be equal in both phases. As the water and glycerol concentrations are known in the polar phase, the mole fraction of

all components in the two-phases can be calculated using mass balances and the UNIFAC activity coefficients. Once all mole fractions and activity coefficients are known, the activity based equilibrium constant can be calculated.

The program TREP

When the equilibrium constant is known, the equilibrium concentrations can be estimated for different situations. Given a certain quantity of substrate and solvent, phase equilibrium is calculated using UNIFAC and mass balances (figure 1). The two-phases are at equilibrium when the activity of a component equals the activity of that component in the other phase. If the phases are at phase equilibrium, then the computer program calculates whether the system has reached chemical equilibrium. Chemical equilibrium is obtained when for the calculated activities and the given standard free energy change ΔG^0 the free energy change ΔG equals zero for each reaction step (equation 5). The standard free energy change ΔG^0 is calculated using the given equilibrium constant K (equation 6). If the system reaches chemical equilibrium the calculations are terminated. Otherwise, a reaction step is calculated, esters and water are produced and glycerol and fatty acids are consumed, or the other way around. After this reaction step, again phase equilibrium is calculated, and so on, untill the point when both phase and chemical equilibrium is obtained.

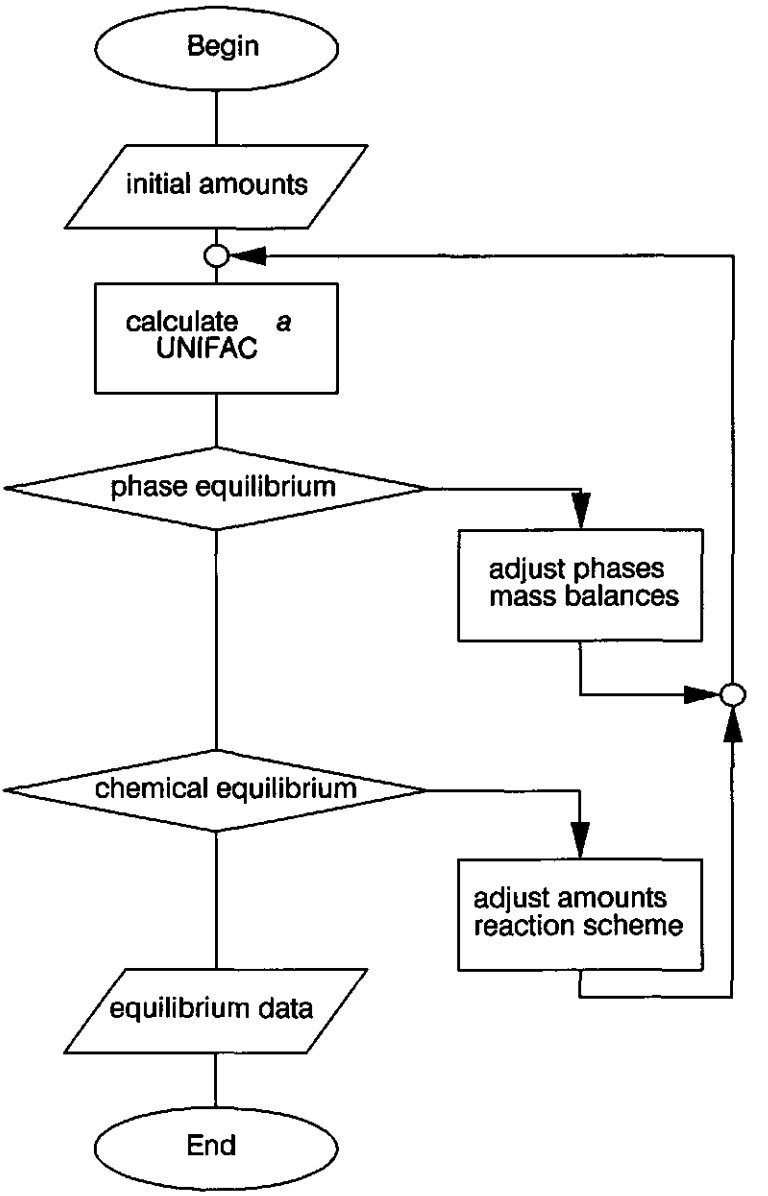


Figure 1 *Scheme of the computer program to calculate the equilibrium concentration at given initial concentrations.*

Results and Discussion

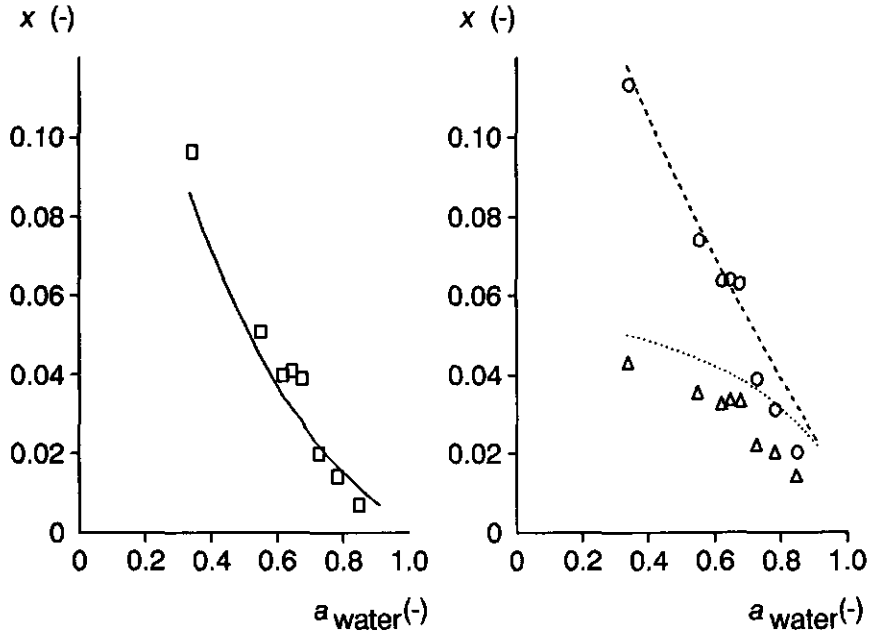


Figure 2 *Equilibrium mono- (□), di- (○) and triester (△) concentration in the non-polar phase as a function of the water activity. Drawn lines are the calculated concentration of mono- (———), di (- - - -) and triester (· · · · ·). Initially, the non-polar phase consists of 1.26 mole decanoic acid and 0.80 mole hexadecane, the polar phase varies from 1.41 mole glycerol and 8.29 mole water to 3.00 mole glycerol and 1.79 mole water.*

Ester synthesis in relation to water activity was measured at 25°C, in a system with hexadecane as solvent. A typical set of emulsion experiments is given in figure 2. This figure shows the measured equilibrium ester concentration in the non-polar phase as a function of the equilibrium water activity. An increase of the ester concentration with decreasing water activity is found. Instead of a surplus of one of the esters, a

mixture of mono-, di- and triester is found. At low water activity no data are available since lipase from *Candida rugosa* is inactivated by high glycerol concentrations (chapter 3).

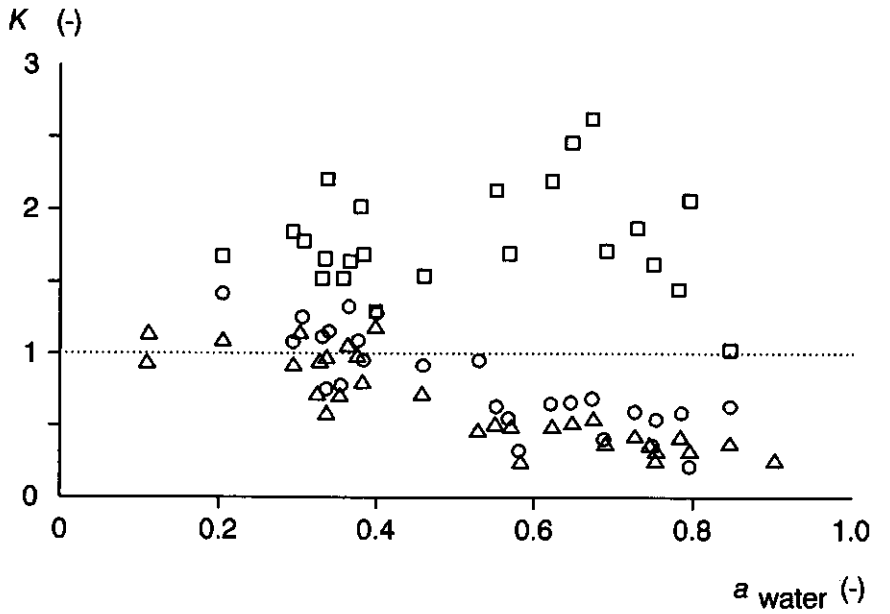


Figure 3 *The equilibrium constant of the mono- (□), di- (○) and triester (△) synthesis as a function of water activity.*

From the equilibrium concentration measurements and the activity coefficients calculated by UNIFAC, the equilibrium constants can be calculated. Data are shown in figure 3. The data show a large scatter but there is a tendency for a decrease in the di- and triester equilibrium constant and increase in the monoester equilibrium constant, with increasing water activity. K should be constant at a given temperature. However, when the negative logarithm of K is calculated, *i.e.* the standard free energy change for the reaction, ΔG^0, over the gas constant times temperature (equation 6), it is observed that for each component, K does not vary by more than a factor of ten.

This variation is often accepted for equilibrium constants. The variation could be caused by the inaccuracy in the analysis. A small difference in measured mole fraction could cause a large difference in calculated activities.

For the K values of mono-, di- and triester, the average of the measured values is chosen, these are 1.6, 0.8 and 0.6, respectively. Using these values for the equilibrium constants, equilibrium concentrations can be calculated for different systems using TREP. In figure 2, the calculated mole fractions are given as lines. This plot shows a good agreement between the measured and calculated values.

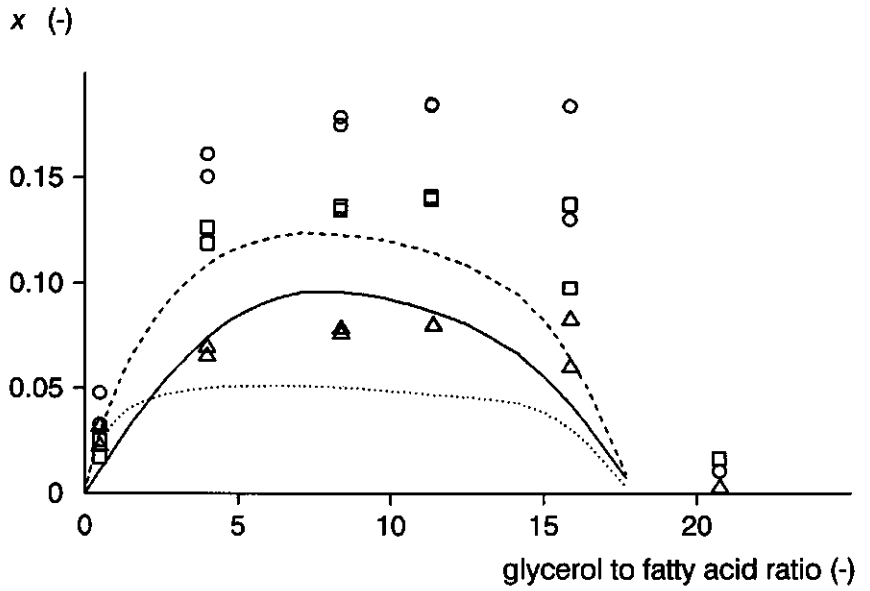


Figure 4 *Equilibrium mono- (□), di- (○) and triester (△) concentration in the non-polar phase as a function of the glycerol to fatty acid ratio, drawn lines, the calculated concentration of mono- (——), di (- - - -) and triester (· · · · ·). The initial amount fatty acid, solvent hexadecane and water is kept constant, 1, 0.7 and 0.4 mole, respectively, and glycerol is added.*

For a closed batch system, ester production is calculated for different initial glycerol to fatty acid ratios, starting from an initial glycerol to fatty acid ratio of 0 up to 23. This means that the polar phase increases whilst the non-polar phase remains constant. The calculated equilibrium concentrations in the non-polar phase and the activity of the esters are given in figure 4. A set of experiments is done to verify the calculations. Figure 4 shows that the measured and the calculated values do differ although only within a factor of about 2. A difference can be expected because the UNIFAC data are not exact for complicated mixtures such as those used here and these equilibrium measurements are not used for the calculation of the equilibrium constants. However, it is clear that even at high glycerol to fatty acid ratios the trend is predicted. Figure 4 shows that none of the initial conditions leads to pure monoester production nor is high triester production obtained. The low ester concentration at low glycerol to fatty acid ratios is due to the high water activity; in this case a low ester activity is also obtained. At high glycerol to fatty acid ratios, a high ester activity is calculated, however, figure 4 shows that a low ester concentration is obtained in the non-polar phase. This is due to the experimental conditions. In this case, ester production is limited by the availability of the fatty acid, over 90 % of the fatty acids are esterified. By increasing the glycerol to fatty acid ratio both the volume of the polar phase as well as the glycerol concentration increases. At high glycerol concentrations, the monoester will be extracted into the glycerol phase. When the volume of the glycerol phase is large, a low mole fraction is obtained in the polar phase, which is in equilibrium with the non-polar phase. Hence low ester concentrations are found in the non-polar phase. All of these phenomena are included in the model, including a predicted decrease in mole fraction (figure 4). At high glycerol to fatty acid ratios the emulsion becomes very viscous, which leads to inaccurate measurements because of phase separation problems.

These calculations show that it will never be possible to obtain pure monoesters in a closed two-phase system containing hexadecane as solvent. In the literature different monoester production systems have been presented. In accordance with the calculations shown above, they are all based on the extraction of monoester during synthesis. McNeill and coworkers (1991) have precipitated the monoesters while Van

der Padt and coworkers (chapter 6) have adsorbed the monoesters onto a silica column. Another approach is to use solvents in which the activity coefficients are favourable for high monoester concentrations (Graille 1985, Janssen *et al* 1993).

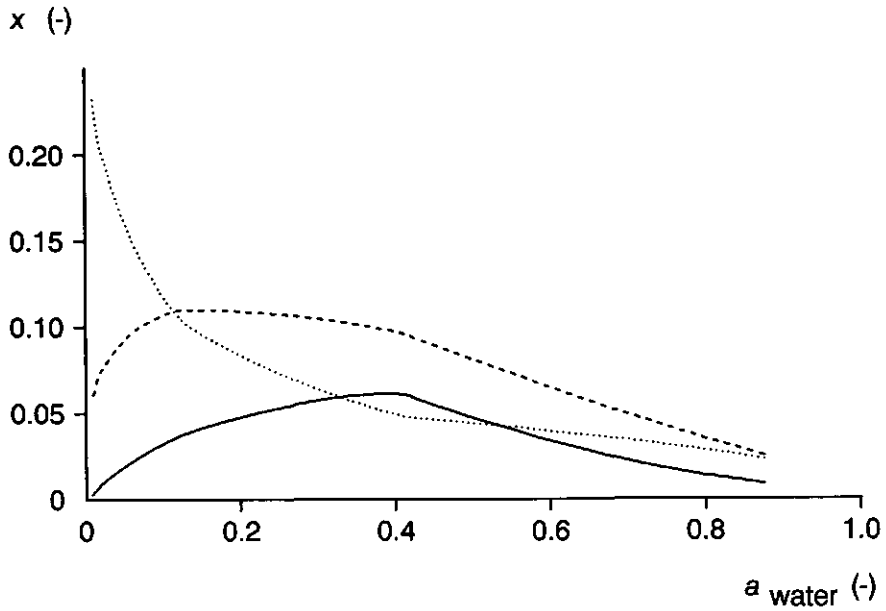


Figure 5 *Calculated ester formation with continuous water removal as a function of the water activity. The initial glycerol to fatty acid mole ratio is 1 to 3, the solvent hexadecane is kept constant at 0.7, different amounts of water is added; mono- (——), di (- - - -) and triester (· · · · ·).*

As mentioned in the literature, pure triester production will be successful only if water is removed during the production (Ergan *et al.* 1990, chapter 5). Indeed, figure 4 shows that a high triester concentration can not be obtained in a closed two-phase system. Calculations have been made excluding water production during synthesis, thus simulating conditions where the water produced is removed instantaneously (figure 5). The initial glycerol to fatty acid mole ratio is 1 to 3. This means that theoretically all

the fatty acid can be incorporated into triesters with a maximum mole fraction of triester of 0.3 mole.mole^{-1}. At low water activities ($a_w < 0.4$) a one-phase system is obtained. Above a water activity of 0.4, a two-phase system is obtained. Phase split occurs at the maximum monoester concentration. These calculations show that only at extremely low water activity conditions a surplus of triesters is formed.

Conclusions

Acylglycerol equilibria can be described using the activity-based equilibrium constant and it has been shown that the ester production equilibrium constant approximates 1. It has been calculated that neither pure monoesters nor pure triesters can be obtained in a closed two-phase system with hexadecane as solvent. This is in agreement with measurements in the literature. However, monoesters can be produced by removing the esters during synthesis. An excess of triesters is formed only under conditions of extremely low water activity.

Acknowledgement

The authors wish to thank H.M. Van Sonsbeek for the development of the computer programs.

Symbols

a	:	activity	(-)
K	:	activity based equilibrium constant	(-)
R	:	gas constant	(J.K^{-1}.mole^{-1})
T	:	temperature	(K)
x	:	mole fraction	(-)
ΔG	:	free energy change for the reaction	(J.mole^{-1})

ΔG^0	: standard free energy change for the reaction	(J.mole^{-1})
γ	: activity coefficient	(-)

The subscripts A, B, C and water refer to the components A, B, C and water, respectively.

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5 Triacylglycerol synthesis

Abstract

Triacylglycerols can be synthesized from glycerol and fatty acids. During this equilibrium reaction water is produced, therefore a mixture of mono-, di- and triesters is obtained. One way to produce an excess of triacylglycerols is to remove the water produced during synthesis. This can be realized in an immobilized enzyme pervaporation system.

The enzyme is immobilized onto the lumen side of a cellulose membrane where the organic phase is present. Air circulates at the shell side and the water activity is controlled with the use of a condenser. The lipase catalyzed esterification of decanoic acid and partial glycerides is studied in this reactor. The system is reaction limited. Only at low water activity conditions, an excess of triacylglycerols is obtained. The enzyme activity at the start of the experiments is independent of the water activity within the range studied. Stability is influenced: After 600 hours the activity is 26 % of the activity at the start at a_w = 0.1 and 71 % at a_w = 0.45.

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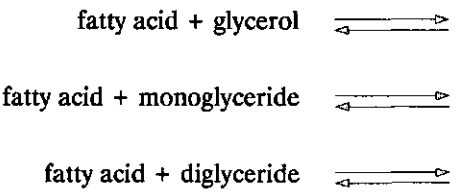
Introduction

Tailor made triglycerides can be used to adjust the melting range of foods and cosmetics. In contrast to the chemically catalyzed esterification, the enzymatic ester synthesis already proceeds at moderate temperatures, thus avoiding the polymerization of unsaturated fatty acids. An attendant advantage is that the enzymatically synthesized ester can usually be qualified as a natural ingredient. Triglycerides can be enzymatically synthesized from glycerol and fatty acids:



When long-tail fatty acids are used a two-phase system is present, a polar glycerol phase and a non-polar fatty acid phase. During esterification water is produced, the water produced accumulates in the polar phase, the esters accumulate in the non-polar phase. As a result of the water production and alcohol consumption, the water activity of the system will increase. Therefore, at equilibrium a mixture of fatty acid, mono-, di- and triesters is formed, even if the reaction is carried out using initially stoichiometric amounts of fatty acid and glycerol (chapter 4). One way to enhance the production of triglycerides is the removal of the by-product water during synthesis (Ergan *et al.* 1990). This can be executed by keeping the system at constant water activity. In this case, the system changes from a two-phase system into a one-organic phase system, at the moment that most of the glycerol is converted into mono-, di- or triesters because the water produced has been removed.

When for the enzymatic esterification a mixture of fatty acids, mono-, di- and triglycerides is chosen as starter material, there is no need to add glycerol and a one-phase system is obtained at the start. The free alcohol groups of the mono- and diglyceride can be esterified to form triglycerides and water (Schuch and Mukherjee, 1989).

It is the objective of this study to produce an excess of triglycerides. As starter material partially esterified glycerol is chosen which has been prepared in a two-phase system starting with fatty acids and glycerol. The mixture of glycerides is used in a second system, in which system lipase catalyses the triester synthesis at constant water activity conditions. To that purpose, a new membrane reactor concept is developed: The enzyme is immobilized onto the membrane surface. At this side of the membrane, the non-polar phase is present, being in contact with the enzyme. At the other side of the membrane, an extraction phase is pumped through to remove the water produced thus avoiding water accumulation in the immobilization carrier. In this way the reaction should proceed to an equilibrium state of almost pure triglycerides. This paper describes the performance of the second system. The choice of the extraction phase and the membrane material will be discussed in terms of extraction rate and enzyme stability. The activity and the equilibrium ester concentration will be discussed reviewing the experimental results.

Selection criteria

The choice of the extraction phase and the membrane material is crucial for the success of this type of membrane reactor. A proper reactor design should lead to a membrane reactor in which the oil phase and the drying agent are kept separated. The enzyme should be immobilized onto the membrane surface and should remain active for a long period of time. Last but not least, the membrane has to withstand water activity conditions low enough to synthesize an excess of triglycerides.

Membrane material

The oil phase and the drying agent are kept separated if one of the two phases preferentially wets the membrane and if the transmembrane pressure is below the maximum Laplace pressure. The Laplace pressure increases upon a reducing pore radius. Therefore, a membrane with a small pore radius has to be preferred, because this results in a high allowed transmembrane pressure and no accurate hydraulic pressure control is necessary. Furthermore, the membrane has to withstand low water activity conditions, in this research as low as 0.1.

Enzyme activity and stability

The hydrophobicity of the immobilization carrier can influence the activity of an immobilized enzyme (Mozhaev *et al.* 1990). For the enzymatic esterification, it has been shown that lipase is active when immobilized onto hydrophobic polypropylene as well as onto hydrophilic cellulose (Hoq *et al.* 1984 and chapter 2, respectively). For hydrolysis it has been shown that different immobilization carriers show different specific enzyme activity (Geluk *et al.* 1992). Lipase showed a higher specific activity on polymethylmethacrylate than on cellulose, the last one being higher than on polystyrene. This can be a criterium for the choice of membrane material. For a given material, a coating method (Keurentjes *et al.* 1990) can be used to alter the hydrophobicity of the membrane. Besides immobilization, enzyme activity is water activity dependent as reported by Valivety and coworkers (1992).

Immobilization can stabilize the enzyme also at low water activity conditions (Mozhaev *et al.* 1990). An immobilized enzyme can employ activity even in anhydrous media (Carta *et al.* 1991). Enzyme stability is not only influenced by the immobilization carrier but also by the water activity. Boyer and coworkers (1990) and Goldberg *et al.* (1990) have shown enzyme inactivation at low water activity conditions for free enzyme systems. They stated that at low water activity conditions there is not enough water available to stabilize the active conformation of the enzyme.

Drying medium

The extraction medium should allow control of the water activity, should be non-miscible with the non-polar acylglycerol phase and should not influence negatively the esterification reaction. The water activity of water can be reduced by adding a polyol to the solution such as glycerol or polyethylene glycol. However, lipase is capable to catalyse the esterification of the alcohol groups of those components (Okumura *et al.* 1979). When salts are used to adjust the water activity, it can be expected that the enzyme is salted out thus loosing its activity (Lehninger 1975). Another possibility is the use of air. With this medium the water activity can be controlled with a condenser used at the appropriate temperature. In this study air is used.

Materials

Lipase of *Candida rugosa* is used. This enzyme preparation (Lipase OF 360,000) is obtained from Meito Sangyo (Japan). Glycerol 99+ % is from Janssen (Belgium). The decanoic acid from Unichema is 95 % pure. Polymethylmethacrylaat (PMMA) and polyethyleneoxide (PEO) are of Aldrich (Belgium). All other chemicals are from Merck (Germany). Demineralized water is used throughout the study. For the membrane bioreactor a polypropylene, a celluloseacetate and a cellulose membrane respectively are chosen. The membrane characteristics are given in table 1. The flat sheet membrane test unit is the Megaflow TM 100 (New Brunswick Scientific Co. Inc. USA). The hollow fibre units are commercially available (Organon Teknika, Akzo group, The Netherlands).

Table 1 Membranes tested; FS = flat sheet, HF = hollow fibre

Membrane	Type	Pore Size Cut Off	Area (m^2)	Manufacturer
Polypropylene	FS	0.1 μm	0.56×10^{-4}	Enka (Germany)
Polypropylene	HF	0.2 μm	0.07	Enka (Germany)
Celluloseacetate	HF	70000 D	0.12	Enka (Germany)
Cellulose*	HF	10000 D	0.77	Enka (Germany)

* fibre diameter = 0.2 mm[b]; fibre length = 0.22 m[b]; number of fibres = 6000[b]; porosity = 0.65[b]; tortuosity = 1.9[a]; dry membrane thickness = 8×10^{-6}m[b]; wetted membrane thickness = 15×10^{-6}m[a] (data [a]) from Keurentjes *et al.* 1992 or [b]) from the manufacturer)

Experimental

Membrane coating

A flat sheet polypropylene membrane has been coated with PMMA as described by Keurentjes *et al.* (1990). A hollow fibre polypropylene unit has been coated using PEO as follows: The module has been rinsed with nitric acid vapour under vacuum for 60 seconds. Afterwards, the module has been cleaned with water and a 0.75 % w/w PEO solution is applied as feed solution at 0.2×10^{-6} $m^{-3}.s^{-1}$ for 2 hours. Finally, the module has been flushed with water.

Water removal rate measurement

The water removal rate is measured at steady state using a membrane reactor without immobilized lipase. At the inlet of the inner fibre side of the cellulose membrane, a non-polar oil phase (see below, density = 900 $kg.m^{-3}$) having a water activity a_w of approximately 1 is pumped through (1.45 $g.s^{-1}$). As extraction phase at the shell side an air stream having a water activity of 0.15 up to 0.4 is used (10 up to 100×10^{-6} $m^3.s^{-1}$). The water activity of the air at the outlet of the condenser is determined by measuring the dew-point using a silicon sensor (Mitchell Instruments U.K.). The water content of the oil-phase at the outlet is measured by Karl Fischer titration. The water flux through the membrane is calculated from mass balances at the oil side. To verify the mass balance, the water flux is also measured by determining the mass of the water in the condenser with time. When both measurements differ less than 5 % then the average value is used, otherwise the measurement is rejected.

The distribution coefficient m (-) of water over the oil and air phase is measured as follows. The non-polar phase is equilibrated above salt solutions with established water activity values. At equilibrium, the water concentration is measured in the non-polar phase. The distribution coefficient m (-) of water over the oil and air phase is defined by $m = C^{eq}_{w,air} / C^{eq}_{w,oil}$ where $C^{eq}_{w,air}$ and $C^{eq}_{w,oil}$ are the equilibrium water concentration of the non-polar- and air phase ($kg.m^{-3}$), respectively.

The membrane reactor

The membrane bioreactor (figure 1) consists of a membrane unit, with a non-polar oil phase circuit (approximately 100 g) and an air circuit. In case of a hollow fibre unit,

the oil phase is pumped through the inner fibre side. The biocatalyst is immobilized on the membrane at the oil side with the method described in chapter 2. This immobilization procedure takes place at a_w = 1, after immobilization it takes 150 hours to remove the surplus of water needed for the immobilization. At the start of each experiment the oil phase is replaced. The conversion takes place at 25°C. The air circuit consists of a pump and a condenser which is placed in a Colora WK3 cryostat.

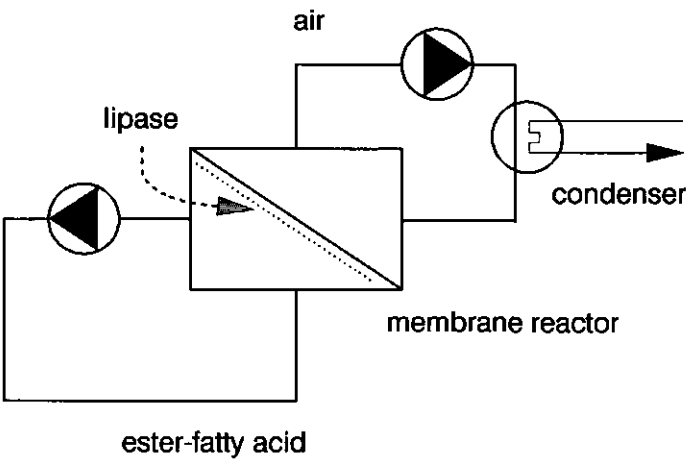


Figure 1. The membrane bioreactor.

After the replacement of the oil phase at 150 hours after the immobilization of the enzyme, the initial fatty acid conversion rate is measured in batch mode from the fatty acid concentration change in time. Equilibrium is assumed when the ester concentrations do not change any more with time. A prerequisite is then that the enzyme is still active. The enzyme activity is tested by either increasing the fatty acid

concentration or by replacing of the non-polar phase.
In a repeated batch experiment the oil phase is replaced just before each determination of the initial activity.

The starting oil is enzymatically esterified decanoic acid and glycerol with hexadecane as a solvent. The synthesis of the starting material has taken place in a two-phase membrane system or in an emulsion system (chapter 2). The initial conditions for the experiments were a 0.74 mole.mole^{-1} decanoic acid in hexadecane non-polar phase, a 0.4 mole fraction glycerol water phase (a_w = 0.5) and *Candida rugosa* lipase. This resulted in a starting material with a composition of 0.30 mole.mole^{-1} decanoic acid, 0.10 mole.mole^{-1} monoester, 0.14 mole.mole^{-1} diester, 0.05 mole.mole^{-1} triester, 0.39 mole.mole^{-1} hexadecane and 0.02 mole.mole^{-1} glycerol.

Fat phase analysis
The composition of the non-polar phase is determined by capillary gas chromatography. Each sample is diluted (400 times) in hexane, 1 μL is injected cold on column of a 5 m CP-Sil-5-CB column (Chrompack, the Netherlands) placed in a Carlo Erba gas chromatograph. The oven temperature is 80°C at the moment of injection. After one minute the temperature is increased with 20°C.min^{-1} up to 320°C. The F.I.D. detection of decanoic acid, mono-, di- and tridecanoate occurs at 370°C. Helium is chosen as carrier gas (4 mL/min). All concentrations are calculated as mole fractions based on the actual amount of moles.

Results and discussion

Membrane selection
Since the hydrophobicity of the membrane can influence enzyme activity, several types of membrane have been tested included two coated with polymers. The effectiveness of the coating has been tested qualitatively by measuring the oil flux before and after the coating procedure. A distinct decrease was observed, indicating the presence of a coating layer. As shown in table 2, only the non-modified polypropylene and the cellulose membrane can keep the oil and air phase separated. However, after

immobilization of lipase, both the oil and air phase permeate through the polypropylene membrane. Therefore, the cellulose hollow fibre unit has been chosen for the reactor.

Table 2 Capability to keep the two phases separated before and after immobilization of lipase and enzyme activity.

Membrane	Type / Coating	Separation before immobilization	Separation after immobilization	Activity
Polypropylene	FS / PMMA	−	n.t.	n.t.
Polypropylene	HF / PEO	−	n.t.	n.t.
Polypropylene	HF	+	−	−
Celluloseacetate	HF	−	n.t.	n.t.
Cellulose	HF	+	+	+

− no; + yes; n.t. not tested

Water removal rate

For the pervaporation system, the mass transfer coefficient can be calculated using mass balances:

$$J = k_{ov} \cdot A \cdot (C_{w,oil} - C_{w,air}/m) \quad (4)$$

where J : transmembrane flux (kg.s^{-1})
k_{OV} : overall mass transfer coefficient (m.s^{-1})
A : membrane area (m^2)
$C_{w,oil}$: water concentration of the oil phase (kg.m^{-3})
$C_{w,air}$: water concentration of the air (kg.m^{-3})

The distribution coefficient m is measured to be 10 within the range of variables. At mass transfer conditions, the measured concentration in the non-polar phase is over 1000 times the concentration at the air side thus the term with m can be neglected. At steady state, the overall mass transfer coefficient k_{ov} has been measured at different water activities (□, figure 2). For $C_{w,oil}$ the average value of the water concentration at the inlet and outlet of the fibres is chosen. At water activities below 0.3, k_{ov} is 2.3×10^{-6} m.s^{-1}. The mass transfer coefficient of the membrane wall is the diffusion coefficient times porosity over the membrane thickness times tortuosity (table 1). When the membrane is completely wetted, the mass transfer coefficient of the membrane wall equals 0.5 m.s^{-1} (diffusion coefficient water in air = 2.2×10^{-5} m^2.s^{-1}). It is shown in literature, that the transmembrane flux of water vapour through cellulose drops a factor 2 when the membrane changes from a wetted, hence swollen, matrix to a dry matrix configuration (Sheng and Mora). The mass transfer coefficient of the membrane wall then should drop to 0.2 m.s^{-1}. Since the measured overall mass transfer coefficient is orders of magnitude smaller, the transfer resistance should be in an other part of the reactor. The diffusion coefficient of water in air is orders of magnitude larger than that in oil which together with $m >> 1$ makes it plausible that the mass transfer limitation occurs in the oil phase. With the diffusion coefficient of water in peanut oil ($2.5.10^{-10}$ m^2.s^{-1} Creemers *et al.* 1983), the mass transfer coefficient at the inner fibre side is calculated to be 3.8×10^{-6} m.s^{-1} (Yang and Cussler 1986). This is the same order of magnitude as the measured overall mass transfer coefficient. This again indicates that diffusion limitation occurs in the oil phase. At these water activity conditions and reactor volume V (m^3), the characteristic time of mass transfer $\tau_m = V/A \cdot k_{ov}$ equals 40 seconds.

If the water activity exceeds 0.3, a lower value of k_{ov} of 0.36×10^{-6} m.s^{-1} is measured (□, figure 2). Also, at $a_w > 0.3$, it was observed that the fibres stick together, which results in a reduction of the effective membrane area. The same phenomenon was found by Keurentjes *et al.* (1992). In their case an effective membrane area of 0.045 m^2 has been found instead of 0.77 m^2 for the same type of membrane unit, during diffusion experiments using methanol, ethanol water mixtures. This value approximates the outer surface area of the bundle of fibres. When this effective membrane area of 0.045 m^2 is used, the calculated overall mass transfer coefficient has a value of

$(6 \pm 2)\times10^{-6}$ m.s^{-1} (○, figure 2). This makes it very plausible that the low mass transfer coefficient is caused by the fact that the fibres stick together. In this case the characteristic time of mass transfer τ_m equals 300 ± 100 seconds.

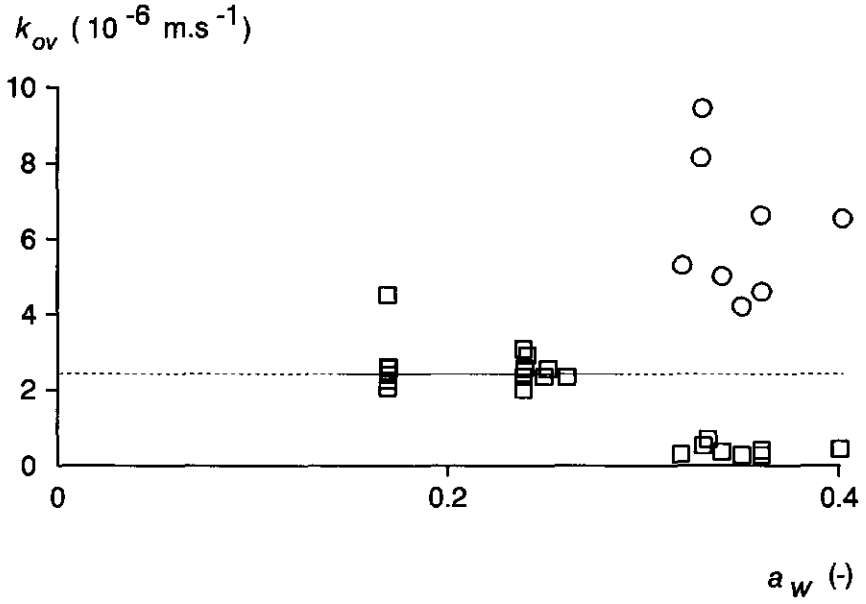


Figure 2. The overall mass transfer coefficient k_{ov} based on the membrane area of 0.77 m^2 (□) and based on an effective membrane area of 0.045 m^2 (○) as a function of the water activity.

Enzymatic triester production

The water activity of the reactor system is changed by changing the water activity of the gas phase. The triester equilibrium concentration is measured as a function of the water activity (figure 3b). The triester mole fraction increases upon a decreasing water activity. However, even at low water activity conditions (a_w < 0.1), there is still a relatively high diester concentration present while at first sight equations 2 and 3 would lead to mainly triester. The reason is that when starting with the mixture given in

experimental, the total amount of fatty acids is smaller than the amount needed for a complete conversion to triester. At a_w = 0.1, 80 % of the fatty acid is included in triesters, 19 % in diester and only 1 % is yet present in free fatty acids or monoesters.

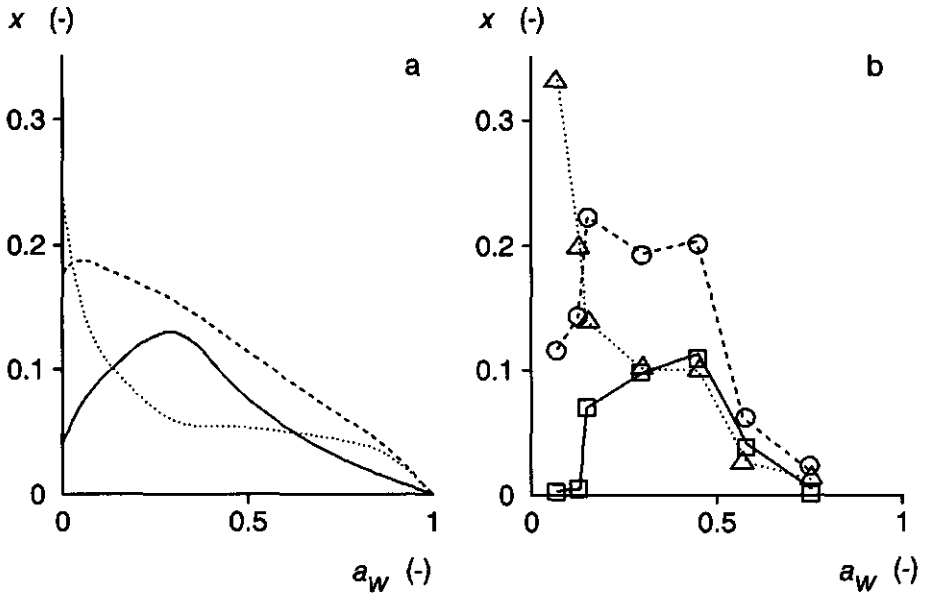


Figure 3. The calculated (a) and measured (b) equilibrium mono- (□, ——) di- (○, - - - -) and triester (△, · · · · ·) mole fraction in the non-polar phase as a function of the water activity. The curves in figure 3b only connect the datum points.

The equilibrium concentrations can be calculated using the program TREP (Two-phase Reaction Equilibrium Prediction) (Janssen *et al.* 1993 and chapter 4). This program calculates the thermodynamic activity of the components in the non-polar oil phase and the glycerol-water phase, if present, using the UNIFAC group contribution method (Fredenslund *et al.* 1977, Magnussen *et al.* 1981). For given equilibrium constants and initial amounts the equilibrium concentrations are calculated using mass

balances and the reaction equations 1, 2 and 3. In the calculations for each experiment, the water-activity is kept constant. For the esterification of decanoic acid and glycerol in hexadecane, the equilibrium constants are measured to be 1.6, 0.8 and 0.6 for the mono-, di- and triester production equilibrium, respectively (chapter 4). The calculations are shown in figure 3a. This figure demonstrates, that the calculated and measured mole fractions do vary within a factor of about 2. A deviation can be expected since the UNIFAC data are not accurate for complex mixtures. Yet, as concluded also in previous studies, the trends are predicted very clearly.

Both graphs show a maximum monoester concentration at a water activity around 0.4. In this a_w range TREP calculates a phase split. At $a_w > 0.3$ a two-phase system is predicted having a fatty acid-ester-hexadecane non-polar phase and a glycerol-water polar phase. Approximately 3 % v/v polar phase is predicted which should correspond with 3.3×10^{-6} m^{-3}. However, during the experiments no phase split is found. A possible explanation might be that the polar phase is absorbed by the membrane matrix, which has a volume of 4×10^{-6} m^{-3}.

Calculations and measurements show an increasing triester concentration upon decreasing water activity. At the conditions of the experiment, an excess of triesters can be achieved only at low water activity conditions $a_w < 0.1$. Another approach is not to reduce the water activity, but to increase the initial fatty acid concentration. At $a_w = 0.30$, 0.5 mole fatty acid was added to the equilibrium mixture. The equilibrium composition then changed from 0.11 : 0.19 : 0.10 $mole.mole^{-1}$ to 0.14 : 0.09 : 0.02 $mole.mole^{-1}$ tri-, di- and monoester, respectively. Indeed a higher triester concentration is found.

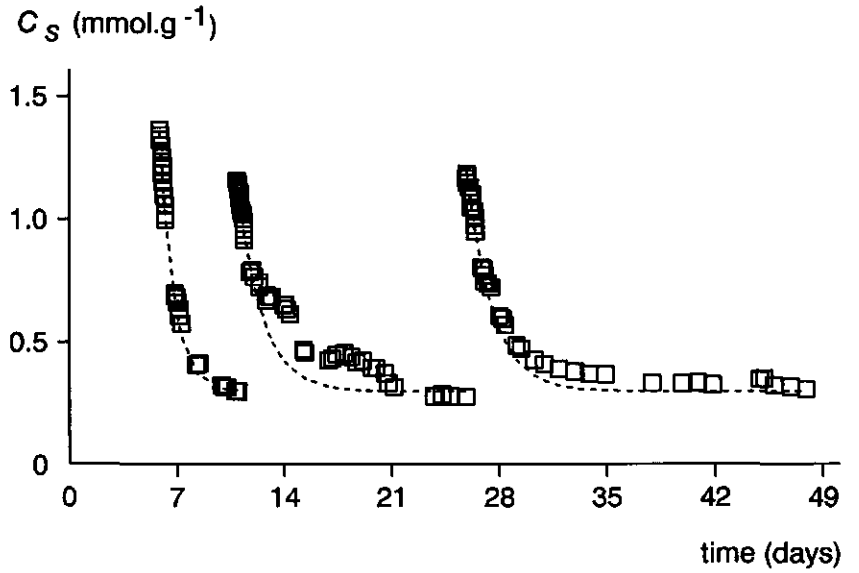


Figure 4. The measured decanoic acid concentration (□) of three repeated batches with time, and the fitted concentration using a pseudo first order model (- - - -) at a_w = 0.15.

Enzyme activity and stability

Often the initial substrate conversion rate is used to describe the enzyme activity. However, in case of an equilibrium reaction, a kinetic description has to be made to describe the enzyme activity (Van Erp *et al.* 1991). For the system described in this paper, a sequence of three second order reactions must be used to describe the fatty acid conversion rate (Pronk *et al.* 1992). In our kinetic experiments, the initial substrate concentrations are the same. However, the water activity differs which results in different equilibrium positions and by that a difference in driving force for the reaction. It is assumed that initially the fatty acid concentration minus its equilibrium concentration is the only rate determining factor. With that, the influence of the second substrate, mono- or diester, on the enzyme kinetics is neglected. Doing this, the reaction

constant can be used only for the optimization of this particular system, since the concentration of the available alcohol groups is part of the reaction constant. When the reaction kinetics is described with a pseudo first order equilibrium kinetics the equation then becomes:

$$\frac{d(C_s - C_s^{eq})}{dt} = k_s \cdot (C_s - C_s^{eq}) \qquad (5)$$

where C_S : the fatty acid concentration (mmole.g^{-1})
k_S : substrate reaction rate constant (s^{-1})

A typical repeated batch experiment is shown in figure 4. It can be seen that pseudo first order is a good approximation. The reaction rate constants measured 150 hours after immobilization are shown in figure 5 (○) as a function of the water activity. A value of $k_S = (1.5 \pm 0.4) \times 10^{-5}\ s^{-1}$ is found. The characteristic time of reaction $\tau_r = 1/k_s$ equals 18 hours. Since the characteristic time of mass transfer τ_m is calculated to be less than 400 seconds, it can be concluded that no mass transfer limitations are occurring during the ester synthesis.

Since a pseudo first order reaction kinetics is assumed, one has to keep in mind that the influence of the alcohol groups is neglected. Then the reaction constant of the membrane system with a glycerol-water phase should be different. The rate constant has been calculated for the membrane system containing a glycerol-water phase (chapter 2). In this case the concentration of the available alcohol groups is extremely high which should result in a higher reaction rate constant (□, figure 5). Indeed, at a water activity of 0.5, the reaction rate constant of the glycerol-water system is two times the reaction rate constant of the pervaporation system. However, at low water activity conditions, the enzyme immobilized in the pervaporation system remains active contrary to the situation with glycerol where it is inactivated during the experiment. This measurement supports our finding that the inactivation of *Candida rugosa* lipase, in a glycerol-water system, is caused by the high glycerol concentration and not by the reduced water activity (chapter 3).

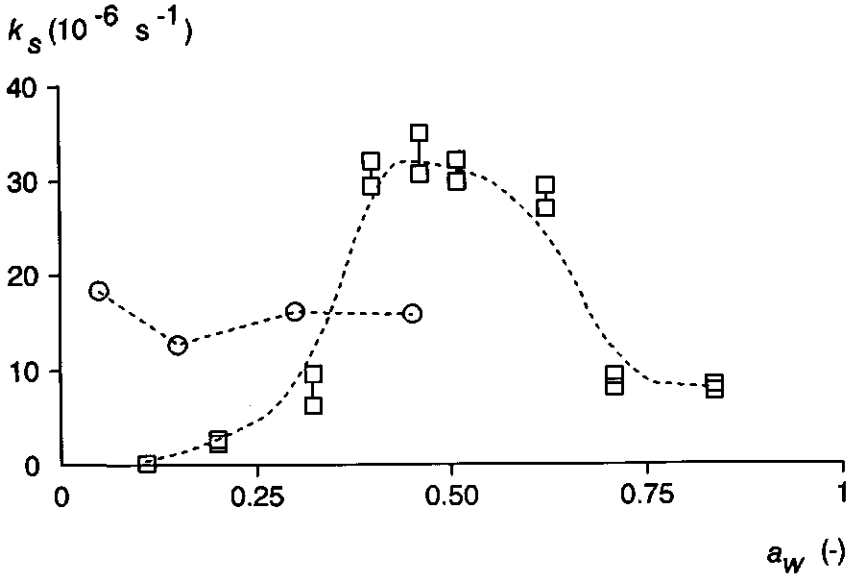


Figure 5. The pseudo first order reaction constant as a function of the water activity of the pervaporation system (○) and the glycerol-water membrane system (□).

Preliminary experiments have been performed concerning the enzyme stability of the system. The pseudo first order reaction constant is measured at different water activity conditions for a series of three batches at 150, 240 and 650 h, respectively (figure 6). At low water activity conditions, the enzyme has a higher inactivation rate compared to the system at a_w = 0.5. After 600 hours the activity at a_w = 0.1 is only 26 % of the first measured activity and 71 % at a_w = 0.45. However, for the first batch, it can be seen that the enzyme activity is independent of a_w between $0.05 < a_w < 0.45$ (○, figure 5). This is in contrast with the findings of Valivety *et al.* (1992). They found an increase of the enzyme activity upon an increase of the water activity ($0 < a_w < 0.5$). Inactivation at low water activity conditions influences the kinetics measurements. Measurements always start at a specific time after the system is prepared. The reaction

system in this study had to equilibrate for 150 hours, and meanwhile the enzyme inactivates. Therefore, different experimental procedures can lead to different results about the water activity influence upon the reaction kinetics.

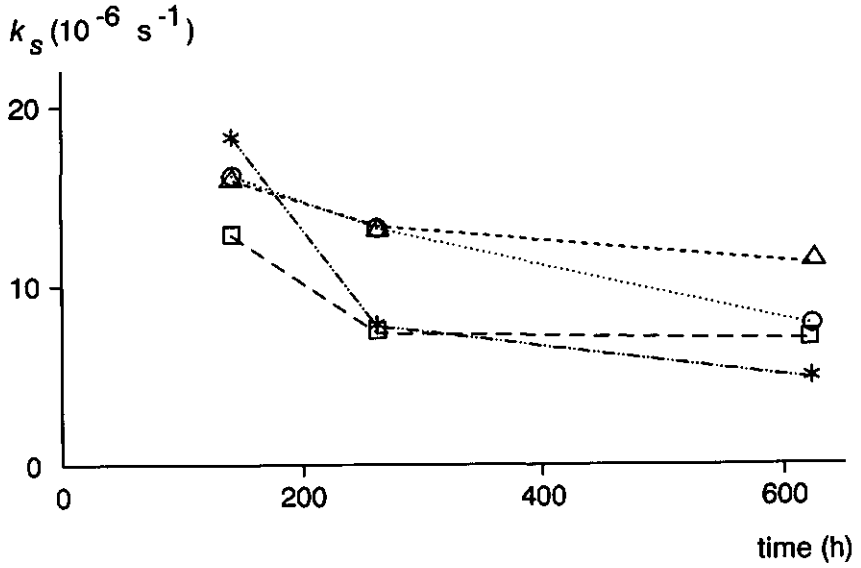


Figure 6. Reaction constant k_s at different water activities as a function of the time; aw = 0.05 (✳); aw = 0.15 (□), aw = 0.30 (○); aw = 0.45 (△).

Conclusion

An excess of triesters can be produced in an immobilized enzyme pervaporation system. A cellulose hollow fibre membrane unit can be used as immobilization carrier. The mass transfer is limited by the water diffusion through the oil phase. The process is reaction limited.
The enzyme activity is hardly influenced by the water activity. The enzyme stability is influenced: The immobilized lipase has lost 74 % of its activity within 600 hours at a_w = 0.05, however, the activity is only 29 % reduced at a_w = 0.45.

Acknowledgement

The authors wish to thank T. Folkerts and S. Debon for performing experiments and A.E.M. Janssen and H.M. Van Sonsbeek for the helpful discussions.

Symbols

A	membrane area	(m^2)
a_w	water activity	(-)
C_s	fatty acid concentration	($mmole.g^{-1}$)
$C_{w,air}$	water concentration of the air phase	($kg.m^{-3}$)
$C_{w,oil}$	water concentration of the oil phase	($kg.m^{-3}$)
J	transmembrane flux	($kg.s^{-1}$)
k_{ov}	overall mass transfer coefficient	($m.s^{-1}$)
k_s	substrate reaction rate constant	(s^{-1})
m	distribution coefficient	$((kg/m^3_{air})/(kg/m^3_{water}))$
V	reactor volume	(m^3)
x	mole fraction	(-)

τ_m	characteristic time of mass transfer	(s)
τ_r	characteristic time of reaction	(s)

Subscript *air*, *oil*, *s* and *w* indicates air phase, oil phase, the substrate fatty acid and water, respectively. The superscript *eq* implies equilibrium.

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6 Monoacylglycerol synthesis.

Abstract

The chemical synthesis of monoglycerides requires high temperatures, which may lead to the polymerization of unsaturated fatty acids. The enzymatic synthesis of these esters is performed at moderate temperatures and, hence, polymerization is avoided. However, enzymatic processes often end up with a mixture of the product, by-product, substrate and enzyme. An alternative process is an immobilized enzyme membrane reactor equipped with an in-line adsorption column to adsorb the monoglycerides preferentially onto the adsorbate. A silica 60 column has shown preferential adsorption of monocaprinate.

The adsorption of a mixture of decanoic acid, mono- and diglycerides is based on two different mechanisms. The decanoic acid will interact with hydroxyl groups at the silica gel surface, which results in a noncompetitive decanoic acid adsorption onto 25 % of the silica gel surface. On the remaining part of the silica gel surface, mono- and diglycerides adsorb competitively. When a mild eluant is used, such as 5 % ethanol in hexane, only the competitively adsorbed molecules are desorbed. This results in a purification factor of approximately 90 % after desorption.

The column can be desorbed off-line in a continuous membrane/repeated batch column process. This results in an estimated production of monoglycerides of 60 mole (15 kg) of monoester per gram enzyme.

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Introduction

Monoglycerides (monoacylglycerols) are used as emulsifiers in food and in cosmetics. The chemical production of these compounds involves an inorganic catalyst and is performed at high temperatures. The major disadvantage of the chemical synthesis is polymerization of unsaturated fatty acids at high temperatures (Oberkobusch 1990). Therefore, the chemical production of monoglycerides is limited to the incorporation of saturated fatty acids.

Enzymatic synthesis of monoesters overcomes the problem mentioned above. Moreover, when natural substrates are used, the enzymatically synthesized ester usually can be qualified as a natural ingredient for cosmetic and food products. To achieve enzymatic monoglyceride production, three routes have been presented in the literature. One way is to hydrolyse triglycerides with a 1,3-specific lipase. Holmberg and Osterberg (1988) have reported hydrolysis in a micro-emulsion system and yields up to 80 % were obtained. Downstream processing of the monoesters, however, is troublesome due to the mixed surfactant system.

Another method is the alcoholysis of a triglyceride and glycerol. In this case a mixture of glycerol, a trace of water and triglyceride are emulsified and lipase is added. The reaction is started at 45 °C and, after a while, the temperature is lowered, allowing the monoglycerides to precipitate (McNeill and Yamane 1991). In the end, a solid fat-enzyme phase is obtained with a monoglyceride concentration larger than 90 % w/w. A disadvantage of this process is that efficient methods are not available to separate the enzyme and the monoglyceride, while maintaining enzyme activity.

A third way to produce monoesters is to perform esterification of glycerol and fatty acid. Monoesters are the first product of the reaction chain, but esterification will proceed and monoglycerides will be converted into diglycerides. In case a lipase without positional specificity is used, the diesters are subsequently converted into triglycerides. Weiss (1990) has presented esterification in a system in which a solid fatty acid phase is dispersed in a glycerol phase. The enzymatic conversion of the fatty acids is over 85 %. The formation of di- and triesters can be minimized by using an organic solvent to extract the monoglyceride. A 100 % monooleate ester yield has

been reported with dichloromethane as a solvent (Graille 1985). The concentration in the extraction phase is approximately 2 % w/w and the monoester easily can be obtained by evaporation of the solvent. Miller and coworkers (1988) have published a 100 % monoester yield by using a derivatized glycerol, in which two of the three hydroxyl positions have been blocked by acetone. Once the esterification is completed, the blocking group can be removed by mild acid treatment.

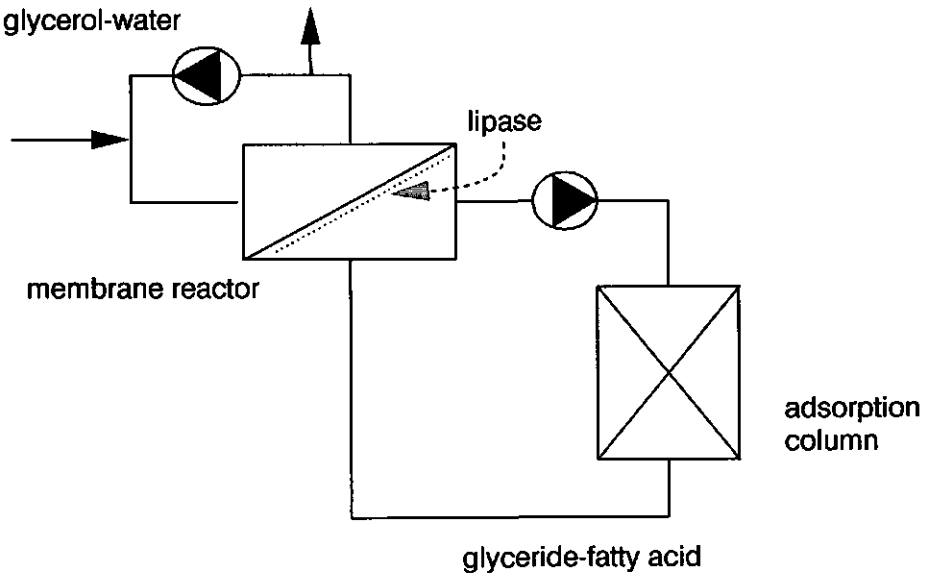

Figure 1. The membrane bioreactor system combined with an in-line adsorption column.

The esterification of a long-chain fatty acid and glycerol is discussed in this paper. The enzymatic reaction requires a two-phase reaction system. This can be either an emulsion system or a membrane reactor. In a previous paper we have described a membrane two-phase reactor for the production of glycerides (chapter 2). In this reactor, the oil and water phases are kept separated. This allows a simple, in-line

removal of monoglycerides by placing an adsorption column in the oil phase of the reactor system (figure 1). The produced monoglycerides should adsorb preferentially onto the column. This results in a low monoglyceride concentration in the oil phase, and represses further esterification to di- and triglycerides. When the adsorption column is saturated with monoglycerides, the column can be replaced and can be eluted off-line. This study deals with the development of a silica gel-based adsorption column for preferential adsorption of monoglycerides.

Theory

Adsorbent

For a porous adsorbent, the specific surface area as well as the pore size distribution is of importance, because this affects the capacity of the adsorbent in two ways. First, pores must be large enough to allow entrance of adsorbate molecules. Second, the adsorbate molecule can only enter a pore when it is filled with the adsorbate-containing phase. Whether a pore is filled is related to the pore size, the surface tension of the liquid and the wetting capacities of the adsorbent. For nonwetting conditions, a pore is filled when the applied pressure exceeds the Laplace pressure, which is a measure for the pressure difference at the two sides of the curved gas-liquid interface (Gregg and Sing 1982). In this way, the available area for adsorption can be calculated when the pore size distribution is measured and the physical properties of both the liquid and the adsorbent are known.

Multicomponent adsorption

Assuming reversible adsorption at local adsorption sites and neglecting lateral interactions between adsorbate molecules, a monolayer is created. The adsorption equilibrium can then be described with the Langmuir equation (Langmuir 1918). The Langmuir equation has been adapted for a bisolute system by Butler and Ockrent (1930).

One of the Langmuir premises is that the adsorption energy has the same value at all the adsorption sites. However, silanol groups are present at the silica gel surface.

These groups can adsorb molecules by H-bond interaction, which results in a relatively high adsorption energy (Hau and Nawar, 1985). Therefore, two different types of adsorption are possible: adsorption onto the silanol groups and adsorption onto the silanol free sites. The different mechanisms can cause a difference in competitive character of the two sites. Jain and Snoeyink (1973) have extended the bisolute Langmuir model for this case. The adsorbent area is divided into a competitive part, where the components *i* and *j* compete for adsorption and a noncompetitive part, where only component *i* will adsorb. When one component adsorbs noncompetitively and competitive multicomponent adsorption takes place for another three components, the amount adsorbed can be calculated as:

$$\Gamma_i = \frac{(\Gamma_{max1} - \Gamma_{max2}) \cdot b_i \cdot C_i}{1 + b_i \cdot C_i} + \frac{\Gamma_{max2} \cdot b_i \cdot C_i}{1 + b_i \cdot C_i + b_j \cdot C_j + b_k \cdot C_k + b_l \cdot C_l} \qquad (1a)$$

$$\Gamma_n = \frac{\Gamma_{max2} \cdot b_n \cdot C_n}{1 + b_i \cdot C_i + b_j \cdot C_j + b_k \cdot C_k + b_l \cdot C_l} \qquad (1b)$$

$$\text{for } n = j \vee n = k \vee n = l$$

where:	$b_{i,j,k,l}$	: affinity constant	($m^3.mole^{-1}$)
	$C_{i,j,k,l}$	: equilibrium concentration	($mole.m^{-3}$)
	$\Gamma_{i,j,k,l}$	: amount adsorbed	($mole.kg^{-1}$)
	Γ_{max1}	: maximum attainable amount adsorbed of component *i*	($mole.kg^{-1}$)
	Γ_{max2}	: maximum attainable amount adsorbed of component *n*	($mole.kg^{-1}$)

The first term on the right-hand side of the adsorption equation of component *i* (equation 1a) is the Langmuir expression for noncompetitive adsorption. The second

term of this equation is the amount of component *i* at the adsorbent surface that is adsorbed competitively with components *j*, *k* and *l*. If Γ_{max1} equals Γ_{max2}, the equation changes into the competitive multicomponent Langmuir model.

Column characteristics

The amount adsorbed in an adsorption column is not only determined by the adsorbent properties, but also is related to the column characteristics, such as flow conditions. The major problem in column operation is the occurrence of channelling. If channelling can be avoided, an estimation of the adsorption capacity can be made. In a system where intraparticle diffusion can be neglected, the rate-determining step is diffusion of the adsorbate molecule from the bulk liquid through the stagnant film to the adsorbent surface. An approximation of the film mass transfer coefficient can be made by using the Chilton-Colburn factor. This factor is a function of the bed porosity, throughput, liquid viscosity, particle diameter and diffusion coefficient of the adsorbate molecules (Costa 1990). Once the transfer coefficient is known, the mass transfer zone in a column can be estimated (Beverloo *et al.* 1991), which leads to the adsorbent load. This estimation refers to the column load under ideal conditions. However, it can occur that a load of 50 % of the calculated load is measured, due to channelling (Costa, 1990).

Assuming laminar flow and neglecting intraparticle diffusion, the diffusion rate is determined only by the diffusion of the adsorbate through the stagnant film layer. This diffusion rate can be calculated from the Sherwood number that is known for a packed bed (Costa, 1990). Once the mass transfer coefficient is known, the mass transfer zone shape can be calculated as shown by Beverloo *et al.* (1991) for the case of high affinity adsorption, the adsorption can be described by the saturation equation:

$$\begin{aligned} \Gamma_n &= \Gamma_{max2} \quad \text{for} \quad C_n > 0 \\ C_n &= 0 \quad \text{for} \quad \Gamma_n < \Gamma_{max2} \end{aligned} \tag{2}$$

When the adsorbate particles are taken as rigid spheres, they have shown that the concentration in the mass transfer zone is described by:

$$C_n(z) = C_{n,in} \cdot e^{\left(\frac{3 \cdot k_f \cdot (z_{av} - z)}{r_p \cdot u \cdot (1 - \epsilon_b)} - 1 \right)} \quad (3)$$

in which	$C_n(z)$	: adsorbate concentration at place z	(mole.m^{-3})
	$C_{n,in}$	: adsorbate concentration at the inlet	(mole.m^{-3})
	k_f	: film mass transfer coefficient	(m.s^{-1})
	r_p	: particle radius	(m)
	u	: superficial velocity	(m.s^{-1})
	z	: axial length coordinate	(m)
	z_{av}	: axial average position of the mass transfer zone	(m)
	ϵ_b	: bed porosity	(-)

When breakthrough occurs, z equals H, and in this case the axial average position z_{av} can be estimated from:

$$z_{av} = H + \frac{r_p \cdot u \cdot (1 - \epsilon_b)}{3 \cdot k_f} \cdot \left(1 + \ln\left(\frac{C_{n,out}}{C_{n,in}} \right) \right) \quad (4)$$

where	H	: column height	(m)
	$C_{n,out}$	: adsorbate concentration at the outlet	(mole.m^{-3})

From this equation the breakthrough time can be calculated. Equation 3 can be integrated from the start of the mass transfer zone (z = 0) to the height of the column (z = H), which results in the average concentration in the mass transfer zone. At a

given outlet concentration, both the average adsorbate concentration in the column, $C_{n,av}$ (mole.m^{-3}), as well as the amount of adsorbate that has passed the column, M_{passed} (mole), can be calculated:

$$C_{n,av} = \frac{C_{n,in}}{H} \cdot \left(z_{av} - \frac{r_p \cdot u \cdot (1-\epsilon_b)}{3 \cdot k_f} \cdot e^{1-N} \right) \quad (5)$$

$$M_{passed} = \frac{C_{n,in} \cdot \pi \cdot R^2}{H} \cdot \frac{r_p \cdot u \cdot (1-\epsilon_b)}{3 \cdot k_f} \cdot e^{1-N} \quad (6)$$

and

$$N = \frac{3 \cdot k_f \cdot (H - z_{av})}{r_p \cdot u \cdot (1-\epsilon_b)} \quad (7)$$

Where N (-) is the number of transfer units between z_{av} and H, and R is the radius of the packed bed. When the column load is known, the amount adsorbed onto the adsorbent can be calculated from the mass balance.

Materials

Decanoic acid (95 % pure) is obtained from Unichema (Germany). Ethanol (100 %), formic acid, hexadecane, phenolphthalein, silica 60 (0.063-0.200 mm) and sodium hydroxide (0.1N) are obtained from Merck (Germany). Glycerol (99$^+$ %) is obtained from Janssen (Belgium), hexane is from Rathburn (U.K.) and monocaprinate (99 %) is from Sigma (U.S.A.). The silica gel No. 1 is obtained from Crosfield Chemicals (U.K.). Lipase of *Candida rugosa* is obtained from Meito Sangyo (Japan), and the membrane module (Andante) is purchased from Organon (The Netherlands).

Experimental

BET and pore size distribution

Nitrogen adsorption is measured at different pressures at 77 K. When the adsorbed volume N_2 (STP) is known in relation to the relative pressure, the specific surface can be calculated (Gregg and Sing, 1982). The relative pressure P/P_0 is the actual pressure over the saturated vapour pressure.

The BET adsorption and desorption isotherms may differ for a porous adsorbent. This so-called hysteresis loop only appears if mesopores (2 nm < r_p < 20 nm) exist. Assuming only cylindrical pores exist, it is possible to estimate the pore size distribution (Gregg and Sing, 1982). The smallest detectable radius is 1.6 nm, which is three to four times the diameter of the nitrogen molecule.

Adsorption measurements.

Silica gel is washed with hexane and dried under vacuum. The adsorption isotherms for decanoic acid and monocaprinate, respectively, are determined by depletion measurements from a hexadecane solution. Multicomponent depletion measurements are performed by adding different amounts of silica gel to a mixture of decanoic acid, mono-, di- and tricaprinate in hexadecane. Concentrations are determined by gas chromatographic analysis and fatty acid titration.

Desorption measurements

The adsorbed column is rinsed with hexane to remove glycerides and fatty acids present in the void volume. Afterwards the column is eluted with different solvents.

Adsorption membrane bioreactor system

The membrane bioreactor consists of a cellulose hollow fibre membrane module, an internal oil circuit (*circa* 80 mL) and an external glycerol-water circuit. The glycerol concentration is kept constant by making use of a feed and bleed system (75 % w/w). The oil circuit is operated batch wise. The conversion takes place at 25°C. The biocatalyst is adsorbed at the inner fibre side (chapter 2). The membrane unit contains 6000 fibres with an internal diameter of 0.2 mm and a wall thickness of 8 μm.

The total membrane surface is 0.77 m^2. Monoglyceride production is started with 50 % w/w decanoic acid in hexadecane. At the start of the experiment, an adsorption column is placed in the oil circuit. Samples are taken at the outlet of the column.

Concentration measurements
The composition of the oil is determined by gas chromatography. Each sample is diluted (400 times) in hexane, 1 μL is injected cold on a 5 m CP-Sil-5-CB column (Chrompack, The Netherlands). The oven temperature of the Carlo Erba system is 80°C at the moment of injection. After one minute the temperature is increased at 20°C.min^{-1} up to 320°C. The F.I.D. detection of decanoic acid, mono-, di- and tricaprinate occurs at 370°C. Helium is chosen as carrier gas (4 mL/min).

The concentration of decanoic acid also can be measured by dilution with ethanol followed by titration of the acid with sodium hydroxide against a phenolphthalein indicator.

Results and discussion

Specific surface area of silica gel
The specific surface area of silica 60 is calculated according to the BET equation from the N_2 desorption isotherm and is approximately 500×10^3 $m^2.kg^{-1}$. The pore size distribution has been derived and shows that the surface area belonging to macropores approximates 115×10^3 $m^2.kg^{-1}$. The smallest pore detected has a pore diameter of 3.2 nm. When this value is compared to the estimated tail length of the decanoic acid molecule (1.3 nm, calculated from bond length data given by Kennard 1978), it can be concluded that the adsorbate molecules fit even in the smallest pores.

To calculate the smallest pore that will be filled with liquid, the Laplace pressure has to be compared to the applied pressure. The surface tension of an oil is approximately 30×10^{-3} $N.m^{-1}$ (Weast 1978) and the contact angle with silica gel is about 32° (Busscher *et al.* 1986). When a static pressure of 0.5×10^5 Pa is applied, the radius of the smallest pore filled with the oil phase will be 0.5 μm. This value suggests that no

pore will be filled at all, although all pores are large enough to allow passage of adsorbate molecules. Only a part of the surface area belonging to the macropores (115×10^3 $m^2.kg^{-1}$) is available for adsorption.

The specific surface area available for adsorption also can be derived from adsorption isotherms. For both decanoic acid and monocaprinate in hexadecane the adsorbed amounts *versus* the equilibrium concentrations are given in figure 2. The maximum attainable amounts adsorbed are 1.2 ± 0.1 mole.kg^{-1}. The fact that these values are equal for both components is in agreement with their molecular sizes, which are approximately the same. In a vertical head-down position estimates for the molecular surface are between 0.17 and 0.24 nm^2 (Orr and Dallavalle 1959), resulting in a specific surface for Silica 60 of 120×10^3 to 170×10^3 $m^2.kg^{-1}$. This value is in good agreement with the measured specific surface of the macropores from the gas desorption data. In the following paragraphs a value of 120×10^3 $m^2.kg^{-1}$ will be used.

Adsorption measurements

Figure 2 shows that both decanoic acid and monocaprinate isotherms appear to be high-affinity isotherms (figure 2). The affinity constant *b* cannot be determined from these one-component adsorption isotherms, due to the fact that the smallest detectable equilibrium concentration still yields the maximum attainable adsorption of 1.2 mole.kg^{-1}. To obtain the affinity constants, multicomponent experiments are required. These multicomponent adsorption measurements are performed with a mixture of decanoic acid, mono-, di- and tricaprinate, dissolved in hexadecane. This mixture is brought into contact with different amounts of silica gel. As an example, the mono- and dicaprinate adsorption is given as a function of the monocaprinate equilibrium concentration (figure 3). Of course the adsorption is not only a function of the monocaprinate concentration, but also of the decanoic acid, di- and tricaprinate concentration. Figure 2 shows that for monosolute adsorption the maximum attainable amount is 1.2 mole.kg^{-1}. Figure 3 shows, contrary to this result, that the maximum amount of ester adsorbed at multicomponent conditions is 0.9 mole.kg^{-1}. Thus only 75 % of the available adsorption area is occupied with esters, despite the high-affinity character of both components. This can be explained with the formation of H-bonds between decanoic acid and the silanol groups of the silica. Glycerides are incapable of forming H-bonds. On this thesis we can assume that

decanoic acid is noncompetitively adsorbed onto that part of the silica gel surface having the silanol groups ($\Gamma_{max1} - \Gamma_{max2}$ = 0.3 mole.kg^{-1}), while the remaining surface (Γ_{max2} = 0.9 mole.kg^{-1}) is available for competitive adsorption.

In all our experiments the esters have been adsorbed onto a column loaded with decanoic acid at solute concentrations below the detection limit. This means that the affinity of decanoic acid is orders of magnitude lower than that of the glycerides. In terms of equation 1, this means that $b_a \cdot C_a \ll b_m \cdot C_m + b_d \cdot C_d + b_t \cdot C_t$. Now equation 1 can be rewritten as:

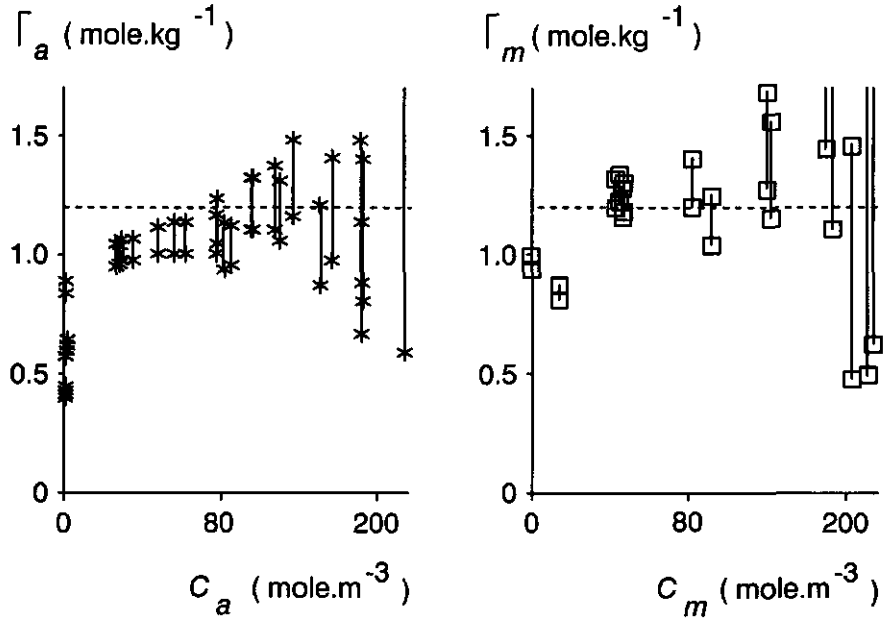

Figure 2. Adsorption isotherm of decanoic acid (*) and monocaprinate (□) on Silica 60.

$$\Gamma_a = \Gamma_{max1} - \Gamma_m - \Gamma_d - \Gamma_t \tag{8a}$$

$$\Gamma_n = \frac{\Gamma_{max2} \cdot b_n \cdot C_n}{1 + b_m \cdot C_m + b_d \cdot C_d + b_t \cdot C_t} \tag{8b}$$

for $n = m \vee n = d \vee n = t$

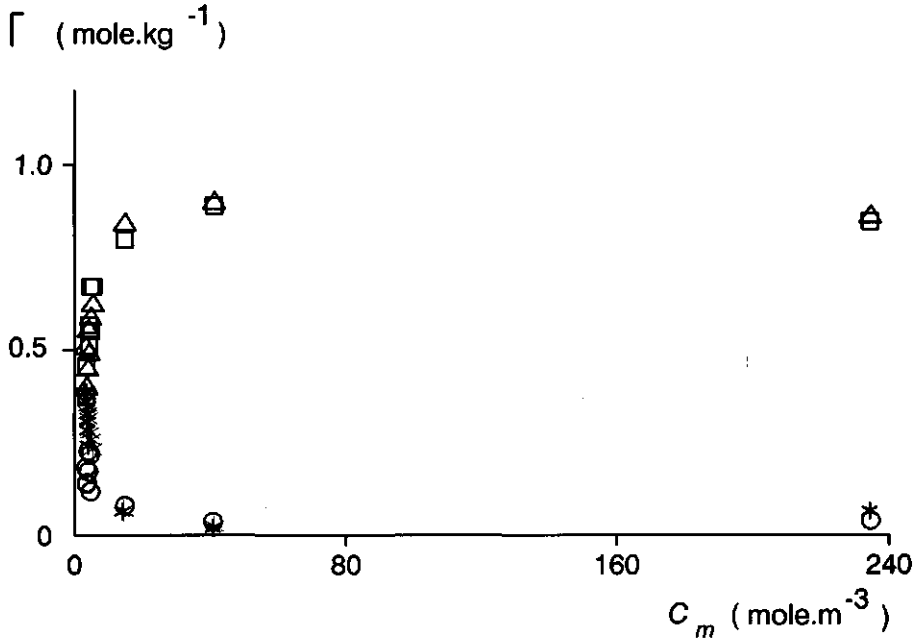

Figure 3. Adsorption of monocaprinate (□) and dicaprinate (○) from different oil mixtures onto silica gel versus the equilibrium monocaprinate concentration and the calculated noncompetitive and competitive adsorption for monocaprinate (△) and dicaprinate (✳), respectively.

Based on equation 8a and 8b and the measured multicomponent adsorption data, the affinity constants for mono-, di- and tricaprinate are calculated as 5.31, 0.35 and 0.07 $m^3.mole^{-1}$, respectively. All adsorption data can thus be estimated by using the last set of affinity constants (figure 3). In this figure, the calculated points are given because at all points the decanoic acid, di- and triester vary. Therefore, it is not possible to interpolate.

Desorption

Several batches of pre-adsorbed silica gel are brought into contact with different solvents to elute the adsorbed compounds. With water as elution solvent, this efficiently removes adsorbed fatty materials from silica gel. However, it cannot be used without drying of the silica gel afterwards. Once silica gel is adsorbed with water, no adsorption of fatty materials will occur at all. When hexadecane is used as eluant, esters are found in the eluate phase. The monoester concentration in hexadecane, however, does not exceed 200 $mole.m^{-3}$ (6 % w/w), which approximates saturation. The hexadecane elution shows that the adsorption is a reversible process, which is one of the Langmuir premises.

A homologeous series of alcohols also is tested as eluant. Good results have been obtained with ethanol. All the adsorbed compounds are desorbed in a 100 % ethanol solution. This technique is used for repeated use of the adsorbent. With a decreasing alcohol in hexane concentration, the desorption of decanoic acid becomes less complete. At 5 % ethanol in hexane solution, decanoic acid is only partially removed from the adsorbent surface. We assume that only the competitively adsorbed amount of decanoic acid is removed, while the solvent silica gel interactions are too weak to break the H-bonds between decanoic acid and silica gel.

The partial desorption of decanoic acid is favourable as it provides the opportunity to increase product concentration. Desorption as described above is measured as a function of the volume of eluant that has passed the column (figure 4). The maximum monocaprinate concentration equals 7.5 % w/w in the eluate phase, which approximates saturation in this solvent. The purification factor in this particular

fraction (20 to 60 mL) is over 98 % as compared to the remaining decanoic acid concentration in the eluate, the average purification factor of all the fractions is 95 %, and the average concentration is about 2.6 % w/w.

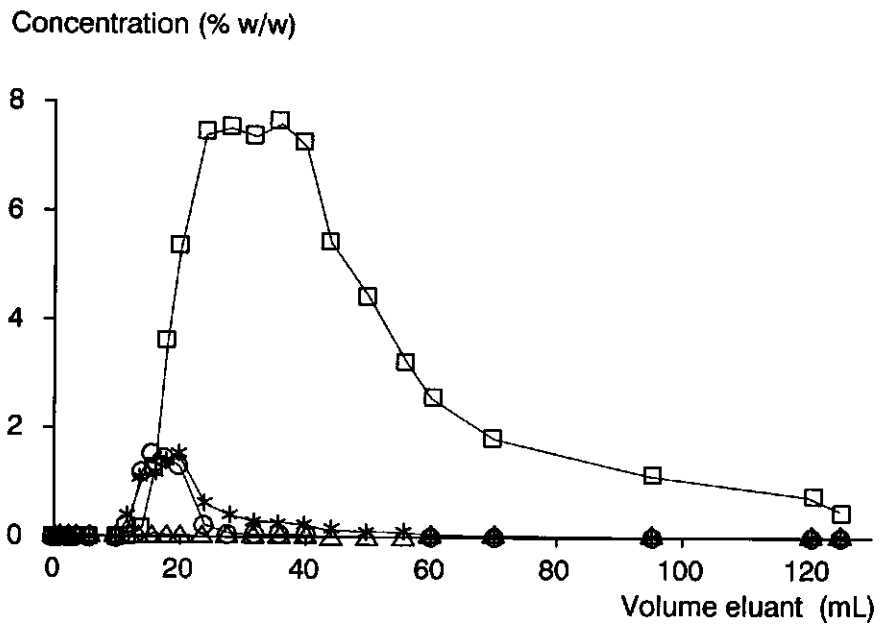

Figure 4. Concentration of the components in 5 % ethanol in hexane during desorption of decanoic acid (*), mono- (□), di- (○) and tricaprinate (△) from a silica gel column.

Membrane bioreactor with an in-line adsorption column

To recover the monoesters produced, a silica 60 column is placed in the oil phase of a membrane reactor. Initially, decanoic acid adsorbs onto the column and no decanoic acid is measured at the outlet of the column. Within a few minutes, the column is saturated with decanoic acid and the acid concentration increases to 2.5 kmole.m^{-3}. During the first two hours of the enzymatic process, only triglycerides are measured at the outlet of the column (figure 5). This indicates that the produced

mono- and diglycerides do adsorb onto the silica. After 2 hours, dicaprinate is measured and after 4.5 hours, monocaprinate also is measured. As soon as monoesters are detected in the column outlet flow, the column is removed (figure 5a). At this moment, the monoester production rate in the membrane reactor is measured as 1.9 μmole.s^{-1}. Assuming that this production rate occurs during the adsorption, the adsorbed amount of monoester should be 0.56 mole.kg^{-1} silica gel.

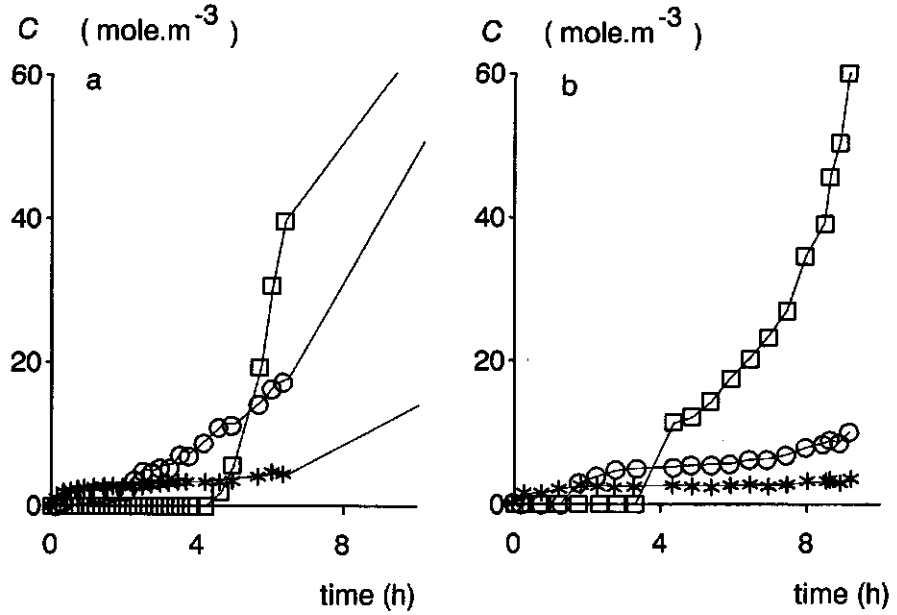

Figure 5. The mono- (□), di- (○) and tricaprinate (∗) concentration at the outlet of a silica gel column in an adsorption membrane bioreactor system: a) the column is removed directly after detection of monoesters at the outlet; b) the column is removed after 8 hours.

At the moment the column has been removed from the oil phase, close examination of the column revealed that about three-quarters of the material is wetted by the oil phase. A completely wetted sample from the column is desorbed with ethanol as

eluent to determine the adsorbed amounts. Values are given in table 1. The remaining silica gel is eluted with ethanol, for which the desorption data are given in table 1. The total amount of monocaprinate in the eluate is 32 mmole, which equals 0.57 mole.kg^{-1}. This value agrees with the measured production rate, resulting in 0.56 mole.kg^{-1}.

Table 1: *Desorption data of the Silica 60 column placed in the oil phase of a membrane reactor after 4.5 hours of adsorption.*

compound	column average (mole.kg^{-1})	correction for channeling (mole.kg^{-1})	thoroughly wetted sample (mole.kg^{-1})
Decanoic acid	0.19	0.29	0.26
Monocaprinate	0.57	0.87	0.79
Dicaprinate	0.05	0.08	0.07
Tricaprinate	0.01	0.02	0.01
Amount silica gel (kg)	55.8×10^{-3}	36.3×10^{-3}	

Column characteristics

The mass transfer coefficient k_f for this system is calculated to be between 8.5 and 9.5×10^{-6} m.s^{-1}. This range is caused by variations in flux (table 2). The calculated affinity constants and concentrations in the experiment given in figure 3 show that we can assume a high-affinity adsorption of monocaprinate. Thus, equations 4-7 can be applied and both the adsorption of monocaprinate onto the column and the breakthrough time of the column can be calculated. Parameter values are given in table 2. The breakthrough time is calculated to be 8.3 hours (equation 4) and average monocaprinate adsorption equals 0.81 mole.kg^{-1} (equations 5-7). The experimentally determined breakthrough time of 4.5 hours is 55 % of the calculated value. The adsorption is about equal to the amount determined for the fully wetted sample.

Table 2: Column parameters.

column porosity	ϵ_b	0.780	(-)
particle radius	r_p	0.13×10^{-3}	(m)
kinematic viscosity	ν	0.36×10^{-6}	($m.s^{-2}$)
diffusion coefficient	D	10^{-10}	($m^2.s^{-1}$)
column radius	R	0.013	(m)
column height	H	0.21	(m)
specific surface	A_{sp}	120×10^3	($m^2.kg^{-1}$)
column load		628	($kg.m^{-3}$)
maximum attainable adsorption	Γ_{max2}	0.9	($mole.kg^{-1}$)
inlet concentration monoester	$C_{m,in}$	12.3	($mole.m^{-3}$)
flux	Φ	0.16×10^{-6}	($m^3.s^{-1}$)

The reduction in breakthrough time can be caused by channelling in the column. Assuming that channelling results in a decrease of the column radius, the apparent column radius at which the calculated breakthrough time equals the experimentally measured time can be estimated. This apparent radius equals 0.01 ± 0.001 m: in this case the wetted amount of silica gel is 0.6 to 0.7 of the silica present in the column. This apparent radius is chosen to calculate the amount of ester that is adsorbed onto the wetted part of the column, and this results in an adsorption of 0.42 to 0.53 $mole.kg^{-1}$. After correcting for channelling, the adsorbed amounts do correspond with the measured values of the thoroughly wetted silica gel sample (table 1). It can be concluded that the low adsorption is caused by channelling and adsorption onto the thoroughly wetted silica gel can be described with the adsorption model.

Monocaprinate production

The immobilized nonspecific lipase catalyses not only the production of monoesters, but also diesters and triesters are obtained. When no adsorption column is used, the monoglyceride production is about 18 % w/w (chapter 2).

If a silica gel adsorption column is placed in the oil circuit of the membrane bioreactor, the produced monoesters are preferentially adsorbed onto the silica gel. The downstream processing of the adsorbed monoesters is handled off-line. This implies that the membrane reactor can be used to produce monoesters in a continuous process, which then adsorb onto a sequence of columns. A half-life time of the immobilized enzyme of 50 days should be possible and a rough estimation of the production capacity then is 100 mole (15 kg) of monoester for a gram of enzyme.

The purification factor of 90 % based on the competitive adsorbed compounds is about the same as presented in literature (Graille 1985, Holmberg and Osterberg 1988, McNeill and Yamane (1991), Miller *et al.* 1988 and Weiss 1990). However, in our case the eluate phase contains only mono-, diesters and fatty acids, and neither enzyme nor other emulsifiers are present. Downstream processing of the eluate only includes saponification of the fatty acids and evaporation of the solvent. Holmberg and Osterberg (1988) present a microemulsion system to produce monoglycerides. This system will end up with a mixture of emulsifier, enzyme, solvent, fatty acid and esters. Downstream processing involves several extraction steps and, unfortunately, denaturation of the enzyme takes place. Therefore, the enzyme can be used for one batch only, which results in a production of 45 mmole monoester per gram enzyme. Downstream processing of the solid fat system as presented by Weiss (1990) and McNeill and Yamane (1991) involves a heating step to melt the monoglycerides produced, which also results in inactivation of the enzyme. The extraction system of Graille (1985) resembles our system in one way: no enzyme is present in the organic phase. Downstream processing of the organic phase is analogous to downstream processing of the eluate of the membrane system. The enzyme preparation can be used 20 times, and production becomes 220 mmole per gram enzyme. The process developed by Miller and coworkers (1988) can not be compared with the other systems because no data are available.

The purification factor of the membrane bioreactor with in-line adsorption equals those of the systems shown in the literature, while enzyme-based productivity in continuous production is at least 250 times the productivity shown in the literature. This high productivity combined with off-line downstream processing of the monoesters is the major advantage of this system.

Conclusion

When monoglycerides are adsorbed onto porous silica gel, the specific surface area of the adsorbent is determined by wetting characteristics of the surface and the adsorbate containing phase. In this case only the surface of the macropores is available for adsorption. The adsorption of a mixture of decanoic acid, mono- and diglycerides is based on two different mechanisms. The decanoic acid will interact with hydroxyl groups at the silica gel surface, which results in a noncompetitive decanoic acid adsorption onto 25 % of the silica gel surface. On the remaining part of the silica gel surface, mono- and diglycerides adsorb competitively, the decanoic acid acts as solvent and covers the remaining surface. When a mild eluant is used, such as 5 % ethanol in hexane, only the competitively adsorbed molecules are desorbed. This results in a purification factor of approximately 90 % after desorption.

The enzymatically produced monoglycerides can be adsorbed onto a silica gel column. While the adsorption column is eluted off-line, the immobilized enzyme system has a productivity of 60 mole of monoester per gram enzyme, much larger than that of any other method published.

Acknowledgement.

The nitrogen adsorption and desorption isotherm data are kindly provided by the Department of Physical and Colloid Chemistry of our University. This work is funded by the Dutch Ministry of Economical Affairs and the Unilever Research Laboratory Vlaardingen.

Symbols

A_{sp}	:	specific surface area	($m^2.m^{-3}$)
b_i	:	Langmuir affinity constant	($m^3.mole^{-1}$)
C_i	:	equilibrium concentration	($mole.m^{-3}$)
$C_{n,av}$	:	average adsorbate concentration in the column	($mole.m^{-3}$)
$C_{n,in}$	:	adsorbate concentration at the inlet	($mole.m^{-3}$)
$C_{n,out}$	:	adsorbate concentration at the outlet	($mole.m^{-3}$)
$C_i(z)$	:	adsorbate concentration at place z	($mole.m^{-3}$)
D	:	diffusion coefficient	($m^2.s^{-1}$)
H	:	column height	(m)
k_f	:	film mass transfer coefficient	($m.s^{-1}$)
M_{passed}	:	amount of adsorbate which has passed the column	(mole)
N	:	number of transfer units	(-)
P/P_0	:	relative pressure	(-)
r_p	:	particle radius	(m)
R	:	bed radius	(m)
u	:	superficial velocity	($m.s^{-1}$)
z	:	axial length coordinate	(m)
z_{av}	:	average position of the mass transfer zone	(m)
Γ_i	:	amount adsorbed	($mole.kg^{-1}$)
Γ_{max}	:	maximum attainable amount adsorbed	($mole.kg^{-1}$)
Γ_{max1}	:	maximum attainable total amount adsorbed	($mole.kg^{-1}$)
Γ_{max2}	:	maximum attainable competitive amount adsorbed	($mole.kg^{-1}$)
ϵ_b	:	bed porosity	(-)
Φ	:	flux	($m^3.s^{-1}$)
ν	:	kinematic viscosity	($m.s^{-2}$)

The subscripts i, j, k, l, a, m, d and t refer to components i, j, k, l, decanoic acid, mono-, di- and tricaprinate, respectively, n is a component.

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7 General discussion

Introduction

This thesis presents the enzymatic esterification of decanoic acid and glycerol in hexadecane as solvent. The two-phase membrane reactor of Pronk and coworkers (1990) is used as basic concept for this study (figure 1). In this membrane reactor, the membrane surface acts as immobilisation carrier and as reaction interface at the same time. Lipase from *Candida rugosa* is immobilized at the same side of the hydrophilic membrane as where the non-polar phase is present. For esterification, the water produced is extracted to the polar glycerol-water phase and can subsequently be removed by any conventional dehydration technique. Chapter 2 compares the two-phase membrane reactor with an emulsion system with respect to the initial conversion rate.

Chapters 3 and 4 describe the activity and stability of *Candida rugosa* lipase and the equilibrium position of this esterification reaction. It is concluded from these chapters that the enzyme stability at a given temperature is determined by the glycerol concentration only. Furthermore, always a mixture of mono-, di- and triesters will be obtained in a two-phase system using hexadecane as solvent. However, only an excess of one of these esters is of commercial interest, especially pure tri- or monoesters. The enzymatic synthesis of an excess of tri- and monoesters is presented in chapter 5 and 6, respectively. The systems make use of a membrane reactor with an in-line removal of the product (ester) or by-product (water).

A part of this chapter have been published as a section of the paper "membrane reactors" in: NATO ASI series "Chromatographic and membrane processes in biotechnology" C.A. Costa, J. Cabral [eds.] (1990)

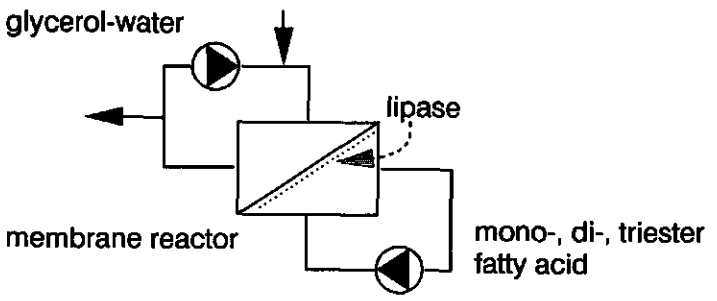

Figure 1 The two-phase membrane reactor.

A series of two membrane reactors is used for the synthesis of an excess of triesters relative to the other reaction components (figure 2). In a two-phase membrane device, a mixture of mono-, di- and triacylglycerols is produced. A pervaporation membrane reactor is placed in series. Lipase is immobilized onto the membrane surface. At one side of the membrane, the non-polar phase is brought into contact with the enzyme. At the other side of the membrane an extraction phase, air, is pumped through to remove the water produced, thus avoiding water accumulation in the immobilization carrier. This results in a reaction proceeding towards an equilibrium state with an excess of triglycerides.

Figure 3 shows a two-phase membrane reactor for the synthesis of monoesters. The monoesters produced adsorb preferentially onto a silica column and can be processed downstream off-line.

In this chapter, the choices made during this study are discussed reviewing the results and in comparison with other systems presented in literature. Potentials and limitations of the enzymatic tri- and monoester synthesis will be discussed.

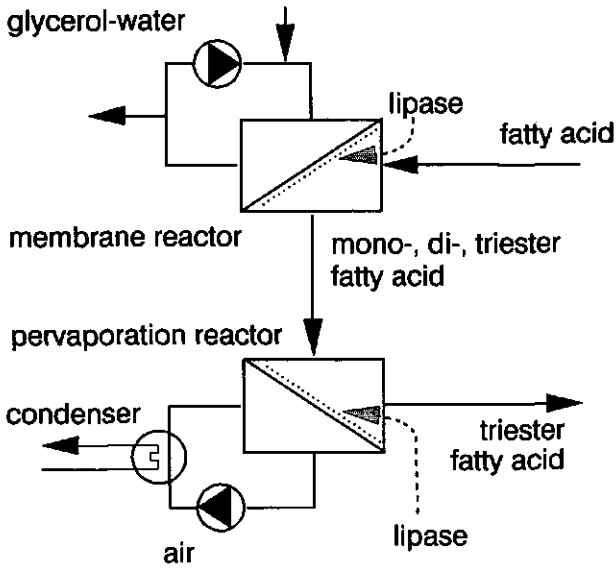

Figure 2 *A membrane reactor concept for the synthesis of an excess of triacylglycerols. A pervaporation membrane reactor is placed in series with a two-phase membrane reactor.*

Choice of the enzyme

The choice of enzyme does alter the reaction rate of the system and the enzyme stability differs for different types of enzymes. Not only the enzyme source, even the same source of lipase from different manufacturers shows a different behaviour (chapter 3). For each enzyme the optimum load, the amount of enzyme per membrane surface area (chapter 2) and the enzyme stability (chapter 3) have to be determined. The equilibrium composition should be catalyst independent. An exception could be the use of a selective enzyme. This paragraph discusses the possibility of using a selective lipase and model predictions for equilibria are presented. Some remarks are made about the consequences of stability data of the enzyme in esterification media.

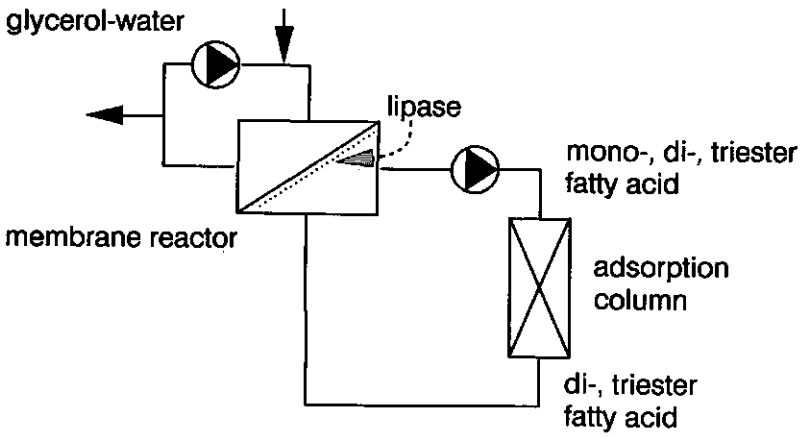

Figure 3 *The two-phase membrane bioreactor combined with an in-line adsorption column for the synthesis of monoacylglycerols.*

For the case that a 1,3 regioselective lipase is used, one should expect that only 1,3-diacylglycerol will be formed. However, the (chemical) acylmigration reaction can also occur. It has been shown that for *Mucor miehei* lipase the acylmigration rate can be as fast as the average esterification rate at 60 °C (Ergan and Trani 1991). This means that triesters will be formed although a 1,3 regioselective lipase is used. However, when the enzymatic conversion rate is at least an order of magnitude higher than the acylmigration rate, a pseudo equilibrium situation can be reached in which mainly mono- and diesters are formed. This is shown in literature for lipase from *Penicillium camembertii* at the esterification of oleic acid and glycerol at 40 °C (Yamaguchi and Mase, 1991). The reaction was terminated before the acylmigration had reached equilibrium and an excess of mono- or diesters was found. This implies that the ester concentration and composition can be controlled by varying the residence time, the choice of the enzyme and the temperature (Bloomer *et al.* 1991). This thesis does not present the kinetic data to calculate the progress of the esterification reaction. A model based on thermodynamic activities (chapter 4) combined with a kinetic model (Pronk

et al. 1992) should result in an overall model to calculate the ester formation with time. This model should also be able to predict the steady-state ester concentrations during synthesis in a continuous reactor.

Assuming that the acylmigration is slow as compared to the esterification rate, mono- and diesters are formed at pseudo equilibrium concentration. An approximation of the mono- and diester concentration can be made using the program TREP (Two-phase Reaction Equilibrium Prediction, chapter 4). The equilibrium constants of mono- and diester synthesis as given in chapter 4 can be used to calculate the apparent equilibrium concentrations. The triester formation equilibrium constant should then equal zero. Figure 4b shows the calculated ester pseudo equilibrium concentrations as a function of the glycerol to fatty acid ratio when a 1,3 regioselective lipase is used. These data can be compared with the equilibrium concentrations calculated assuming a non-selective catalysis, as given in figure 4 of chapter 4 (figure 4b). There are two essential differences between the 1,3 regioselective and the non-selective catalysed reaction: A relative difference in substrate concentration and of course no triester synthesis occurs in the 1,3 regioselective case. With a 1,3 regioselective lipase, there are only 2 hydroxyl groups per glycerol molecule which can be esterified, in case of a non-selective lipase, 3 hydroxyl groups are available. This results in a relative higher fatty acid concentration relative to the concentration of hydroxyl groups which could be esterified. Furthermore, no mono- and diesters will be converted into triesters. This could explain the relatively higher mono- and diester pseudo equilibrium concentration predicted when a 1,3 regioselective lipase is used at initial glycerol to fatty acid ratio < 12. It is worth to be noticed that at equilibrium the fraction incorporated fatty acids as function of the water activity is the same for both cases at initial glycerol to fatty acid ratio < 12. A consequence of the relative high monoester concentration is the enhanced extraction towards the glycerol-water phase because monoesters are relatively well soluble in the glycerol-water phase. Since the monoester concentration is higher for the 1,3 regioselective lipase case, relatively more esters will be extracted to the glycerol-water phase. This phenomenon explains the low ester mole fraction in the non-polar phase at an initial glycerol to fatty acid ratio > 12 for the 1,3 regioselective case as compared to the non-selective lipase. For the non-selective catalysed case, the

extraction occurs at an initial glycerol to fatty acid ratio > 15. These calculations show that it is theoretically possible to synthesize a mixture of mono- and diesters in a two-phase system using a 1,3 regioselective lipase.

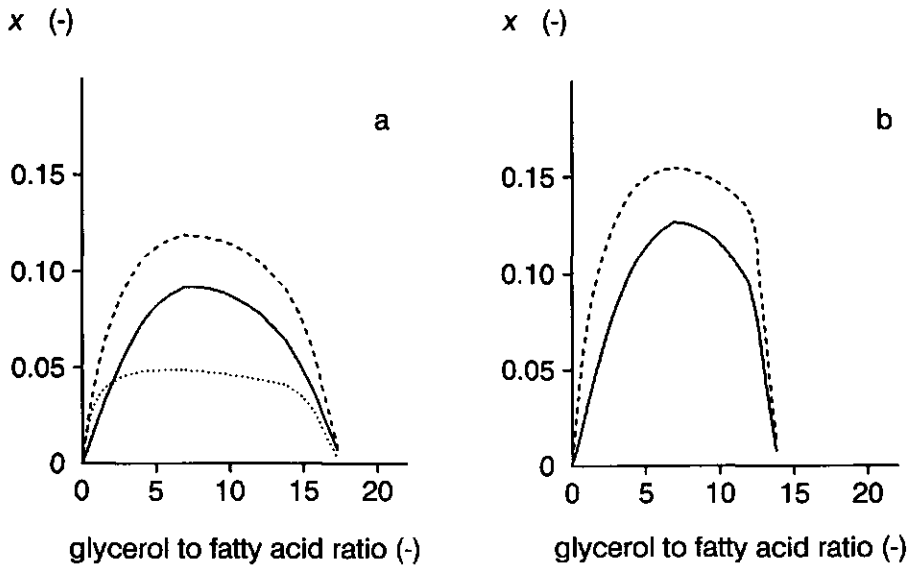

Figure 4 *Calculated ester mole fractions in the apolar phase as a function of the initial glycerol to fatty acid ratio, at equilibrium (a) (figure 4, chapter 4) and at pseudo equilibrium (b) when a 1,3 regioselective lipase is used. In the latter case K_{tri} is assumed to be 0.02. The initial amount of fatty acid, hexadecane and water is 1, 0.7 and 0.4 mole, respectively, for both cases. The quantity of glycerol is variable; mono- (——), di- (- - -) and triester (· · · · ·).*

Of course, the enzyme must be active and stable at esterification conditions, *i.e.* at the interface of a non-polar phase and a glycerol rich polar-phase. As discussed in chapters 2 and 3, the glycerol concentration is of main importance, in combination with the enzyme source for a successful esterification. Hoq and coworkers (1985) did not find

any activity at all using *Candida rugosa* for the esterification of oleic acid with glycerol (Hoq *et al.* 1985). In these experiments a high glycerol concentration of 0.87 mole glycerol per mole of polar phase was used, resulting in a rapid inactivation and consequently no esterification was measured. Glycerol-water mixtures can be classified as non-conventional media, especially at high glycerol concentrations. Generally, these media might increase the inactivation of enzymes. It is known from literature that heat resistant enzymes are expected to be more stable in non-conventional media (Von Stockar 1992). Another advantage of these enzymes is that the synthesis can take place at higher temperatures which should increase the esterification rate, while enzyme stability is hardly affected. This could lead to a better performance of the membrane reactor.

It could be concluded that the enzyme choice influences the esterification rate and the enzyme stability could be different for different types. Especially heat resistant lipases are expected to be more stable at high glycerol concentrations. The equilibrium concentration is independent of the enzyme choice. One exception is the use of regioselective lipases. If the esterification rate is high compared to the acylmigration rate, equilibrium predictions show that at pseudo equilibrium, higher mono- and diester concentrations are calculated, relatively to the non-regioselective predictions. This offers the opportunity to control the ester concentration and composition by varying the residence time, the choice of the enzyme and the temperature. A model based on thermodynamic activities combined with a kinetic model should be developed to calculate the ester formation with time and the steady-state ester concentrations during synthesis in a continuous reactor.

Choice of the fatty acid and solvent

For commercial production, the choice of the fatty acid is of great importance. The type of fatty acid determines the physical properties of the ester produced. The chain length of the fatty acid(s) and the degree of unsaturation determine the emulsifying properties of the monoester. In the case of triester synthesis the melting properties of the product are affected by the fatty acid composition (Chapter 1). For a model study

as presented in this thesis the choice of the fatty acid is arbitrary. Decanoic acid is chosen for its relative low melting point (32 °C) and the fact that it is commercially available in high purity as well as reasonably priced. This is in contrast with for example commercially available oleic acid.

The choice of the solvent will be discussed with respect to the enzyme stability and the equilibrium ester concentration which is reached with and without the solvent. Since all experiments are performed at 25 °C the addition of a solvent is necessary. At the start of this study it was known that the solvent can influence the enzyme stability. A guide-line is the log P value of the solvent, log P being the logarithm of the partition coefficient in a standard octanol/water two-phase system. Solvents having a log P > 4 do not influence the enzyme stability (Laane *et al.* 1987, Reslow *et al.* 1987). The solvent choice was based upon this log P criterion, since the relation between solvent and equilibrium concentration was not known at that time. Hexadecane was chosen because it has a log P value of 8.8, furthermore, hexadecane has a relatively high flash point (135 °C). A log P value of 8.8 should mean that the enzyme activity is not influenced by the addition of this solvent. Chapter 3 shows indeed that there is no difference in stability of an enzyme-glycerol-water system and a membrane system in which hexadecane is present. This leads to the conclusion that the enzyme inactivation as described in this thesis is only glycerol concentration dependent.

The influence of solvents upon the equilibrium concentrations can be predicted with the TREP method (Janssen *et al.* 1993, chapter 4). The thermodynamic equilibrium concentrations can be calculated as a function of the glycerol to fatty acid ratio, assuming that the equilibrium constant remains constant upon a temperature raise of 10 °C.

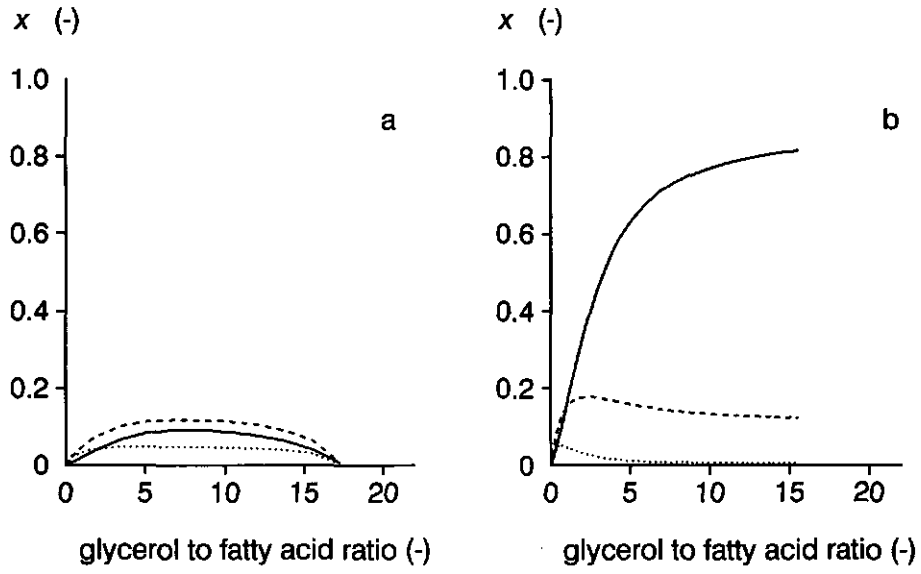

Figure 5 *Calculated equilibrium ester mole fraction in the apolar phase as a function of the initial glycerol to fatty acid ratio in the presence of hexadecane (a) (figure 4, chapter 4) and without the solvent (b). The initial amount of fatty acid, hexadecane, if any, and water is 1, 0.7 and 0.4 mole, respectively, for both cases. The amount of glycerol is varied; mono- (——), di- (- - - -) and triester (· · · · ·).*

Figure 5 shows the calculated equilibrium concentrations with and without hexadecane as solvent (Chapter 4, figure 4). Three differences clearly are present between the solvent and the solvent-free predictions. First of all, an excess of monoesters is predicted in the solvent-free system, at glycerol to fatty acid ratio higher than 7, an eightfold or more mole fraction of monoester is found as compared to the hexadecane case. Even if the concentration is corrected for the addition of the solvent, still a fourfold mole fraction of monoester is predicted. Secondly, in the solvent-free system, almost no triester is present at equilibrium, except at very low glycerol to fatty acid ratio.

Finally, without solvent the total ester concentration continuously increases upon an increase of the glycerol to fatty acid ratio even at high ratios. This is in agreement with the measurements of Janssen and coworkers (1993).

The addition of the non-polar solvent hexadecane decreases the solvability of the relatively polar monoester and increases the solvability of the relatively non-polar triester in the non-polar phase. As a result, a lower equilibrium monoester mole fraction and except at very low ratios a higher equilibrium triester mole fraction is obtained when hexadecane is used as solvent (Janssen *et al.* 1993). As discussed in chapter 4, the monoester is extracted out the hexadecane-rich phase to the glycerol-water phase at high glycerol to fatty acid ratio. This results in a decrease of the monoester concentration at high glycerol to fatty acids ratio. Since the monoester is solubilised better in the non-polar hexadecane-free phase, this extraction is not predicted in the solvent-free case.

These calculations show that it is not possible to obtain pure monoesters in a two-phase system, either with or without hexadecane (figure 5). Product removal is needed, for instance as done by the adsorption process given in chapter 6. Although a higher monoester equilibrium concentration is predicted in the system without solvent, it does not necessarily imply that the monoester synthesis process as described in chapter 6 will be improved. The process described in chapter 6 is based on the removal of monoester during synthesis using an adsorption column and it is shown to be reaction rate dependent. Solvents not only affect the equilibrium position but also the reaction rate. For example, Valivety and coworkers (1992) showed for immobilized lipase from *Mucor miehei* that the esterification rate decreases when a more polar solvent was chosen. Therefore, conclusions can only be drawn for the process given in chapter 6 when research is done on the influence of solvents on the reaction rate. Not only the esterification rate is affected by the addition of a solvent, also the adsorption of monoesters onto the silica surface can be solvent dependent. The effectiveness of the monoester synthesis system is also dependent on the selectivity of the adsorption. Therefore, research has to be done also on the influence of the solvent upon the adsorption rate and selectivity. This will reveal whether the process is reaction limited for all solvents and whether the monoester yield can be improved with the use of a solvent.

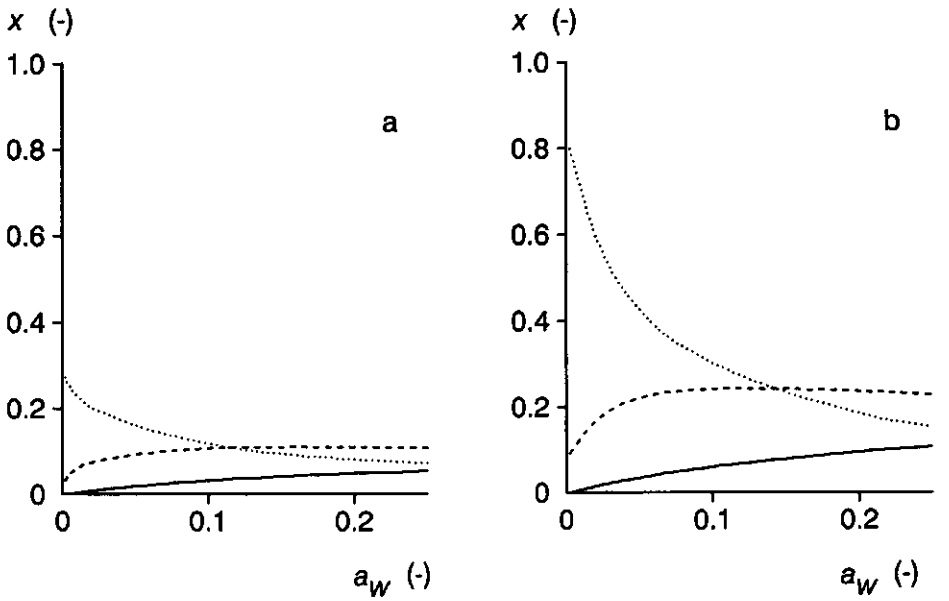

Figure 6 *Calculated equilibrium ester mole fraction with continuous water removal as a function of the water activity in the presence of hexadecane (a) (figure 5, chapter 4) and without hexadecane (b). The initial amounts of glycerol, fatty acid and hexadecane, if any, are 1, 3 and 0.7, respectively, for both cases, the amount of water is varied; mono- (——), di- (- - - -) and triester (· · · · ·).*

Similar calculations are made for the equilibrium prediction with continuous water removal. Figure 6 shows the calculated equilibrium concentrations with (Chapter 4, figure 5) and without hexadecane. Again, higher ester concentrations are obtained for the case without hexadecane. In contrast to the two-phase calculations, this difference is caused solely by the reduced concentration due to the addition of the solvent. The relative ester fraction, the amount of ester per mole of esters and fatty acid, is more or less equal for the solvent-free and solvent-rich system. This suggests that in this case the equilibrium concentrations are a function of the water activity mainly.

For a maximum conversion, triacylglycerol synthesis should reach equilibrium. Calculations show that the predicted fatty acid concentration at equilibrium is still 3.5 % w/w at a water activity (a_w) of 0.001. It has to be noted, however, that the predictions are fatty acid dependent. Although different fatty acids have the same equilibrium constant value, the difference in carbon chain length implies different activity coefficients which will lead to different equilibrium concentrations (Janssen *et al.* submitted). In this study decanoic acid in hexadecane is used while Ergan and coworkers (1990) used oleic acid. At equilibrium at a_w < 0.001 (60 °C at 1.4 kPa), they found only 1 % w/w oleic acid when initially a 3 to 1 ratio of fatty acid to glycerol was chosen. From these data, it is difficult to quantify to which extent the fatty acid tail length influences the equilibrium position at extreme water activity conditions.

This section shows the effect of the solvent hexadecane upon the equilibrium ester concentration in a two-phase system. Without hexadecane, a higher monoester concentration is predicted. Whether the monoester synthesis process as described in chapter 6 could be improved by changing the solvent or solvent concentration cannot be estimated at this time. The influence of solvents upon the reaction rate, adsorption rate and adsorption selectivity should be measured. This should point out whether the monoester production rate and monoester yield could be improved by the use of solvents.
At low water activities, the solvent hardly affects the equilibrium concentrations in one-phase systems. In this case, the equilibrium concentration mainly depends upon the water activity.

Membrane reactor design

For process development it is of importance to know whether reaction or diffusion limitation occurs. Therefore, the characteristic time for reaction and mass transfer should be calculated and compared.

Optimum esterification rate is obtained at a_w = 0.45. At this water activity, the characteristic time for reaction, defined as one over the first order reaction rate

constant, is 33000 s (see chapter 2 and 5). There are three regions in the membrane reactor where diffusion occurs, namely at the inner fibre side, in the membrane matrix and at the shell side. Assuming that the characteristic time for mass transfer at the inner fibre side is equal for the two-phase membrane reactor and the pervaporation membrane reactor, this value equals 40 s. The characteristic time for diffusion through the membrane matrix (square of the membrane thickness over the diffusion coefficient) is 18 s when the diffusion coefficient of water in glycerol is chosen as $D = 2.10^{-11}$ $m^2.s^{-1}$ (Perry 1963).

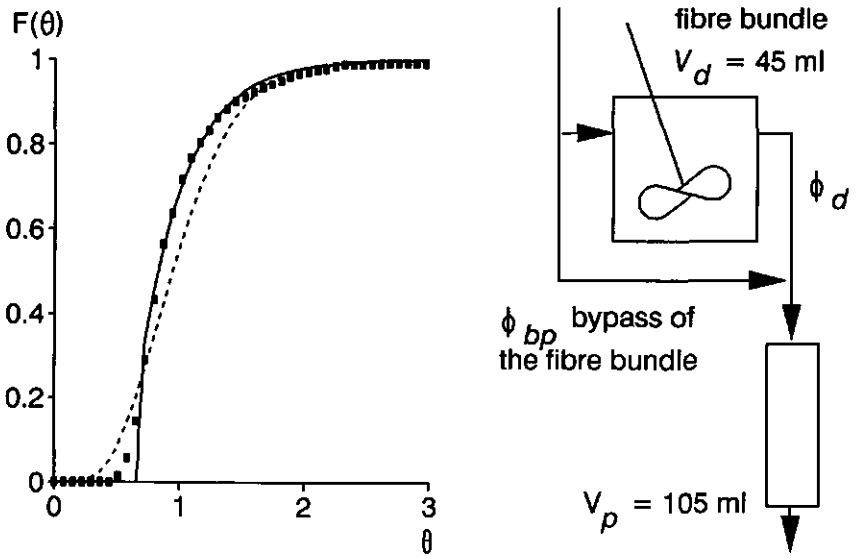

Figure 7 *Measured F-curve of the flow at the shell side of the hollow fibre device (■). ——: model curve using a dead volume V_d = 45 ml with by-pass (Φ_{bp}/Φ_d = 0.43) and a plug flow reactor V_p = 105 ml in series model (rss = 0.03). - - - -: model curve for six CSTR in series (rss = 0.13).*

To deal with the shell side of the membrane unit, a residence time distribution has been measured (figure 7). A model consisting of a cascade of 6 ideally stirred vessels does not describe the measurements adequately (residual sum of squares *rss* = 0.13). Therefore, the shell is modelled with two subsystems in series consisting of a subsystem with a dead volume representing the volume between the fibres (V_d = 45 ml) with a bypass followed by a subsystem with a plug flow reactor representing the flow outside the fibre bundle (V_p = 105 ml; *rss* = 0.03). This model indicates that about one third of the glycerol-water circuit passes the fibre bundle without any exchange of mass with the fibre bundle. The mean residence time in the volume between the fibres (the ratio of the volume and the flow through the vessel) is 180s.
Since the reaction rate time constant of 33000 s exceeds both the mean residence time between the fibres as well as the characteristic time for mass transfer, it can be concluded that the membrane bioreactor is reaction rate limited in the case of acylglycerol synthesis.

Since the characteristic times for mass transfer and reaction respectively differ several orders of magnitude, the esterification process can be improved only by decreasing the characteristic time of reaction. For most cases the membrane is the cost limiting factor, therefore, one should aim to the same order of magnitude for the characteristic time for reaction and mass transfer. The characteristic time for reaction is dependent on the amount of enzyme and on the specific membrane surface area. For low loading, it is inversely proportional to the amount of enzyme. If the amount of lipase per membrane surface area is higher than the optimum load, then the maximum activity per surface area is obtained (chapter 2). In that case the reaction rate is proportional to the specific membrane area. Hence, the characteristic time for reaction can be reduced by increasing the specific membrane surface area. The characteristic time for mass transfer is also inversely proportional to the specific membrane surface area and if the membrane area is increased the characteristic time of transport will be reduced with the same ratio as the characteristic time of reaction. This means that further improvement of the activity per surface area is possible.

The amount of immobilized enzyme has to be uncoupled from the membrane surface area. This can be done by placing a packed column with immobilized enzyme resin in the recirculation stream (figure 8). However, during esterification water is produced

and this could accumulate in the immobilization beads (Goldberg *et al.* 1988). This results in a reduced ester production. Therefore, an immobilisation carrier should be chosen which cannot contain water, for example nylon (Carta *et al.* 1991). Then the water produced will accumulate in the non-polar phase and the water activity of the non-polar phase increases. The column outlet stream is led into the membrane reactor. The surplus of water is removed during the passage through the membrane reactor and the oil phase with the required water activity is brought into the packed column again. This will improve the reaction rate of the system, while the equilibrium ester concentrations can be controlled with control of the non-polar phase of the membrane reactor.

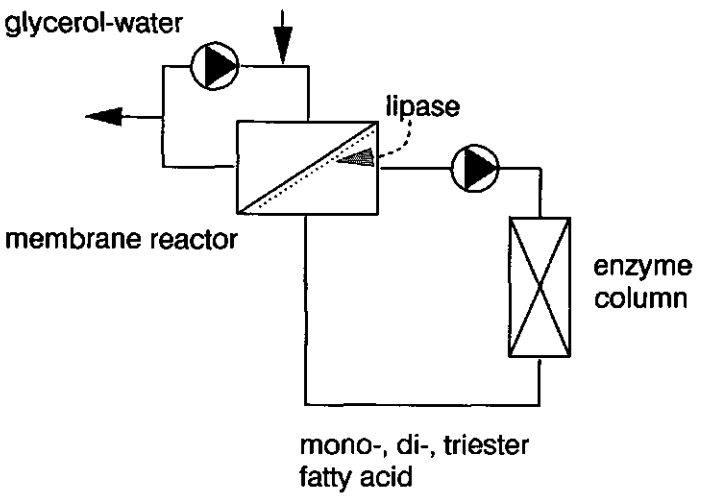

Figure 8 A membrane reactor with an in-line enzyme column.

Kloosterman and coworkers (1988) have worked out an economical evaluation of a membrane bioreactor. They stated, that the production rate should exceed

2.7 μmole.s^{-1}.m^{-2} and the total productivity should exceed 200 mole per kilogram enzyme. The maximum fatty acid removal rate which is measured in this thesis is 12 to 14 μmole.s^{-1}.m^{-2} at a_w = 0.45 (chapter 2). At this water activity the enzyme half-life time is measured to be 62 days in a membrane reactor. This results in a productivity of 70 kmole per kilogram of enzyme when the system is operated three times the half-life time. These calculations show that the reactor should be feasible with respect to reaction rate and enzyme stability. Product yield, however, is far below 90 %, a minimum according to Kloosterman and coworkers (1988). Therefore, a downstream process should be developed. The triacylglycerol yield can be improved by using a membrane reactor and a pervaporation membrane reactor in series. Monoacylglycerol recovery can be enhanced by an in-line adsorption column for product removal.

Triacylglycerol synthesis

A membrane reactor concept is developed for the production of an excess of triglycerides relative to the other reaction components (figure 2). Two disadvantages arise, the process is reaction limited and the maximum triester concentration obtained is the equilibrium value belonging to the applied a_w.

Chapter 5 shows that the system is reaction limited, the characteristic time for mass transfer equals 40 s while the characteristic time of reaction is 65000 s. As discussed above, the system can be improved by placing a column packed with immobilized enzyme in the recirculation loop. The characteristic times for reaction and transport of this system have to be measured and compared with the economical data provided by Kloosterman and coworkers (1988).

The equilibrium ester composition is related to the water activity and fatty acid concentration (see section 'Choice of the fatty acid and solvent'). There it has been shown that no pure triesters can be obtained in the case of decanoic acid, when a fatty acid to glycerol ratio of 3 to 1 is used. Of course the equilibrium can be forced to triester synthesis by adding an excess of fatty acids. However, after the reaction has reached equilibrium, the surplus of fatty acids has to be removed without any losses of triacylglycerols. This can be performed by using saponification in a two-membrane system (Keurentjes *et al.* 1990) or by using a silica adsorption column (Hau and Nawar 1985). As described in chapter 6, fatty acids have a high affinity adsorption behaviour

onto a silica column if no monoesters are present. Since it can be expected that at the outlet of the pervaporation membrane reactor the monoester concentration approximates 0, the fatty acids will adsorb preferentially onto the column.

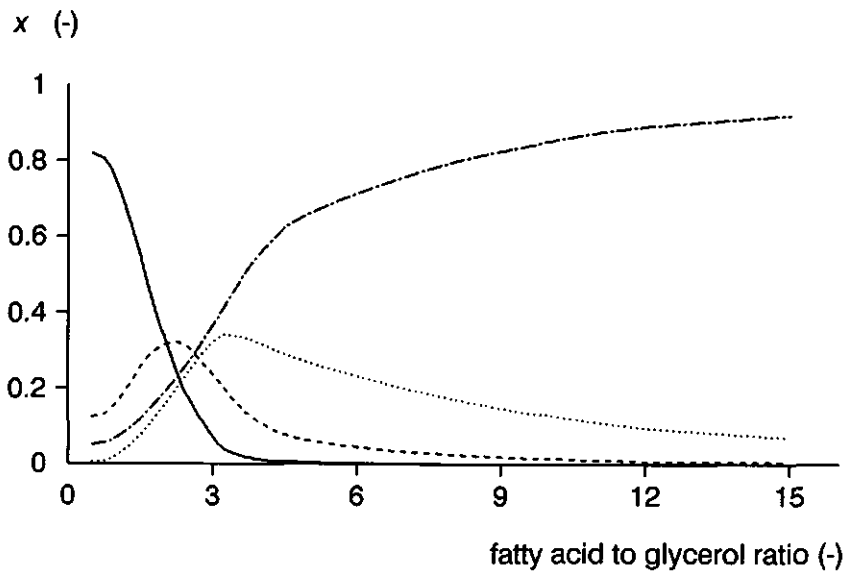

Figure 9 *Predicted equilibrium ester concentration as a function of the initial fatty acid to glycerol ratio at a constant water activity of 0.1; mono- (——), di- (- - -), triester (· · · · ·) and fatty acids (– · – · – ·).*

Figure 9 shows the predicted equilibrium concentration as a function of the initial fatty acid to glycerol ratio at a_w = 0.1. Below a fatty acid to glycerol ratio of 0.5, a two-phase system is predicted. The non-polar phase of this two-phase system has approximately the same composition as the one-phase system at a glycerol to fatty acid ratio of 1. At low ratios a high monoester yield is predicted. Addition of fatty acids enhances the triester production relative to the mono- and diester for the whole range of ratios used. At the maximum ratio of 15 to 1 fatty acid to glycerol, a 0.93 triester mole fraction

relative to mono-, di- and triester is calculated. However, this effect is completely nullified by the high fatty acid concentration, only 0.07 mole triester per mole of mixture is formed. Optimum triester synthesis conditions are at an initial fatty acid to glycerol ratio around 3.5. This is in agreement with the findings of Lortie and coworkers (1992). They found a maximum triester concentration ($mmol.ml^{-1}$) at an initial fatty acid to glycerol ratio between 3 and 3.5. From these calculations it can be concluded that the addition of a surplus of fatty acids leads to a relatively high triester fraction with respect to the other esters, however, the triester concentration drops as a result of the addition of the fatty acids.

The pervaporation membrane reactor can be used also for the interesterification or acidolysis. During interesterification two types of esters are mixed in the presence of a catalyst. At equilibrium, a random mixture is obtained. In the case of acidolysis, an ester is mixed with fatty acids in presence of the enzyme. The esterified acids will be released partly while the added fatty acid will be incorporated partly. This reaction is also called transesterification. A problem of interesterification and transesterification is the water activity of the system. At moderate water activity conditions, hydrolysis will take place (Bloomer *et al* 1991, Goderis *et al.* 1987). Therefore, the water activity should be kept at low value, which is one of the features of the pervaporation membrane reactor system. To predict the equilibrium position of this type of reactions the program TREP has to be extended.
A special case of the acidolysis is the 're-esterification' of natural oils and fats. During transport of the oil seeds, hydrolysis occurs and fatty acids are liberated (Applewhite 1980). The pervaporation membrane system (with an in-line column packed with enzyme) can be used to esterify the fatty acids and mono- or diesters back to triesters. However, it has to be noticed that the final fatty acid content of the oil is a function of the water activity. In the case that decanoic acid is used, even at a_w = 0.001, still 3.5 % w/w fatty acid remain at equilibrium. For edible oils such a percentage is yet unacceptable. Of course, natural oil often consists of fatty acids with longer carbon chains compared to decanoic acid and therefore, a higher triester equilibrium concentration could be expected (Janssen *et al.* submitted). Research has to point out whether it is possible to 're-esterify' natural oils to only triesters.

Monoacylglycerol synthesis

The charm of the monoester synthesis system is that no equilibrium has to be reached (chapter 6). During the synthesis the reaction intermediate, the monoester, is adsorbed onto silica. Therefore, the monoester concentration in the non-polar phase approximates 0 and the reaction proceeds. As discussed before, the conversion rate can be enhanced by placing a packed column with immobilized enzyme in the recirculation loop.

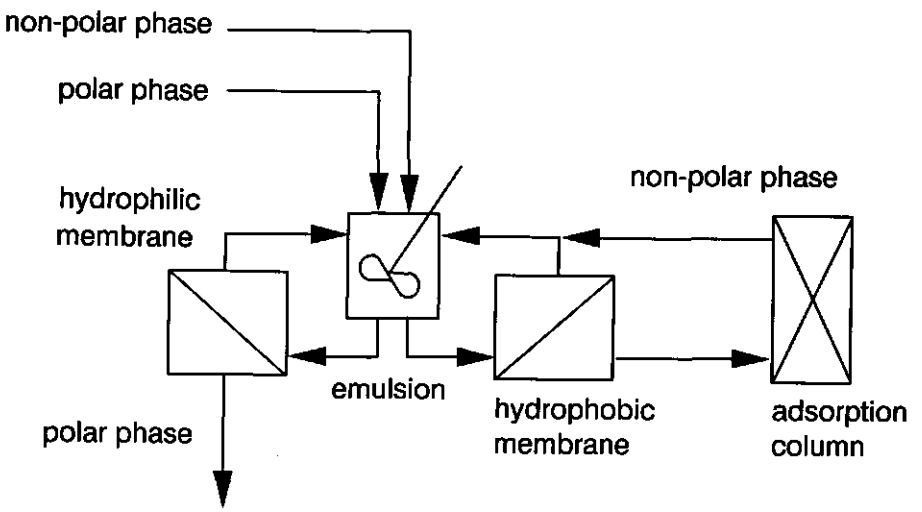

Figure 10 The emulsion membrane reactor for the synthesis of monoacylglycerols.

Another possible reaction is the glycerolysis of natural oils. In this case the triesters of a natural oil and glycerol are converted to monoesters (McNeill and Yamane, 1991). Again, the monoesters should adsorb onto the column. The conversion rate of such a membrane reactor system can be enhanced by making use of a free-enzyme emulsion system. The reaction takes place in an emulsion with an in-line phase separation step

using centrifuges (Bühler and Wandrey 1988) or a sequence of membranes (Pronk *et al.* 1991, Schroën *et al.* 1992). In the process which makes use of centrifugal separation, the oil phase is removed while the enzyme solution is reused. The other process uses a hydrophilic membrane to remove the glycerol-water phase while rejecting the enzyme and a hydrophobic membrane to remove the oil phase. With this process the enzyme remains in the emulsion. An advantage of this system is that a large specific area is created in the emulsion and a high volumetric activity can be expected. However, in these processes the stability of the enzyme is the limiting factor. This system can be used for the monoacylglycerol synthesis also. In the oil stream an adsorption column must be placed, the column outlet should be recirculated to the emulsion (figure 10).

Scale-up

At our laboratory, some preliminary scale-up experiments are performed for the hydrolysis of triesters (Sewalt *et al.* 1992). These experiments show that the hydrolysis rate is proportional to the membrane surface area. This makes scaling-up very easy. However, during hydrolysis, membrane fouling occurred during these experiments and the volumetric activity decreased with time. This fouling should be avoided. Up till now, it is not clear which components are responsible for the membrane fouling or even fibre blocking. One possibility is the crystallisation of reaction intermediates during the start up of the process. This could be avoided by increasing the process temperature. However, one has to keep in mind that the half-life time of the enzyme will decrease upon an increasing temperature. Therefore, the choice of enzyme could be different and must be studied as a function of temperature.

This section discusses the performance of the membrane bioreactor. The membrane reactor is reaction rate limited. Without influencing the characteristic time for mass transfer, the characteristic time for reaction can be decreased by placing a packed column with immobilized enzyme resin in the recirculation stream.

Calculations show that the membrane reactor should be feasible with respect to reaction rate and enzyme stability. Product yield, however, is far below 90 % a minimum according to Kloosterman and coworkers (1988). In-line product or by-product removal improves the product yield.

A pervaporation membrane reactor is developed for triester synthesis. The equilibrium triester concentration is water activity dependent. At extreme low water activity

conditions still 3.5 % w/w decanoic acid remains present at equilibrium. Whether natural oils can be 're-esterified' to only triesters must be investigated. Of course the equilibrium can be forced to triester synthesis by adding an excess of fatty acids. However, for triester synthesis the excess of fatty acids itself dilutes the triester concentration. When this type of reactor is used for the acidolysis, an excess of fatty acids must be present and the reaction can be forced to an excess of triesters. To predict the equilibrium concentrations of the acidolysis the program TREP has to be extended for the case that different types of fatty acids are present.
It is discussed that monoester synthesis can be enhanced using an emulsion membrane reactor with an in-line adsorption column.
Preliminary scale-up shows membrane fouling during hydrolysis. One solution could be an increase of reaction temperature to solubilise the reaction intermediates. Therefore, the effect of temperature upon the performance of the reactor has to be studied.

General conclusions

Although the enzyme and fatty acid are arbitrary chosen, the methods used in this thesis give insight in the possibilities and limitations of enzymatic ester synthesis. When another type of enzyme is chosen, the reaction rate and enzyme stability could change, nevertheless, the thermodynamic equilibrium remains the same. However, a 1,3 regioselective lipase could lead to a pseudo equilibrium at which a different ester composition is obtained. To predict this pseudo equilibrium, the enzyme kinetics must be described. This model could also be used to predict the steady-state concentration of a continuous reactor.
Equilibrium calculations show that only at extreme low water activity conditions an excess of tridecanoylglycerol can be obtained. However, the fatty acid carbon chain length does influence the equilibrium concentration. Research has to point out whether it is possible to 're-esterify' natural oils to triesters only.
The membrane reactor is reaction limited, the reaction rate can be improved by placing a packed column with immobilized enzyme in the non-polar phase. Not only

esterification can be performed in the pervaporation system, this system could also be suitable for interesterification or transesterification. Then the program TREP should be extended for reactions with different types of fatty acids.

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Summary

Up till twenty years ago, only chemical modifications of agricultural oils for novel uses were studied. Because of the instability of various fatty acids, enzymatic biomodifications can have advantages above the chemical route. Nowadays, enzymatic catalysis can be used for the modification of oils and fats. One way of biomodification is the enzymatic esterification of glycerol with fatty acid for the synthesis of mono- and triacylglycerols. Monoesters (monoacylglycerols) are used as emulsifiers in food and in cosmetics, tailor made triesters (triacylglycerols) are used to adjust the melting range of foods and cosmetics. This thesis describes a number of membrane reactor systems for the enzymatic esterification of glycerol with decanoic acid in hexadecane as solvent. Description and modelling of the kinetics and thermodynamic equilibrium have resulted in reactor concepts to reach the objective of mono- and triester synthesis.

The basic reactor studied is a two-phase immobilized enzyme membrane reactor. In the membrane reactor, lipase from *Candida rugosa* is immobilized at the inner fibre side of a hydrophilic hollow fibre module. Decanoic acid in n-hexadecane is circulated at the same side, meanwhile a water-glycerol phase is circulated at the shell side. The glycerol diffuses through the membrane matrix allowing the synthesis to take place at the interface. The water produced diffuses backwards.

Chapter 2 describes the enzymatic esterification of decanoic acid with glycerol for an emulsion system and for a hydrophilic membrane system. In a two-phase system, the enzyme activity is related to the oil-phase volume, the interface area and the enzyme load. The rate per unit interface area of the membrane system approximates the rate measured in an emulsion system. This implies that the cellulose membrane does not affect the esterification. Another consequence is that the activity per oil-phase volume is only specific surface area related, therefore a hollow fibre device is desirable. The optimum enzyme load in the membrane system is half of that in the emulsion system.

The enzyme stability in glycerol-water mixtures is described in chapter 3. The activity of lipase from *Candida rugosa* with time can be described with a two-step model, assuming the native lipase reversibly altering its conformation to a form having no activity. The reversibility is experimentally verified. Both, the native and inactive form do inactivate irreversible at the same time to a completely inactive form. The inactivation is a function of the glycerol concentration. The activity of immobilized enzyme is reduced to the same level of activity as is found for free lipase.

Not only activity and stability of the enzymatic system are of importance, also the equilibrium ester concentrations must be known in the non-ideal two-phase system. Chapter 4 presents the program TREP (Two-phase Reaction Equilibrium Prediction). With the use of measured thermodynamic activity based equilibrium constants, mass balances and the UNIFAC group contribution method, TREP predicts the equilibrium product and substrate concentrations for given initial amounts. Equilibrium predictions show that an excess of triesters can be obtained only at low water activity conditions, in this case an one-phase system is predicted. Predictions show that pure monoesters cannot be obtained in a two-phase system of decanoic acid-hexadecane phase and a glycerol-water phase, even with a high glycerol to fatty acid ratio. This is experimentally verified.

From the knowledge gathered in these chapters, two membrane reactor systems are designed, one membrane reactor for the triester production and a second membrane reactor system equipped with an in-line adsorption column for the synthesis of monoesters.

Chapter 5 describes a pervaporation system in which an excess of triesters can be synthesized at low water activity conditions. Lipase is immobilized onto the lumen side of a cellulose membrane where the organic phase is present. At the shell side, air circulates and the water activity is controlled with the use of a condenser. The lipase catalyzed esterification of decanoic acid with partial glycerides is studied in this reactor. In agreement with the predictions made in chapter 4, an excess of triacylglycerols is obtained at low water activity conditions only.

A second membrane reactor concept is described in chapter 6, the organic-phase is led over an adsorption column in order to adsorb the monoglycerides onto the adsorbate. When the column is saturated with monoesters, the column can be desorbed off-line in a continuous membrane/repeated batch column process. If a 5 % ethanol in hexane solution is used as desorption solvent, monoesters are desorbed selectively leading to a 90 % purity.

Finally, in chapter 7, the potentials and limitations of the enzymatic esterification are discussed. To predict the steady-state concentration of a continuous reactor, the enzyme kinetics must be described. The membrane reactor is reaction limited, this could be overcome by placing a column packed with immobilized enzyme in the organic phase recirculation loop. Not only esterification can be performed in the pervaporation system, this system could also be suitable for interesterification or transesterification. Then the program TREP should be extended for reactions with different types of fatty acids.

Samenvatting

Nog geen twintig jaar geleden werd alleen de chemische modificatie van natuurlijke oliën en vetten bestudeerd. Omdat veel vetzuren instabiel zijn, zou enzymatische modificatie voordelen kunnen hebben boven de chemische route. Tegenwoordig kunnen oliën en vetten ook enzymatisch worden gemodificeerd. Een methode is de enzymatische verestering van glycerol met vetzuur voor de synthese van monoacylglycerolen en triacylglycerolen. Mono-esters (monoacylglycerolen) worden aan levensmiddelen en farmaceutica toegevoegd als emulgator, tri-esters (triacylglycerolen) worden toegevoegd om het smeltgedrag van levensmiddelen en farmaceutica te beïnvloeden. Dit proefschrift beschrijft een aantal membraan-bioreactorsystemen die ontworpen zijn voor de enzymatische verestering van glycerol met in hexadecaan opgelost decaanzuur. De beschrijving en de modellering van de kinetiek en het thermodynamisch evenwicht heeft geleid tot reactorontwerpen die het mogelijk hebben gemaakt een overmaat mono- of tri-ester te synthetiseren, wat het doel was van het in dit proefschrift beschreven onderzoek.

Het basistype reactor is een tweefasen-membraanreactor met een geïmmobiliseerd enzym. In de reactor is het lipase van *Candida rugosa* geïmmobiliseerd aan de binnenkant van de vezels van een hydrofiel-holle-vezel-membraaneenheid. Decaanzuur opgelost in n-hexadecaan wordt door de vezel heen rondgepompt, tegelijkertijd wordt een water-glycerolfase om de vezels heen rondgepompt. De glycerol diffundeert door de membraan matrix, zodat de synthese kan plaatsvinden aan het grensvlak. Het geproduceerde water diffundeert terug.

In hoofdstuk 2 wordt de enzymatische verestering van decaanzuur met glycerol in een emulsiesysteem vergeleken met de verestering in een membraansysteem. In een tweefasensysteem is de enzymactiviteit gerelateerd aan het volume van de oliefase, de oppervlakte van het grensvlak en de enzymbelading. De reactiesnelheid per eenheid van grensvlakoppervlakte van het membraansysteem benadert deze van het emulsiesysteem. Dit impliceert, dat het cellulosemembraan de veresteringsreactie niet beïnvloedt. Een andere consequentie is dat de activiteit per reactorvolume enkel

afhangt van het specifieke oppervlak, daarom is het gunstig om een holle-vezel-membraaneenheid te gebruiken. In het membraansysteem is de optimale enzymbelading de helft van de waarde voor een emulsiesysteem.

De enzymstabiliteit in glycerol-water mengsels is beschreven in hoofdstuk 3. De activiteit van het lipase van *Candida rugosa* kan in de tijd beschreven worden met een tweestappenmodel. Verondersteld wordt dat het lipase reversibel verandert van de actieve conformatie naar een inactieve vorm. De reversibiliteit is experimenteel getoetst. Het enzym, zowel in de actieve conformatie alsook in de inactieve vorm, wordt tegelijkertijd irreversibel omgezet in een blijvend inactieve vorm. De inactivatie is afhankelijk van de glycerolconcentratie. De activiteit van het geïmmobiliseerde enzym wordt in de zelfde mate verlaagd als die van het opgeloste enzym in een glycerol-water oplossing.

Naast de activiteit en de stabiliteit van het enzymsysteem, moeten ook de evenwichtsconcentraties bekend zijn in dit niet-ideale tweefasensysteem. In hoofdstuk 4 staat het programma TREP beschreven (TweefasenReactieEvenwichts-voorsPelling). Dit model maakt gebruik van de gemeten evenwichtsconstante, gebaseerd op thermodynamische activiteiten, massabalansen en de UNIFAC-groepsbijdrage-methode. TREP voorspelt de evenwichtssubstraat- en -produktconcentraties bij gegeven begin hoeveelheden. Evenwichtsvoorspellingen tonen aan dat alleen bij lage wateractiviteit een overmaat tri-ester gevormd wordt, in dit geval wordt een eenfasesysteem berekend. De voorspellingen tonen aan dat in een tweefasensysteem, decaanzuur-hexadecaan / glycerol-water, geen pure mono-esters gemaakt kunnen worden, zelfs niet bij een grote overmaat aan glycerol. Dit is experimenteel getoetst.

Op basis van de kennis die in deze hoofdstukken vergaard is, zijn twee membraan-reactorconcepten ontworpen, één voor de tri-esterproduktie en één voor de mono-esterproduktie.

Hoofdstuk 5 beschrijft een pervaporatiesysteem waarin een overmaat tri-esters wordt gesynthetiseerd bij een lage wateractiviteit. Het lipase is geïmmobiliseerd op de binnenzijde van een holle-vezel-cellulosemembraan, aan deze kant bevindt zich ook

de organische fase. Aan de schaalzijde wordt lucht rondgepompt, de wateractiviteit van deze fase wordt met behulp van een condensor geregeld. In deze reactor is de enzymatische verestering van mono- en di-esters met decaanzuur bestudeerd. In overeenstemming met de berekeningen in hoofdstuk 4 wordt enkel bij lage wateractiviteit een overmaat tri-ester gevormd.
In het tweede reactorconcept wordt de organische fase over een adsorptiekolom geleid (hoofdstuk 6). Op het moment dat de kolom verzadigd is met mono-esters, wordt de kolom uit de kringloop voor de organische fase gehaald en gedesorbeerd. Indien een 5 % ethanol in hexaanoplossing wordt gebruikt bij de desorptie, zullen de mono-esters selectief worden gedesorbeerd. Dit leidt tot een 90 % puur produkt.

Tenslotte worden in hoofdstuk 7 de mogelijkheden en beperkingen van de enzymatische verestering besproken. Om tot een voorspelling te komen van de uitgaande concentraties van een kontinu-proces in stationaire toestand, moet de enzymkinetiek verder worden beschreven. Het membraansysteem is reactie-gelimiteerd, dit kan worden verholpen door een gepakte kolom met enzym in de organische fase te plaatsen. Een andere optie voor de reactor is om de transveresteringsreactie er in uit te voeren. Dan zal het programma TREP moeten worden uitgebreid zodat ook reactie-evenwichten met verschillende vetzuren te kunnen worden berekend.

Curriculum vitae

Albert van der Padt was born on May 3rd 1960 in Eindhoven, The Netherlands. He graduated from high school (*atheneum B*) at the *Eindhovens Protestants Lyceum* in 1979. In the same year he began his studies Food Science and Technology at the Wageningen Agricultural University (formerly called the *Landbouw Hogeschool Wageningen*). He specialized in process engineering and graduated (*Ir.*) in June 1986. From August 1986 to January 1990 he worked as a research assistant at the Food and Bioprocess Engineering Group of the Wageningen Agricultural University. In January 1990 he became a faculty member at the same group. His field of special interest is separation and reaction.