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Causevic, Ariana

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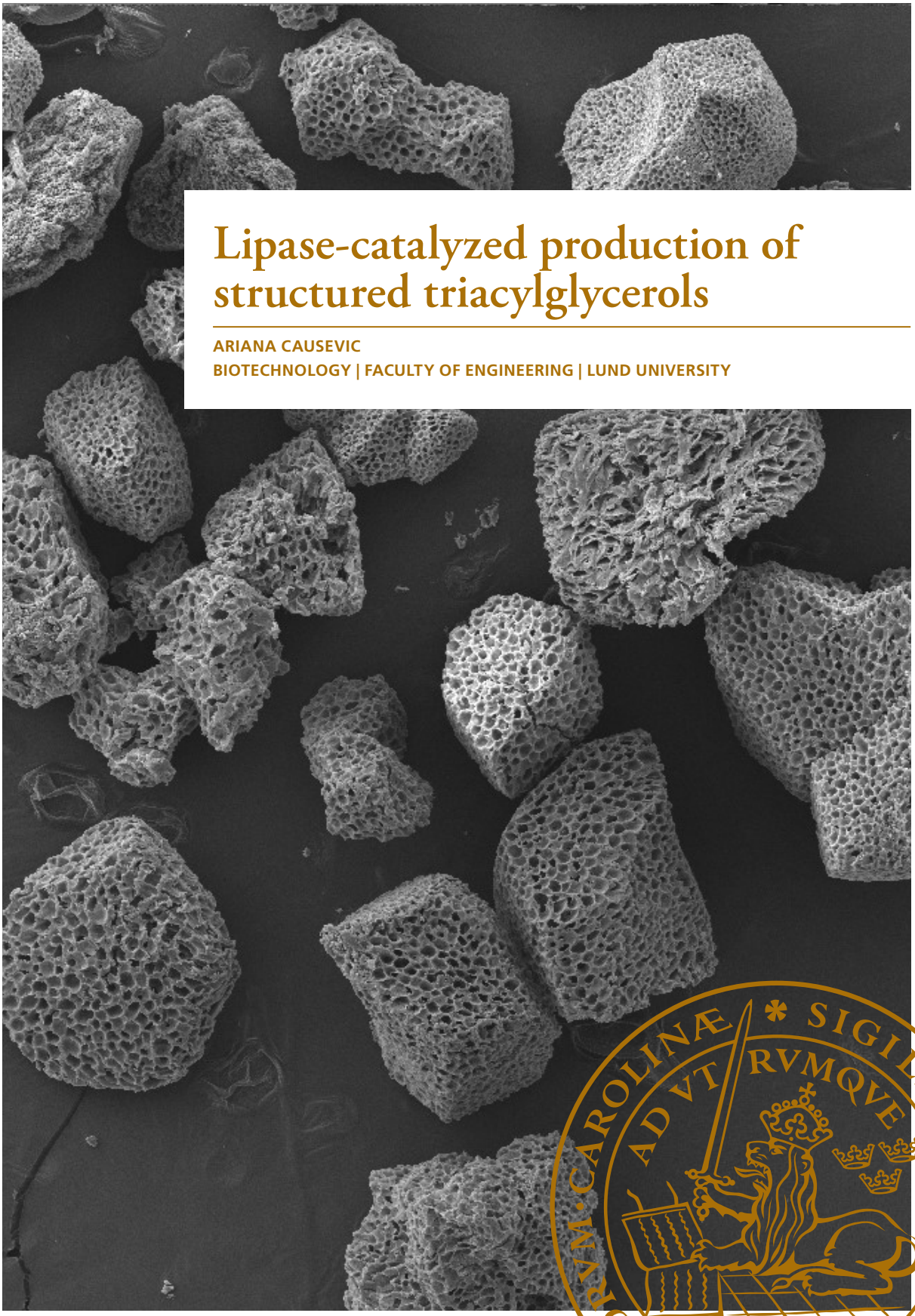

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
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Abstract
Vegetable oils and fats are major energy sources but also play an essential part in the physical and chemical properties of food products. The current change in the climate is affecting harvest yields, leading to a shortage and increased prices of raw materials that are difficult to replace. In addition, there is an increasing demand for new products with positive health effects.

Triacylglycerols (TAGs) with specific properties can be produced using synthesis methods controlling which fatty acids are present in each position of the molecule. These are called structured triacylglycerols (STAGs) and enzymatic transesterification is a good method to produce them. Immobilized lipases are the catalyst of choice for STAG production as they can selectively tailor the fatty acid composition in the triacylglycerol to obtain the desired properties. Few industrial processes have been developed despite the positive aspects of lipase-catalyzed synthesis and the continuously growing application areas. Further implementation of industrial processes requires highly efficient and stable catalysts and optimized reactor systems to overcome the limitations of too high production costs. This dissertation attempts to improve the production of STAGs by the development of a comprehensive lipid analysis method and effective lipase catalysts, and optimization of reaction conditions.

Analysis of reaction samples is a central part of research and quality control. There is thus a great need for efficient and cost-effective methods for the analysis of the composition of vegetable oils and fats. In this work, a high-performance liquid chromatography method with charged aerosol detection was developed for complete analysis of lipid samples. Within 80 minutes the method manages to separate free fatty acids, fatty acid esters, monoacylglycerols, diacylglycerols (DAGs) and TAGs, including several regioisomers. The developed method facilitated the performance of the succeeding research.

The properties of an immobilized preparation will depend on the combination of lipase and support. In this work, it was found that the immobilization conditions and support material had profound effects on the catalyst activity. Immobilization by adsorption on highly hydrophobic supports resulted in significantly higher immobilization yield and activity, especially when compared to covalent binding. We managed to develop immobilized preparations with activities equal to commercially available preparations and even higher regioselectivity. This shows the possibility of STAG synthesis with high productivity and purity.

Transesterification for the synthesis of STAGs is a multistep reaction with DAGs formed as intermediate products. Many factors influence the initial reaction rate, product yield and quality. The effects of water activity, temperature, substrate ratio and ethanol concentration were studied in the reaction between high oleic sunflower oil and ethyl stearate producing 1,2-stearin-3-olein (SOS) using immobilized lipase from Rhizopus oryzae (Lipase DF “Amano” IM). It was found that the factors influencing the enzymatic activity (water activity, ethanol addition, substrate ratio, and temperature) were highly important initially, whereas factors affecting the thermodynamic equilibrium (water activity, ethanol addition, and substrate ratio) dominated later. This emphasizes the importance of controlling the reaction parameters. The product quality was negatively affected by temperature, as an increased temperature promoted acyl migration.

A water activity control system based on dry/wet nitrogen gas sparging was developed and implemented in a SpinChem® rotating bed reactor to control the water activity in non-aqueous media during enzymatic reactions. With a stepwise change in water activity from high (0.65) to low (drying of reaction solution), it was possible to increase the initial DAG and product formation while reducing the final DAG content to 3.1%. In addition, a final yield corresponding to 78% of the theoretical maximum was obtained using 0.5% (w/w) enzyme and 7 h of reaction. Excellent regioselectivity of Lipase DF “Amano” IM and minimized acyl migration resulted in only 0.11% undesired incorporation in the sn-2-position.

Key words
Structured lipids, lipase, acyl migration, immobilization, lipid analysis, transesterification, water activity

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For a better future

Table of Contents

Abstract

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from *Rhizopus oryzae* (Lipase DF "Amano" IM). It was found that the factors influencing the enzymatic activity (water activity, ethanol addition, substrate ratio, and temperature) were highly important initially, whereas factors affecting the thermodynamic equilibrium (water activity, ethanol addition, and substrate ratio) dominated later. This emphasizes the importance of controlling the reaction parameters. The product quality was negatively affected by temperature, as an increased temperature promoted acyl migration.

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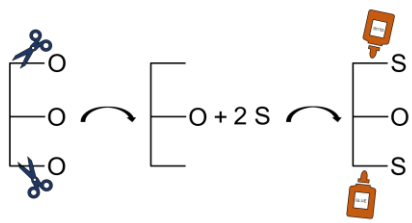
Popular science summary

Almost all food products we eat contain fat. Fat is an energy-giving nutrient needed to build and repair cells, create hormones and absorb fat-soluble vitamins. Besides being required for human health and well-being, fat also contributes to the product's taste, texture and consistency. A well-known example of this is the role of fat in chocolate. The chocolate gets its glossiness, distinct snapping sound when broken and characteristic melting properties so that it melts in the mouth but still offers some chewing resistance thanks to the unique properties of the cocoa butter. The composition of the fat governs the properties. Fats mainly consist of triacylglycerols, formed of a glycerol molecule with three fatty acids. The fat will behave differently depending on which fatty acids and where on the glycerol molecule the fatty acids sit.

Within the food and pharmaceutical industry, there is an increased need for fats with specific properties to meet the demand for new products with positive health effects. Unfortunately, fats with the desired properties are rarely found in nature. In addition, climate change is expected to reduce agricultural productivity, leading to a shortage of raw materials with unique properties, such as cocoa butter for chocolate. There is thus a need for a method for producing fats with a specific composition and properties, so-called structured triacylglycerols (STAGs).

STAGs are created by replacing one or more fatty acids in a fat molecule with another fatty acid until the desired composition is achieved. This is best performed using enzymes. Enzymes are nature's catalysts that make reactions go faster without being consumed. In nature, there are many different enzymes able to catalyze almost any type of reaction. A group of enzymes called lipase is used for the production of STAGs. The natural function of lipases is to break down fats, and they are therefore important in our digestion. However, under certain conditions, lipases can also do the reverse reaction, meaning that they can build bonds in fats. This is used during the production of STAGs, which takes place in two steps. In the first step, the lipase acts like a scissor and removes unwanted fatty acids on the original fat molecule. In the second step, the lipase acts as a glue that attaches the new fatty acids to give a fat with the desired properties.



The lipase acts like a scissor in the first step and like glue in the second to remove the undesired and attach the desired fatty acids to the triacylglycerol.

The work in this dissertation has been carried out in a collaboration between Lund University and AAK. We have investigated several important aspects in the development of a process for the production of STAGs. First, a method for efficient and precise analysis of the fat composition and, thus, the lipase's ability to produce the desired product was developed. The new method facilitated the performance of the succeeding research.

Lipases are effective catalysts but very small and, therefore, difficult to handle. They are often also expensive and can contribute to a significant part of the production cost. To facilitate the handling and re-use of the lipase and to lower the lipase cost, they can be attached to a larger support material in a process called immobilization. We have investigated how different support materials affect the catalytic ability of the lipase to try to understand which is the best in this process. Highly hydrophobic materials, that is, materials that repel water, were found to be best for achieving a high degree of immobilization and high activity of the lipase. We succeeded in creating new immobilized lipases with higher activity and selectivity than commercially available preparations.

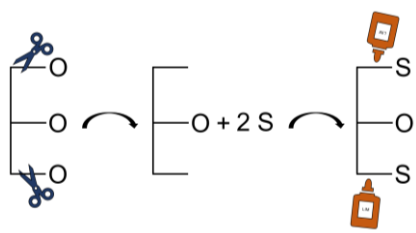
Many other parameters affect the formation of the desired product. This dissertation investigated the effect of water, temperature and the ratio between the original fat and the new fatty acid. All parameters were found to be important. We then tried to find the optimal combination for maximum product formation in the shortest possible time. The aim was to make the process as efficient as possible. We developed a process using immobilized lipase from *Rhizopus oryzae* with high lipase activity, high yield and low byproduct formation. This resulted in exceptionally high product purity and productivity compared to previously obtained results.

Populärvetenskaplig sammanfattning

Nästan alla matprodukter vi äter innehåller fett. Fett är ett energigivande näringsämne som behövs för att bygga och reparera celler, tillverka hormon och ta upp fettlösliga vitaminer. Förutom att fett behövs för hälsa och välmående så bidrar det även till produktens smak, textur och konsistens. Ett välkänt exempel på detta är fettets roll i choklad. Chokladen får sin glansighet, tydliga knäppande ljud när den bryts och karakteristiska smältegenskaper så att den smälter på tungan men trots det ger lite tuggmotstånd tack vare kakaosmörets unika egenskaper. Egenskaperna styrs av fettets sammansättning. Fetter består främst av triacylglyceroler som byggs upp av en glycerolmolekyl med tre stycken fettsyror. Beroende på vilka fettsyror och var på glycerolmolekylen de sitter kommer fettet att bete sig på ett visst sätt.

Inom mat- och läkemedelsindustrin finns ett ökat behov för fetter med specifika egenskaper för att möta efterfrågan på nya produkter med positiva hälsoeffekter. Tyvärr hittas sällan fetter med de önskade egenskaperna i naturen. Dessutom väntas klimatförändringar minska jordbrukets produktivitet vilket kan leda till brist på råvaror med unika egenskaper så som kakaosmör till choklad. För att kunna tillgodose behovet behövs ett sätt att producera fetter med specifik sammansättning och således egenskaper, så kallade strukturerade triacylglyceroler (STAGs).

STAGs skapas genom att byta ut en eller flera fettsyror i en fettmolekyl till en annan fettsyra tills den önskade sammansättningen har uppnåtts. Detta görs allra bäst med enzymer. Enzymer är naturens egna katalysatorer som gör att reaktioner går snabbare utan att själva förbrukas. I naturen finns det många olika enzymer för att katalysera nästan alla typer av reaktioner man kan tänka sig. För produktion av STAGs används en grupp av enzym som kallas lipas. Lipasers naturliga funktion är att bryta ner fetter och de är därför viktiga i vår egen matsmältning. Lipaser kan dock under vissa förhållanden även göra den omvända reaktionen, det vill säga bygga upp bindningar i fetter. Detta utnyttjas under produktionen av STAGs som sker i två steg. I det första steget fungerar lipaset som en sax och klipper bort de oönskade fettsyrorna på det ursprungliga fettet. I det andra steget fungerar lipaset istället som ett lim som sätter fast de nya fettsyrorna för att ge ett fett med de önskade egenskaperna.



Lipaset agerar sax i första steget och lim i andra för att klippa bort de oönskade och sätta fast de önskade fettsyrorna på triacylglycerolen.

Arbetet i denna avhandling har genomförts i ett samarbete mellan Lunds Universitet och AAK. Vi har undersökt flera viktiga aspekter i utvecklingen av en process för produktion av STAGs. Först utvecklades en metod för att på ett effektivt och precist sätt analysera fetters sammansättning och därmed lipasets förmåga att producera den önskade produkten. Den nya metoden möjliggjorde utförandet av den efterföljande forskningen.

Lipaser är effektiva katalysatorer men mycket små och därför svåra att hantera. De är ofta också dyra och kan stå för en betydande del av produktionskostnaden. För att underlätta hanteringen och återanvändandet av lipaset och på så sätt sänka kostnaden kan man sätta fast dem på ett större bärarmaterial i en process som kallas immobilisering. Vi har undersökt hur olika bärarmaterial påverkar lipasets katalytiska förmåga för att försöka förstå vilket som är det bästa i just denna process. Mycket hydrofoba material, det vill säga material som repellerar vatten, visade sig vara bäst för att åstadkomma en hög grad av immobilisering och aktivitet av lipaset. Vi lyckades skapa nya immobiliserade lipaser med högre aktivitet och specificitet än vad som idag finns kommersiellt tillgängligt.

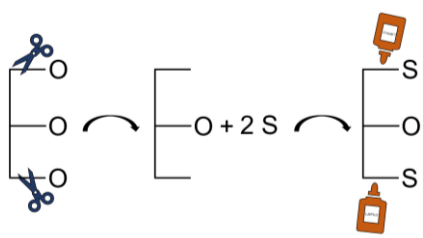
Många parametrar påverkar bildandet av den önskade produkten. I denna avhandling undersöktes bland annat effekten av vatten, temperatur och förhållande mellan det ursprungliga fettet och den nya fettsyran. Alla parametrar var viktiga och vi försökte finna den optimala kombinationen för maximal produktbildning på kortast möjliga tid. Målet var att skapa en så effektiv process som möjligt. Vi utvecklade en process som använder ett immobiliserat lipas från *Rhizopus oryzae* med hög aktivitet, högt utbyte och låg bildning av biprodukter. Detta resulterade i en exceptionellt hög produktrenhet och produktivitet jämfört med tidigare uppnådda resultat.

Sažetak na bosanskom jeziku

Gotovo svi prehrambeni proizvodi sadrže masti. Masnoća je hranljiva materija koja daje energiju i koja je potrebna za izgradnju i popravku ćelija, stvaranje hormona i apsorpciju vitamina rastvorljivih u mastima. Osim što je mast potrebna za zdravlje, ona doprinosi i ukusu, teksturi i konzistenciji proizvoda. Dobro poznati primjer za to je uloga masti u čokoladi. Čokolada dobija svoj sjaj, jasan zvuk pucanja kada se lomi i karakteristična svojstva topljenja tako da se topi u ustima ali i dalje pruža otpor pri žvakanju zahvaljujući jedinstvenim svojstvima kakao putera. Svojstva su kontrolisana sastavom masti. Masti se uglavnom sastoje od triacilglicerola, koje se sastoje od molekule glicerola sa tri masne kiseline. U zavisnosti od toga koje masne kiseline i gdje se na molekulu glicerola nalaze masne kiseline, mast će se ponašati na određeni način.

Unutar prehrambene i farmaceutske industrije postoji povećana potreba za mastima sa specifičnim svojstvima kako bi se zadovoljila potražnja za novim proizvodima sa pozitivnim zdravstvenim efektima. Nažalost, masti sa željenim svojstvima rijetko se nalaze u prirodi. Osim toga, očekuje se da će klimatske promjene negativno utjecati na poljoprivrednu produktivnost, što bi moglo dovesti do nedostatka sirovina s jedinstvenim svojstvima kao što je kakao puter za čokoladu. Da bi se zadovoljilo tržište, potrebno je iznači metodu za proizvodnju masti specifičnog sastava, a time i svojstava, takozvanih strukturiranih triacilglicerola (STAG).

STAG se stvaraju zamjenom jedne ili više masnih kiselina na molekulu masti drugom masnom kiselinom dok se ne postigne željeni sastav. To se najbolje radi sa enzimima. Enzimi su prirodni katalizatori koji ubrzavaju reakcije bez da se i sami troše. U prirodi postoji mnogo različitih enzima koji kataliziraju gotovo svaku vrstu reakcije koja se može zamisliti. Enzimi koji se zovu lipaze koristi se za proizvodnju STAG-ova. Prirodna funkcija lipaza je razlaganje masti i stoga su važne i u našoj probavi. Međutim, pod određenim uslovima, lipaze mogu izvršiti i obrnutu reakciju, odnosno izgraditi veze u mastima. Ovo se koristi tokom proizvodnje STAG-ova, koja se odvija u dvije faze. U prvom koraku, lipaza djeluje kao makaze i uklanja neželjene masne kiseline na izvornoj masti. U drugom koraku, lipaza djeluje kao ljepilo koje vezuje nove masne kiseline dajući mast sa željenim svojstvima.



U prvom koraku lipaza djeluje kao makaze kako bi odsjekla nezeljene masne kisiline sa triacilglicerola. U drugom korako lipaza djeluje kao ljepilo da prikaci nove masne kisiline.

Rad u ovoj doktorskoj tezi izveden je u saradnji između Lund univerzitet i firmi AAK. Istražili smo nekoliko važnih aspekata u razvoju procesa za proizvodnju STAG-ova. Prvo je razvijena metoda za efikasnu i preciznu analizu sastava masti, a time i sposobnosti lipaze da proizvede željeni proizvod. Nova metoda omogućila je provođenje naknadnih istraživanja.

Lipaze su efikasni katalizatori, ali vrlo maleni i zbog toga ih je teško rukovati. Često su i skupi i mogu činiti značajan dio cjene proizvodnje. Da bi se olakšalo manevriranje i ponovna upotreba lipaze i stime da se smanji trošak, mogu se pričvrstiti na veći materijal u procesu koji se zove imobilizacija. Istražili smo kako različiti materijali utiču na katalitičku sposobnost lipaze da pokušamo da shvatimo koja je najbolja u ovom konkretnom procesu. Utvrđeno je da su hidrofobni materijali, odnosno materijali koji odbijaju vodu, najbolji za postizanje visokog stepena imobilizacije i visoke aktivnosti lipaze. Uspjeli smo stvoriti nove imobilizirane lipaze s većom aktivnošću i specifičnošću od onih koji su trenutno komercijalno dostupni.

Mnogi parametri utiču na formiranje željenog proizvoda. U ovom radu, između ostalog, istražen je utjecaj vode, temperature i omjera između izvorne masti i nove masne kiseline. Svi parametri su važni i pokušali smo da pronađemo optimalnu kombinaciju za maksimalno formiranje proizvoda u najkraćem mogućem roku. Cilj je bio stvoriti što efikasniji proces. Razvili smo proces koji koristi imobiliziranu lipazu od *Rhizopus oryzae* s visokom aktivnošću lipaze, visokim prinosom i niskim stvaranjem nusproizvoda. To je rezultiralo u iznimno visoku čistoću proizvoda i produktivnost u odnosu na ranije postignute rezultate.

List of papers

The dissertation is based on the following papers, referred to by their Roman numerals in the text and provided at the end of the dissertation. Published papers are reprinted with the permission of their respective publishers.

I. **Non-aqueous reversed phase liquid chromatography with charged aerosol detection for quantitative lipid analysis with improved accuracy**

A. Causevic, K. Olofsson, P. Adlercreutz and C. Grey

Journal of Chromatography A, 1652 (2021) 46237

II. **Effects of lipase immobilization conditions and support materials on the production of structured triacylglycerols**

A. Causevic, K. Olofsson, P. Adlercreutz and C. Grey

(Submitted 2022)

III. **Impact of critical parameters influencing enzymatic production of structured lipids using response surface methodology with water activity control**

A. Causevic, E. Gladkauskas, K. Olofsson, P. Adlercreutz and C. Grey

Biochemical Engineering Journal, 187 (2022) 108610

IV. **Improved production of structured triacylglycerols by reaction condition control**

A. Causevic, K. Olofsson, P. Adlercreutz and C. Grey

(Manuscript)

My contribution to the papers

All work described in this dissertation was performed under the supervision of Dr. Carl Grey and Prof. Patrick Adlercreutz at the Division of Biotechnology and Dr. Kim Olofsson at AAK AB.

I. I planned and designed the study together with my co-authors. I carried out the experimental work and wrote the first draft of the manuscript. I revised the manuscript together with my co-authors.

II. I planned and designed the study together with my co-authors. I carried out the experimental work and wrote the first draft of the manuscript. I revised the manuscript together with my co-authors.

III. I, together with Carl Grey, supervised the master thesis of Eimantas Gladkauskas where he developed and programmed the first version of the water activity control system. I further developed and validated the water activity control system. I planned and performed the design of experiment trials and evaluated the data. I wrote the first draft of the manuscript and revised the manuscript together with my co-authors.

IV. I planned and designed the study together with my co-authors. I carried out the experimental work and wrote the first draft of the manuscript. I revised the manuscript together with my co-authors.

List of abbreviations

CAD	charged aerosol detector
DAGs	diacylglycerols
ECN	equivalent carbon number
FAEs	fatty acid esters
FFAs	free fatty acids
HOSO	high oleic sunflower oil
HPLC	high-performance liquid chromatography
MAGs	monoacylglycerols
NARP	non-aqueous reversed phase
OOO	triolein
PBR	packed bed reactor
RBR	rotating bed reactor
RF	response factor
RP	reversed phase
RSM	response surface methodology
SOO	1,2-olein-3-stearin
SOS	1,3-stearin-2-olein
SSO	1,2-stearin-3-olein
SSS	tristearin
STAGs	structured triacylglycerols
STR	stirred tank reactor
TAGs	triacylglycerols

1 Introduction

The oleochemical food industry is facing numerous challenges as an effect of climate change and shifting demographics. The change in the climate, with increasing global temperatures and variations in precipitation patterns, is affecting harvest yields, resulting in large product fluctuations and increasing prices (1–3). There is a constant concern about an inadequate supply of raw materials that are difficult to replace. Furthermore, an aging population, the rising prevalence of obesity and an increasingly health-conscious consumer population call for products with beneficial properties for new applications (4–6). Lipids with such desired properties are rarely found in nature. There is thus a great need for tools that enable the production of lipids with specific properties, also known as structured lipids.

Structured lipids are lipids with modified fatty acid composition and distribution resulting in the desired physical, chemical and/or nutritional properties. Modified triacylglycerols (TAGs) are more specifically referred to as structured triacylglycerols (STAGs). STAGs can be synthesized by chemical modification or enzyme technology. A comparison of chemical and enzymatic modification can be found in Table 1.

Table 1: Comparison of chemical and enzymatic modification of vegetable oils (7–9).

Conditions	Chemical modification	Enzymatic modification
Catalyst	Chemical catalyst (*e.g.*, sodium methoxide)	Lipase
Reaction conditions	Harsh reaction conditions (high temperature and low pressure)	Mild reaction conditions (low temperature and normal pressure)
Reaction	Random reaction	Random or specific reaction
Byproduct formation	High	Low
Sustainability	Low	High
Technical challenge	Extensive downstream processing is necessary, catalyst sensitive to substrate impurities	Low stability of catalyst
Cost	Lower catalyst cost, high energy cost	High catalyst cost

Chemical modification processes are well established within the oleochemical industry but have several drawbacks (7–9). Enzyme technology using lipases as catalysts has shown to be a better, safer and more environmentally friendly alternative to chemical catalysis (10). Compared to chemical catalysts, lipases are highly specific and selective. Lipases are active under mild conditions such as low temperature and pressure, reducing the destruction of temperature-sensitive

substrates and products. Processes using lipases also produce fewer byproducts giving improved product yields and quality.

STAGs have many applications in the food, medical and nutraceutical industries such as the production of cocoa butter equivalents and human milk fat substitutes. Furthermore, various health promoting STAGs with lower energetic value, faster energy release and increased amount of fatty acids with health benefits have been developed to meet the growing need for healthier foods and to prevent obesity (11–14). A number of these products are already implemented on an industrial scale. Human milk fat substitutes have been produced by Bunge Loders Croklaan (15) and Advanced lipids (16), a joint venture of AAK and IFF, under the names Betapol® and INFAT® for more than 30 years. Cocoa butter equivalents are manufactured by Fuji Oil among others and Healthy Resetta™ is a low-calorie fat produced by Nisshin Oillio since 2003. Fats for margarine and baked goods such as NovaLipid™ and SansTrans™ (17) produced by random modification of oils and fats using enzymatic methods are also commercially available (7).

Despite the long-standing industrial production of STAGs using enzymes, further implementation of industrial processes is limited by too high production costs. Furthermore, STAGs for food applications are rarely high-value products. For this reason, an economically feasible and successful production of these products requires a highly productive and efficient catalyst and optimized reactor system.

1.1 Aim and scope of the dissertation

The presented dissertation focuses on some of the key steps in the development of a lipase-catalyzed process for synthesis of STAGs from vegetable oils and fats. The dissertation addresses three major challenges: determination of the total lipid composition in complex oil samples, development of efficient immobilized lipase preparations and optimization of reaction parameters for improved STAG formation. Transesterification of high oleic sunflower oil (HOSO) and ethyl stearate for the synthesis of 1,3-stearin-2-olein (SOS) was used as a model reaction in all studies.

In **Paper I**, we present a non-aqueous reversed phase (NARP) high-performance liquid chromatography (HPLC) method with charged aerosol detection that can be used to separate and quantify different free fatty acids (FFAs), fatty acid esters (FAEs), monoacylglycerols (MAGs), diacylglycerols (DAGs) and TAGs, including regioisomers, using a reduced calibration approach.

Paper II evaluated the impact of immobilization conditions and support material on the immobilization yield, activity and regioselectivity of the immobilized lipase.

In **Paper III** the effect of water activity, temperature and substrate ratio on the product yield and quality was investigated. A water activity control system based on dry/humid nitrogen gas sparging was developed for control of water activity during enzymatic reactions in non-aqueous media.

Paper IV evaluated the effect of water activity, temperature, substrate ratio and ethanol concentration on the initial rate formation of intermediate and final products. Furthermore, the results obtained in **Papers III** and **IV** were utilized to improve the product yield in a SpinChem® rotating bed reactor by reaction condition control.

2 Lipid chemistry and analysis

2.1 Lipid chemistry

Lipids are a diverse group of organic compounds that are insoluble in water. In human nutrition, the most important lipids are TAGs (~95% of dietary fats) (18). TAGs are the main components in vegetable oils and fats, but lipids from other classes such as FFAs, MAGs and DAGs can also be found. MAGs, DAGs and TAGs contain one, two or three fatty acids, respectively, esterified to the hydroxyl groups of a glycerol molecule at distinct stereospecific positions denoted *sn*-1, 2 and 3 (Figure 1).

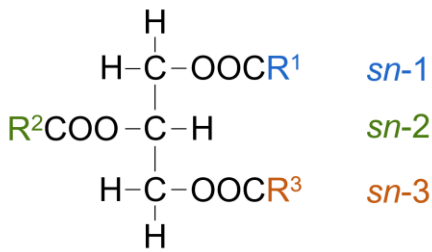


Figure 1: Triacylglycerol structure with different fatty acids, R^1, R^2 and R^3, at stereospecific positions *sn*-1, *sn*-2 and *sn*-3.

Fatty acids can differ in chain length described by their carbon number and the number of double bonds. The fatty acids can either be saturated (*i.e.*, having no double bonds), monounsaturated (*i.e.*, having one double bond) or polyunsaturated (*i.e.*, having two or more double bonds). Furthermore, the double bonds can differ in position on the carbon chain and have a *cis*- or *trans*-configuration. In *cis*-configuration the two adjacent carbon atoms are on the same side of the double bond, while in *trans*-configuration, the carbon atoms are on the opposite sides. The *cis*-configuration is found in almost all naturally occurring unsaturated fatty acids. Fatty acids commonly found in vegetable oils and fats, their abbreviations and melting points are presented in Table 2. The systematic names are based on the carbon number and number, position and configuration of the double bonds.

Table 2: Systematic and trivial names of common fatty acids with their abbreviations, carbon number (CN), number of double bonds (DB) and melting point (°C).

Systematic name	Trivial name	Abbreviation	CN:DB	Melting point (°C)
Hexanoic acid	Caproic		6:0	-3.4
Octanoic acid	Caprylic		8:0	16.7
Decanoic acid	Capric	C	10:0	31.6
Dodecanoic acid	Lauric	L	12:0	44.8
Tetradecanoic acid	Myristic	M	14:0	54.4
Hexadecanoic acid	Palmitic	P	16:0	62.9
Octadecanoic acid	Stearic	S	18:0	70.1
c-9-octadecenoic acid (n-6)	Oleic	O	18:1	16.3
c-9,*c*-12-octadecadienoic acid (n-6)	Linoleic	Li	18:2	-5.0
c-9,*c*-12,*c*-15-octadecatrienoic acid (n-3)	α-Linolenic	Le	18:3	-11.0
Icosanoic acid	Arachidic	A	20:0	76.1
Docosanoic acid	Behenic	B	22:0	80
c-13-docosenoic acid (n9)	Erucic	Er	22:1	33.8

The fatty acids can vary in the positional distribution on the glycerol backbone (*sn*-1, 2 and 3). This results in the formation of regioisomers, which is molecules that have the same functional groups but attached at different positions. The composition of saturated and unsaturated fatty acids and fatty acid chain length as well as the distribution of fatty acids within the TAG molecule affects the nutritional, physical and chemical properties. The human metabolism of fats is dependent on the fatty acid distribution due to the specificity of metabolic lipases (18). This is of special importance in infant nutrition where palmitic acid in the *sn*-2-position and unsaturated fatty acids in the outer positions of the glycerol backbone is essential for proper fat uptake (18, 19). Human milk fat has been found to contain up to 70 % of palmitic acid in the *sn*-2-position (20).

The fatty acid composition and distribution affect the physical properties of solid fats by changing the crystallization pattern and melting temperature. A higher degree of saturation and longer carbon chain length increases the melting temperature of TAGs. Furthermore, fats can solidify into more than one crystal form, α, β′ and β, with increasing stability and melting point, due to a more densely packed structure (21, 22). The crystallization rate and structure depend on the TAG regioisomers (23). Thus, the presence of the right regioisomer is highly important in *e.g.*, the production of cocoa butter equivalents (21). For cocoa butter equivalents, the desired product, SOS, has a melting point of 36.5-38.5 °C in β′-form. The regioisomer, 1,2-stearin-3-olein (SSO), crystallizes differently and has a melting temperature of 41.4-43.4 °C in the same crystal form (24). Thus, significant amounts of the undesired regioisomer will affect the product properties.

Due to the importance of fatty acid distribution, TAGs are named after the composition using the structure XYZ, where X is the FA in the *sn*-1-position, Y is the FA in the *sn*-2-position, and Z is the FA in the *sn*-3-position. However, since

most analysis methods do not distinguish between the *sn*-1 and *sn*-3 positions, TAG XYZ is considered to be the same as ZYX. Abbreviations used for fatty acid denomination are presented in Table 2.

2.2 Lipid analysis

Vegetable oils and fats contain several lipid classes that, in turn, consist of numerous lipid species. This results in a highly complex composition with a large variation in chemical structure. Due to the complexity, the qualitative and quantitative analysis of oils and fats constitutes a challenging problem. Traditionally, oil characterization has been performed by combining various methods, depending on the lipid classes investigated (25–27). However, this is often difficult, time-consuming and costly. There is thus a great need for a harmonized approach for the analysis of lipid species and regioisomers from several lipid classes.

2.2.1 Lipid separation

HPLC is the primary separation technique used to analyze lipids (26). The challenge of HPLC analysis of vegetable oils and fats is to obtain separation of the lipid species. Two modes of liquid chromatography can be used: reversed phase (RP) and NARP. RP- and NARP-HPLC are similar modes of operation, both working under the principle of hydrophobic interactions. The hydrophobic stationary phase of the column will form interactions with the hydrophobic sample components. Components with higher hydrophobicity will form stronger interactions and thus have longer retention times. The mobile phase is initially more polar but increases in hydrophobicity to alter the partitioning of the components between the stationary and mobile phase. In RP-HPLC the most polar component is water, while NARP-HPLC often uses solvents such as acetonitrile or methanol.

Using RP- (28, 29) and NARP-HPLC (30–34) the separation of acylglycerols (MAGs, DAGs and TAGs) with different carbon chain lengths and degree of unsaturation can be achieved. The separation and elution order is typically based on the concept equivalent carbon number (ECN), calculated as the total carbon number minus the number of double bonds of the fatty acids (35). For a long time, separation within an ECN group was not possible. However, using improved columns with higher separation power and optimized column temperature and mobile phase composition, separation of lipids within an ECN group (36, 37) and even a few regioisomers has been shown possible (38–40).

Other methods are generally used to analyze FFAs and FAEs. However, simultaneous analysis of different lipid classes using RP- and NARP-HPLC has been investigated. Most published methods have been able to separate MAGs,

DAGs, TAGs and either FFAs or FAEs, but no regioisomers (41–43). In **Paper I**, we present a NARP-HPLC method that can be used to analyze the complete lipid profile of FFAs, FAEs, MAGs, DAGs and TAGs, as well as a number of regioisomers, within 80 minutes (Figure 2). The lipids were separated using two 25 cm Nucleodur C18 ISIS columns connected in series and an optimized mobile phase gradient composed of acetonitrile, acetic acid, isopropanol and heptane (**Paper I**). The column temperature was found to affect the separation. A sub-ambient temperature gradient was implemented to separate the regioisomers and lipid species from different lipid classes. The temperature was kept at 9 °C for the first 18 minutes and then 15 °C for the rest of the run. While sub-ambient temperatures allowed for baseline separation of regioisomers, it had a negative impact on the peak shape. This resulted in longer retention times and broader peaks. Acylglycerols with longer and saturated fatty acids were more affected by the lower temperatures due to their hydrophobic character and low crystallization point.

The elution order of the lipids correlated to their ECN. For regioisomers having the same ECN, the elution order was decided by the distribution of the fatty acids on the glycerol backbone. An unsaturated FA in the *sn*-2-position resulted in an earlier elution, which is consistent with previously presented results (39, 44, 45). This could be explained by a weaker interaction with the stationary phase of an unsaturated fatty acid in the *sn*-2-position compared to the *sn*-1/*sn*-3-position. The FFAs, FAEs and MAGs eluted within the first 40 minutes, DAGs between 40-45 minutes and TAGs between 49-75 minutes (Figure 2). The method was used to separate 33 lipids from the different lipid classes (5 FFAs, 4 FAEs, 5 MAGs, 6 DAGs and 13 TAGs), including 7 regioisomeric pairs.

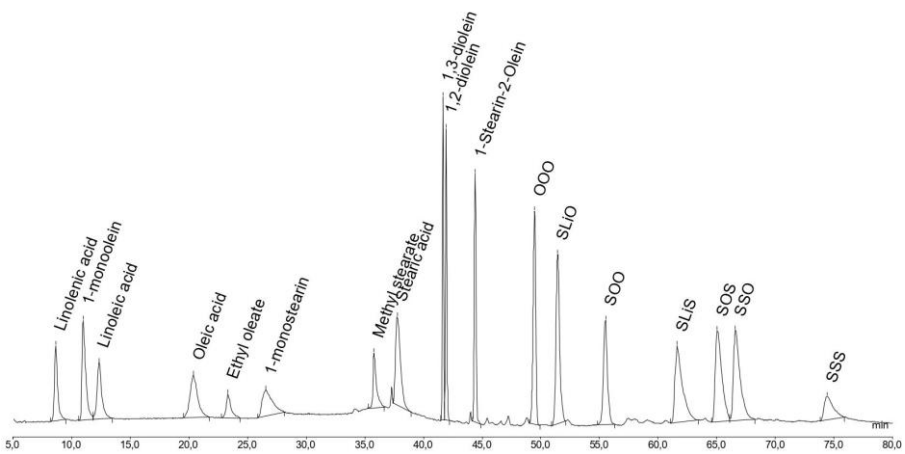


Figure 2: Representative separation of a mixture of lipid standards containing FFAs, FAEs, MAGs, DAGs and TAGs using the developed NARP-HPLC method with gradient elution, sub-ambient temperature gradient and charged aerosol detection (as described in **Paper I**).

2.2.2 Lipid quantification

The complex composition of vegetable oils and fats also complicates the quantification. The large variation in lipid structure between and within the lipid classes leads to significant differences in detector response with commonly used detectors such as UV, refractive index and mass spectrometry (26, 36, 46). The charged aerosol detector (CAD) is considered a universal detector as the response is independent of the analyte structure of non-volatile compounds. In the CAD the analyte molecules are firstly converted into dry particles. The number of particles increases proportionally with the amount of analyte. Secondly, the particles are charged by collision with charged nitrogen gas. Finally, the charged particle flux is measured by an electrometer, generating a signal in proportion to the quantity of the analyte. This results in a uniform response increasing with the amount of the analyte (46, 47).

The CAD is sensitive to changes in mobile phase composition resulting in response differences and loss in sensitivity. Using a second pump delivering the opposite mobile phase gradient to compensate for the change during gradient elution has been found to circumvent this issue in RP-HPLC (48–50). However, conflicting results have been obtained during NARP-HPLC. Lísa *et al.* (2007) found that the variation in response could be decreased to only 5% using mobile phase compensation, while we obtained no significant improvement for the developed method in **Paper I** (31). Large variations in response factor (RF) were obtained both with and without mobile phase compensation. The variation in RF was 2-3 times within each lipid class and at most 28 times when comparing all calibrated components (**Paper I**). The response was dependent on the peak shape. The peak shape was, in turn, influenced by the column temperature and the volatility of the components. Thus, the CAD response was greatly affected by the physical properties of the specific lipid regardless of the mobile phase composition.

For reliable quantification, calibration and determination of RFs must be performed for all components. This is tedious, expensive and might not be possible when standards are unavailable. Therefore, it is common in lipid analysis to neglect RFs and quantify the components based on the peak area relative to the total peak area (relative area). A lipid with a relative area of 25% would thus constitute 25% of the total sample. Quantification using relative area can result in large errors, depending on the detector used (**Paper I**) (36). Significant differences in quantification using RFs for all detected components (full calibration of 33 components) and relative area could be seen for a sample from enzymatic transesterification of HOSO and ethyl stearate using CAD (Figure 3). The largest difference was observed in the quantification of FAEs that were underestimated by a factor of 5.5. This can be explained by their higher volatility resulting in lower response in the detector. TAGs and DAGs were instead highly overestimated. The results emphasized the importance of RFs, but the calibration issues remained.

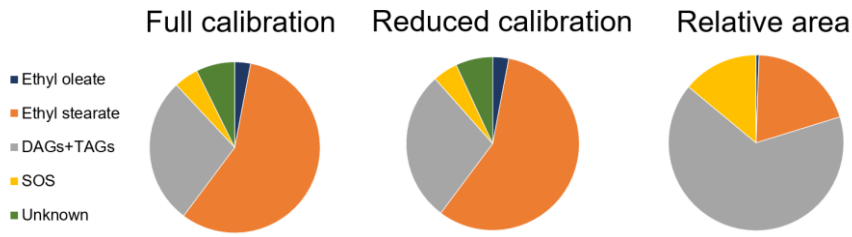


Figure 3: Composition of sample from enzymatic transesterification of ethyl stearate and HOSO (2 w/w) using Novozym® 40086. Comparison of three quantification approaches: full calibration with 33 components (right), reduced calibration with 6 components (middle) and relative peak area (right) as described in **Paper I**.

A reduced calibration approach was developed to circumvent the problems with calibration (**Paper I**). The approach uses the correlation between peak width at half peak height divided by the retention time (PW(50%)/RT) and RF to determine the RFs of non-calibrated components (**Paper I**). The reduced calibration approach was compared with full calibration of all components (Figure 3). Almost identical results were obtained with the reduced calibration approach, using only 6 components. The developed method and quantification approach was used to analyze and quantify the lipid composition of samples from several enzymatic transesterification reactions (**Paper I-IV**). Overall, the method significantly reduces the analysis time and cost compared to performing several methods for full characterization of oils and fats.

3 Lipases as catalysts

3.1 Lipases

Lipases (EC 3.1.1.3) are ubiquitous enzymes found in microorganisms, plants and animals. Lipases belong to a group of serine hydrolases that incorporate a catalytic triad of serine, histidine and aspartate or glutamate in their active site. Their natural function is to hydrolyze ester bonds of TAGs forming DAGs, MAGs, FFAs and glycerol. TAGs have low solubility in water and the reaction often occurs at an organic-aqueous interface. Interestingly, most lipases express higher catalytic activity under such conditions, a mechanism referred to as interfacial activation (51). The mechanism is based on the existence of a large hydrophobic pocket surrounding the active site to which the substrate can bind. In an aqueous solution, the active site of lipases is covered by an amphiphilic flexible peptide loop referred to as the lid. The lid isolates the active site from the environment, making the lipase inactive. Interaction with a hydrophobic phase can cause opening of the lid to make the active site accessible and activate the enzyme (52).

Structural determinations have found that the lid's size and appearance differ between lipases (53, 54). This can affect the ability to undergo interfacial activation. Lipase from *Rhizomucor miehei* has been found to have one lid covering the active site (51, 52, 55), while lipase from *Geobacillus thermocatenulatus* has a highly complex double lid (56). Both these lipases exhibit interfacial activation. However, lipase B from *Candida antarctica* has a small lid that does not fully seclude the active site from the medium, and this enzyme does not have the characteristic of interfacial activation (57).

Lipases are important industrial enzymes in the production of foods, biodiesel, pulp and paper, cosmetics, pharmaceuticals, surfactants and as additives in detergents (53, 58). They are excellent catalysts well suited for commercial applications for several reasons:

- Most lipases used for biotechnological applications are from bacteria (*e.g., Burkholderia cepacia*, *Pseudomonas fluorescens*) or fungi (*e.g., C. antarctica*, *C. rugosa*, *Thermomyces lanuginosus*, *Aspergillus niger*, *R. miehei*, *Rhizopus oryzae*). The lipases are commercially available in large quantities as they can be produced in high yields from natural or recombinant microbial organisms.
- Lipases have excellent regio- and stereoselectivity.

- Lipases can act under mild conditions such as low temperature, neutral pH and normal pressure, which makes them useful in reactions with sensitive substrates and products, results in minimal byproduct formation and a more sustainable process.
- Lipases are active in both aqueous and non-aqueous media, such as organic solvents (59) and ionic liquids (60).
- Lipases are "promiscuous" enzymes with the ability to catalyze alternative reactions that differ from their natural reactions and have a broad substrate specificity (61, 62).
- Lipases are robust enzymes with relatively high stability also in organic solvents.
- Microbial lipases do not require coenzymes.
- The crystal structure of several lipases has been solved. This facilitates protein engineering for the design of improved catalysts (63, 64).

Regioselectivity is the ability of the lipase to distinguish between the positions on the glycerol backbone. Lipases are divided into three groups based on their regioselectivity: *sn*-1,3-selective, *sn*-2-selective and non-selective (65, 66). The *sn*-1,3-selective lipases show a preference for the outer positions of the glycerol backbone and *sn*-2-selective lipases prefer the middle position, while non-selective lipases show no preference for any of the positions. The selectivity and substrate specificity of lipases is determined by the shape of the active site and its substrate binding sites (54). So far, no strictly *sn*-2-selective lipase has been identified, most likely due to steric hindrance in the active site (67). Many lipases instead show strong *sn*-1,3-selectivity, which is important in the modification of oils and fats for the production of STAGs. Lipase from the *Rhizopus* genus, *R. miehei, A. Niger* and *T. lanuginosus* are generally *sn*-1,3-selective.

The activity of most enzymes, including lipases, relies on the secondary and tertiary structure of their peptide chains and individual amino acid residues. The structures are determined by weak interactions such as hydrogen bonds as well as electrostatic and van der Waal forces (64). This makes enzymes sensitive to the conditions in which they are used. Lipases are relatively robust enzymes, but factors such as pH, temperature and organic solvent are still critical and can cause conformational changes resulting in inactivation. This potential disadvantage should be considered in the development of enzymatic processes.

In this dissertation, lipase from the two filamentous fungi *R. oryzae* (**Paper II**, **III**, **IV**) and *R. miehei* (**Paper I**, **II**) were studied. Lipase from *R. oryzae* and *R. miehei* have a relatively high sequence and structure similarity with a long and deep hydrophobic active site (54, 68). Both lipases are *sn*-1,3-selective and show a preference for medium to long-chain fatty acids. The lipases are broadly employed in the industry due to their high selectivity and broad substrate specificity resulting in the potential of synthesizing many different products (69, 70).

3.2 Catalytic mechanism of lipases

Lipases catalyze reactions in two steps referred to as the ping-pong bi-bi or substituted enzyme mechanism, shown in Figure 4. In the first step, the acyl donor binds to the active site. The acidic group of the aspartic acid in the catalytic triad forms a hydrogen bond with the imidazole ring of the catalytic histidine. The histidine becomes a general base that deprotonates the hydroxyl group on the catalytic serine. The deprotonated serine will act as a nucleophile and attacks the carbon of the substrate acyl group. This forms a negatively charged tetrahedral intermediate that is stabilized by the formed oxyanion hole. The serine forms an acyl-enzyme intermediate through an ester linkage between the enzyme and the acyl group of the substrate while at the same time releasing the first product. In the second step, the nucleophilic substrate (the acyl acceptor) is deprotonated by the catalytic histidine and attacks the acyl-enzyme intermediate. This breaks the acyl-enzyme complex and releases the second product.

Many different esters and acids can act as acyl donors due to the broad substrate specificity of the lipases. Likewise, several different hydroxyl-containing compounds, such as water, alcohols and DAGs, can take the role of the nucleophile and de-acylate the acyl-enzyme complex. This leads to the catalysis of many different reactions. In a reaction mixture with several potential acyl donors and nucleophiles, these will compete for reaction with the enzyme.

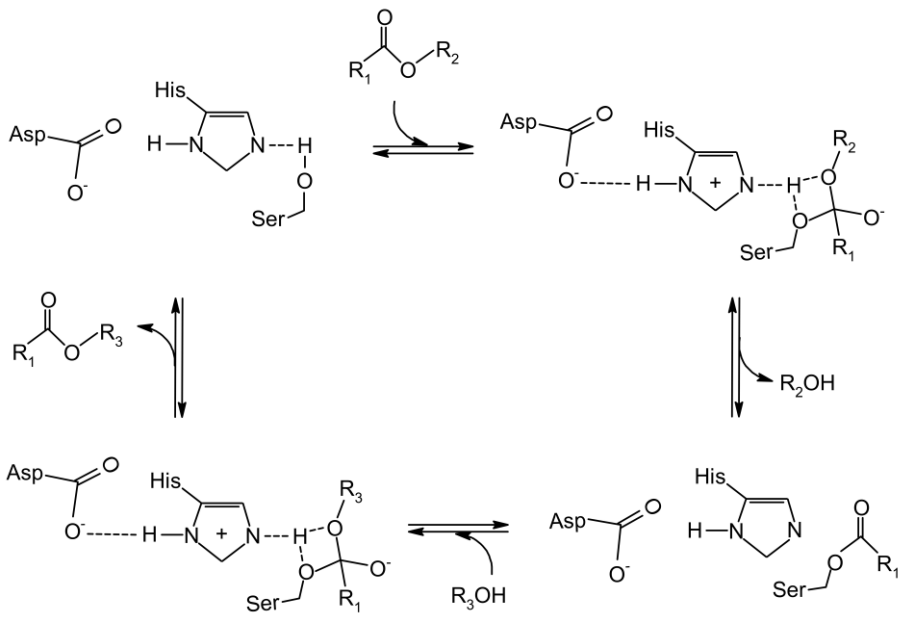


Figure 4: The catalytic mechanism of lipases.

The rate equation for the substituted enzyme mechanism in the absence of product is described in its simplest form by Equation 1, where v is the reaction rate, a and b are the concentrations of acyl donor and acceptor, respectively, and V_m and $K_{mA,B}$ are the kinetic constants. The kinetic constants represent the maximum velocity of the lipase (V_m) and the concentration of substrate required to obtain half the maximum velocity ($K_{mA,B}$). However, since both substrate and product can act as lipase inhibitors, the simplest form of the rate equation has been found to be a rather rough estimate of the lipase behavior. Rate equations, including the effect of inhibition, have been derived (71). These equations are highly complex, and the determination of the kinetic constants is often difficult.

$$v = \frac{V_m \cdot a \cdot b}{K_{mA} \cdot b + K_{mB} \cdot a + a \cdot b}$$

Equation 1: Rate equation for the substituted enzyme mechanism in the absence of products.

3.3 Lipase-catalyzed reactions

The natural reaction for lipases is to catalyze the hydrolysis of ester bonds between the fatty acids and the glycerol backbone of TAGs in the presence of water. However, in low water environments, lipases catalyze the formation of ester bonds through synthesis reactions, such as esterification, alcoholysis, acidolysis and transesterification (Figure 5). Esterification is the reversed reaction of hydrolysis, often referred to as reversed hydrolysis. In esterification, an ester bond is formed by a condensation reaction while water is released. Esterification is used to produce esters for different applications, such as flavor and aroma esters for the food and fragrance industry (72).

Alcoholysis is similar to hydrolysis but the water molecule is replaced with alcohol. This is utilized in the production of new esters from ester substrates by exchanging the alcohol moiety. Alcoholysis can be used in the production of MAGs (73, 74) and biodiesel (75, 76), where the ester substrate is a TAG. Alcoholysis of TAGs produces partial acylglycerols (DAGs and MAGs), glycerol and FAEs depending on the selectivity of the lipase, isomerization of DAGs and MAGs and the extent of the reaction

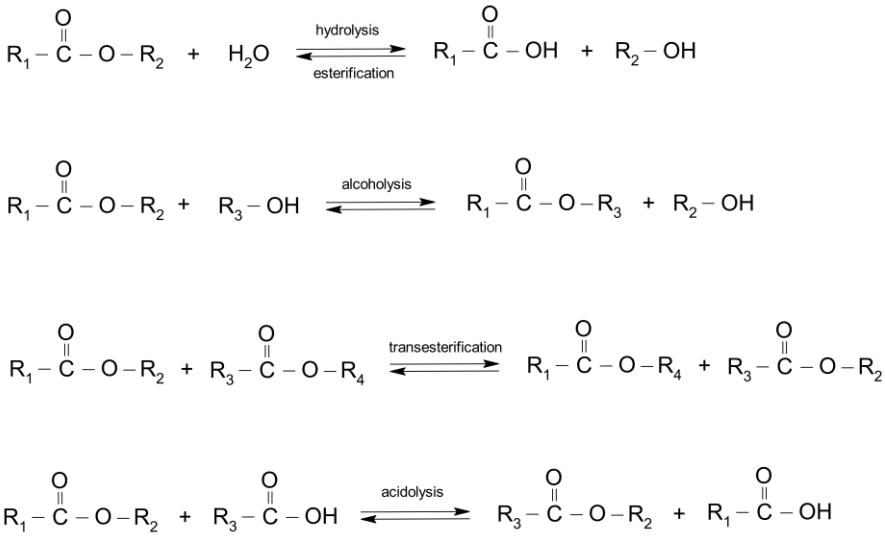


Figure 5: Lipase-catalyzed reactions in non-aqueous media.

Acidolysis is the exchange of the fatty acid moiety between a TAG and FFA, while transesterification is the exchange of the fatty acid moiety between two esters. Acidolysis and transesterification can be used to produce STAGs, depending on the type of substrates available. The production of STAGs by acidolysis and transesterification will be discussed in Chapter 4.

3.4 Effect of water on lipase activity

Water is an important factor in enzymatic reactions as it is a substrate in hydrolysis, but acts as a competing nucleophile in reversed hydrolysis. The amount of water will thus affect the reaction rates and the equilibrium position of hydrolysis and reversed hydrolysis. Furthermore, many enzymes require a certain amount of water to maintain their active conformation and optimal activity (77–79). It has been suggested that water acts as a molecular lubricant for enzymes by enabling proper movement and interaction of different parts of the protein.

The activity of enzymes in non-aqueous media was found to correlate best with the amount of water bound to the enzyme (80). However, this is difficult to both set and measure. The use of water activity to quantify water in non-aqueous media was proposed since it correlates well with the amount of water on the enzyme (81–83). Water activity is defined as the partial pressure of water in a solution divided by the vapor pressure over pure water at a given temperature. The water activity ranges from 0 in a completely dry system to 1 in a water-saturated system. At equilibrium,

the water activity is equal in all phases and can thus be measured in any phase. The major advantage of water activity is that the optimal level for a given reaction is almost the same in different solvents, while the water concentration varies greatly due to differences in solubility (81, 82). In the same way, the use of water activity can reduce the effects on water dependence of immobilization supports with different water absorbing capacity (82). Furthermore, the enzyme activity versus water activity profile has been found to be independent of the reaction (84). This implies that the water activity optimum is an intrinsic property of the specific enzyme (85). Methods for control of water activity during enzymatic reactions will be discussed in Chapter 6.

The optimal water activity varies greatly between lipases. Some lipases, such as lipase from *B. cepacia* and *Pseudomonas* species, work well at water activities close to 1 (86, 87). Other lipases, including lipase from *C. rugosa* exhibit the highest activity at intermediate hydration levels (86, 87). While some, such as lipase from *R. miehei* and lipase B from *C. antarctica*, work optimally in very dry conditions.

The water activity optimum for immobilized lipase from *R. oryzae* was determined in an alcoholysis reaction at 40 °C in **Paper IV**. The water activity optimum was found to be 0.3 (Figure 6). Interestingly, only a small difference in activity was observed in the range of 0.1-0.5. This correlates with previously presented results that lipase from *Rhizopus* species express maximal catalytic activity at water activities between 0.2-0.35 but maintain activity at very low water amounts (86). This rather unique feature of many lipases emphasizes why they are excellent catalysts for synthesizing reactions in non-aqueous media.

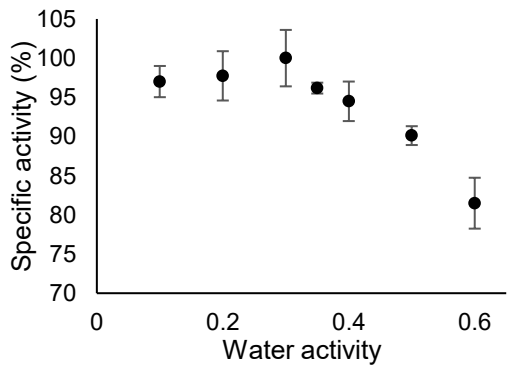


Figure 6: Water activity profile at 40 °C for immobilized lipase from *R. oryzae* (Lipase DF "Amano" IM). The evaluated reaction was the alcoholysis of methyl laurate and 1-octanol, producing octyl laurate (**Paper IV**).

The water activity optimum appears because of the dual effect of water. On the one hand, some water is essential for the enzyme's function. The increase in activity up to the water activity optimum could be a general activation of the lipase due to

increased internal flexibility and facilitated proton conductivity in the active site (79). On the other hand, high water activities shift the equilibrium position towards hydrolysis, as water outcompetes the nucleophile (88, 89). In addition, water accumulation on the lipase surface can create a diffusion barrier for hydrophobic substrates (88). The substrate can then not reach the active site and reduced catalytic activity is observed.

4 Synthesis of structured triacylglycerols using lipases

4.1 Acidolysis

Acidolysis is the reaction between FFAs and TAGs for the exchange of the fatty acid moiety. This is a two-step reaction involving hydrolysis and esterification. In the first step, TAGs act as acyl donors and are hydrolyzed by water for the formation of DAGs. In the second step, the formed DAGs act as nucleophiles and are esterified with the added FFAs. Both reaction steps are reversible, and the equilibrium constant will govern the yield. A high excess of FFA must be used to achieve a high degree of incorporation. Factors such as temperature, reaction time, water content and enzyme load are also important for acidolysis (90–94).

Acidolysis is the most used approach for the production of STAGs and numerous studies have been published (95–98). FFAs are basic oleochemicals produced by chemical hydrolysis of TAGs at high a temperature and pressure. This is followed by the removal of the formed glycerol. Using FFAs as acyl donor is thus beneficial due to their easy availability and low price.

4.2 Transesterification

Transesterification is the exchange of the fatty acid moiety between two esters. For STAG production this includes the reaction of a TAG and FAE or two TAGs, often referred to as interesterification (99, 100). In this dissertation, the transesterification between a TAG and FAE has been studied. This is, just like acidolysis, a multistep reaction with DAGs formed as intermediate products.

The transesterification of HOSO and ethyl stearate for SOS production will be used to explain the synthesis of a symmetrical STAG. A reaction scheme of the complete reaction is presented in Figure 7. The total fatty acid composition of the HOSO used in **Papers I-IV** was 82% oleic acid, 8.9% linoleic acid, 4.2% palmitic acid and 2.8% stearic acid. Furthermore, oleic acid constituted approximately 88% of all fatty acids in the *sn*-2-position, making triolein (OOO) the main TAG (66%). The HOSO composition will therefore be simplified to OOO in the reaction scheme (Figure 7).

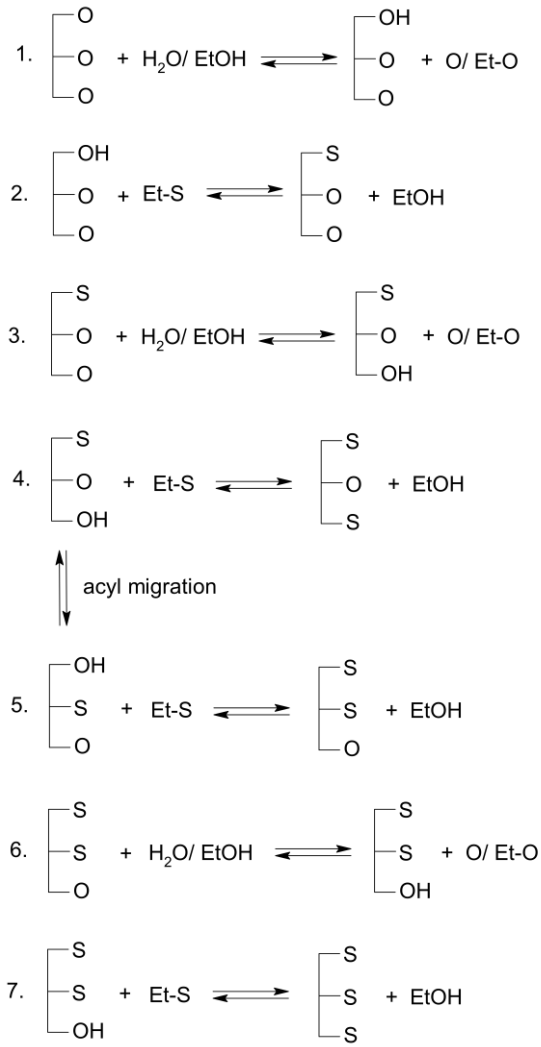


Figure 7: Reaction scheme for possible reactions during transesterification of HOSO (OOO) and ethyl stearate for production of SOS. Abbreviations: EtOH – ethanol, O – oleic acid, S – stearic acid, Et-O – ethyl oleate, Et-S – ethyl stearate.

The transesterification reaction is initiated by the formation of a DAG from a TAG (acyl donor) (reaction 1, Figure 7). In the second step, the DAG act as a nucleophile forming a new TAG while alcohol is released from the FAE (acyl donor) (reaction 2, Figure 7). DAGs are initially formed by hydrolysis with the release of FFAs. However, due to the formation of alcohol from the FAEs, DAGs can also be formed through alcoholysis. The reaction progresses through a combination of hydrolysis, esterification and alcoholysis. For the synthesis of a symmetrical TAG, the first and second steps must be performed two times, with both DAGs and TAGs formed as

intermediate products. This means that a minimum of four reactions must occur to form the desired product (reactions 1-4, Figure 7).

Transesterification of a TAG and FAE is a complex reaction system due to the many potential acyl donors and nucleophiles and the reversibility of all reactions. This can result in many competing and unproductive reactions. The reaction conditions should be optimized to minimize unproductive reactions for efficient product synthesis. Water activity, temperature, substrate ratio and addition of ethanol were found to significantly affect the transesterification reaction of HOSO and ethyl stearate (**Paper III, IV**). A detailed discussion of the effect of different parameters can be found in section 4.4.

The advantage of using FAEs as acyl donor is their lower melting point and higher volatility compared to FFAs. This allows for lower reaction temperatures and easier separation of the FAEs by distillation. Furthermore, using FAEs has resulted in higher reaction rates (101, 102) and fewer side reactions, such as acyl migration (102). The disadvantage of FAEs is that they must be chemically or enzymatically synthesized. This result in higher substrate costs compared to FFAs.

4.3 Acyl migration

The formation of DAGs during acidolysis and transesterification is essential for the progress of the reactions. However, DAGs are subject to something called acyl migration that can lead to the loss of positional specificity and byproduct formation. Acyl migration is the spontaneous intra-molecular transfer of a fatty acid from one hydroxyl group to an adjacent free hydroxyl group. The proposed mechanism is presented in Figure 8 (103–106). In the first step, the free hydroxyl group in the *sn*-1(3)-position performs a nucleophilic attack on the carbonyl carbon in the *sn*-2-position. This forms a five-membered intramolecular cyclic intermediate. In a second step, the intermediate hydroxyl oxygen attacks the carbon again, resulting in the opening of the cyclic intermediate and migration of the fatty acid moiety to the *sn*-1(3)-position. The mechanism of acyl migration is believed to be the same for MAGs.

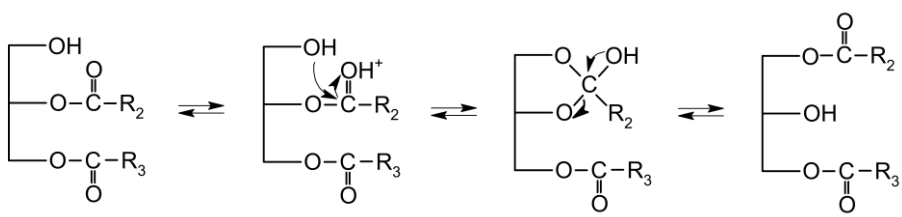


Figure 8: Mechanism of acyl migration of a diglyceride.

Acyl migration from *sn*-2- to *sn*-1(3)-position is favored due to the higher thermodynamic stability in the outer positions of the glycerol. Therefore, the isomerization equilibrium is pushed to the more thermodynamically stable 1,3-DAG and 1(3)-MAG (103, 105, 106). The equilibrium state of a pure DAG and MAG mixture contains about 60% 1,3-DAG and 90% 1(3)-MAG, respectively. The MAGs are clearly more sensitive to acyl migration and have been found to have a higher acyl-migration rate (106, 107).

Several studies have evaluated factors that promote acyl migration. The reaction is found to be catalyzed by high temperature, solvents, acid, bases and different materials such as silica gel (**Paper III**) (75, 103–105, 108). The migrating group also influences the rate of acyl migration. Short-chain fatty acids migrate faster than long-chain fatty acids (109) and unsaturated fatty acids move faster than saturated fatty acids (106).

Acyl migration is a big issue in selective lipid modification, such as the synthesis of STAGs, and must be minimized to produce the desired product. In the reaction of HOSO and ethyl stearate, acyl migration leads to the formation of SSO and tristearin (SSS) (reactions 5-7, Figure 7). These byproducts are highly undesired due to the loss of yield and their negative impact on the crystallization properties of the product. A commonly used measure of product quality is the ratio SOS to SSO. A ratio above 100 is desired to avoid any impairment of the crystallization properties.

4.4 Factors affecting enzymatic transesterification

The production rate, product yield and purity of STAGs produced by enzymatic transesterification will depend on many parameters, such as temperature, water activity, substrate ratio, reaction time and enzyme load. The effects can be evaluated using traditional approaches, investigating one factor after the other, or simultaneous evaluation using statistical methods. Statistical tools such as response surface methodology (RSM) make it possible to evaluate the single and combined effects of each factor on the response while reducing the number of experiments (110). At the same time, RSM provides sufficient information for statistically acceptable results, estimates the experimental error and simplifies outlier determination (111).

Models describing the response as a function of the factors are developed within the experimental space (*i.e.*, the evaluated range of the factors). The influence of the factors can be estimated from the regression coefficients of the models. Furthermore, the developed models can be used to predict the observed response in points within the experimental space where no experiments were performed or to optimize the responses based on the evaluated factors. Choosing an appropriate experimental design can reduce the experimental time and cost, which explains the

general use in research and development (91–94, 108, 112, 113). RSM was used in **Papers III** and **IV** to evaluate the effect of water activity, temperature, substrate ratio and ethanol concentration on the transesterification of HOSO and ethyl stearate.

4.4.1 Water activity

The importance of water on the lipase activity in non-aqueous media has been discussed in section 3.4. Water acts as a substrate in hydrolysis and is essential for optimal enzyme activity. In the transesterification reaction, water is needed in the initial stage to form DAGs. As expected, a higher water activity increases the DAG formation (**Paper IV**) (114). The DAGs are necessary intermediate products for the production of the final product, and the DAG content positively correlates with the incorporation of the desired fatty acid (**Paper IV**) (114). The reason for the positive effect of increased water activity on the product yield in Figure 9 is thus an increased hydrolysis rate and DAG formation. However, the water activity only has a positive effect up to a certain point, as seen by the water activity optimum (Figure 9).

There are many possible reasons for the decreased product yield at higher water activities. As seen in the water activity profile for Lipase DF "Amano" IM (Figure 6, section 3.4), the lipase activity decreases above water activity 0.3. This can be due to the accumulation of water on the enzyme surface and/or an increased hydrolysis rate. The amount of water present in the system controls the equilibrium between DAGs and TAGs. A higher water activity will thus push the equilibrium towards DAG formation and reduce the final product yield (**Paper III**) (115). Furthermore, the stability of enzymes has been found to decrease with higher water activity due to increased flexibility of the enzyme (77, 116–120). Loss of activity could also explain the decreased yield at higher water activities.

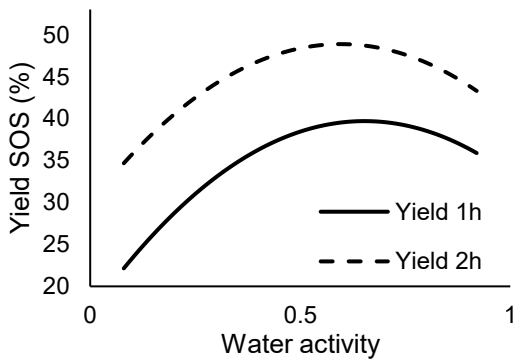


Figure 9: Effect of water activity on SOS yield after 1 and 2 h of reaction using Lipase DF "Amano" IM. Adapted from **Paper III**.

Ideally, the water activity should be higher initially to promote DAG formation and decreased at the end to promote acylation of the DAGs and push the equilibrium towards TAG formation. This would maximize the initial reaction rate and increase the final product yield and quality. In **Paper IV** we performed transesterification reactions of HOSO and ethyl stearate with water activity control. The initial DAG formation was increased 2.6 times by increasing the initial water activity from 0.3 to 0.65. In addition, the initial SOS formation increased by 10%. The reaction with the higher water activity was subjected to drying after 4 h, to remove the water. This resulted in a final DAG content of 3.1% compared to 7.9% for the reaction performed at water activity 0.3 (7 h of reaction). The same effect of drying was observed by Irimescu *et al.* (2000) (102). It can be concluded that drying the reaction solution is a successful method for reducing the final DAG content.

The effect of water activity on acyl migration during transesterification has been evaluated in numerous studies (**Paper III**) (107, 108, 113, 115, 121). The results are inconclusive as the different studies found that high water activity reduced acyl migration (107, 115) or enhanced acyl migration (108, 113, 115, 121). In addition, we found that water activity had a negative effect on the product quality by enhancing acyl migration after reaction equilibrium was reached (**Paper III**). Higher water activity has been suggested to reduce acyl migration by increasing the polarity of the reaction medium and thereby reducing the stability of the intramolecular cyclic intermediate formed during acyl migration (107). At the same time, a higher water activity increases hydrolysis and the DAG formation (**Paper III**). The DAG content should correlate with acyl migration (see mechanism in Figure 8) and has also been confirmed experimentally during transesterification (114). Thus, higher water activity can indirectly promote acyl migration by forming more DAGs. It can be concluded that water activity is a key parameter factor during transesterification as it affects the initial activity, final product yield and product quality.

4.4.2 Temperature

The reaction temperature increases the enzyme activity by increasing the flexibility of the lipase. A rule of thumb is that doubling the temperature will result in a 10-fold increase in activity. Higher reaction temperatures are also beneficial as it increases the solubility of most substrates and is crucial to ensure that saturated lipid substrates are entirely liquefied to yield a homogenous substrate mixture. Considering this, the use of high temperatures should be economically advantageous. However, thermal inactivation at elevated temperatures is the most critical mode of enzyme inactivation, resulting in the loss of activity (122). An increase in reaction temperature will thus have a positive effect on the enzyme activity but only until enzyme inactivation starts to dominate (92, 123, 124).

The effect of thermal inactivation was observed in the transesterification reaction of HOSO and ethyl stearate for immobilized lipase from *R. oryzae* (Lipase DF "Amano" IM) (**Paper III**). As expected, an increase in reaction temperature positively affected the activity resulting in a higher product yield but only up to a certain point. Above 57 °C the enzyme was subjected to inactivation already after 1 h of reaction. This resulted in a decrease in product yield and the formation of a temperature optimum (Figure 10). The obtained temperature optimum is dependent on the reaction time due to the combined effect of temperature and enzyme inactivation (**Paper III**) (124).

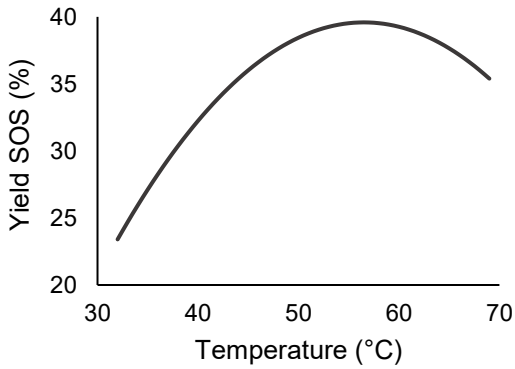


Figure 10: Effect of temperature on SOS yield after 1 h of reaction using Lipase DF "Amano" IM. Adapted from **Paper III**.

Temperature also strongly affects acyl migration during enzymatic transesterification (**Paper III**) (93, 107, 108). Acyl migration is a chemical reaction that is considerably more temperature dependent than the enzymatic reaction. Therefore, a decrease in temperature should be beneficial to suppress acyl migration for higher product quality (**Paper III**) (108, 125).

In the case of transesterification for STAG production, the temperature should be kept as low as possible to increase the operational stability of the lipase and reduce the acyl migration to obtain a high-quality product. However, the lowest temperature possible will be limited by the melting point of all substrates and products as they must be in liquid form. Using substrates with lower melting points, such as FAEs instead of FFAs, is thus beneficial. For this reason, ethyl stearate was used instead of stearic acid for SOS synthesis in **Papers I-IV**, lowering the melting point by more than 35 °C.

4.4.3 Substrate ratio

The substrate ratio between the two substrates is of major importance in obtaining the desired product. The substrate ratio controls the equilibrium position of the reactions and, thus, the maximal product yield. A high substrate ratio, *i.e.*, excess of FAEs, will result in a higher degree of incorporation of the desired fatty acid (**Paper III**) (13).

Excess of one substrate also shifts the equilibrium towards synthesis and increases the reaction rate. This was observed for the formation of SOS in **Paper IV**. However, both substrates act as acyl donors and will compete to acylate the enzyme. With an excess of FAEs, this will acylate the enzyme more often and outcompete the TAG. This will result in a reduced DAG formation and negatively affect the product formation rate at too high ratios (Figure 11). The highest formation rate of symmetrical STAGs should occur when neither of the intermediate products are limiting and all four necessary reactions proceed at the same and highest possible rate. An optimal substrate ratio must therefore exist.

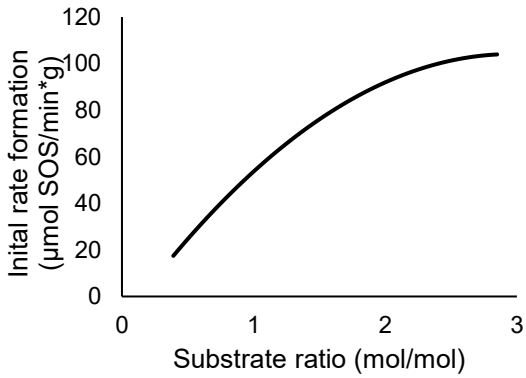


Figure 11: Effect of substrate ratio (FAE/TAG) on initial rate formation of SOS. Adapted from **Paper IV**.

The use of FAEs results in the release of alcohol during transesterification. An increase of the FAEs will thus result in more ethanol release. In **Paper IV**, it was found that an increase in substrate ratio above a certain level resulted in an increased DAG formation. The surprising effect was reasoned to be a result of the released ethanol. The ethanol boosted the DAG formation by acting as a highly potent nucleophile in the de-acylation of the acyl-enzyme complex. This suppressed hydrolysis and promoted DAG formation via alcoholysis, also observed in the high and instant formation of ethyl oleate. A faster formation of the intermediate DAG products will promote the synthesis of the final product as long as the alcohol does not outcompete the DAG as nucleophile. The release of ethanol from the FAEs is likely the reason for the higher reaction rates when using FAEs instead of FFAs, previously observed (101, 102).

The benefits of high substrate ratio are many, but a number of drawbacks can be identified. After the reaction the excess substrate must be removed to obtain the product. In an industrial process, the viable substrate ratio will be limited by the cost of the substrate and the ease and cost of separation. Recirculation can improve the efficiency of the process but can result in large circulation streams and require larger equipment. The chosen substrate ratio will be a compromise between product yield and productivity per volume of processed substrate.

4.4.4 Ethanol addition

Ethanol is formed as a byproduct in the transesterification reaction between TAGs and fatty acid ethyl ester. In **Paper IV** the results implied that the released ethanol was a better nucleophile than water in the formation of DAGs. Furthermore, MAG production is usually performed by alcoholysis using ethanol as it gives higher reaction rates and yields (73, 126). It was hypothesized that the addition of ethanol could increase the initial formation rate of DAGs and thereby the total reaction rate of the transesterification reaction for STAG production.

A study was performed to compare the suitability of ethanol and water as nucleophiles in the formation of DAGs. From the obtained apparent maximal velocity (V^{app}) and Michaelis constant (K_{mB}^{app}) it could be concluded that ethanol is a better nucleophile than water (Table 3). The major problem with water is the very low solubility in oil. Even at a concentration corresponding to the obtained K_{mB}^{app} the oil would be over-saturated, resulting in a two-phase system. This implies that one-phase reactions with water operate at velocities far from the maximum.

Table 3: Apparent maximal velocity (V^{app}) and Michaelis constant (K_m^{app}) for hydrolysis and alcoholysis with ethanol.

Reaction	Substrate A	Substrate B	V^{app} (U/g)	K_{mB}^{app} (mol/L)
Hydrolysis	HOSO	Water	1030	0.154
Alcoholysis	HOSO	Ethanol	1580	0.068

The main advantage of ethanol as nucleophile is the possibility to use higher concentrations due to the higher solubility in oil. This results in higher alcoholysis rates. However, one major drawback of ethanol is that it can cause inhibition of the enzyme. The inhibitory effect of ethanol and other alcohols has been shown in several studies (127, 128). Inhibition occurs when the ethanol binds to the enzyme and forms a non-productive enzyme-substrate complex. This is visualized by the decreasing specific activity at higher ethanol concentrations in Figure 12. It is essential to keep the concentration below this inhibitory level for optimal enzyme utilization.

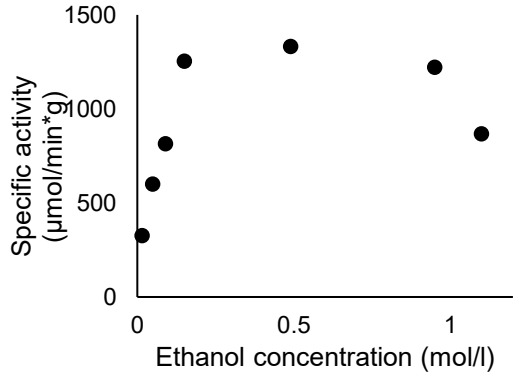


Figure 12: Effect of added ethanol on the enzymatic activity in the alcoholysis of HOSO using Lipase DF "Amano" IM as catalyst. Specific activity (U/g) determined at different ethanol concentrations (mol/L) and constant concentration of HOSO, 55 °C and water activity 0.15.

The effect of ethanol addition on the transesterification reaction for STAG production was evaluated in **Paper IV.** The water activity was equilibrated to the water activity optimum of the lipase, while the ethanol concentration and substrate ratio were varied. The addition of ethanol resulted in significantly higher initial DAG formation compared to hydrolysis. As expected, the DAG formation also increased with an increasing ethanol concentration. However, the SOS formation decreased in the reactions with added ethanol. The reason was that the ethanol outcompeted the DAGs as nucleophile in the de-acylation of the substrate-enzyme complex. Furthermore, the large formation of both DAGs and MAGs increased the risk of acyl migration and byproduct formation. The ethanol amount will also affect the equilibrium position between DAGs and TAGs and thereby the final product yield. In conclusion, the added ethanol amount must be optimized to balance the two reaction steps and potentially increase the rate of the total transesterification and to avoid excessive DAG formation.

5 Lipase immobilization

The natural environment of enzymes, including lipases, is aqueous. The strong interactions between the water and the hydrophilic enzyme surface make the enzyme soluble in an aqueous environment. However, in non-aqueous media, the interactions between the hydrophilic groups and the solvent are lost, making the enzyme insoluble. Therefore, adding lipases to non-aqueous media will cause the enzyme molecules to aggregate into a solid phase. The lipase will act as a heterogeneous catalyst where the catalyst and the substrate are in two distinct phases. The lipase molecules on the aggregate surface will be exposed to the substrate and could express high activity. However, the substrate concentration in the core will drop due to mass transfer limitations, resulting in an overall reduced lipase activity compared to homogenous catalysis in water. The use of the enzyme will be highly unproductive (82, 129).

Immobilization on a support limits the impact of these issues by spreading the enzyme on a large surface. This prevents the enzyme from aggregating and reduces mass transfer limitations. Furthermore, immobilization increases the reusability due to facilitated separation from the reaction mixture, enables the use in continuous processes and increases the operational stability of the enzyme (89, 122, 130–133). The lipase cost is often of high importance for the process economy and can thus significantly be improved by immobilization. Facilitated separation of the enzyme also prevents contamination of the final product. This is important in cases where enzymes are unacceptable, such as in the production of foods and pharmaceuticals.

Although beneficial in most cases, a few disadvantages of enzyme immobilization have been identified. Firstly, immobilization can cause conformational changes to the enzyme, which can alter or even remove its catalytic activity (89, 131–134). Secondly, mass transfer limitations, inherent to heterogeneous catalysis, can also decrease the observed reaction rate. The mass transfer limitations will highly depend on the enzyme loading and activity, particle size and rate of substrate diffusion through the particle (135–137). Furthermore, immobilization on different supports has been found to reduce the regioselectivity of the lipase by enhancing acyl migration (115, 138, 139).

Characteristics such as catalytic activity, stability, and regio- and substrate selectivity will be related not only to the intrinsic properties of the lipase but to the preparation as a whole when the lipase is used in an immobilized form. Thus, no

universal immobilization strategy works in all situations, and it is essential to include the choice of support and preparation of the immobilized lipase in the optimization of the process in which they are used.

5.1 Immobilization strategies and choice of support material

Various lipase immobilization strategies for non-aqueous media have been developed, including adsorption, deposition, covalent binding, entrapment and cross-linking (89, 134, 136). Adsorption by hydrophobic interactions between the hydrophobic areas of the lipase and the hydrophobic support is a frequently applied method. Hydrophobic interactions are relatively weak forces, but in non-aqueous media the risk of enzyme leakage is low since the lipase generally is insoluble in the reaction solution. Covalent binding can reduce the risk of leakage by creating a more stable link between the lipase and the support. However, this often causes lipase inactivation due to multipoint attachment and conformational changes (89, 131). The choice of immobilization strategy will thus depend on the risk and consequence of enzyme leakage and the desire to maintain as much catalytic activity as possible.

Many factors must be considered when choosing immobilization support. The support characteristics can significantly affect the process set-up and economy. The support's mechanical resistance may condition the reactor where the preparation can be used. In addition, properties such as particle size and uniformity can affect the packing and flow properties in continuous processes and separation from the reaction medium in batch processes. The support will increase the final cost of the biocatalyst, and the cost should also be considered during catalyst development.

5.1.1 Immobilization by adsorption

Adsorption to hydrophobic supports is perhaps the simplest method for immobilization of lipases. Lipases dissolved in an aqueous solution will spontaneously be adsorbed by hydrophobic interactions between the hydrophobic areas on the lipase and the support. Large hydrophobic areas on the protein surface are unique for lipases and will often result in a simultaneous purification and immobilization of lipases from other proteins (140). This is reflected in a higher activity immobilization yield compared to the protein immobilization yield for commercial lipase preparations containing many different proteins (**Paper II**).

Adsorption allows for gentle immobilization under mild conditions with high retention of the activity. It is believed that contact with the hydrophobic support causes interfacial activation of the lipase (*i.e.*, opening of the lid covering the active

site) and immobilization of the lipase in its active conformation (141, 142). This results in hyperactivation of the lipase.

Common materials used for hydrophobic supports are polyethylene, polypropylene, styrene and acrylic polymers. However, a range of supports with varying degrees of hydrophobicity have been developed and evaluated for lipase immobilization (**Paper II**) (138, 141, 143–147). The specific activity was found to increase with enhanced surface hydrophobicity due to greater hyperactivation of the lipase (**Paper II**) (141, 143, 148).

Adsorption by hydrophobic interactions is reversible. This can be both an advantage and disadvantage. The disadvantage is the risk of enzyme leakage from the support, however low in non-aqueous media. The advantage of reversible immobilization is the possibility of reusing the support. When the catalytic activity of the preparation becomes too low, it can be regenerated using detergents. Detergents break the hydrophobic interactions between the lipase and the support but leave the support material unchanged for immobilization of new batches of lipase (132, 145, 147). Reuse decreases the cost of new support and reduces waste. This allows for the use of more expensive supports with favorable properties and increases the sustainability of the process.

5.1.2 Immobilization by covalent binding

Immobilization by covalent binding is a common method for enzyme immobilization. Covalent binding couples the functional groups present on the enzyme surface to reactive groups on the surface of the support by irreversible bonds. Multiple attachments between the support surface and the enzyme are often created. This results in activity loss during the immobilization but gives a preparation with high stability and minimal risk of enzyme leakage even under harsh conditions (89, 131).

Immobilization by covalent binding can be tedious and difficult as it, in most cases, requires several steps. Firstly, many supports must be activated prior to immobilization. Secondly, free reactive groups must be inactivated after immobilization. In addition, the immobilization conditions can affect the attachment rate or even inactivate the reactive groups. Optimization of the immobilization procedure is crucial for obtaining an active preparation.

Many methods for covalent immobilization of enzymes have been developed and several supports are commercially available. Commonly used methods involve coupling of free amino groups on the enzyme surface to epoxy-groups (**Paper II**) (149), amino groups activated with glutaraldehyde or glyoxyl-activated hydroxyl groups (134, 150). The two latter supports require activation before immobilization.

5.1.3 Comparison of different lipase preparations

Among the available immobilization techniques, adsorption has the highest commercial potential because of the simple and relatively cheap immobilization procedure. High retention of the activity, improved mass transfer and hyperactivation by adsorption on hydrophobic supports resulted in significantly higher activity compared to "free" lipase suspended in non-aqueous media (59, 132) and immobilization by covalent binding (**Paper II**) (132, 146). Immobilization by covalent binding instead resulted in a loss of activity compared with "free" lipase (132). Covalent binding is often employed due to the minimal risk of enzyme leakage and is considered the most powerful immobilization method for increased enzyme stability. However, several studies have shown that preparations with even higher thermostability can be created by adsorption on highly hydrophobic supports (132, 146). The results clearly imply that adsorption is a better immobilization strategy for lipases in non-aqueous media where the risk of enzyme leakage is low.

Another parameter to consider in the choice of immobilization support is the effect on the regioselectivity, as certain materials have been found to enhance acyl migration (**Paper II**) (115, 139, 151). A regioselective lipase can therefore be turned into non-selective depending on the immobilization matrix. In **Paper II**, the effect on activity and regioselectivity of five commercial supports (four adsorbents and one epoxy-activated support for covalent binding) and two commercially available immobilized preparations (Novozym® 40086 and Lipase DF "Amano" IM) was evaluated. Immobilization by adsorption resulted, as expected, in higher activity than covalent binding, but the choice of adsorbent had profound effects on the activity. The best preparations exhibited activities equal to or higher than the commercial preparations. Furthermore, only the support of Novozym® 40086 enhanced acyl migration, resulting in significantly higher byproduct formation. This is highly undesired in the synthesis of STAGs but can be necessary for applications where non-specificity is needed, such as margarine and biodiesel production. The obtained results showed the potential of the developed immobilized preparations and emphasized the importance of choosing the right combination of lipase and support material for successful synthesis of the desired product.

5.2 Choice of immobilization buffer

Enzymes are usually immobilized from buffer solutions. With the choice of immobilization buffer, it is possible to alter the pH, salt type and ionic strength. Variation of these parameters can affect the immobilization yield and/or the specific activity of the preparation. Immobilization at optimal pH for the specific enzyme has been found to greatly increase the catalytic activity of lipase in non-aqueous media (80, 152). The enzyme will acquire an ionization state that is dependent on

the pH in the buffer solution. When added to non-aqueous media, the ionization state remains unchanged. This is referred to as the "pH-memory" of enzymes in non-aqueous media (78, 152).

Adsorption of lipases on hydrophobic supports is primarily related to hydrophobic interactions and is thus not sensitive to the pH of the immobilization solution (140). Hydrophobic interactions are instead favored by high ionic strength and specific salts. This is utilized in protein purification *e.g.*, in precipitation by ammonium sulfate (153). Hypothetically, higher ionic strength or specific salts should increase the hydrophobic interactions between the lipase and the hydrophobic support, resulting in higher immobilization yields. However, the results are contradictory, and the effects of salt type and ionic strength seem not to be fully understood. In **paper II** we found that using ammonium sulfate instead of sodium phosphate increased the immobilization yield and specific activity of both RML and ROL on Accurel® MP1000. On the contrary, Bastida *et al.* (1998) showed that the presence of ammonium sulfate decreased the immobilization rate on octyl-agarose at all concentrations, with a more profound effect at higher concentrations (142). Manoel *et al.* (2015) obtained similar results for sodium phosphate, but the effect was not as significant for all evaluated lipases (154).

It is believed that high salt concentrations could increase the hydrophobic interactions but disfavor the opening of the lipase lid involved in the adsorption to hydrophobic supports (155). Therefore, an optimum ionic strength promoting the opening of the lid covering the active site and hydrophobic interactions should exist. This correlates with previously presented results (**Paper II)** (156, 157). Increasing the ammonium sulfate concentration from 50 mM to 500 mM in the immobilization of ROL on Accurel® MP1000 resulted in a 26 % increase in activity (**Paper II**). However, further increasing the activity to 1 M had a strong negative effect resulting in only 24-34 % activity compared to the lower salt concentrations. Indeed, an optimum concentration must exist. Unexpectedly, the ammonium sulfate and sodium phosphate concentration had no significant effect on the specific activity of ROL in the range of 0.05-0.5 M, once again indicating that the effect of ionic strength is lipase specific. Kanasawud *et al.* (1992) also found that the optimum salt concentration for maximal specific activity was substrate specific (157).

The results show that pH, salt type and ionic strength can influence the immobilization yield and specific activity. High ionic strength could favor hydrophobic interactions between the lipase and support but could, at the same time, disfavor the opening of the lipase lid. The effect has also been shown to be highly enzyme and support specific. This highlights the difficulty of foreseeing the effect on specific immobilized enzymes and emphasizes the importance of optimizing the immobilization buffer type, ionic strength and pH for each specific enzyme, support and reaction.

6 Process development

6.1 Industrial processing of oils and fats

Neutral taste, long shelf-life, bright color and suitable crystallization and melting behavior are important properties of vegetable oils and fats for food applications. Several processing steps are needed to obtain a product that meets the quality requirements. The oil is first recovered by simple pressing or solvent extraction, depending on the type and amount of oil in the crop. The extracted oil is then treated with water and centrifuged to remove phospholipids, which will partition into the water phase. This process is referred to as degumming (158). The degummed oil still contains many undesired components from the crop such as FFAs, oxidation products and compounds with strong taste, odor or color. The oil must therefore be further refined to remove the contaminants. The refining steps should be gentle not to damage natural nutrients while at the same time offering an efficient purification.

The first refining step removes the FFAs by physical refining using heat or chemical neutralization by treating the oil first with acidified water and then diluted lye. This forms a soap that neutralizes the FFAs. The second refining step is bleaching. The oil is mixed with bleaching earth that is a naturally occurring clay with the capacity to absorb impurities. Bleaching removes colored compounds such as chlorophyll, metals, some oxidation products and phosphorous (158). The final step of refining is deodorization. This is a process using steam, high temperature and low pressure to remove various volatile components with undesired flavor and odor (159). After deodorization, the quality and oxidative stability of the oil is significantly improved. Depending on the refining process the oil is referred to as RBD (physically refined, bleached and deodorized) or NBD (neutralized, bleached and deodorized).

The melting and crystallization properties of oils can be modified using different processes, including fractionation, hydrogenation, chemical and enzymatic modification. Fractionation separates the oil into fractions based on their crystallization temperature. The oil is subjected to controlled cooling, which causes the high melting TAGs to crystallize and be filtered off. This can be performed several times to obtain several fractions from one raw material. Fractionation can be accomplished with or without solvent. Solvent-free fractionation is less efficient but cheaper, simpler and more sustainable than solvent-based fractionation. Solvent-free fractionation is therefore preferred in industrial processes (158).

Hydrogenation modifies the oil by adding hydrogen to the double bonds. This saturates the fatty acids in the oil and results in increased melting temperature. The use of a catalyst, such as nickel or palladium, reduces the necessary reaction

temperature (160). Partial hydrogenation can result in the formation of *trans*-double bonds. Due to the adverse health effects of these, only full hydrogenation for complete saturation is performed today.

The use of chemical and enzymatic modifications for alteration of the oil properties has been discussed in Chapter 1. The new properties are obtained by rearrangement of the fatty acids on the glycerol backbone. Chemical modifications use a chemical catalyst and high temperature to rearrange the fatty acids in a random fashion, while enzymatic modifications can be performed specifically or randomly depending on the enzyme preparation used.

6.1.1 The STAG production process

The production process of STAGs is exemplified in this dissertation by the transesterification reaction of HOSO and ethyl stearate for SOS production. The complete process flowsheet is presented in Figure 13.

The enzymatic production process can be divided into upstream processing, enzymatic reaction and downstream processing. The upstream part aims to prepare the substrates for the enzymatic reaction. Lipases are sensitive to impurities, such as short FFAs and oxidation products (161–163). Therefore, refinement of the substrate by neutralization and bleaching is necessary for minimized enzyme inactivation and improved operational stability.

The second step of the process is the enzymatic reaction. The enzymatic reaction can be performed by different modes of operation, in different types of reactors and with or without reaction condition control. This is discussed further in section 6.2.

The last part of the process is the downstream processing, which includes the purification of the product. The excess ethyl stearate and formed ethyl oleate are separated from the TAG-phase by distillation. This is possible as the FAEs are more volatile than the TAGs. The ethyl oleate can also be converted to ethyl stearate by hydrogenation. The ethyl stearate can then be recirculated to the enzymatic reaction to reduce the substrate cost and FAE byproduct.

The residue from the distillation will contain the SOS, intermediate products such as DAGs and non-reacted TAGs from the HOSO. The TAG-phase must be fractionated to obtain a product with high enough concentration of SOS. The SOS will end up in the high melting fraction due to the large content of stearic acid. However, byproducts formed as a result of acyl migration such as SSO, SSS and distearin will co-crystallize with SOS at higher temperatures. Thus, it is essential to keep acyl migration under control to obtain a high-quality product.

The low melting fraction will contain TAGs and DAGs with a higher degree of unsaturation. These can be recirculated to the ingoing HOSO to improve yield and reduce the substrate cost further. Finally, the product stream from the fractionation is bleached and deodorized to meet the quality requirements.

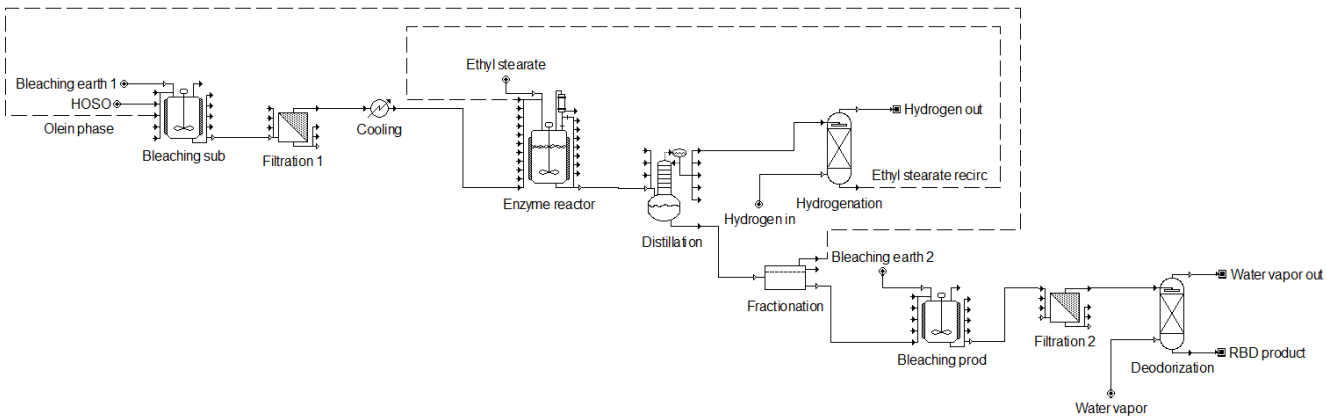


Figure 13: Process flowsheet of an industrial production process of SOS from HOSO and ethyl stearate. The refined, bleached and deodorized (RBD) product is the final product stream containing SOS.The solid line represents the process flow. The dashed line represents optional recirculation of substrate.

6.2 Reactor operation

6.2.1 Reactor configuration

Reactions can be run in different configurations including batch, continuous and fed-batch. Batch mode is the most common in small-scale laboratory experiments performed in vials and flasks as it allows for very small volumes. This was utilized in **Papers II**, **III** and **IV** to evaluate different immobilized preparation and the effect of different factors on the transesterification reaction. In an industrial process, batch mode is preferred if several products are to be produced using the same equipment. This reduces the loss of products and substrates during the interchange between products. However, it is not necessarily the most efficient for large-scale production of a single product due to the long downtime for filling, emptying and cleaning and difficulties with separation of the enzyme particles.

Continuous processes operate with a constant addition and removal of substrate and product. This is preferred where manufactured volumes are large, and productivity is a key factor. One example is the enzymatic interesterification reaction for production of plastic fats, such as margarine, modified butters and shortenings (164).

Fed-batch is similar to batch mode but with continuous or stepwise addition of one or more substrates. This is a highly useful tool in the case of substrate inhibition of the enzyme. The concentration can be maintained at a low but constant level by matching the addition of the substrate with the consumption.

6.2.2 Reactor types

The stirred tank reactor (STR) is one commonly used reactor for enzyme catalysis. The STR has many benefits as it is cheap, simple to operate and although batch mode is the most common, it can be adapted to different reactor configurations. The main drawback of the STR is that the stirring can cause abrasion of the immobilized enzyme particles and reduce the lifetime of the enzyme.

Packed-bed reactors (PBR) with a continuous flow are often the reactor of choice in industrial lipid modifications due to their high activity and easy application to large-scale production. The immobilized enzymes are packed in a tube or column reactor and the substrate is pumped through the enzyme bed. Compared to the STR, this leads to easier separation and less physical damage to the enzyme particles. The shorter reaction time can lead to less side reactions such as acyl migration (165). The disadvantage with the PBR is the risk of internal and external mass transfer limitations, creation of substrate channels in the bed and a large pressure drop, harming the enzyme particles by compressing them. Processing of large volumes of

substrate can also lead to the accumulation of byproducts and contaminants in the reactor. These can decrease the enzyme stability and cause inactivation. In addition, possible modes of operation are highly limited, and the reaction conditions cannot be altered during the reaction.

An alternative reactor is the rotating bed reactor (RBR). The RBR combines components from both the STR and the PBR. It is comprised of an agitated tank, just like the STR, but the enzyme particles are packed in the stirring element. Rotation of the packed bed offers simultaneous stirring and filtration of the substrate solution through the enzyme bed, which resembles the packed bed in a PBR. Due to centrifugal forces, the liquid in the bed is repeatedly thrown out and new liquid is drawn in from the bottom and the top (166). The efficient stirring reduces mass transfer limitations and makes the reactor suitable for highly viscous solutions. The packed bed also facilitates the separation of the enzyme particles from the reaction solution and enables the reaction to be run as a batch, continuous or fed-batch process (167). Furthermore, the packed bed protects the enzyme particles from being harmed by the mixing and keeps the pressure drop considerably lower compared to the PBR. This allows for the use of softer and friable particles. A SpinChem® RBR reactor and the rotating bed used for stirring and compartmentalization of the enzyme is shown in Figure 14.

The SpinChem® RBR was used for the implementation of a water activity control system based on sparging with dry or wet nitrogen gas in **Paper III**. The efficient mixing in the RBR improved the dispersion of the gas and the mass transfer rate of water. The developed water activity control system will be further discussed in section 6.2.4. The SpinChem® RBR was also used for enzymatic production of SOS with reaction condition control in **Paper IV**.

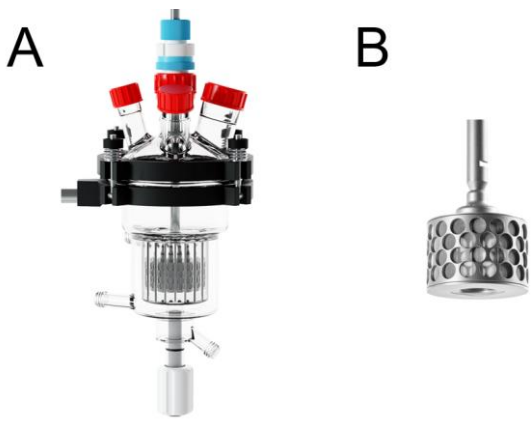


Figure 14: A) SpinChem® RBR S2 (120-300 mL). B) Rotating bed for enzyme compartmentalization and stirring.

6.2.3 Solvent-based and solvent-free processes

Lipase-catalyzed reactions in non-aqueous media can be performed with (168, 169) or without solvents as reaction medium (101, 157, 170). Solvent-free systems are attractive from an industrial perspective as running highly concentrated reactions increases the efficiency and simplifies downstream processing. In addition, reaction volumes are decreased and thereby reactor size and cost.

The use of organic solvents is also problematic from an environmental perspective. Organic solvents are often volatile and hazardous and can cause major problems if released into the environment. Therefore, the use of solvents puts large requirements on the equipment and product purification for safe production and products. This is especially important for food and pharmaceutical applications. In general, solvents will increase the process cost and should be avoided.

Solvents can be necessary for practical reasons when the reactants are highly viscous or solid. Increasing the reaction temperature to melt the reactants might not be possible as it can lead to undesired side reactions and enzyme inactivation. In some cases, the substrates and substrate ratios can be chosen so that a solid or viscous reactant can be dissolved or diluted by the other. This strategy was used in **Papers II**, **III**, and **IV**, where the melting point of ethyl stearate is decreased when mixed with HOSO.

6.2.4 Water activity control

Several methods for water removal have been suggested, including pervaporation (171, 172), vacuum evaporation (173, 174), dry gas bubbling (174) and molecular sieves (170, 174, 175). However, precise control of water activity using these methods is not possible and other approaches must be considered to both change and control the water activity.

The most commonly used method to change the water activity in small-scale reactions is pre-equilibration with saturated salt solutions (169). However, the water activity is not maintained in reactions where water is either produced or consumed. Methods for continuous control using saturated salt solutions have been suggested, such as allowing saturated salt solutions (176–178) or air equilibrated with saturated salt solutions (179, 180) flow through silicon tubing or hollow fiber membranes in contact with the reaction solution. However. these methods are limited due to the slow mass transfer of water and are not feasible on a larger scale. The use of salt hydrate pairs for control of water activity has also been evaluated but was found to have many drawbacks (181, 182). Problems with the removal of the salt result in impurities in the product, and difficulties in re-use and limited buffering capacity also restrict their use on a larger scale. Additionally, salt hydrates have been shown to have a negative effect on enzyme stability.

The perhaps most straightforward and most promising method for large-scale application is sparging of the reaction solution with dry or humid air or nitrogen gas (**Paper III**) (183–185). The water activity control is based on the measurement of the relative humidity in the headspace of the reactor using a sensor and applying feedback control to either supply or remove water depending on the offset from the set-point. Air and nitrogen gas have many benefits as they provide high water transfer rates due to a large contact area between the gas bubbles and the substrate, have a limited effect on the enzyme and nitrogen gas can help to protect the substrate from oxidation by removal of oxygen.

A simple, low-cost and effective water activity control system based on humid and dry nitrogen gas was developed in **Paper III**. A schematic representation of the control system can be seen in Figure 15. The control system could be used to control the water activity in the range of 0.05-0.92 with less than 5% deviation from the set-point for more than 16 h. The developed control system was implemented in a SpinChem® RBR and used for pre-equilibration and continuous water activity control of substrate solution and immobilized lipase preparation (**Paper II-IV**).

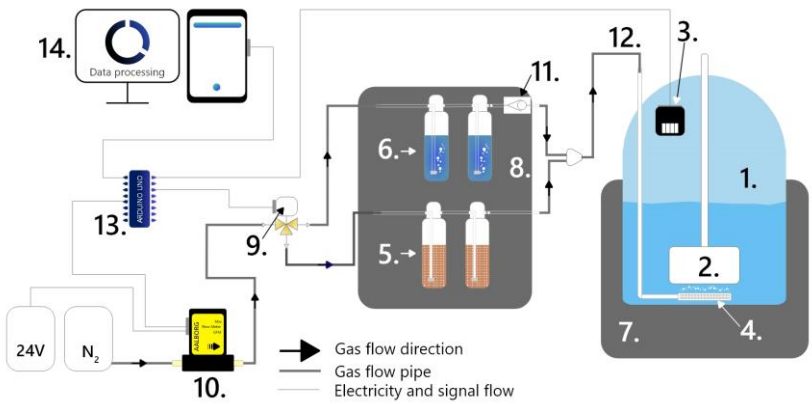


Figure 15: Schematic representation of developed water activity control system implemented in a SpinChem® RBR (**Paper III**). 1: reactor, 2: rotating bed, 3: temperature and relative humidity sensor, 4: gas sparger, 5: silica gel in gas washing bottles, 6: deionized water in gas washing bottles, 7: water bath for reactor, 8: water bath for heating of gas, 9: three-way solenoid valve, 10: mass flow controller, 11: check valve, 12: nitrogen gas inlet, 13: programmed Arduino control unit, 14: PC.

6.3 Comparison of different STAG production processes

Comparison of transesterification reactions for the production of STAGs can be difficult as many factors can vary between the processes and significantly affect the results. In addition, the analysis methods and presented data are often different,

further complicating the comparison. However, an attempt was made to compare the productivity and minimal undesired incorporation in the *sn*-2-position of different processes (Table 4).

We developed a process in a SpinChem® RBR with a stepwise water activity change (**Paper IV**). High initial water activity (0.65) increased the DAG and product formation while drying the reaction solution for the last 3 h reduced the DAG content. A final DAG content of 3.1% and a yield corresponding to 78% of the theoretical maximum was obtained with 0.5% (w/w) enzyme and 7 h of reaction. The productivity was found to be 3.1 times higher and the incorporation in the *sn*-2-position was 17 times lower compared to the second best studies. It can be concluded that the developed process can be used to produce SOS with a high yield, productivity and purity. Furthermore, Lipase DF "Amano" IM was an excellent catalyst with high activity and regioselectivity in the production of symmetrical STAGs.

Table 4: Minimal undesired incorporation in *sn*-2-position (%) and productivity (mmol product/g enzyme*h) for reactions producing symmetrical STAGs.

Product	Substrates	Lipase	Reaction type	Temperature (°C)	Incorp. in *sn*-2-position (%)	Productivity (mmol/g enzyme*h)	Ref.
1,3-stearin-2-olein (SOS)	HOSO and ethyl stearate	Lipase DF "Amano" IM[b]	Batch, solvent-free	40	0.11	3.7	**Paper IV**
S-U-S[a]	HOSO, stearic acid and palmitic acid	Lipozyme RM IM[b]	Batch, solvent-free	65	2.9	0.36	(90)
S-U-S[a]	HOSO, ethyl stearate and ethyl palmitate	Lipozyme RM IM[b]	PBR	60	6.1	-	(186)
1,3-arachidin-2-palmitin (APA)	Tripalmitin and arachidonic acid	Immobilized *R. delemar* lipase	Batch, solvent-free	40	3.2	-	(187)
SOS	Triolein and ethyl stearate	Immobilized *C. antarctica* lipase	Batch, hexane	55	11	0.10	(188)
1,3-olein-2-palmitin (OPO)	Palm stearin and oleic acid	Immobilized *A. oryzae* lipase	Batch, solvent-free	65	-	1.2[d]	(189)
OPO	Tripalmitin and oleic acid	Lipozyme TL IM[b]	Batch, hexane	50	4.7	0.30	(190)
OPO	Tripalmtitin and oleic acid	Lipozyme RM IM[b]	Batch, solvent-free	60	11	0.24	(190)
OPO	Tripalmitin and oleic acid	Lipozyme RM IM[b]	Batch, hexane	45	7.1	-	(191)
OPO	Tripalmitin and methyl oleate	Lipozyme RM IM[b]	Batch, hexane	45	12	-	(191)
OPO	Tripalmitin and ethyl oleate	Lipozyme TL IM[b]	Batch, solvent-free	50	6.1	0.39	(192)
1,3-caprylin-2-eicosapentaenoin (CEC)	Trieicosapentaenoin and caprylic acid	Immobilized *R. delemar* lipase	Batch, solvent-free	30	1.9[c]	0.04[d]	(193)
CEC	Trieicosapentaenoin and ethyl caprylate	Lipozyme RM IM[b]	Batch, solvent-free	30	6.2[c]	0.05[d]	(193)

[a] S-U-S is a TAG with saturated fatty acids in the *sn*-1,3-position and an oleic acid in the *sn*-2-position.

[b] Lipase DF "Amano" IM – immobilized lipase from *R. oryzae*, Lipozyme RM IM – immobilized lipase from *R. miehei*, Lipozyme TL IM – immobilized lipase from *T. lanuginosus.*

[c] Includes only the formation of tricaprylin (CCC)

[d] Includes both product and regioisomer formation.

7 Concluding remarks and future perspectives

The high regioselectivity and catalytic power of lipases under mild conditions make them good catalysts for STAG production. However, high production costs limit the development of industrial processes. This dissertation has addressed three major challenges in the development of a successful and economically feasible STAG production process.

Determination of total lipid composition in complex oil samples

A central part of research and industrial processes is the analysis of reaction samples for determination of the product formation and quality control. Oils and fats contain numerous lipids of varying structures, complicating both the separation and quantification. Samples from enzymatic transesterification are especially complex due to the formation of lipids from several lipid classes. In addition, regioisomers can be formed as a result of acyl migration. Reliable analysis methods for the determination of the lipid composition are of crucial importance as it affects the nutritional, physical and chemical properties.

Traditionally, tandem procedures are necessary for comprehensive analysis of lipid samples. However, we developed a single NARP-HPLC method using charged aerosol detection for simultaneous analysis of five different lipid classes. The method manages to within 80 minutes separate and quantify FFAs, FAEs, MAGs, DAGs, TAGs, and several regioisomers. Furthermore, we developed a reduced calibration approach to circumvent many issues associated with calibration.

The developed method shows great potential for analysis of different oils and fats and samples from enzymatic transesterification. The method is more time- and cost-efficient, without compromising the analytical quality, than separate analyses for complete characterization of oils and fats.

Development of efficient immobilized lipase preparations

Productive and stable catalysts are essential for the development of viable enzymatic processes. Immobilization of lipases is necessary to avoid aggregation in non-aqueous media, and it facilitates reuse, enables the use in continuous processes and can increase stability. Immobilization can significantly improve the process economy by reducing the lipase cost.

The properties of the preparation will be dependent on the combination of the lipase and the support. The immobilization strategy can have profound effects on the lipase activity and the support material can promote acyl migration. We evaluated the effect of immobilization conditions and support material on the immobilization of lipase from *R. miehei* and *R. oryzae* for STAG production. The results emphasized the importance of choosing the right combination of enzyme, support material and immobilization conditions in the synthesis of STAGs. Adsorption was found to be the best immobilization strategy and the highest activity was obtained on highly hydrophobic supports. The developed preparations exhibited activities equal to commercial preparations and even better regioselectivity. Determination of the operational stability still has to be performed to evaluate their suitability in industrial processes. In addition, protein engineering can be utilized to improve the lipase stability further. To decrease the cost of the biocatalyst and increase the sustainability, reuse of the support should be explored.

Optimization of reaction parameters for improved productivity and product yield

Lipase-catalyzed transesterification for the production of symmetrical STAGs is a complex multistep reaction with the formation of several intermediate products. The risk of unproductive reactions is big due to the reversibility of all reactions and many possible acyl donors and nucleophiles. Furthermore, acyl migration negatively affects the product quality and excessive formation of DAGs affects the product yield and quality. How to optimize these reactions for maximal product formation and minimal byproduct formation is neither straightforward nor intuitive.

Evaluation of the effect of water activity, temperature, substrate ratio and ethanol concentration made it possible to understand the reaction better. The results showed that the optimal reaction conditions for maximal product formation change throughout the reaction. The water activity should be high initially to promote hydrolysis but low at the end to push the equilibrium towards TAG formation. The initial substrate ratio should instead be low initially to reduce acyl donor competition and increased at last for high degree of fatty acid incorporation. Finally, the chosen temperature will be a compromise between product quality, lipase stability and activity. This indicates the importance of controlling reaction conditions for an efficient production of STAGs.

A water activity control system for enzymatic reactions in non-aqueous media was developed. The control system was implemented in an RBR, a non-conventional reactor in regard of STAG production. Promising results with high productivity and low degree of undesired incorporation in the *sn*-2-position were obtained in the RBR with stepwise water activity change. Scale up of the RBR and water activity control to an industrial scale production of STAGs and evaluation of the economic and technical feasibility would be an interesting continuation of this work.

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