Evaluation of Lipases for Enzymatic Catalysis of Vegetable Oils and Fats

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Master thesis in Biotechnology KBTM05
Division of Biotechnology
May 2024

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Populärvetenskaplig sammanfattning

Enzymer för en grönare och mer effektiv tillverkning av vegetabiliska oljor och fetter.

Fett är en vanlig komponent i mängder av produkter, både naturligt förekommande och tillsatt. I kroppen är fett viktigt för normal funktion då det används som energi, men även funktioner som uppbyggnad och reparation av celler, tillverkning av hormoner och upptag av fettlösliga vitaminer kräver fett. En del fetter är till och med essentiella, alltså livsnödvändiga att få i sig via kosten eftersom vi inte kan tillverka dem själva i kroppen. Fett associeras ofta med mat och hälsa, men fett är också en viktig beståndsdel i produkter såsom kosmetika, hudvård och värmeljus.

Beroende på användningsområdet för fetter så är olika egenskaper önskvärda. Inom chokladtillverkning vill man ha ett fett som smälter i munnen, medan man för modersmjölksersättning vill ha ett högt näringsvärde. Huvuddelen av fett består av triacylglyceroler, vilket är tre fettsyror som är kopplade till en glycerolmolekyl. Egenskaperna hos ett fett styrs av kompositionen av fettsyrorna och deras positioner på glycerolmolekylen. Kompositionen kan varieras med olika längd och mättade eller omättade fettsyror. Positionen för fettsyrorna kan variera mellan den inre positionen och de två yttre positionerna på glycerolmolekylen. Ett sätt att framställa fett med önskade egenskaper är att modifiera naturligt förekommande fetter med enzymer. Enzymer är protein som snabbar kemiska reaktioner utan att själva förbrukas. De kan liknas vid kockar som använder ingredienser för att skapa en önskad produkt. Enzymer fungerar olika bra med olika substrat, eller ingredienser, och det gäller att hitta ett enzym som passar för ändamålet. Detta examensarbete har gjorts i samarbete med AAK AB och fokuserar på att utvärdera en grupp enzymer för att ta reda på dess möjliga användningsområden och på så sätt lägga grunden till ett enzymbibliotek.

En viktig aspekt gällande användning av enzymer är att de är olösliga i oljor och fetter, de kommer därför att klumpa ihop sig om de är fria. En vanligt förekommande lösning är att fästa enzymerna på ett bärarmaterial, en process som kallas immobilisering. Immobilisering av enzymerna kommer även möjliggöra återanvändning av enzymerna, då de enklare kan separeras från oljan. I detta projekt immobiliserades enzymerna på en porös, tvättsvampsliknande plast innan de användes till de önskade reaktionerna. Enzymerna utvärderades på fem olika substrat med varierande storlek och form. Enzymernas aktivitet mättes på de enskilda substraten, men även på en mix av alla substrat. Detta gjordes för att kunna observera skillnader hos enzymerna mellan en kompetitiv och icke kompetitiv miljö.

I undersökningen utvärderas fem kommersiella enzymer, samt två kontrollenzym för att säkerställa att metoden fungerar. Resultaten visade att X1, Y2 och Y3 fungerade bäst på korta fettsyror. Kontrollenzymerna TL och TL IM, samt Y1, visade sig föredra medellånga fettsyror, men fungerar även bra på övriga fettsyror. Ingen slutsats kring X2 och dess preferens kunde dras på grund av stora variationer i mätningarna. Utöver resultaten för enzymerna så är även det utvecklade protokollet för undersökning av fettsyrapreferens ett viktigt resultat för framtida undersökningar. Förhoppningen är att AAK AB i framtiden kan använda detta arbete för att bygga ut enzymbiblioteket med fler intressanta enzym.

Abstract

As part of a switch towards more green and environmentally friendly processes, together with a need for highly specific reactions, AAK AB wants to broaden their use of lipases in the production of structured triacylglycerols (STAGs). AAK are working on creating a lipase library where several lipases are evaluated, this master thesis aims to contribute to this lipase library by characterizing a selection of commercially available lipases. The description of the lipases contains their origin, GMO-status, possible reactions, temperature- and pH range, and specificity towards fatty acid chain length and saturation. The specificity of the lipases was studied experimentally in this thesis while the other parameters in the library were determined by literature studies.

To determine the specificity of the lipases, transesterification reactions were carried out on fatty acid esters and the change in product concentrations with time were measured by gas chromatography. From the product formation rate, the transesterification activities could be estimated and compared. The substrates used in this thesis ranged from 4 to 18 carbons long fatty acid esters (FAEs) and included both unsaturated and saturated FAEs. The activity of each lipase was investigated in two different situations, both on pure substrates and on a mixture of all substrates.

The results showed that X1, Y2 and Y3 had the best activity on short chain fatty acids (SCFAs), while the control lipases TL and TL IM were concluded to have the highest activity on medium chain fatty acids (MCFAs). Y1 showed the highest activity on both medium chain- and long chain fatty acids, however, Y1, TL and TL IM had relatively high activities on all substrates. Looking at saturated- versus unsaturated fatty acids, it was shown that X1 and Y2 had higher activity on a monounsaturated fatty acid (MUFA), while X2, TL and TL IM preferred saturated fatty acids (SFAs). Y1 showed equal specificity towards SFAs and MUFAs. No conclusion could be made for the preference of carbon chain length for X2 or the preference of SFAs versus MUFAs for Y3 due to uncertainties in the data.

Sammanfattning

Som en del i utvecklingen mot grönare och miljövänligare processer, samt ett behov av ytterst specifika reaktioner, vill AAK AB utvidga sin användning av lipaser i sin produktion av strukturerade triacylglyceroler (STAGs). AAK vill bygga upp ett bibliotek av utvärderade lipaser. Syftet med detta examensarbete är att bidra till detta bibliotek genom att karakterisera en grupp kommersiellt tillgängliga lipaser. Beskrivningen av lipaserna innehåller deras ursprung, GMO status, möjliga reaktioner, temperatur- och pH intervall, samt specificitet mot kolkedjelängd och mättnad på fettsyror. Lipasernas specificitet bestämdes experimentellt i detta examensarbete medan de andra parametrarna i biblioteket bestämdes genom litteraturstudier.

För att bestämma lipasernas specificitet utfördes transesterifieringsreaktioner på fettsyraestrar av olika längd där produktkoncentrationen sedan analyserades med gaskromatografi. Från bildningen av produkt över tid kunde sedan lipasernas transesterifieringsaktivitet bestämmas och jämföras. Substraten som användes i detta examensarbete varierade från 4 till 18 kol långa fettsyraestrar och inkluderade både mättade och omättade fettsyror. Lipasernas aktivitet undersöktes på både rena substrat och en mix av alla substraten.

Resultaten visade på att X1, Y2 och Y3 hade bäst aktivitet på korta fettsyror, medan kontrollenzymerna TL och TL IM hade högst aktivitet på medellånga fettsyror. Y1 visade sig fungera bäst på både medellånga och långa fettsyror, dock så hade Y1 samt TL och TL IM relativt hög aktivitet på alla substrat. Undersökningen gällande preferens för mättade eller omättade fettsyror visade att X1 och Y2 hade bättre aktivitet på omättade fettsyror medan X2, TL och TL IM föredrog mättade fettsyror. Y1 i sin tur visade liknande aktivitet på de mättade och omättade fettsyrorna. Inga slutsatser kunde dras för vilken kolkedjelängd som X2 föredrog eller om mättade eller omättade fettsyror föredrogs av Y3, detta berodde på mätosäkerhet i datan.

Preface

This master thesis was completed during the period of January 2024 - May 2024 as a part of the Master of Science program at LTH, Lund University. This master thesis has been a collaboration between AAK AB, Sweden, and the Division of Biotechnology at LTH, Lund. We would like to thank AAK AB for providing us with essential material, knowledge and an interesting study visit at their production plant in Karlshamn. Thanks to the Division of Biotechnology for letting us perform the study at the department.

We would like to extend a big thanks to our supervisor Ariana Causevic at AAK AB, who has made this master thesis possible by providing invaluable support and feedback throughout the project. We would also like to thank our supervisor at LTH, Carl Grey, for helpful discussions and his deep insights in enzyme technology. We want to thank our examiner, Patrick Adlercreutz, for valuable input when needed. Lastly, we would also like to direct a thanks to all the people at the Division of Biotechnology for helping and treating us well during the project. A special thanks to Lars Dahlgren for lending us his lab, sharing his office and helping us on numerous occasions with the gas chromatograph.

Planning, laboratory work and analysis have been conducted together. Oliver Andersson has written the main parts of 1., 3.1, 3.2, 3.3, 3.5, 4.1, 5.1 and 5.4 and Hampus Pradon has written the main parts of 3.4, 5.2, 5.3, 5.6, and 7. However, good teamwork has taken place and no part has been written without input from the other group member. The parts not mentioned above have been written in collaboration.

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List of Abbreviations

FFAs - Free fatty acids

FAEs - Fatty acid esters

SCFAs - Short chain fatty acids

MCFAs - Medium chain fatty acids

LCFAs - Long chain fatty acids

SFAs - Saturated fatty acids

MUFAs - Monounsaturated fatty acids

PUFAs - Polyunsaturated fatty acids

STAGs - Structured triacylglycerols

TAGs - Triacylglycerols

DAGs - Diacylglycerols

MAGs - Monoacylglycerols

CBB - Coomassie brilliant blue

GC - Gas chromatograph

p-NPB - *para*-nitrophenyl butyrate

U - Units (micromole per minute)

1. Introduction

1.1 AAK

The history of AAK AB started in 1871 in Aarhus, Denmark, when Aarhus Palmkærnefabrik was founded and started to process palm kernels to extract palm oil. The oil was sold and used in a wide range of industries while the remaining press cake was used for animal feed. In 1892 the company changed name to Aarhus Oliefabrik and the main product became vegetable oils used for margarine production. Another important product was speciality fats for chocolate. These products became successful and in 1940 Aarhus Oliefabrik was a global company accounting for 10% of Denmark's exports. During the 1980s, new production plants were built and the company gathered all operations under the name Aarhus United in 2003. At the same time as the expansion of Aarhus Oliefabrik, a company called Karlshamns AB was established in Karlshamn, Sweden, that produced soybean meal for animal feed and soybean oil for oil-containing products. Karlshamns AB developed over the years and started to produce and export speciality fats for margarine-, ice cream- and chocolate production. In 2005 Aarhus United and Karlshamns AB merged under the name AarhusKarlshamn, AAK. These two organizations completed each other and AAK is today a world-leading global company specialized in customized and sustainable plant-based oils and fats solutions. Today AAK has their headquarters in Malmö, Sweden, and is listed on Nasdaq Stockholm. They have around 4000 employees working at 25 regional sales offices, 16 customer innovation centers and more than 20 production facilities (AAK, n.d.).

AAK has a Co-Development approach, meaning that their experts together with the customers create value-adding vegetable oil solutions within the field of chocolate and confectionery, dairy, plant-based foods, special nutrition, food service and personal care. AAK's business model is suited to help customers meet today's and tomorrow's challenges. Everything is about Making Better Happen™ (AAK, n.d.).

1.2 Background

According to a NASA study using advanced climate and agricultural models, the increasing global temperature, increased surface carbon dioxide concentrations and shift in rainfall patterns will lead to decreased harvest yields (Gray, 2021). Furthermore, consumers nowadays become more and more health conscious and are looking for products helping them reach their nutrition and health goals (Solan, 2020). These progressions create challenges in the oleochemical industry and call for innovation and new solutions for new applications. In today's market, products such as cocoa butter equivalents, human milk substitutes and low-calorie fats already exist. Many of these applications require fats with properties rarely found in nature and thus have to be produced by modifications of natural fats where the fatty acid composition and distribution are changed. When customizing the fatty acid composition and distribution on TAGs to acquire desired physical, chemical or nutritional properties, structured triacylglycerols (STAGs) are obtained (Causevic, 2022). Synthesizing of STAGs can be performed either chemically or enzymatically, both with its advantages and disadvantages. Enzymatic modifications are carried out under mild reaction conditions with low temperature and normal pressure, produce low amounts of byproducts, are sustainable and above all, carry out specific reactions in contrast to chemical modifications. However, enzymatic modification is often more costly and chemical modification is already well established in the oleochemical industry (Sivakanthan,

2020). The implementation of enzymes in industrial processes have therefore been limited to high-value products. Thus, to implement enzymatic processes on an industrial scale, the reactor system must be optimized and the enzymatic catalysts must be highly productive and efficient.

As climate change proceeds, the quest for more environmentally friendly processes increases. As a part of this switch towards more green processes, AAK wants to broaden their use of lipases as a complement to chemicals in the modification processes of TAGs. However, enzymatic modification of TAGs is not only used because of environmental reasons, it is also advantageous due to its high specificity and selectivity. Today AAK uses lipases for production of bulk fats used in for example margarine production, but also in production of specialty fats for infant formulas. This requires active and appropriately specific lipases performing the desired reactions. AAK are today working on creating a lipase library where several lipases are evaluated based on their origin, GMO status, possible reaction, temperature- and pH range and specificity. This master thesis aims to contribute to the lipase library by characterizing a selection of commercially available lipases according to the aforementioned properties.

1.3 Disposition

The report starts with a section of scientific background, including information needed to understand the methods used, obtained results and discussion of the results. The next part of the report deals with materials and methods used, in which the working process and experimental trials are described. Thereon comes the results and discussion section where all results from the experiments are presented, analyzed and discussed, before the report ends with conclusions and suggestions for further research.

2. Aim

The aim of this master thesis is to contribute to the construction of a library of commercial lipases available to AAK. The library will contain the lipases' origin, GMO status, possible reactions, temperature- and pH range and specificity towards fatty acids with varying carbon chain length and saturation.

3. Scientific Background

3.1 Fatty acids and lipids

Fatty acids are aliphatic monocarboxylic acids and are the main building blocks of lipids. The chain length can vary from 2 to over 80 carbon atoms, but the most common ones range from 4 to 22 carbons. The fatty acids can either be saturated, monounsaturated or polyunsaturated, depending on the presence of none, one, or two or more double bonds respectively. Fatty acids can be divided further based on the carbon chain length, where short chain fatty acids (SCFAs) are shorter than 8 carbons, medium chain fatty acids (MCFAs) are between 8 to 14 carbons and long chain fatty acids (LCFAs) are between 16 and 22 carbons (Gunstone et al., 2007). The name of the fatty acid esters (FAEs) used as substrates in this project, together with their number of carbons, number of double bonds and melting point are presented in Table 1.

Table 1. Names, number of carbons (nC), number of double bonds (nDB) and melting point of the fatty acid esters used in this project.

Name	**nC:nDB**	**Melting point (°C)**
Methyl butyrate	4:0	-95 °C
Methyl octanoate	8:0	-40 °C
Methyl laurate	12:0	5 °C
Methyl oleate	18:1	-20 °C
Methyl stearate	18:0	39 °C

Acylglycerols consist of a glycerol backbone with fatty acids esterified to the hydroxyl groups of the glycerol. In vegetable oils and fats, triacylglycerols (TAGs) are most common, however diacylglycerols (DAGs), monoacylglycerols (MAGs) and free fatty acids (FFAs) are also present in smaller amounts. When drawing a glycerol molecule in Fischer projection with the secondary hydroxyl group to the left of the central prochiral carbon, the positions are numbered top to bottom. An example can be seen in Figure 1, where R$^{'}$, R$^{''}$ and R$^{'''}$ are three fatty acids. Since the molecule is stereospecifically numbered, the prefix *sn* is used to describe the molecule (Gunstone et al., 2007).

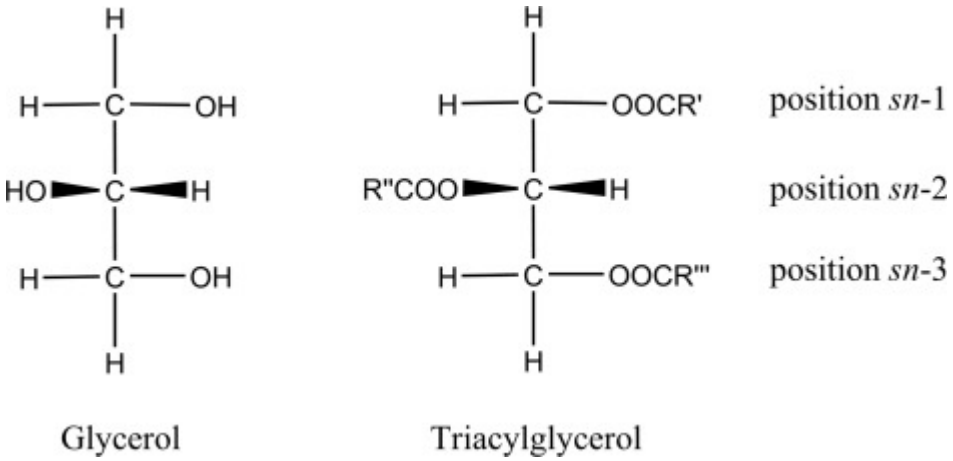


Figure 1. Fischer projection of a glycerol molecule and a generic triacylglycerol with *sn*-1, *sn*-2 and *sn*-3 positions marked (Indelicato, 2017).

Since the distribution of the fatty acids on the glycerol backbone can vary, regioisomers are formed. The regioisomers have the same functional groups, but attached to different positions and will affect the physical and chemical properties (Zhang, 2022). Some applications require lipids with properties not available or insufficiently occurring in nature. For example, cocoa butter equivalents for chocolate production and human milk fat substituents for infant formulas require specific regioisomers of TAGs and can be synthesized by enzymatic catalysis (Dicko et al., 2022).

3.2 Lipases

Lipases (E.C.3.1.1.3) are enzymes catalyzing the hydrolysis of ester bonds in fats and oils (Brockman, 2013; Reetz, 2002). They belong to a group of enzymes called hydrolases, which includes proteases, carbohydrate active enzymes and lipases (Dicko et al., 2022). Lipases are found in animals, plants and several microorganisms, but only microbial lipases are used commercially (Patel et al. 2019). The most common lipase-producing microorganisms are bacteria and fungi, where the most frequently used are native or recombinant strains of *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas* (Gupta et al., 2004). Most lipases have a similar tertiary structure which comprises two folding domains. One of the domains is larger and called the N-terminal,, while the smaller domain is called the C-terminal. The N-terminal is responsible for the catalysis, but the C-terminal is needed to bind the substrates and enzyme cofactors. The C-terminal consists of several β-sheets folded into a sandwich-like structure, while the N-terminal has a more complex structure and contains the active site (Doolittle & Péterfy, 2010). The active site is often covered by a helical oligopeptide acting like a lid, which opens when the enzyme is associating with the substrate. Different lipases have different characteristics of their lids, however they are all activated by a lipid-water interface (Brzozowski et al. 1991; Reetz, 2002; Verger, 1997). The interaction between the interface and the lid, causes the lid to undergo movement and exposes the active site to the surroundings, which provides free access for the substrate (Brzozowski et al. 1991; Reetz, 2002).

The catalytic mechanism of lipases is a two-step mechanism and is referred to as the ping-pong bi bi- or substituted enzyme mechanism. The active site of lipases generally consists of the catalytic triad serine, histidine and aspartate. Serine reacts with the acyl donor in the first step and creates an acyl-enzyme intermediate through an ester linkage, via a tetrahedral intermediate. In this step the first product is also released. In the next step of the ping-pong bi bi mechanism, an acyl acceptor reacts

with the acyl-enzyme intermediate and dissociates the complex. This leads to release of the second product of the reaction. The lipase, at the same time, returns to its original state (Causevic, 2022; Mitchell & Krieger, 2022). An illustration of the catalytic mechanism can be seen in Figure 2, where R_1, R_2 and R_3 are carbon chains of varying length.

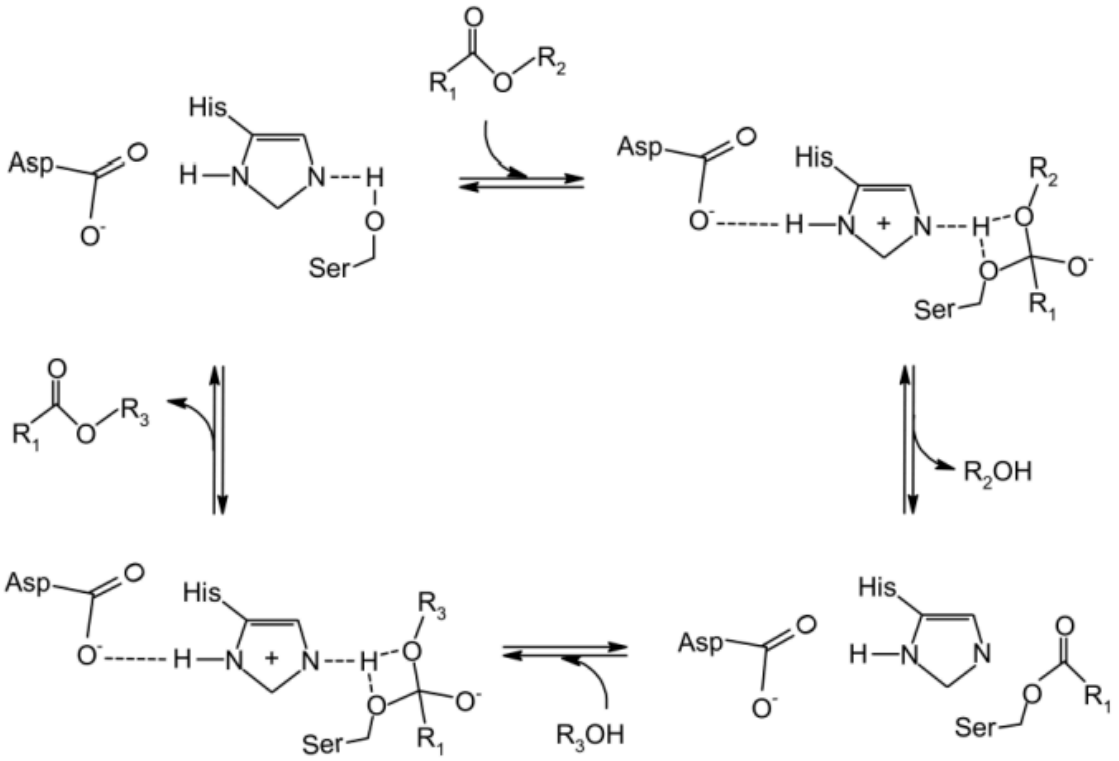


Figure 2. The catalytic mechanism of lipases (Causevic, 2022).

Lipases play a crucial role in living organisms. However, they are also used as biocatalysts in several industrial processes. They can be used in oil and fat production, as additives in detergents, cheese making, baking, pharmaceutical production as well as paper and leather processing (Reetz, 2002). Lipases are robust enzymes that can execute their catalytic activity under mild reaction conditions, use a wide range of substrates, have high pH- and temperature stability while also having high regio- and stereoselectivity (Angajala et al., 2016). Another property of lipases is that they can be used in both aqueous and non-aqueous media (Angajala et al., 2016; Persson et al., 2002).

The regioselectivity of a lipase means that they can distinguish between the three different positions on the glycerol backbone of a triglyceride, *sn*-1, *sn*-2 and *sn*-3. Depending on which position they prefer, they are divided into three groups: preferring the outer positions (*sn*-1,3-selective), preferring the middle position (*sn*-2-selective) or non-selective. *sn*-1,3-selective lipases is the most common type. The substrate selectivity on the other hand, relates to the preference of substrate characteristics like fatty acid chain length and degree of unsaturation (Castillo et al., 2016). It is the geometry of the binding site and the active site that determines the substrate selectivity of the lipases (Pleiss et al., 1998).

3.2.1 Lipozyme®TL 100 L - Thermomyces lanuginosus (Control 1)

Lipozyme® TL 100 L contains a lipase from the fungus *Thermomyces lanuginosus*, produced in genetically modified *Aspergillus oryzae* by Novozymes A/S. According to the distributor, the lipase effectively performs transesterification, interesterification, ester hydrolysis and desymmetrization of esters. Lipozyme® TL 100 L is stated to have a substrate specificity for esters and diesters and is *sn*-1,3 specific, allowing bulky and LCFAs on the alcohol and acid parts of the molecule. The optimum usage conditions were specified to be at a pH between 7.0 and 10.0 and a temperature in the range of 20-50 ℃ (Strem Chemicals, Lipozyme® TL 100 L, n.d.).

3.2.2 Lipozyme® TL IM - Immobilized Thermomyces lanuginosus (Control 2)

Lipozyme® TL IM is a lipase from the fungus *Thermomyces lanuginosus*, produced in genetically modified *Aspergillus oryzae*, which is immobilized on non-compressible silica gel. The immobilized preparation is produced by Novozymes A/S. According to the distributor, the lipase acts hydrolytically on ester bonds and catalyzes interesterification and rearrangement of fatty acids on triglycerides with specificity on the first and third position, however it can also act on the second position. Lipozyme® TL IM is said to work with a variety of different groups on the alcohol and acid part of the molecule, including bulky and LCFAs. The optimal pH range for Lipozyme® TL IM is stated to be between 6.0 and 8.0 and the optimal temperature was specified to range from 50-75 ℃ (Strem Chemicals, Lipozyme® TL IM, n.d.).

3.2.3 X1

X1 is a lipase that, according to the producer, is effective in hydrolysis of triacylglycerols, esterification reactions, transesterification and alcoholysis. X1 is stated to have hydrolytic activity on short-, medium- and long chain fatty acids and on all three positions on the triacylglycerol molecule. The optimum pH range for X1 is understood to range from 5.0 to 8.0 and the optimum temperature is said to range from 40-55 ℃. The lipase is said to be classified as non-GMO since no modified microorganism was used in the production and no modification of the lipase has occurred.

3.2.4 X2

X2 is a lipase with microbial origin and has high hydrolytic activity on particularly dairy fats, according to the producer. It has also been stated that the lipase has a high specificity towards release of SCFAs, especially butyric acid and caproic acid, from fat substrates. X2 is understood to have an optimum pH range between 5.5 and 7.5, and an optimum temperature range between 30-45 ℃. The lipase is said to be produced by a genetically modified microorganism, but the microorganism is not present in the final enzyme preparation and no GMO-labeling is required on food products.

3.2.5 Y1

Y1 is a lipase that has, according to the producer, highest specificity for MCFAs, second highest specificity for SCFAs and lowest specificity for LCFAs. It is said to have a much higher affinity for position 1 and 3 compared to position 2. The lipase is understood to prefer a temperature range of 30-45 ℃ and a pH value between 4.0 to 7.0. Y1 is stated to be most suitable for transesterification reactions, but can be used for both hydrolysis- and esterification reactions as well. It is said to be used in applications such as oil processing, dairy industry and baking and it is classified as non-GMO.

3.2.6 Y2

Y2 is a lipase that has highest specificity for SCFAs and MCFAs, according to the producer. It is said to have no positional specificity and to prefer a temperature range of 30-40 ℃ and a pH range of 3.0 to 8.0. Y2 is stated to be applicable in oil processing and performs hydrolysis- and esterification reactions.

3.2.7 Y3

Y3 is a lipase that has, according to the producer, the highest specificity for SCFAs and MCFAs and it acts specifically on position 1 and 3 on TAGs. The lipase is stated to prefer a temperature of 30-40 ℃ and a pH between 6.0 to 8.0. Y3 is understood to be used in the dairy industry and performing hydrolysis-, esterification- and transesterification reactions.

3.3 Enzymatic reactions of lipids

3.3.1 Hydrolysis and reversed hydrolysis

Hydrolysis is the reaction where bonds in a larger molecule are broken to form smaller molecules by addition of water. In the enzymatic hydrolysis of TAGs, it is the ester bonds between the fatty acid chains and the glycerol backbone that is broken by the enzyme. The ester bond is attacked by the serine in the active site and the result is an acyl-enzyme and a DAG. The second substrate, water, enters the active site and deacylates the enzyme, forming the second product, a fatty acid (Dicko et al., 2022). The overall reaction is visualized in Figure 3.

The hydrolysis reaction can occur reversibly as well. This reaction, called esterification or reverse hydrolysis, is also catalyzed by hydrolases. The reactants in esterification of TAGs are an alcohol moiety and a fatty acid moiety while the products are a TAG and water (Figure 3). Whether the reaction goes in the direction of the hydrolysis or the reverse hydrolysis depends on initial concentrations of the reactants and the equilibrium constant (Dicko et al., 2022).

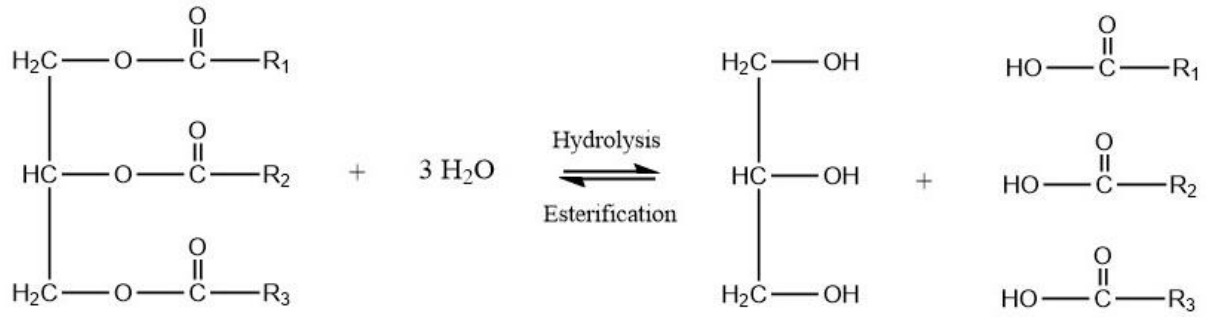


Figure 3. Enzyme-catalyzed hydrolysis and esterification of TAGs.

3.3.2 Transesterification

Transesterification is a reaction where the fatty acid moiety is exchanged between two esters. It can be the exchange between a TAG and FAE or two TAGs and are then referred to as interesterification (Zhang, 2001). It can also occur between a FAE and an alcohol and are then generally referred to as alcoholysis (Causevic, 2022). In this project, the transesterification between methyl esters with

different fatty acids and 1-propanol have been studied. The generic reaction can be written as in Figure 4, where R_1 is a fatty acid with varying carbon chain length, Me is a methyl group and Pr is a propyl group.

Figure 4: Transesterification between a methyl ester and 1-propanol.

Interesterification between a TAG and FAE is a multistep reaction where hydrolysis of TAGs to form DAGs and FFAs is the first step. After that, the DAG will act as a nucleophile and form a new TAG with the fatty acid from the FAE while alcohol is released. The reaction can be seen in Figure 5. The alcohol formation makes it possible to form DAGs in another way, through alcoholysis. These reactions are complex due to the different potential nucleophiles and acyl donors, and also the reversibility of the reactions (Causevic, 2022). The reactions are kinetically controlled reactions and the direction and extent of the reactions depend on the concentrations of the substrates, reaction times and enzyme.

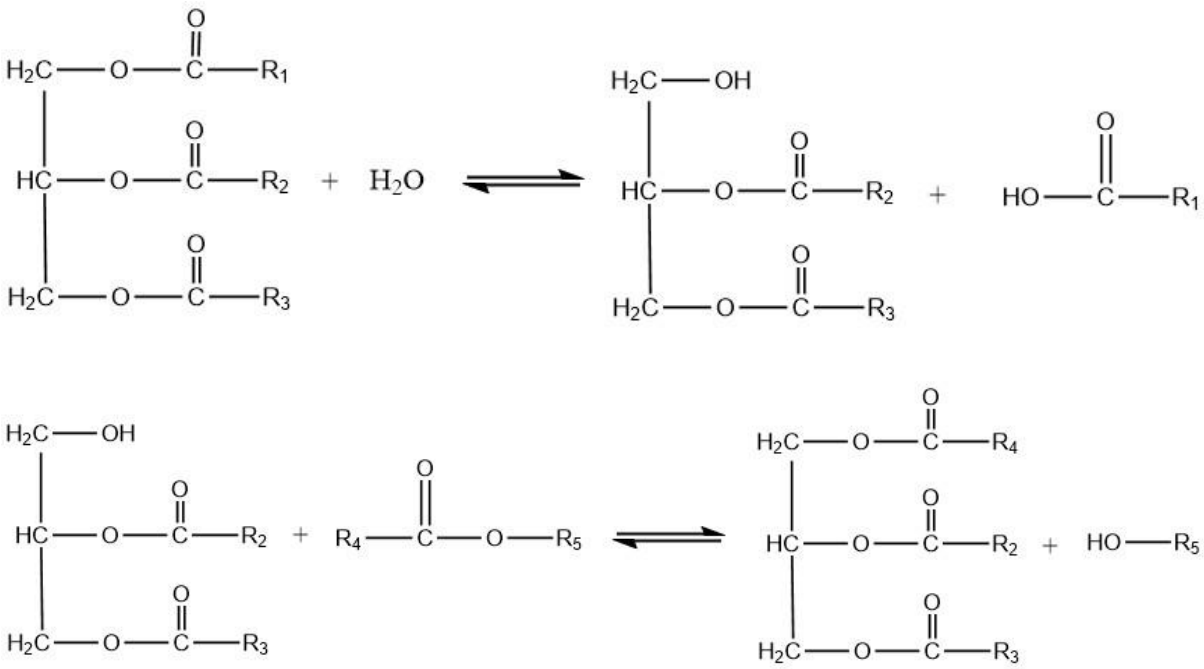


Figure 5: Interesterification of a TAG.

Whenever DAGs are present, a side reaction called acyl migration can take place. In the initial stage of the interesterification reaction a lot of DAGs are formed, making it susceptible to this unwanted side reaction. Acyl migration is a spontaneous intramolecular process where a fatty acid is transferred to an adjacent free hydroxyl group. The reaction mechanism is shown in Figure 6. The reaction is initiated by a nucleophilic attack on the carbonyl carbon on the *sn*-2-position by the free hydroxyl group, which forms an intramolecular cyclic intermediate. In the next step, the hydroxyl oxygen on the cyclic intermediate attacks the carbon again. This leads to opening of the cyclic intermediate and thus the fatty acid has migrated from the *sn*-2-position to the *sn*-1(3)-position (Dicko et al., 2022; Causevic, 2022). The problem with this side reaction is that it causes changes in the *sn*-2-position although the enzyme used for transesterification is *sn*-1,3 specific (Dicko et al., 2022). Acyl migration

from the *sn*-2-position to the *sn*-1(3)-position is a result of the higher thermodynamic stability for *sn*-1,3 DAGs compared to *sn*-1(3),2 DAGs. The reaction is more probable to occur for SCFAs and unsaturated fatty acids (Boswinkel et al., 1996) and is favored by high temperature, acidic- or basic conditions, solvents and particular materials such as silica gel. Acyl migration is an unwanted reaction during selective lipid synthesis and should be minimized (Causevic & Gladkauskas et al., 2022).

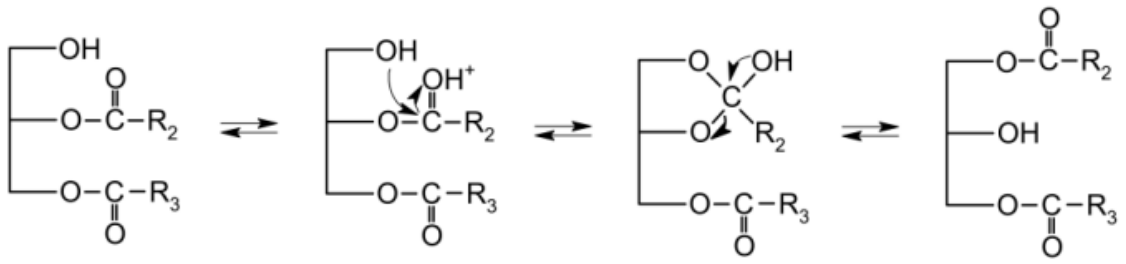


Figure 6. Reaction mechanism of acyl migration of a DAG (Causevic, 2022).

3.3.3 Water and temperature effects on enzyme activity

Factors such as amount of water and temperature will affect the activity of enzymes and also the product yield and purity (Dicko et al., 2022; Causevic, 2022).

The amount of water will affect the hydration level of the enzyme, which affects its flexibility. Water acts as a lubricant and therefore the flexibility is often increased with increased water content. This leads to increased enzymatic activity (Dicko et al., 2022). However, water also acts as a substrate in hydrolysis reaction and will determine the equilibrium between transesterification products and hydrolysis products. Therefore, the water content should only be increased to a certain degree if the transesterification reaction is wanted (Dicko et al., 2022; Causevic, 2022). The water in the system will be distributed between the enzyme, solvent and support material. This means that the hydration level of the enzyme is better correlated to water activity, defined as the partial vapor pressure of water in a solution divided by the vapor pressure of pure water at a given temperature, than to water concentration. It is thus favorable to measure water activity when investigating water effects on enzyme activity (Dicko et al., 2022). Studies have shown that there is a maximum in enzymatic activity at a certain water activity. At low water activities the enzyme is too rigid to work optimally, and at higher water activities water can accumulate on the enzyme surface, causing reduced enzymatic activity due to substrate transport limitations (Dicko et al., 2022; Causevic, 2022). It does not seem to matter for the enzyme activity versus water activity profile if it is esterification, transesterification or hydrolysis (Wehtje & Adlercreutz, 1997). The optimal water activity is independent of the reaction and implies that optimal water activity is individual for each enzyme (Valiveti et al., 1994). The stability of enzymes has also been suggested to decrease at higher water contents since it facilitates partial unfolding and cleavage of peptide bonds, which in turn leads to decreased enzyme activity (Dicko et al., 2022).

Temperature influences all chemical reactions and the flexibility of enzymes and thus affects the activity of enzymes. Higher temperature leads to higher flexibility and activity. However, it is only true to a certain degree until thermal inactivation becomes the dominating factor. This means that increasing the temperature is beneficial, but only up to a certain point. As mentioned in section *3.3.2 Transesterification*, the unwanted acyl migration during transesterification is also favored by high temperature. Lower temperatures can therefore be used to obtain high purity products. The limiting

factor for lowering the temperature is that all substrates must be in liquid form to be accessible for the enzyme (Causevic, 2022).

3.4 Immobilization of enzymes

3.4.1 Advantages with immobilization

Enzymes have many advantages, like their ability to catalyze reactions under mild conditions and with a high substrate specificity. However, enzymes are relatively unstable and exposure to unfavorable conditions can cause denaturation and loss of catalytic activity. These unfavorable conditions include high temperatures, high or low pH, water-miscible organic solvents and presence of proteases. The low stability of enzymes in these conditions is limiting their use. The reason for why enzymes are so unstable is because they need a flexible structure to function. In living cells, some enzymes need to be transferred over membranes to reach their intended destination and cells must also be able to break down the enzymes relatively easily to not interfere with the body's regulation, two functions that require flexibility. This flexible structure is kept together by weak, non-covalent bonds that are easily broken. Further, the amino acid side chains are relatively reactive and can cause the attraction in the structure to change (Dicko et al., 2020).

In the technical industry the restrictions that apply for the living cell do not apply in the same way and a more rigid structure can still function. By immobilizing the enzymes, their stability can be enhanced, which consequently enhance their technical performance. In most immobilization methods additional bonds are introduced, occurring between the enzyme and the support. The bonds help the enzyme retain its structure even under altered conditions. It is important that the support does not itself alter the structure of the enzyme by attaching at too many points to the enzyme. More bonds increases the stability, but too many will lead to distortion of the enzyme which causes a modified enzymatic activity or even complete inactivation. For porous supports, retention of the enzyme structure is not the only advantage with immobilization. These supports contain pore systems where the immobilization occurs and this pore system will protect the enzymes from temporary condition changes in the medium. The pore system also protects against degradation from microbial proteases since they are too large to enter the narrow pores where the enzymes are located (Dicko et al., 2020).

Immobilization of enzymes will improve their stability in multiple unfavorable conditions, but it will also improve the storage properties as well as their operational lifetime. This will make the enzyme more useful, for example operating at a higher temperature will accelerate the reaction rate, lower reaction times and make enzymes a viable catalysator option for substrates that are not soluble at lower temperatures. This will also make the enzyme more economically beneficial, for example longer operational lifetime will reduce the amount of enzyme needed and less replacing leads to a reduced amount of time where the production is standing still (Dicko et al., 2020). Another advantage with immobilized enzymes over their free counterpart is that they are easier to handle in many situations, they can be reused and enzymes and products from immobilized enzyme systems are more easily recovered. This leads to a product free from the contaminating enzyme which saves purification processes in industries where purity is highly desired, such as pharmaceuticals (Homaei et al., 2013). When it comes to applications using non-conventional media, such as oils and fats, the enzymes should be immobilized to function optimally. Free enzymes tend to aggregate and thus decrease the enzyme's activity (Dicko et al., 2022).

3.4.2 Problems with immobilization

The immobilization procedure is not without its disadvantages. Inactivation from too strong bond formation between support and enzyme has already been mentioned, but even if enzyme activity is retained, immobilizing the enzymes has drawbacks. Just as steric hindrance keeps microbial proteases out of the pores, larger substrates will have reduced accessibility to the enzymes, which leads to a loss of catalytic activity (Brena et al., 2013). Additionally, the interaction between enzyme and substrate takes place in a microenvironment created by the support and this can differ from the environment in the medium. The occurrence of partitioning effects of H^+, causing a local change in pH, means that the measured pH of the medium might not be the same as the pH experienced by the enzymes in the pores of the support. Often the pH of the medium is set knowing the optimum pH of the enzyme. If this is not the pH experienced by the enzyme, a decrease in catalytic activity might be observed (Dicko et al., 2020).

Another factor that might affect the immobilized enzyme's activity negatively is the presence of mass transport limitation. Mass transport limitations occur when the rate at which substrate is transported from the medium to the enzyme site, relative to the enzymatic reaction rate, cannot be neglected. Mass transport limitations can be divided into two problems: external diffusion limitations and internal diffusion limitations. External diffusion limitations are the obstruction of substrate transport through a stagnant layer close to the support surface, resulting in longer times for substrate to enter the pore system of the support. Internal diffusion limitations are the restricted diffusion of substrate and product through the pore system of the support, meaning that different substrate and product concentrations will be experienced by enzymes at different parts of the support. Enzymes immobilized closer to the middle part of the support will have less substrate available and therefore not operate at maximum capacity and more product will accumulate close to the enzyme, leading to a lower reaction rate (Illanes et al., 2020).

The problems and drawbacks encountered when working with immobilized enzymes can be minimized by designing the system in a way that limits the negative effects and by choosing an appropriate support. This will be more thoroughly discussed below in section *3.4.4 Important immobilization parameters*.

3.4.3 Methods of immobilization

There are multiple methods used to immobilize enzymes: covalent binding to support, cross-linking, entrapment, membrane confinement and adsorption to support.

Covalent binding to support and cross-linking are two methods that both involve powerful, covalent bonds. In covalent binding to support, the bonds are between enzyme and support, whilst in the cross-linking method the bonds are between multiple enzyme molecules. Cross-linking is a carrier-free method and therefore does not require any support. The cross-linking is induced by reagents such as glutaraldehyde. The free amino group of the lysine residue on the enzyme surface reacts with polymers of the reagent (Mohamad et al., 2015). Covalent binding to support requires the support to be activated before coupling to the enzyme. Activation of the support can be achieved by addition of a number of substances, which one to use depends on what chemical groups the support contains (hydroxyl, carboxyl, thiols, etc.) and what kind of conditions that are desired (Dicko et al., 2022). Because of the strong covalent bonds, both methods create irreversible immobilizations which enhances their reusability compared to other methods (Mohamad et al., 2015). Another method is

entrapment. Entrapment is a method where the enzymes are entrapped in a support or a polymeric network. The enzyme's movements are restricted, but substrate and product can move freely in and out of the support/network (Sirisha et al., 2016). Entrapment is also an irreversible immobilization method (Mohamad et al., 2015). An additional method, membrane confinement, is a method where the enzymes are not attached to any support, but instead confined in a protective space. The enzyme appears free, but still shares many of the advantages the other immobilization methods possess since the enzymes are still isolated. Common confinement strategies are the use of microcapsules, membrane devices and constructing a two-phase system where the enzymes can be confined to the aqueous phase (Dicko et al., 2022).

The last method, adsorption to support, is the method used in this thesis. In adsorption to a support, the enzymes bind to the surface of the support by weak bonds, such as van der Waals, hydrophobic interactions or affinity interactions. Incubation is necessary to allow the enzymes to be adsorbed and enzymes that have not been adsorbed can be washed away afterwards. The use of adsorption generates weaker bonds than other immobilization methods and this can be both advantageous and disadvantageous. The weak bonds means that this method is relatively reversible, which can be useful when the immobilized enzymes have lost their activity. The support can then be regenerated and new enzymes can be applied to the support. The weakness of the bonds also leads to better retaining of the enzyme activity, as less distortion of the enzyme occurs. On the other hand, the weak bonds also means that the immobilization is less stable and the risk of enzyme leakage increases (Mohamad et al., 2015).

3.4.4 Important immobilization parameters

There are a number of factors that will affect the success, i.e. the retained catalytic activity, of the immobilization. It is important to take these factors into consideration when planning the system. As mentioned above, this can minimize the drawbacks that come when working with immobilized enzymes.

Choosing a support with the correct properties is very important, especially when the immobilization method used is adsorption to support. A pore size large enough that the enzyme fits is necessary, but at the same time too large pore size may lead to enzyme leakage (Hartmann et al., 2013). Another influencing factor is the surface area of the support, large surface area is desired as it will be able to bind more enzymes. A larger surface area can be obtained by using a porous material (Mohamad et al., 2015; Dicko et al., 2020). The support must of course also be compatible with the enzymes it is going to bind to, the hydrophobicity or hydrophilicity of the support surface is strongly influential to the immobilization. Most enzymes are rather hydrophilic and adsorbs to hydrophilic supports. However, lipases have been proven to adsorb more efficiently to hydrophobic supports. This was explained by the fact that lipases contain hydrophobic areas surrounding the active site, which allows the lipase to be adsorbed to the hydrophobic surface of the support (Hartmann et al., 2013; Fernandez-Lorente et al., 2020). Inorganic support materials such as glass and silica-based materials work very well as a support for immobilization, they are rigid and porous materials that do not promote bacterial growth. Silica-based material, a widely used inorganic support, gives ordered structures, are very stable and has a large surface area. Silica-based materials also contain both hydrophobic and hydrophilic sites which gives it a diverse adsorption profile (Sirisha et al., 2016; Soleimani et al., 2012). However, inorganic materials have weaknesses. Inorganic materials often require additional bonds to be created, as lipases have a low affinity to these materials. The other category of support materials, organic support material, does not have this weakness as these materials

have a higher affinity and bind more easily to lipases (Ismail et al., 2020). Organic support materials refer to both the natural and synthetic supports. Natural organic materials consist of mainly water-insoluble polysaccharides. These materials form inert gels, are easily activated and relatively cheap. This makes them an attractive choice of support (Sirisha et al., 2016). Synthetic polymers on the other hand, are a porous and insoluble material that includes the polymer polypropylene, which the support material used in this thesis, MP1000, is made of. Synthetic polymers help keep the active site of the enzyme safe from distortion after immobilization and are very useful when increased thermal stability or reusability is desired (Ismail et al., 2020; Sirisha et al., 2016).

It is not only the characteristics of the support that determine the efficiency of the support, using the optimal temperature, pH and salt concentration of the buffer during immobilization will improve the hydrophobic interaction between the enzymes and the support. Optimization of these parameters are therefore desired to improve the overall immobilization efficiency (Fopase et al., 2020). Another factor regarding the immobilization that must be taken into consideration is the amount of enzyme immobilized, referred to as enzyme loading. More enzyme loaded onto the support gives a higher overall activity. However, if the support contains a high load of enzyme and their activity is compared to the same amount of free enzyme, the activity will be lower. This is because at high enzyme loading, the enzymes in the center of the support will be starving for substrate and not be working close to their maximum capacity. No actual loss of activity has occurred but it will appear so because of mass transport limitations. Low enzyme loading on the other hand would lead to more substrate available to the enzymes closer to the center and therefore a system where more enzymes are operating closer to their maximum capacity. This also means a lower overall enzymatic activity as less amount of enzymes have been immobilized. Finding the appropriate loading where as much enzymes as possible have been immobilized, while the enzymes still work close to saturation, is necessary to optimize the effectiveness of the process. Mass transfer limitations regarding enzyme loading refers to internal mass transfer limitations. Generally, the external mass transfer limitations are typically less of a problem and can often be circumvented. By ensuring high degree of mixing in the reactions, the stagnant layer outside of the support can be decreased and external mass transfer limitations can be abolished in comparison to the internal (Dicko et al., 2020).

3.5 Analysis methods

3.5.1 Gas chromatography - Flame ionization detector (GC-FID)

Gas chromatography (GC) is an analytical method used to determine presence/absence and quantities of chemical components in a liquid solution. A sample from the solution is taken and injected into the gas chromatograph manually or by an autosampler. Then it is vaporized and transported through an analytical column by the mobile phase, the carrier gas, which often consists of helium since it should neither react with the sample nor the instrument components. The analytical column, which is placed inside an oven where the temperature can be set to desired value, is where separation of the sample components occurs. The separation is based on the volatility of the components and their interaction with the stationary phase coated on the walls of the fused silica column. The size of the column can vary in length from 10-150 m and an inner diameter between 0.1-0.53 mm. The sample components are then eluted from the column and reach the detector at different times (Turner, 2021).

The gas chromatograph is coupled to a detector that responds to the eluted chemical components and converts it to a signal that can be analyzed using a computer program. One type of detector often used

is flame ionization detector (FID) (Hinshaw, 2005). When the components reach the FID together with the carrier gas, they are mixed with hydrogen gas and air to get combusted and produce charged particles. The charged particles are collected in a collector electrode by applying a voltage, the current created in the collector electrode is very small, however proportional to the amount of solute, and has to be amplified to be analyzed. FID is a highly sensitive detector to analyze molecules ionized in a hydrogen-air flame, including hydrocarbons (Hinshaw, 2005; Poole, 2015).

3.5.2 Spectrophotometry

A microplate reader is a type of spectrophotometer, which is an instrument that sends light through a sample coupled with a detector on the other side of the sample, to compare the light intensity before and after the sample. Different spectrophotometers have different sample holders with varying size and number of wells. The microplate reader used in this project had a sample holder with 96 wells. A microplate reader consists of an optical system with a light source that sends light through a monochromator and wavelength selector to emit the selected wavelength through the sample. After the light has passed the sample, it hits a photodetector that sends a signal to a computer software where the data can be analyzed (Excedr, 2023). When the light passes through the sample, it interacts with the matter in the sample and light of a certain wavelength is absorbed depending on the substance in the sample. Different molecules have different absorbance spectra and these are used to select wavelength for measurements (BMG LABTECH, n.d.).

The Bradford assay can be used to analyze protein content in a solution. The Bradford reagent contains Coomassie brilliant blue (CBB) G-250 which is a reddish/brown compound and has an absorbance maximum at 465 nm. When CBB G-250 interacts with amino acids it becomes blue and has an absorbance maximum at 595 nm. This makes it possible to determine the protein concentration since the intensity of the blue color is directly proportional to the protein concentration (Shen, 2023). By doing measurements with solutions of known protein concentrations, a standard curve can be generated. This standard curve can then be used to calculate the protein concentration of solutions with unknown protein concentration.

When analyzing an enzyme's hydrolytic activity, the hydrolysis of *para*-nitrophenyl butyrate (*p*-NPB) might be used. When the enzyme hydrolyses the *p*-NPB, the hydrolysis products become *p*-nitrophenol and butyric acid. The *p*-nitrophenol has an absorbance maximum at 400 nm, hence the hydrolytic activity (rate of release of *p*-nitrophenol) can be determined by continuously measuring the absorbance increase at 400 nm over time (Tsujita, 1989). The formation rate of *p*-nitrophenol together with its extinction coefficient can then be used to calculate the enzyme activity.

When using microplate readers there are some pitfalls. If the volume in the wells are different, the pathlength will vary and the results can be misleading. Also, everything that disturbs the light path will contribute to an absorbance increase and give misleading results. For example air bubbles, dust, scratches or fingerprints (BMG LABTECH, n.d.).

4. Materials and Methods

4.1 Materials

4.1.1 Immobilization

Commercial enzyme powder preparation of the lipases X1, X2, Y1, Y2, Y3 was used in this thesis, as well as a liquid enzyme preparation of the lipase Lipozyme® TL 100 L from Novozymes. The support material used for immobilization of the lipases was porous polypropylene (MP1000) with a diameter between 250 and 500 µm. As an immobilization buffer, a 500 mM sodium phosphate buffer at pH 7 made from NaH_2PO_4 and Na_2HPO_4 was used. For wetting of the MP1000, pure ethanol was used. The incubation took place in baffled flasks of 500 ml from Duran, placed in a shaking incubator called Lab-Therm LT-X. Filter papers from Munktell with porosity of 3 was used for filtration of the immobilized lipases.

4.1.2 Protein content determination

For the protein content determination, Bradford reagent and a 50 mM sodium phosphate buffer at pH 7 made from NaH_2PO_4 and Na_2HPO_4 was used. The creation of a calibration curve required bovine serum albumin (BSA) as a standard. For the analysis, a microplate reader from ThermoScientific called Multiskan Go was used.

4.1.3 Lipase hydrolytic activity assay

A 500 mM sodium phosphate buffer at pH 7 made from NaH_2PO_4 and Na_2HPO_4 was used during the lipase hydrolysis activity assay with *p*-nitrophenyl butyrate mixed with isopropanol with a concentration of 20 mM. For the analysis, a microplate reader from ThermoScientific called Multiskan Go was used.

4.1.4 Transesterification reaction

The transesterification reactions took place in 4.5 ml screw-capped glass vials with teflon lined septums. A Hettich thermoshaker was used for mixing during the reactions and keeping the temperature constant. To take samples, a Hamilton® glass syringe was used. Chemicals used for the reactions were methyl esters, 1-propanol, ethyl laurate and cyclohexane. The methyl esters used in the transesterification reactions can be seen in Table 2 where their purity and producer is stated. The reactions were run on the enzyme preparations mentioned under *4.1.1 Immobilization*, as well as on Lipozyme® TL IM from Novozymes. For analysis of the reactions, a Thermo Scientific TRACE1300 GC with a flame ionization detector and a ZB-FAME colon (20 m x 0.18 mm and 0.15 µm film thickness) was used.

Table 2. Substrate specification of the substrates used in the transesterification reactions. N/A = Not Applicable.

Substrate	Producer	Purity
Methyl butyrate	Sigma Aldrich	99%
Methyl octanoate	Sigma Aldrich	99%
Methyl laurate	Sigma Aldrich	≥98%
Methyl oleate (Individual substrate reactions)	Thermo Fisher Scientific	89.4%
Methyl oleate (Mixed substrate reactions)		N/A
Methyl stearate	Sigma Aldrich	≥96%

4.2 Methods

4.2.1 Immobilization of lipase

Immobilization of the lipases on the support material, MP1000, began with weighing up MP1000 and lipases. Two separate immobilizations were performed, in the first 5 g MP1000 was used and in the second 4 g MP1000 was used, see Table 3 for the exact amounts of lipase and MP1000. The amount of lipase for each immobilization was decided based on the lipases' hydrolytic activities. The next step was wetting the MP1000 with ethanol, as well as dissolving the lipase in a 500 mM phosphate buffer. 3 ml ethanol was used per gram MP1000, while the amount of phosphate buffer had a ratio of 40 ml buffer per gram lipase for the first immobilization and 20 ml buffer per gram lipase for the second, these amounts can also be seen in Table 3. Then, a 500 µl sample from the enzyme solution was taken for further analysis of protein content and hydrolytic activity before mixing it with the wetted MP1000. After the enzyme solution was added to the wetted MP1000 it was left for 17 hours in a shaking incubator at 170 rpm. After the incubation, the enzyme solution was filtered using a filter with a porosity of 3 and vacuum equipment. A 500 µl sample from the permeate was taken for protein content- and hydrolytic activity analysis. Finally, the retained enzyme preparation on the filter was washed with 5 ml 500 mM phosphate buffer per gram MP1000 and further dried with the vacuum equipment. The immobilized lipase was then collected and divided into several petri dishes and dried in a vacuum desiccator for 60 respectively 55 hours, see Table 3 for the time for each lipase.

Table 3. Amount of lipase, MP1000 and buffer used for immobilization and their incubation- and drying time.

Lipase	Amount of enzyme preparation	Amount of MP1000	Amount of buffer	Incubation time	Drying time
X1	4.99 g	5 g	200 ml	17 h	60 h
X2	20.0 g	4 g	400 ml	17 h	55 h

Y1	20.0 g	4 g	400 ml	17 h	55 h
Y2	10.8 g	5 g	430 ml	17 h	60 h
Y3	20.0 g	4 g	400 ml	17 h	55 h
TL	16.7 ml	5 g	268 ml	17 h	60 h

4.2.2 Protein content determination

The enzyme solutions before and after immobilization, described in the above paragraph, were used to determine the protein content of the enzyme preparations and the protein immobilization yield. The protein immobilization yield was determined indirectly by assuming that the difference in protein content between the solution before and after immobilization was immobilized on the support material. To determine the protein content in each of the solutions, the Bradford method was used. Firstly, a calibration curve was made by creating and measuring the absorbance of five standards. To make the standards, 20 mg BSA was mixed with 20 ml of a 50 mM phosphate buffer to make a 1.0 mg/ml solution. It was then diluted to make solutions with concentrations of 0.2, 0.4, 0.6 and 0.8 mg/ml. A volume of 6.9 µl of the standards were then added to the 96-wells microplate before adding 343 µl of Bradford reagent. After incubation for 5 min, the absorbance was measured spectrophotometrically at 595 nm in room temperature (21℃) in triplicates. The calibration curve with its equation used to calculate protein content can be found in Figure A6 in Appendix. After constructing the calibration curve, the enzyme solutions were diluted 3, 9, 27 and 81 times with a 500 mM phosphate buffer before their absorbance were measured in the same way as the standards. From the results of the spectrophotometric measurement, the dilution that fitted best within the calibration curve was chosen and measured in triplicate. The protein content of the lipases could then be calculated from the calibration curve. The amount of protein immobilized and protein immobilization yield could be calculated using Equation (1) and (2), where $c_{p,before}$ and $c_{p,after}$ is the protein content before and after immobilization.

$$Amount\ of\ protein\ immobilized = c_{p,before} - c_{p,after} \quad (1)$$

$$Protein\ immobilization\ yield = \frac{c_{p,before} - c_{p,after}}{c_{p,before}} \quad (2)$$

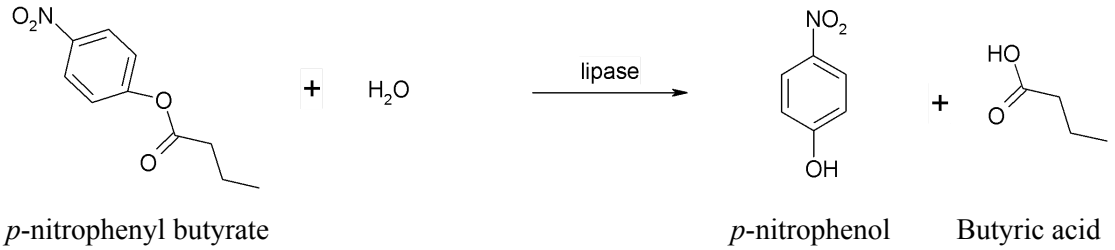
4.2.3 Lipase hydrolytic activity assay with *p*NPB

To determine the lipase's hydrolytic activity, a spectrophotometric assay based on the hydrolysis of *p*NPB was performed. The enzyme solutions from before and after the immobilization, described in *4.2.1 Immobilization of lipase*, were diluted 3, 9, 27 and 81 times to be able to observe the initial reaction rate. They were then mixed with 388 µl of 500 mM phosphate buffer, and just before the measurement, 8 µl of 20 mM *p*NPB in isopropanol was added and the whole sample mixed. Then the absorbance at 400 nm and 30°C was measured with a 2 s interval for 10 min. After a first run, the dilutions where the initial reaction rate was observable were measured in triplicates to make it more reliable. When the lipase hydrolyses *p*NPB, *p*-nitrophenol and butyric acid is formed, which can be seen in Figure 7. *p*-nitrophenol absorbs light at a wavelength of 400 nm and the absorbance increase at 400 nm is therefore used as a measurement of the reaction rate, calculated as the slope of the

absorbance curve (increased absorbance per time). The formation rate (µM/min) of *p*-nitrophenol was then calculated from its extinction coefficient (16000 $M^{-1}cm^{-1}$), sample volume (400 µl) and path length (1.07 cm) and was used to calculate the lipase activity (U/ml) in the stock. The spontaneous hydrolysis of *p*NPB was measured by adding 8 µl of *p*NPB to 392 µl of 500 mM phosphate buffer. After the enzyme solutions hydrolytic activity was calculated, the amount of lipase activity immobilized and activity immobilization yield could be calculated using Equation (3) and (4), where a_{before} and a_{after} is the activity before and after immobilization respectively.

$$Amount\ of\ lipase\ activity\ immobilized = a_{before} - a_{after} \quad (3)$$

$$Activity\ immobilization\ yield = \frac{a_{before} - a_{after}}{a_{before}} \quad (4)$$



p-nitrophenyl butyrate *p*-nitrophenol Butyric acid

Figure 7. Hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol and butyric acid by lipase.

4.2.4 Evaluation of lipase activity on methyl esters

4.2.4.1 Water content optimization

To determine the optimal water concentration for the lipases, reactions with three different water concentrations were run and analyzed. In the optimization, water concentrations of 0.25%, 0.5% and 1.0% (vol%) and one substrate, methyl laurate, was used. Transesterification reactions were carried out for all lipases using the three water activities. This step is more thoroughly explained in *4.2.4.2 Transesterification of methyl esters*. After the reaction, gas chromatography was used to determine the enzymatic activity, this step is more thoroughly explained in *4.2.4.3 Lipase activity determination*.

4.2.4.2 Transesterification of methyl esters

The transesterification reactions for all lipases, except Y1, were performed by mixing 100 mg of immobilized lipase with 1990 µl of a substrate/1-propanol mixture and 10 µl water in a 4.5 ml septum capped vial, corresponding to a water concentration of 0.5%. The substrate/1-propanol mixture was mixed beforehand with a 3:1 molar ratio with the substrate being the excessive component. For Y1, only 20 mg immobilized lipase was used in the reactions. The substrates used were methyl butyrate, methyl octanoate, methyl laurate, methyl stearate and methyl oleate, as well as a mix of these substrates. However, the methyl oleate used in the mix is not the same as the methyl oleate used as pure substrate. A methyl oleate with a purity of 89.4% was used for the pure substrate reactions, while the one used in the mix had a purity 62.6%. Methyl stearate and 1-propanol were not mixed beforehand and were instead mixed right before the lipase was added. The methyl stearate was kept in an oven set at 50℃ before any reactions to ensure it was in liquid phase. For the mixture containing

all five substrates, they were added with the same molar ratio and the overall substrate to 1-propanol ratio was still 3:1. The vials were then put in a Thermoshaker and the reactions were carried out at 55 °C and 900 rpm. 10 µl samples were taken from the vials continuously during the reactions by inserting a syringe through the septum cap of the vials. The reactions were performed for different amounts of time, since only initial activity was desired to be determined. For more active lipases, samples were taken during a shorter amount of time while for less active lipases, samples were taken during a longer time period. Five samples were taken for each reaction regardless of the total reaction time. Samples were taken after 4, 8, 15, 22 and 30 min for all samples except for TL IM and Y1, where samples were taken after 3, 6, 10, 15 and 20 min. For the reactions with methyl stearate, the syringes were kept in an oven set to 80℃ between each sampling to avoid solidification of the stearate. All reactions were run in triplicates and it is stated in the figure captions in the results section how many replicates that were used. After the reactions had been completed, every sample was diluted in 990 µl of ethyl laurate in cyclohexane with a concentration of 27.5 mM. The ethyl laurate was used as an internal standard (ISTD) and the final concentration of the ISTD in the vial was 25 mM.

4.2.4.3 Lipase transesterification activity determination

For initial lipase transesterification activity determination, all samples were analyzed in a gas chromatograph, generating a peak area for each component. An example of a chromatogram showing peaks for the substrate, transesterification product and internal standard can be seen in Figure 8. The temperature programs used for the GC differed between the substrates and they can be found in Appendix Table A1-A6. To be able to convert the obtained peak area to concentration, known concentrations of the products were run in the GC and a calibration curve for each product could be constructed. The obtained calibration curves can be found in Figure A1-A5 in the Appendix. For the calibration curves, a stock of 200 mM and a stock of 20 mM were created for each product, this was done by diluting the products with cyclohexane. From these stocks, vials containing 50 mM, 40 mM, 30 mM, 20 mM, 10 mM, 5 mM and 1 mM were made for each product. The vials also contained 500 µl of 50 mM ethyl laurate (ISTD), and cyclohexane was then added to the vials to make the final volume 1 ml (for vials already containing 1 ml, no cyclohexane was added). A scheme of the dilution for the calibration standards can be seen in Table 4 and Table 5. From the calculated concentrations of every sample, the amount of transesterification product could be determined and plotted versus time, an example can be seen in Figure 9. A linear regression of the quantity of product produced versus time then gives the initial transesterification activity. Further, the initial transesterification activity per gram immobilized carrier and per mg protein immobilized could be calculated. When analyzing the activity in the mixture, the relative specificity factor k_{cat}/K_M was used to interpret the results.

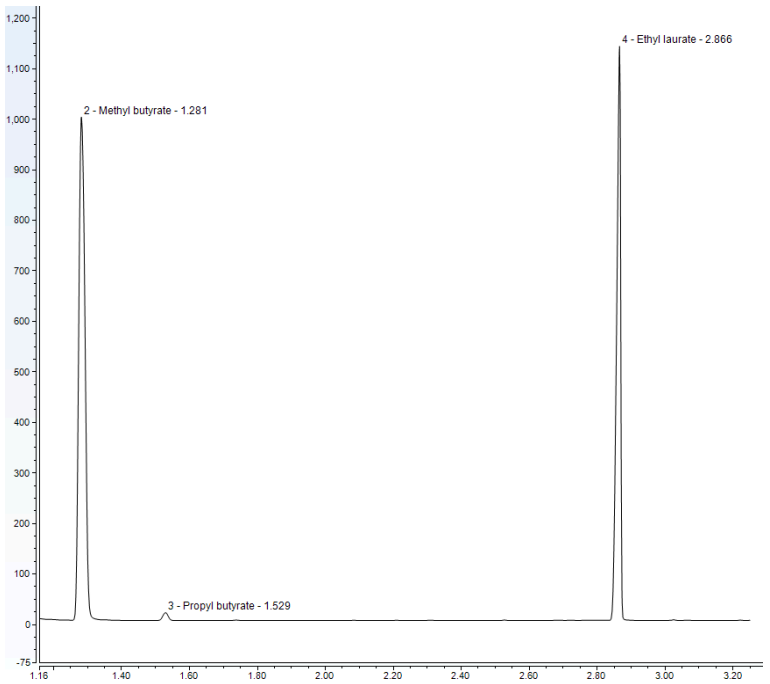


Figure 8. Chromatogram including peak for methyl butyrate, propyl butyrate and ethyl laurate.

Table 4. Dilution scheme for creating calibration standards of 50, 40, 30, 20 and 10 mM for evaluation in the GC.

Product conc. (mM)	**Added 200 mM propyl-ester (µl)**	**Added 200 mM fatty acid (µl)**	**Added 50 mM ISTD (µl)**	**Added cyclohexane (µl)**	**Final volume (µl)**
50	250	250	500	0	1000
40	200	200	500	100	1000
30	150	150	500	200	1000
20	100	100	500	300	1000
10	50	50	500	400	1000

Table 5. Dilution scheme for creating calibration standards of 5 and 1 mM for evaluation in the GC.

Product conc. (mM)	**Added 50 mM propyl-ester (µl)**	**Added 20 mM fatty acid (µl)**	**Added 50 mM ISTD (µl)**	**Added cyclohexane (µl)**	**Final volume (µl)**
5	250	250	500	0	1000
1	50	50	500	400	1000

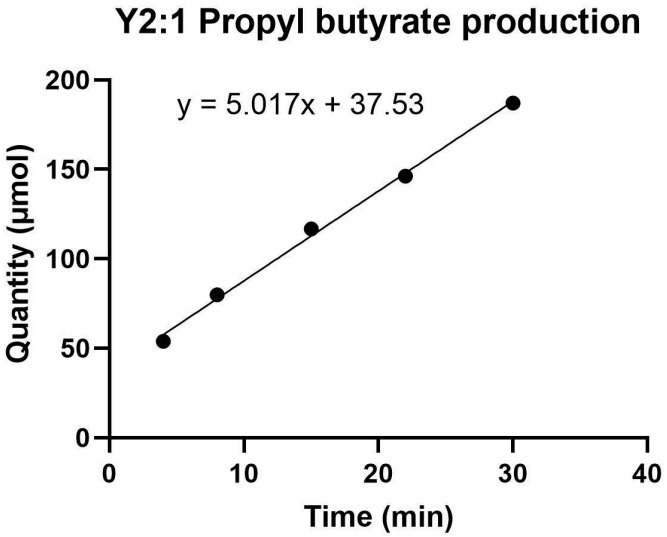


Figure 9. Linear regression of quantity of product produced per time with corresponding equation.

4.2.4.4 Statistical analysis

All reactions were performed in triplicates to increase the statistical reliability, but not all replicates were usable and the number of replicates used is specified in each figure- and table text. To evaluate differences between lipase activities on different substrates, one factor analysis of variance (ANOVA) was done in Microsoft Excel. All statistical analysis and conclusions were based on a significance level of $p<0.05$.

5. Results and Discussion

5.1 Protein content in enzyme preparation

From the Bradford assay, presented in Table 6, it can be seen that the commercial enzyme preparations contain other components than proteins. The results showed that X1 contains the highest amount of protein, while X2 and Y3 contain a significantly lower amount. Y1 and Y2 contain moderate amounts compared to the other lipases. TL is not a powder and therefore the estimated value is expressed in a different unit (mg/ml). The calibration curve constructed for the Bradford assay can be seen in Figure A6.

Table 6. Mean value and standard deviation of protein content in the commercial enzyme preparations expressed as weight percentage. Measurements were made in triplicates.

Lipase	**Protein content in enzyme preparation**
X1	69 ± 2.9 (mg/g)
X2	1.3 ± 0.49 (mg/g)
Y1	14 ± 1.1 (mg/g)
Y2	7.1 ± 0.55 (mg/g)
Y3	1.7 ± 0.31 (mg/g)
TL	1.4 ± 0.095 (mg/ml)

5.2 Hydrolytic activity in enzyme preparation

Originally, immobilizations of similar activity were desired for all lipases as this would minimize the effects a more active lipase might have on the reaction. This would result in more comparable results. The enzymes have different activities, so to be able to normalize the immobilization based on activity, different amounts of enzyme preparation will be needed. To determine the amount of enzyme preparation needed for each enzyme, the hydrolytic activity on *p*NPB was measured for each enzyme preparation and can be seen in Table 7. This activity was assumed to be relatively comparable to the transesterification activity. The results showed that the enzyme preparations had very different hydrolytic activities, making normalization on one activity for all lipases impossible. Instead, normalization in three groups was made: X1 and Y2 had the highest activities and formed group 1; X2, Y1 and TL had intermediate activities and formed group 2; Y3 had significantly lower activity than the rest and formed group 3 alone.

During method optimization, the observed transesterification activities for X2, Y1 and Y3 were very low, therefore a new immobilization with higher enzyme loading was attempted for these lipases. This altered the immobilized activity and therefore the normalization groups were not accurate anymore. This can be an indication that the hydrolytic activity on *p*NPB of a lipase is not very well correlated to the transesterification activity on methyl esters. It can also be a consequence of the lipases now being

immobilized. Since it is two completely different reaction systems, one with free lipases and *p*NPB in water and one with immobilized lipases in a non-aqueous environment, it is hard to draw any conclusions about it.

Table 7. Mean value and standard deviations of hydrolytic activity on *p*NPB in the commercial enzyme preparations expressed in units (U) per mg enzyme preparation. Measurements were made in triplicates.

Lipase	**Hydrolytic activity of enzyme preparation (U/g enzyme preparation)**
X1	3890 ± 425
X2	97.0 ± 21.5
Y1	369 ± 31.4
Y2	1810 ± 252
Y3	3.26 ± 0.412
TL	110 ± 11.6 (U/ml)

5.3 Water activity

As mentioned in *3.3.3 Water and temperature effects on enzyme activity*, the amount of water in the reactions can have a big impact on the activity. To minimize this effect and make the comparison between substrates more accurate, the plan was originally to have the same water activity in all substrates. To ensure that the water activity of the substrates worked for all lipases, an optimization was needed. Three preparations of methyl laurate with different water activities were constructed by incubating the substrate with a saturated salt solution. A saturated magnesium chloride (MgCl) solution was used to obtain a water activity of 0.25, a saturated magnesium nitrate ($MgNO_4$) solution was used to obtain a water activity of 0.50 and a saturated sodium chloride (NaCl) solution was used to obtain a water activity of 0.75. The solutions need a couple of days to achieve the wanted water activity, however, because of delays in the lab work, the solution ended up being incubated for around three weeks. The water content was analyzed using Karl-Fischer titration, it was then observed that the water content of the substrate preparations did not coincide with the intended water activities. The substrate preparation with higher water activity is assumed to have a higher water content than the substrate preparations with lower water activity. However, the water content was not related as expected between the substrate preparations. It was argued that 1-propanol is a volatile compound and that at 55℃, it is possible that the 1-propanol is evaporated from the substrate solution and that some of it dissolved in the salt solution. This would change the composition of the substrate and the saturated salt solution, affecting the water dissolving ability, which consequently affects the water activity.

Methyl butyrate is also a relatively volatile compound, making it susceptible to evaporation at 55℃. Setting the water activity and running the reactions at a lower temperature would diminish the volatility problem with 1-propanol and methyl butyrate. However, as methyl stearate has a melting temperature of 41-45°C, a temperature lower than 55℃ would possibly lead to solidification problems. On the other hand, the other methyl esters, including methyl oleate, have much lower

melting temperatures. It was suggested to set the water activities at a low temperature for the other methyl esters, before raising the temperature for the reactions. It would also be assumed that methyl oleate and methyl stearate dissolves water to a similar extent. If this was the case, the water concentration corresponding to the set water activity in methyl oleate, would be added to methyl stearate. However, it has been proved that the water solubility in methyl esters varies with temperature as water dissolves better at higher temperatures. More water dissolved in the solvent leads to less water available for the enzyme and a lower water activity. This means that unequal water activities will be obtained if the water activity is set at one temperature before raising it for the reactions. It was also shown that the methyl stearate has lower water solubility than methyl oleate (Oliveira, 2008). Therefore, it would be inaccurate to add the same water amount to these substrates and expect similar water activities. It was theorized that another, less volatile alcohol would work better, but it was concluded that re-doing the experiments could not be done within the time frame of this thesis.

Performing the reactions at the same water activity would be the preferred option, but because of volatility issues and time constraints, it was decided to use the same content instead. As explained above, this leads to different water activities for methyl oleate and methyl stearate. It will also lead to different water activities for the other substrates, as the water solubility decreases as the carbon chain of the methyl ester increases (Oliveira, 2008). Thus, this solution is not ideal and this must be kept in mind when evaluating the results.

5.4 Immobilization

The protein immobilization yield and amount of protein immobilized on the support material was determined for the different lipases and are presented in Table 8. Protein immobilization yield and activity immobilization yield are measurements on how successful the immobilization was. It is expected to observe a higher activity immobilization yield than protein immobilization yield since it has been shown by Gitelsen et al. (1997) that lipases are selectively adsorbed to hydrophobic support materials, such as MP1000.

From Table 8, it can be concluded that Y2 and TL were the most successful immobilizations and that immobilization of Y1 and Y3 was very poor. Regarding the immobilization yield results, it is worth noting that the carrier can only bind a certain amount of protein. The carrier was mixed with different amounts of enzyme preparations and some enzyme preparations contain more protein than others. Thus, saturation of the carrier might affect the observed yields. The affinity of the lipases towards the carrier is also a factor that influences the results (Hartmann et al.,2013). The very low activity immobilization yield for Y1, only 16%, suggests that almost no activity has ended up on the support. However, Causevic & Olofsson et al. (2022) hypothesized that low activity immobilization yield can be a consequence of dissolution of aggregated enzymes. When the inactive enzyme aggregates come in contact with the hydrophobic material, they might dissolve. This would result in active enzymes, leading to an increased activity in the enzyme solution after immobilization. This potentially means that more activity has been immobilized than what was observed.

From Table 8, it can be observed that X1 and TL had the most protein immobilized on the carrier. This is reasonable since it was shown in *5.1 Protein content in enzyme preparation* that the enzyme preparation for X1 contained significantly more protein than the other enzyme preparations. Looking at total immobilized activity, X1 again showed the highest results. However, as theorized regarding the low activity immobilization yield of Y1, it is possible that the estimated total immobilized activity of Y1 is undervalued as well.

For lipases X2 and Y3, the protein concentrations both before and after immobilization were too low to fit within the calibration curve. This made the obtained results unreliable and are presented as N/A in Table 8. To be able to measure and calculate the protein concentration in the enzyme solutions of X2 and Y3, they would need to be concentrated.

Table 8. Protein immobilization yields and activity immobilization yields expressed as percentages and amount of protein immobilized and amount of activity immobilized onto the support material expressed as mg protein per g carrier and U per g carrier respectively. N/A = Not Applicable.

Lipase	**Protein yield (%)**	**Amount protein immobilized (mg protein/g MP1000)**	**Activity yield (%)**	**Immobilized activity (U/g MP1000)**
X1	65	44.7	62	43.1
X2	N/A	N/A	51	3.44
Y1	42	28.2	16	2.19
Y2	80	12.2	84	11.2
Y3	N/A	N/A	26	0.253
TL	64	50.2	81	21.9

5.5 Lipase specificity for methyl esters

The initial transesterification activity of the lipases was evaluated for methyl esters with different carbon chain length. This was done to investigate which lipase had the highest activity on the different substrates and which carbon chain length they prefer as their substrates. Experiments were carried out on both the methyl esters individually, *5.5.2. Activity on individual methyl esters*, and all methyl esters mixed together, *5.5.3 Activity on methyl esters in mixture*. Both tables and figures are used to present the findings in this section and statistical analysis was performed to interpret the results.

5.5.1 k_{cat}/K_M as a specificity factor

For the individual substrate experiments, no other substrates were present to compete for the available enzyme and the experiments were carried out in a solvent-free environment. This meant that the substrate itself acted as the solvent. Therefore, it was assumed that the substrate concentration was far greater than the affinity constant, K_M, meaning the experiment would be located to the far right on a Michaelis-Menten curve, see Figure 10. Thus, indicating that the measured activity, presented in Figure 11-17, is maximum reaction rate, V_{max}. The factor V_{max} is related to the turnover number, k_{cat}, and the enzyme concentration according to Equation (5) (AKLECTURES, 2015). However, since the enzyme concentration was constant for all substrates for the lipases respectively, V_{max} is directly related to k_{cat}.

$$V_{max} = k_{cat} * [E]_{total} \quad (5)$$

When an enzyme instead is acting simultaneously on multiple substrates, k_{cat}/K_M is a good indicator of relative specificity of the enzyme (Cornish-Bowden, 2012). When more substrates are present and compete for the available enzyme, the measured activity will no longer be V_{max}. Instead, the measured activity will now be proportional to the factor k_{cat}/K_M, see Equation (6) (AKLECTURES, 2015), taking into consideration the enzyme's affinity for the different substrates. Since the enzymes acted on all substrates simultaneously in the mix experiments, equal enzyme concentration is present for each transesterification reaction. Further, equal concentrations of the substrates are present, therefore the observed differences in activity on the mixture experiments are directly proportional to the factor k_{cat}/K_M.

$$V_0 = \frac{k_{cat}}{K_M} * [E] * [S] \quad (6)$$

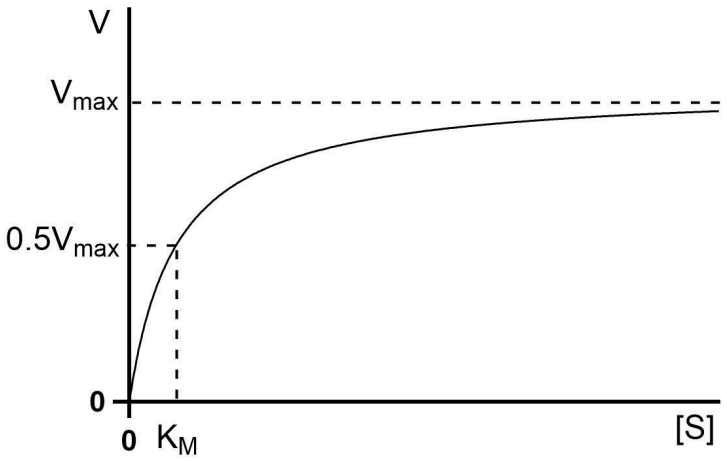


Figure 10. A generic Michaelis-Menten curve, showing the relationship between the velocity of a reaction and the substrate concentration as well as displaying the Michaelis-Menten parameters, V_{max} and K_M.

5.5.2 Activity on individual methyl esters

The initial transesterification activity on individual substrates was determined as total activity per gram immobilized carrier in Table 9 and as total activity per mg protein immobilized in Table 10. However, the total activity per mg protein immobilized could not be calculated for the lipases X2 and Y3 since the amount of protein immobilized could not be estimated, seen in section *5.4 Immobilization*. Also, it was not possible to calculate it for TL IM, as there was no information available about the amount of protein immobilized. Results presented as N/A means that they were inconsistent and could therefore not be determined. Results presented as ND means that no activity was detected. To easily get a detailed view of the activity for each lipase, bar charts for each lipase showing activity per gram immobilized carrier on all substrates have been made and are displayed in Figure 11-17. The results are also discussed adjacent to the figures. A comparison of the activity of the lipases can be seen below Figure 17.

Table 9. Average value of initial transesterification activity per gram immobilized carrier on individual methyl esters with varying carbon chain length where Cn represents the carbon chain length with n carbons. N/A = Not Applicable, ND = Not Detected. Measurements were made in triplicates.

Lipase	Activity on Me-C4 (U/g immobilized carrier)	Activity on Me-C8 (U/g immobilized carrier)	Activity on Me-C12 (U/g immobilized carrier)	Activity on Me-C18:0 (U/g immobilized carrier)	Activity on Me-C18:1 (U/g immobilized carrier)
X1	214	79.4	50.4	1.85	9.49
X2	6.00	ND	4.17	2.11	ND
Y1	198	697	315	N/A	595
Y2	49.4	41.5	33.1	2.12	8.86
Y3	2.08	N/A	0.251	N/A	ND
TL	271	617	491	260	136
TL IM	577	1670	882	398	284

Table 10. Average value of interesterification activities per mg immobilized protein, on individual methyl esters with varying carbon chain length where Cn represents the carbon chain length with n carbons. N/A = Not Applicable.

Lipase	Activity on C4 (U/mg protein)	Activity on C8 (U/mg protein)	Activity on C12 (U/mg protein)	Activity on C18:0 (U/mg protein)	Activity on C18:1 (U/mg protein)
X1	4.78	1.78	1.13	0.0413	0.212
X2	N/A	N/A	N/A	N/A	N/A
Y1	7.03	24.7	11.2	N/A	21.1
Y2	4.04	3.39	2.70	0.173	0.724
Y3	N/A	N/A	N/A	N/A	N/A
TL	5.41	12.3	9.78	5.17	2.71
TL IM	N/A	N/A	N/A	N/A	N/A

In Figure 11 it is statistically shown that X1 has its highest transesterification activity on methyl butyrate and that it decreases with increasing carbon chain length. For exact numbers, see Table 9. This contradicts the information from the producer who stated that X1 has similar activities on fatty acids regardless of carbon chain length, seen in *3.2 Lipases*. This could possibly be explained by the

fact that the shorter methyl esters had higher water solubility and consequently lower water activity (Oliveira, 2008), as explained in *5.3 Water activity*. The transesterification reaction is possibly favored at the lower water activity in this scenario if the higher water activity is above the enzyme's optimum or the hydrolysis reaction is favored at the higher water activity. This would have led to the enzyme activity to appear higher on shorter methyl esters. Figure 11 also shows that X1 has a higher activity on methyl oleate than on methyl stearate which suggests that X1 prefers monounsaturated fatty acids (MUFAs) over SFAs However, this conclusion is drawn on limited results as only one MUFA was used in this thesis. Methyl oleate has also been proven to have better water solubility (Oliveira, 2008), explained in *5.3 Water activity*, therefore the difference in activity between methyl stearate and methyl oleate could be an apparent effect because of the water solubility.

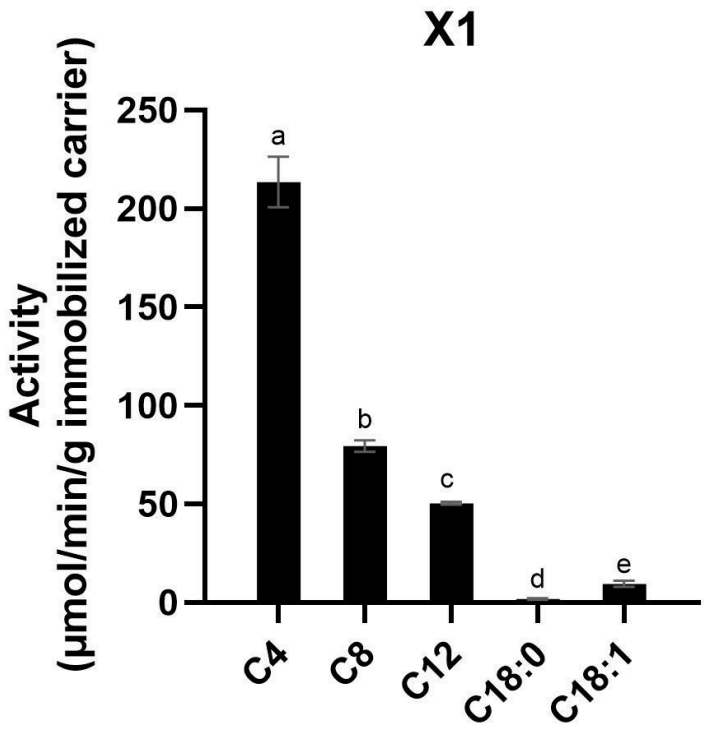


Figure 11. Transesterification activities of X1 on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

In Figure 12 it can be seen that X2 only showed activity on methyl butyrate, methyl laurate and methyl stearate. However, the data for methyl stearate and methyl laurate had large variation, resulting in no statistical difference observed. The activities are also relatively low overall compared to other lipases, making it hard to draw any conclusion on its fatty acid chain length preference. As no activity was detected on methyl oleate, it is indicated that X2 prefers SFAs compared to MUFAs. X2 has been stated by the producer to have higher activity on SCFAs compared to MCFAs and LCFAs. The results in Figure 12 neither agrees with nor contradicts the information from the producer, as the activity on methyl butyrate was only higher than one of the two MCFAs. However, it seems unlikely that the activity on methyl octanoate should be zero when it has activity on methyl butyrate and methyl laurate, indicating that something has maybe gone wrong with the reaction. This has to be investigated further to be able to draw a conclusion regarding its preference for different carbon chain lengths.

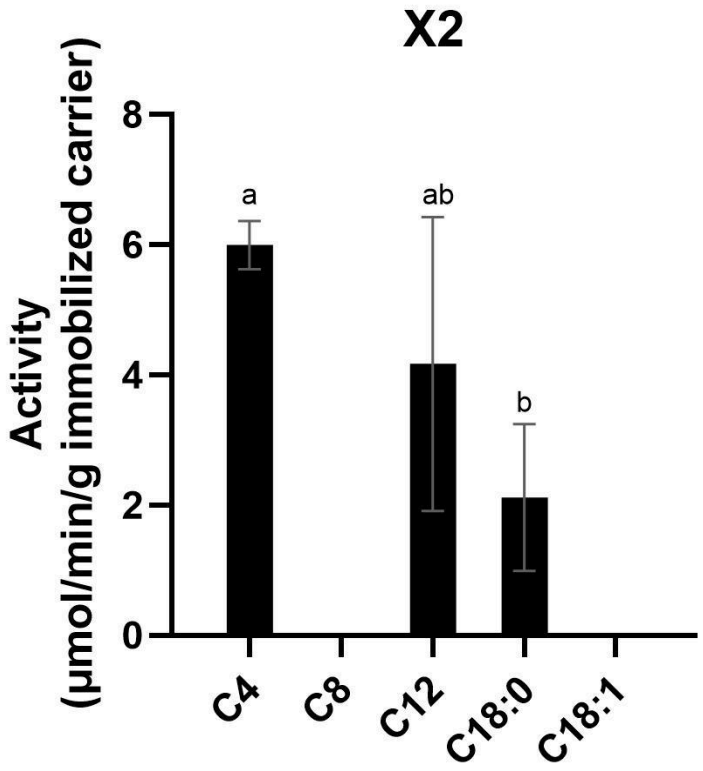


Figure 12. Transesterification activities of X2 on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

Y1 showed higher preference for the MCFA and LCFAs, compared to the short methyl butyrate, as indicated in Figure 13. This results contradicts the data from the producer, as they stated that Y1 has the lowest preference for LCFAs. However, it should be noted that the experiments for Y1 on methyl oleate showed a flattened curve which could mean that saturation occurred. Shorter time and lower amount of immobilized carrier showed similar patterns, which could not be explained. To obtain an activity for Y1 on methyl oleate, the first obtained graph was used and only the first two points were used, the slope was also forced through origo. Therefore, the activity could have been overestimated. Further, the activity for methyl stearate could not be estimated, indicated by the N/A, which is an outcome of the measurements showing inconsistent results with high variance between the replicates and no upgoing trend in product formation during the reaction. Consequently, this also makes it impossible to draw any conclusion on Y1's preference for saturation versus unsaturation.

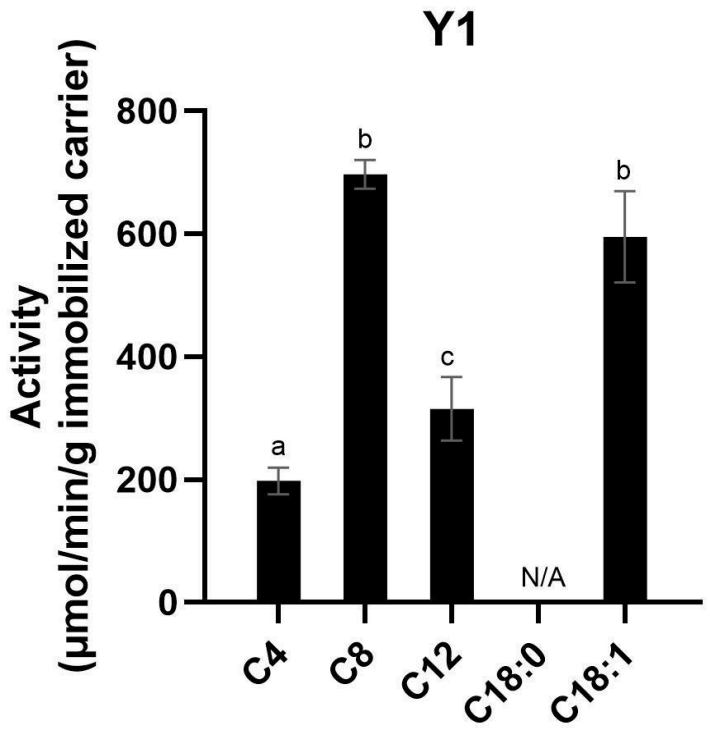


Figure 13. Transesterification activities of Y1 on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference. N/A = Not Applicable.

The results for Y2, presented in Figure 14, indicate that SCFAs are preferred as the activity steadily declines with increased fatty acid chain length, with a very steep decline to the LCFAs. This agrees to some extent with data from the producer that suggests that Y2 has similar activities on SCFAs and MCFAs while lower on LCFAs. The discrepancy might be accredited to the aforementioned differences in water solubility between the fatty acids (Oliveira, 2008). The results in Figure 14 also show a clear preference for the unsaturated methyl oleate over the saturated methyl stearate, but as the case with X1, this could be an apparent effect caused by the higher water solubility of methyl oleate (Oliveira, 2008).

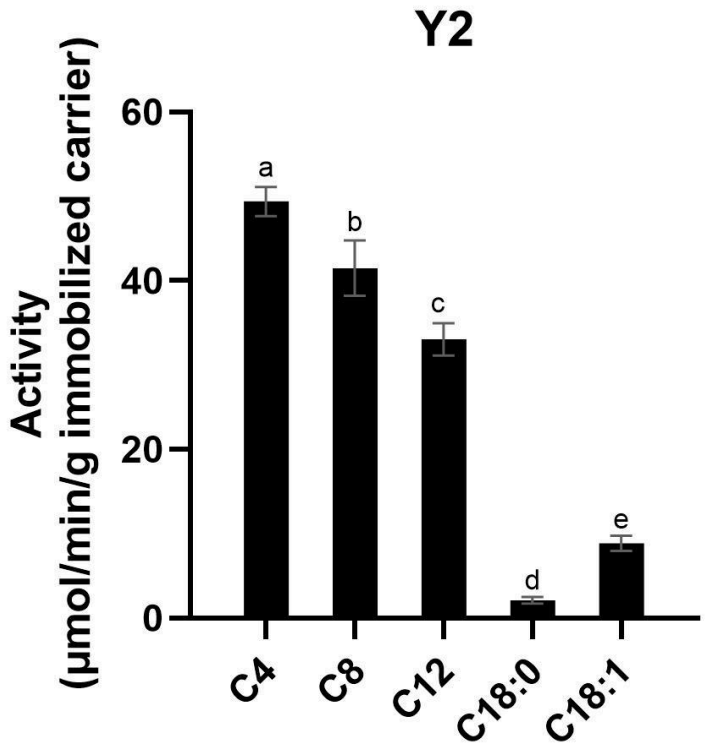


Figure 14. Transesterification activities of Y2 on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

From the results of Y3, presented in Figure 15, it can be seen that the activities were relatively low and that no activity could be obtained on methyl octanoate and methyl stearate. This makes it difficult to draw any conclusion on the activity of Y3. However, there seems to be a significantly higher activity on methyl butyrate than on methyl laurate, contradicting the producer's data stating that Y3 has similar activity on both SCFAs and MCFAs. The information from the producer also suggests that Y3 has the lowest activity on LCFAs, which is backed up by the fact that no activity was detected on methyl oleate. However, as no activity on methyl stearate could be calculated due to uninterpretable results, this conclusion is imprecise. The inconsistency in the results on methyl stearate also resulted in no possible conclusion about the preference of the lipase for either SFAs or MUFAs.

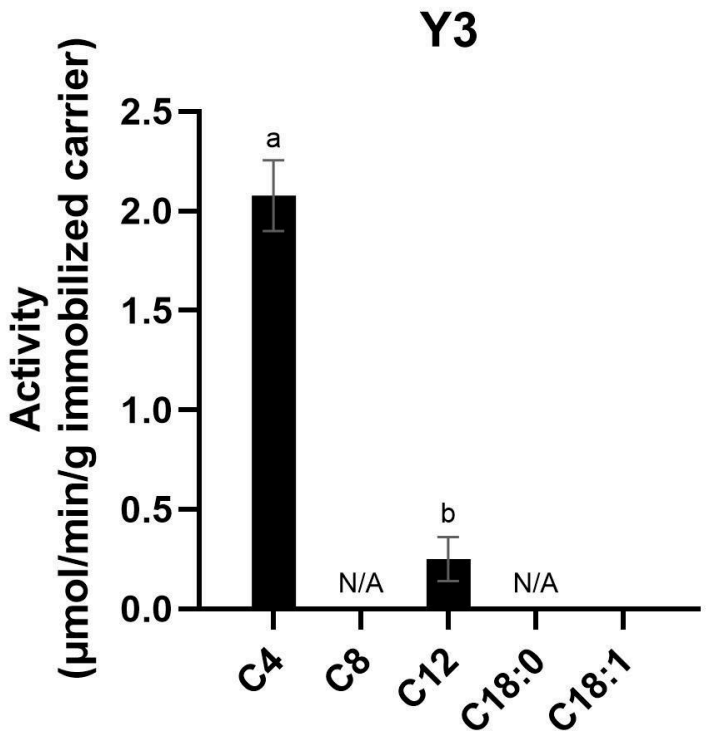


Figure 15. Transesterification activities of Y3 on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference. N/A = Not Applicable.

Although TL and TL IM were used as controls and are not the subject of interest in this thesis, the results on their activities are presented and discussed below. TL showed high activity, seen in Figure 16, relative to the lipases discussed earlier. The results suggest that TL prefers MCFAs compared to SCFAs and LCFAs. Whether TL prefers SCFAs or LCFAs over the other cannot be concluded as the activity on methyl butyrate and methyl stearate are statistically equal. It can also be seen that the activity was higher on methyl stearate compared to on methyl oleate, indicating that TL prefers SFAs.

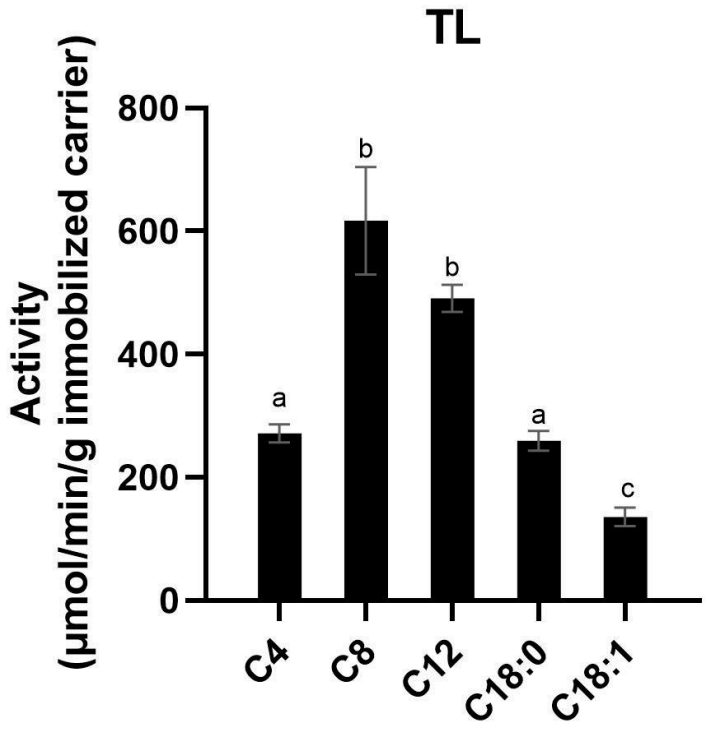


Figure 16. Transesterification activities of TL on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

Just as TL, TL IM showed relatively high activities compared to the previously discussed lipases. The activities for TL IM are presented in Figure 17. TL IM had higher activity than TL on every substrate. As the enzyme loading is unknown for TL IM, no conclusion about the effect of the different carriers can be drawn. The results also indicated that TL IM prefers MCFAs the most, but also SCFAs over LCFAs. It is also indicated that SFAs are preferred over MUFAs. The preference for MCFAs and SFAs was observed for TL as well, however, the preference for SCFAs over LCFAs was not observed. The activity for TL on methyl butyrate was higher than on methyl oleate but equal to methyl stearate, it is possible that the activity of TL on methyl stearate have been overestimated, resulting in this observed discrepancy. Another explanation is the effect of the different carriers between TL IM and TL.

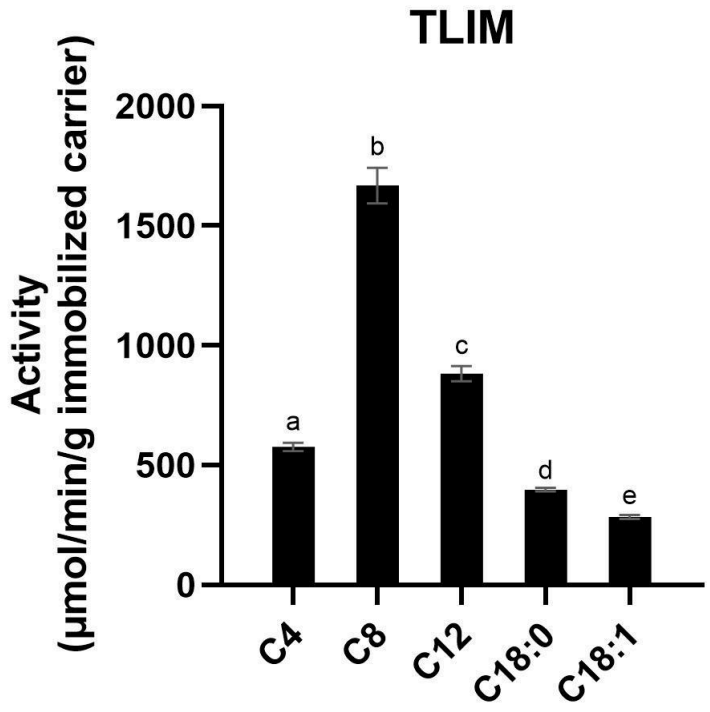


Figure 17. Transesterification activities of TL IM on methyl esters with different carbon chain length where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

From the results above it can also be concluded which lipase works best for each substrate. Outside of the control lipases, X1 and Y1 exhibit the highest activity on methyl butyrate. Y1 shows significantly higher activity on methyl octanoate and methyl laurate than the other non-control lipases and also higher than TL on methyl octanoate. On methyl stearate, activity could only be estimated for X1, X2 and Y2 and they all had similar results, but the activities were also relatively low compared to the activities observed on other substrates. Given that Y1 had the highest activity on methyl- butyrate, octanoate and laurate, it is possible that it would have the highest activity on methyl stearate as well, if it had been able to be determined. Y1 again had the highest activity on methyl oleate, even significantly higher than the control lipases. However, as it has been theorized before, the activity of Y1 on methyl oleate might have been overestimated.

A lot of the presented results were inconsistent, shown as N/A in Figure 11-17 and Table 9 and 10. For some lipases and substrates, the product concentrations obtained from the GC were very low, then the results are more susceptible to fluctuations and data uncertainties. The low activity could be because no optimization of the important parameters mentioned in *3.4.4 Important immobilization parameters*, such as water activity, was conducted. Therefore, the conditions were not optimal for the lipases.

Overall, the results indicated a much lower activity on the LCFAs, methyl stearate and methyl oleate, compared to the other fatty acids. Even though this was expected for many of the lipases after reading the producers information, it is still important to point out that LCFAs are more likely to be steric hindered and therefore have less access to the lipases in the pores of the support (Brena et al., 2013), explained in *3.4.2 Problems with immobilization*. The experiments were also carried out in a

solvent-free environment, meaning that the substrate acted as the solvent. As a solution of LCFAs will have higher viscosity than a solution composed of SCFAs, the properties of the solutions will differ between the reactions (Wedler et al., 2023). A higher viscosity will lead to decreased degree of mixing (Trubiano et al., 2007). This will, as explained in *3.4.4 Important immobilization parameters*, increase external mass transport limitations, as the stagnant layer outside the support thickens (Dicko et al., 2020). The viscosity differences will influence less at higher temperatures and no apparent differences were observed during the experiment. On the other hand, the possible presence of steric hindrance of the substrate through the carrier is difficult to reject, as no evaluation of this was performed and any observation is impossible. It is possible that this is the explanation for the results for X1 not agreeing with the producer's data and it could also be the reason the results for TL and TL IM appear lower for LCFAs. It is also possible that the results for the other lipases, that agree with stated data, are influenced by these limitations.

The methyl oleate used in the individual substrate experiments had, as mentioned in *4.2.4.2 Transesterification of methyl esters*, a purity of 89.4%, unlike the methyl oleate with a purity of 62.6% used in the experiment with a mixture of the substrates. From the chromatogram obtained for the GC runs, the contaminations were estimated to be mostly made up of methyl stearate, methyl linoleate (C18:2) and methyl linolenate (C18:3). The concentration of these substrates was monitored and their conversions were estimated. Relative to the conversion of the methyl oleate, the conversion of the contaminations was very low and it was therefore estimated that they did not interfere with the activity results on methyl oleate.

5.5.3 Activity on methyl esters in mixture

Performing interesterification reactions in a mixture of all substrates makes it possible to evaluate the relative specificity of the lipases. In this case the reaction conditions are the same, meaning that there should be no effect of different water activities or viscosities during the reaction.

In Table 11, the calculated activity for the lipases on the mixed substrate is presented for each lipase and the activity was estimated as total activity per gram MP1000. Results presented as N/A means that they were inconsistent and could therefore not be determined. Results presented as ND means that no activity was detected. To get a more comprehensive view of the lipases' specificity for the different methyl esters, a bar chart of relative k_{cat}/K_M for each substrate is presented for every lipase in Figure 18-24. Setting the k_{cat}/K_M to 1 for methyl butyrate and relating the other factors to this, the relative k_{cat}/K_M can be presented for each substrate.

Table 11. Mean value of initial transesterification activity on methyl esters with varying carbon chain length in a mixture of the methyl esters. N/A = Not Applicable, ND = Not Detected.

Lipase	Activity on Me-C4 (U/g immobilized carrier)	Activity on Me-C8 (U/g immobilized carrier)	Activity on Me-C12 (U/g immobilized carrier)	Activity on Me-C18:0 (U/g immobilized carrier)	Activity on Me-C18:1 (U/g immobilized carrier)
X1	19.1	8.06	4.40	ND	1.45
X2	2.17	N/A	N/A	ND	N/A
Y1	18.5	58.8	50.3	43.8	35.9
Y2	10.7	58.7	2.47	ND	1.27
Y3	3.73	8.56	9.44	N/A	4.11
TL	25.8	116	107	45.3	35.5
TL IM	55.3	100	94.8	76.0	61.2

As can be seen in Figure 18, X1 showed a higher k_{cat}/K_M on SCFAs, compared to the MCFAs, which in turn was higher than for LCFAs. It showed more than twice as high k_{cat}/K_M on methyl butyrate in comparison to methyl octanoate, which in turn was almost twice as high compared to methyl laurate. These results correlate with the previously presented results on individual methyl esters, where the activity decreases with increasing carbon chain length. For the LCFAs, a higher k_{cat}/K_M was detected on methyl oleate than on methyl stearate. This was observed in the results on individual methyl esters as well, strengthening the conclusion that X1 has higher relative specificity for MUFAs over SFAs.

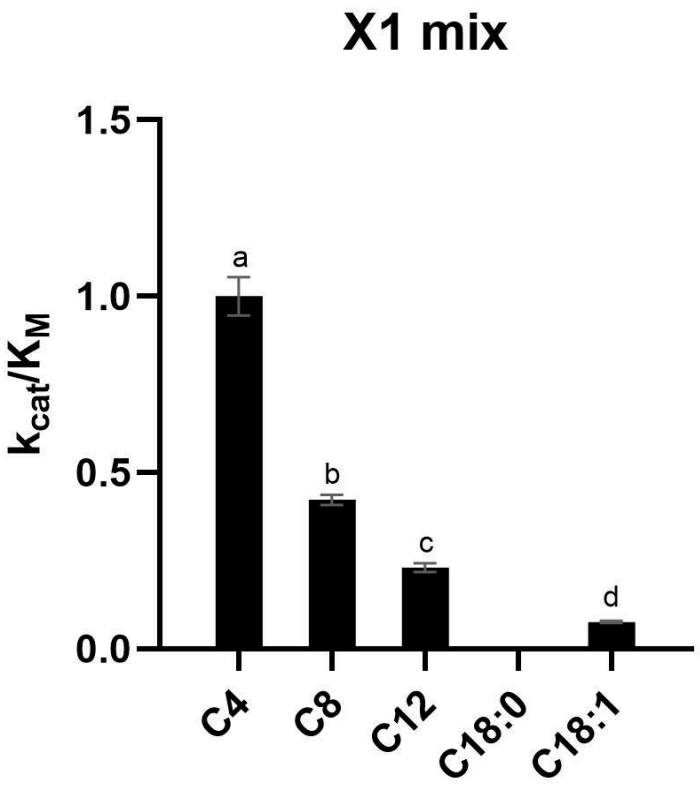


Figure 18. Relative k_{cat}/K_M of X1 on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

The results for the transesterification activity for X2 is inconsistent (Figure 19), which makes it hard to draw any conclusions about its relative specificity. The activity measurements on methyl-octanoate, laurate and oleate showed inconsistency and therefore no k_{cat}/K_M could be estimated. On methyl stearate no activity was detected. The calculated activity on methyl butyrate, seen in Table 11, is relatively low compared to the other lipases, independent of the substrate. The results also had great variation between the replicates, which makes the results questionable.

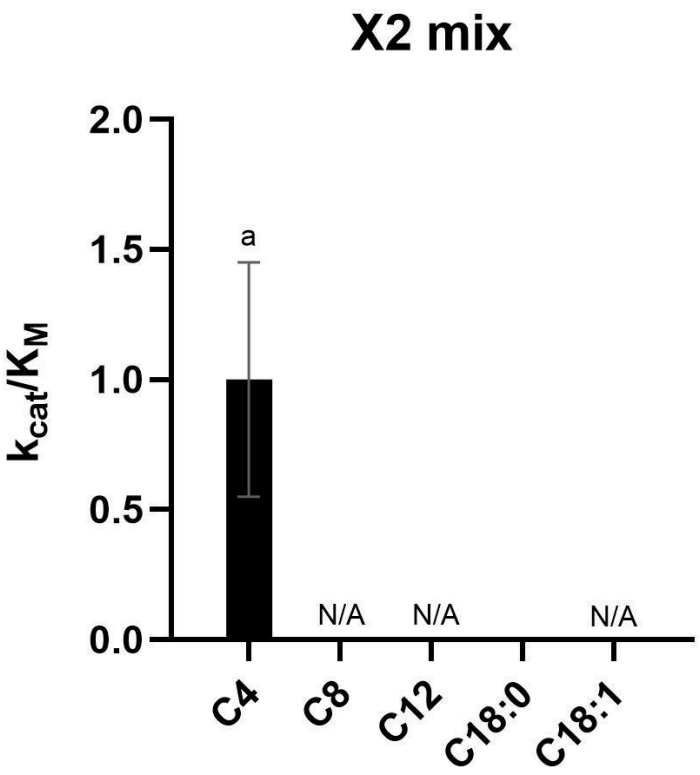


Figure 19. Relative k_{cat}/K_M of X2 on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference. N/A = Not Applicable.

In Figure 20, the results for Y1 are shown. From the bar chart, it can be seen that Y1 had highest k_{cat}/K_M on medium- and long chain fatty acids, and lower on methyl butyrate. This is consistent with the results for the reactions on individual methyl esters. However, it does not correlate with data from the producer, stating that LCFAs are least preferable. Regarding relative specificity for saturation/unsaturation of the 18 carbon long fatty acids, it is not possible to draw a conclusion since the k_{cat}/K_M is statistically equal for both LCFAs.

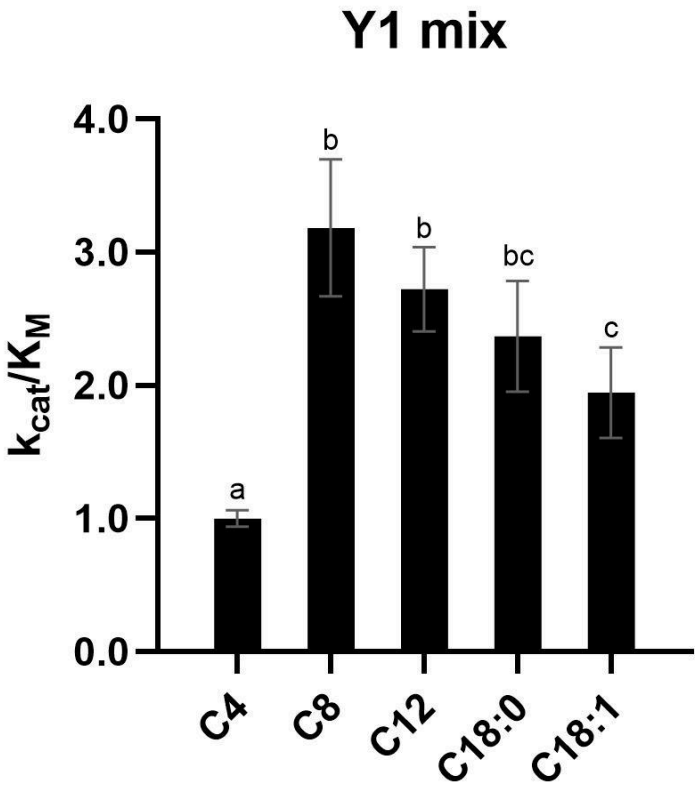


Figure 20. Relative k_{cat}/K_M of Y1 on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

As is shown in Figure 21, Y2 had highest relative specificity towards SCFAs and lower towards MCFAs and LCFAs. As no statistical difference was observed between methyl laurate and methyl oleate, no conclusion about the relative specificity between MCFAs and LCFAs could be drawn. This result agrees with the previous results presented for the individual substrates to some extent, however, the earlier results indicated a higher relative specificity towards MCFAs over LCFAs. Further, the k_{cat}/K_M difference between SCFAs and MCFAs was bigger in the mix than the activity difference for the pure substrates. This may suggest that the lipase allows both SCFAs and MCFAs, but selects SCFAs when both are present. The k_{cat}/K_M on the MUFAs was very low, but the results indicate a relative specificity for unsaturation compared to saturation.

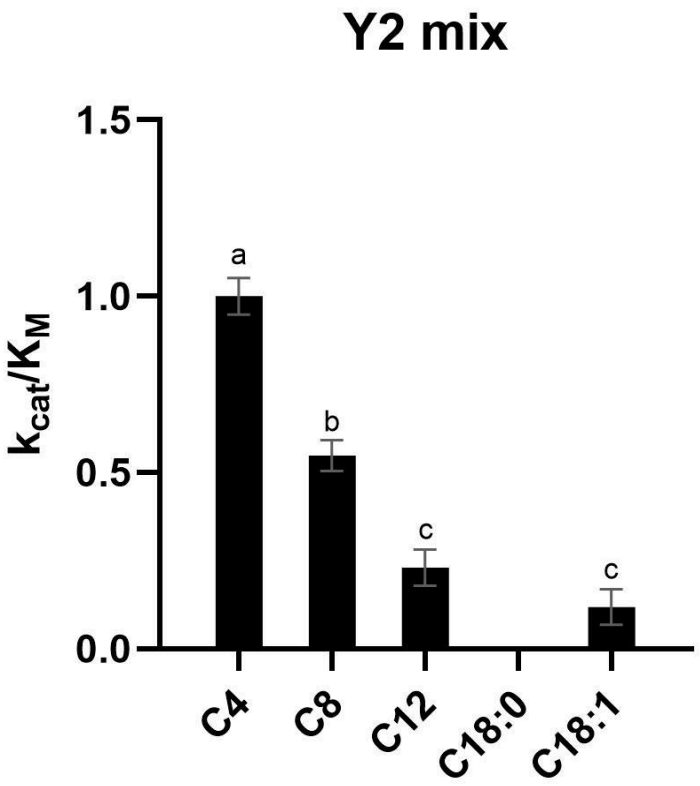


Figure 21. Relative k_{cat}/K_M of Y2 on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements were made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

The results for Y3, presented in Figure 22, have a very high degree of uncertainty, resulting in no statistical difference observed. This makes it impossible to draw a reasonable conclusion about the relative specificity for carbon chain length and for saturation or unsaturation.

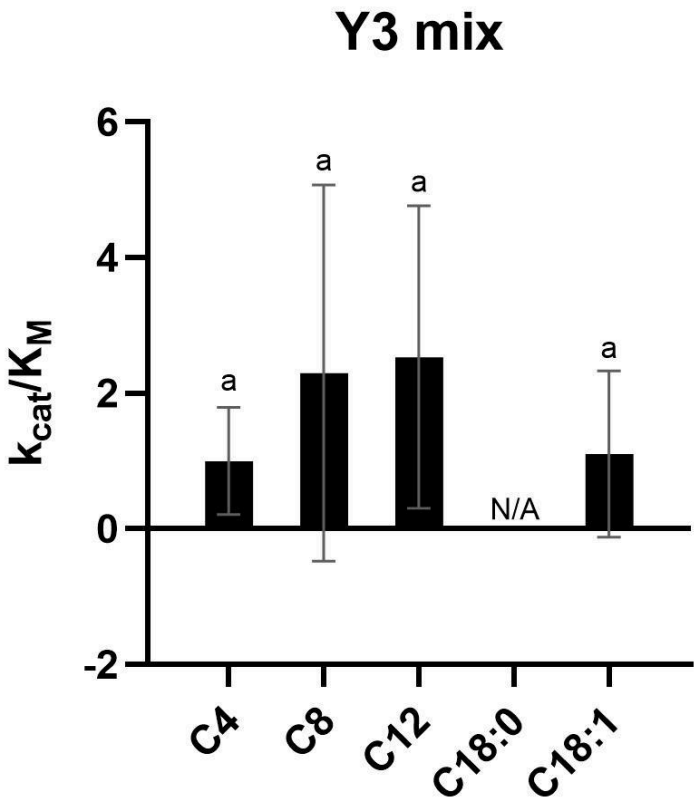


Figure 22. Relative k_{cat}/K_M of Y3 on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements made in triplicates for methyl octanoate and methyl oleate and in duplicates for methyl butyrate and methyl laurate, standard deviation is shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference. N/A = Not Applicable.

From the results of TL, presented in Figure 23, it is visualized that TL had the highest k_{cat}/K_M on the MCFAs compared to the SCFAs and LCFAs. It is also shown that TL had higher relative specificity for LCFAs than SCFAs. The relative specificity for MCFAs was also observed for the reactions on pure substrates, however the higher relative specificity for LCFAs than SCFAs was not observed. Looking at the methyl stearate and methyl oleate, TL selected for saturated LCFAs as the k_{cat}/K_M for methyl stearate was greater than for methyl oleate. This result agrees with the result on individual substrates, strengthening the conclusion about its relative specificity for SFAs.

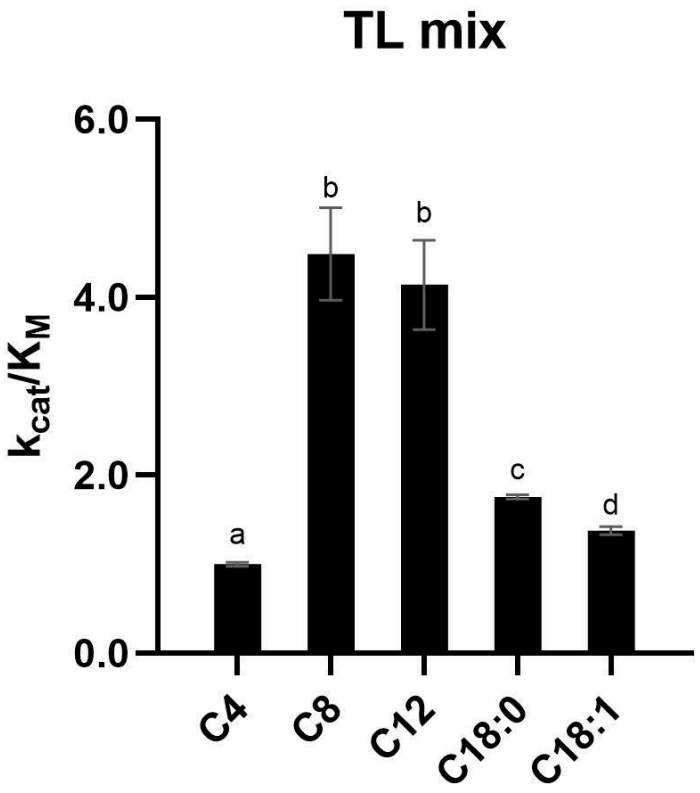


Figure 23. Relative k_{cat}/K_M of TL on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

In Figure 24 it is shown that TL IM exhibited the highest relative specificity for MCFAs, which was also observed for the individual substrates. However, the results from Figure 32 suggest that TL IM had a higher relative specificity for LCFAs over SCFAs, disagreeing with previous results. The results indicate a relative specificity towards SFAs, agreeing with the results for the pure methyl esters. The results for TL IM match the results for TL, indicating that the relative specificity of the lipase does not depend on its support material.

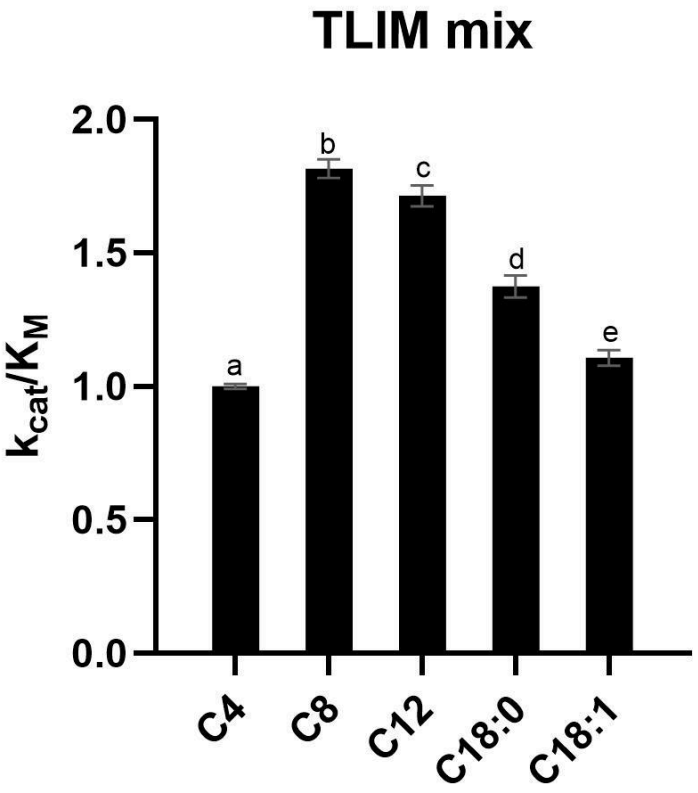


Figure 24. Relative k_{cat}/K_M of TL IM on the methyl esters in a mixture, where nC represents the carbon chain length with n carbons. Measurements made in triplicates with standard deviation shown as error bars. Letters above bars indicate statistical significance or not. Similar letters represent no statistical difference and different letters represent a statistically significant difference.

Comparing the results from the pure substrates to the mixed substrate, it can be seen that they correlate in many cases. As explained in *5.5.1 k_{cat}/K_M as a specificity factor*, the activities from the mixed substrate experiments are proportional to the factor k_{cat}/K_M while the activities from the pure substrate experiments are proportional to the factor k_{cat}. The similar results indicate that the K_M-factors are not affecting the results very much and that the concentration for the substrates in the mix are a lot higher than the K_M-factors for these enzymes. The results that did not correlate could possibly be explained by the different experiment conditions. The pure substrates have varying water solubility capacity and viscosity due to different carbon chain lengths of the substrates, while these parameters are constant in the mixture. However, it could also be an indication that the K_M differs between the substrates and is a determining factor for the enzymes where the results do not correlate.

The inconsistency in some of the results, marked as N/A, could possibly be explained in the same way as for the individual substrates, that the low product concentration makes the results susceptible to small fluctuations and data uncertainties.

An important note to take into consideration is that the methyl oleate used as pure substrate and in the mixed substrate was different, which could cause the comparison between specificity in the mix and pure substrates to be uncertain. The contaminants could not be monitored in these experiments since methyl stearate was both present as a substrate and as a contaminant from the methyl oleate. Therefore, the interference on the results from the contaminations are unknown. The contamination also means that it is likely that more methyl stearate and less methyl oleate is present than assumed,

invalidating the assumption that all substrates are present in equal concentrations. Therefore, the observed differences in activity might not only be proportional to k_{cat}/K_M, but also to the differences in substrate concentration. However, the concentration of the methyl oleate is smaller in the mixture experiments compared to the individual experiments. Therefore, the inhibitory effect of the contaminants will impact less on the mixture and the need for a more pure substrate is lower.

5.6 Sources of error

All results presented in this report come with uncertainties due to time constraints and choice of methods. There are several sources of error found throughout the thesis that influence the accuracy of the results. The effect of water is one source of error which has already been discussed. The water activity is lower for short substrates and higher for longer substrates, which influences the results for the lipases' preference of substrates, presented in Figure 11-17. The experiments are solvent-free, which leads to varying viscosity and mass transport limitations. These variations, together with the possibility of steric hindrance occurring for larger fatty acids, will affect the preference the lipases have for different substrates.

The results comparing the lipases' activity on the same substrate is influenced greatly by the possibility that the system might be less optimal for some lipases compared to others. The aforementioned water activity is assumed to be equally optimal for every lipase, which is most likely not the case. Further, there was no time for a temperature optimization to be performed, meaning that the chosen temperature of 55℃ will have some lipases operating closer to maximum than others. There is also an assumption made that the lipases are not inactivated at this temperature. In general, free enzymes are at risk of inactivation at temperatures higher than 45℃ (Unacademy, n.d.), this critical point is increased when the enzymes are immobilized (Dicko et al., 2020), as explained in *3.4.1 Advantages with immobilization*. However, the extent of the increase has not been studied in this report and therefore it is possible that the chosen temperature of 55℃ leads to partial inactivation. It is also assumed that the pH set in the aqueous enzyme solution before immobilization will be unchanged when the enzyme is introduced to the organic media, a phenomenon called "pH memory". This assumption is made because protonation and deprotonation rarely take place in organic media (Dicko, 2022). It is possible that this assumption is incorrectly made in this situation and that the lipases operate at a different pH than the set value of 7. The lipases might also be operating at a different pH value than the set value because of the partitioning effects (Dicko et al., 2020), mentioned under *3.4.2 Problems with immobilization*. Further, just as with temperature and water activity, it is also possible that the pH is less optimal for some lipases. All these sources of errors and assumptions will influence the comparison of the lipases on a substrate.

The immobilization results for the activity yield are affected by the potential occurrence of aggregation in Y1, as discussed in *5.4.2 Activity immobilization yield*. This could possibly influence the activity yield and total immobilized activity, making the values appear lower than they are. Additionally, as with every experiment, there are human errors affecting the results. Pipetting, weight measurements and timing are all procedures prone to errors by hand. The biggest human source of error in this thesis is likely the sampling from the reaction vials, 10 µl is a very small volume and a minor deviation would affect the results greatly.

Another source of error is that the GC temperature programs were not optimal for every substrate. The GC programs were optimized to a certain extent, but some products were hard to separate from each other in the obtained chromatograms. Especially distinguishing propyl stearate and propyl oleate from

each other and from other impurities in the methyl oleate was difficult. This affects the accuracy of the results obtained for these products on the mix substrate and the results obtained for propyl oleate from the methyl oleate runs. The contamination of the methyl oleate also influenced the results from the mixture experiments. The conversion of the contaminants could not be estimated which means that the effect on the obtained results could not with certainty be neglected. As explained in *5.5.3 Activity on methyl esters in mixture*, it is also possible that the presence of the contaminants invalidates the assumption that the estimated differences in activity is strictly proportional to the factor k_{cat}/K_M.

6. Conclusion

From the results and discussion above, it can be concluded that X1, Y2 and Y3 works better on SCFAs, while TL and TL IM prefer MCFAs. Y1 can be seen to work the best for both MCFAs and LCFAs. No conclusion could be drawn on the preference of carbon chain length for X2.

Looking at the results for SFAs versus MUFAs, it was seen that X1 and Y2 preferred the MUFA, while X2, TL and TL IM preferred the SFA. Y1 was concluded to have similar preference of MUFAs and SFAs and no conclusion could be made for Y3.

Some of the results were inconsistent and some did not correlate between the pure substrate experiments and the mixture experiments. Further investigations should be conducted to lower the uncertainty of the results as well as to possibly draw more conclusions.

The finding of this thesis will function as a base of an enzyme library available to AAK AB. Further, evaluation of other lipases can be added to expand the enzyme library.

7. Further work

The purpose of this thesis was to evaluate a selection of lipases for their suitability in AAK's oil production and as mentioned in *1.2 Background*, that production involves TAGs. Because of limitations in analysis methods, available equipment and time, this report determined activities on FAEs. It was hypothesized that the transesterification activity would correlate on FAEs and TAGs. However, further studies on TAGs would be interesting, as the accuracy of the hypothesis could be evaluated. Running the experiment on TAGs would also allow for evaluation of the regioselectivity, which is an interesting characteristic that would be added to the constructed enzyme library.

To increase the credibility of this report, the activity on additional substrates, a wider range of MUFAs as well as polyunsaturated fatty acids (PUFAs) could be studied. Only a limited group of substrates were evaluated which increases the risk that the conclusions are inaccurate. Additionally, as discussed in *5.6 Sources of error*, parameters of the reactions, such as temperature, pH and water activity are not equally suitable for every lipase. Optimization of these parameters are needed to make it a more accurate comparison. Further, immobilization parameters such as choice of buffer, carrier, ionic strength and ratio of enzyme to buffer to carrier could be evaluated and more optimal conditions could be found.

In *5.3 Water activity*, it was mentioned that similar water contents were used for the reactions, instead of similar water activities, which would have been preferred. The problem with water activity in this report was that the 1-propanol is volatile, using a less volatile alcohol like octanol in the reactions would solve this problem. This would allow for equal water activities to be set in the substrates and the activities between substrates would be more comparable. Substituting 1-propanol with octanol only solves the water activity problem for the MCFAs and LCFAs, as methyl butyrate would still be volatile. Finding a solution to this or designing the experiment in another way would be needed to omit the effects of different water activities completely.

Furthermore, because of time constraints, the reactions on the mix of substrates were only run on the less pure methyl oleate. Therefore, a suggestion would be to re-do these reactions with the more pure methyl oleate, as it could be assumed that the contamination of the more pure methyl oleate did not interfere with the results. Another improvement for future work is a more extensive optimization of the GC temperature programs, as this would allow for better separation between the products, avoiding co-elution, and a more accurate peak area obtained.

The paragraphs above discuss how to increase the credibility of the thesis, and the purpose of this thesis was to evaluate this selection of lipases, however, an important future action is to also evaluate more lipases to expand the lipase library.

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9. Appendix

Table A1: GC temperature program for methyl butyrate.

Time	Temperature	Temperature increase
0 – 1 min	80 ℃	-
1 – 2.25 min	80 – 180 ℃	80 ℃/min
2.25 – 3.25 min	180 ℃	-

Table A2: GC temperature program for methyl octanoate.

Time	Temperature	Temperature increase
0 – 1 min	100 ℃	-
1 – 3 min	100 – 180 ℃	40 ℃/min
3 – 4 min	180 ℃	-

Table A3: GC temperature program for methyl laurate.

Time	Temperature	Temperature increase
0 – 2 min	160 ℃	-
2 – 3 min	160 – 180 ℃	20 ℃/min
3 – 5 min	180 ℃	-

Table A4: GC temperature program for methyl stearate.

Time	Temperature	Temperature increase
0 – 1 min	200 ℃	-
1 – 3.67 min	200 – 280 ℃	30 ℃/min
3.67 – 4 min	280 ℃	-

Table A5: GC temperature program for methyl oleate.

Time	Temperature	Temperature increase
0 – 1 min	200 ℃	-
1 – 3.67 min	200 – 280 ℃	30 ℃/min
3.67 – 4 min	280 ℃	-

Table A6: GC temperature program for the substrate mix.

Time	Temperature	Temperature increase
0 – 1.5 min	90 ℃	-
1.5 – 2.9 min	90 – 160 ℃	50 ℃/min
2.9 – 3.9 min	160 – 165 ℃	5 ℃/min
3.9 – 4.21 min	165 – 190 ℃	80 ℃/min
4.21 – 5.21 min	190 – 210 ℃	20 ℃/min
5.21 – 6.09 min	210 – 280 ℃	80 ℃/min
6.09 – 7.5 min	280 ℃	-

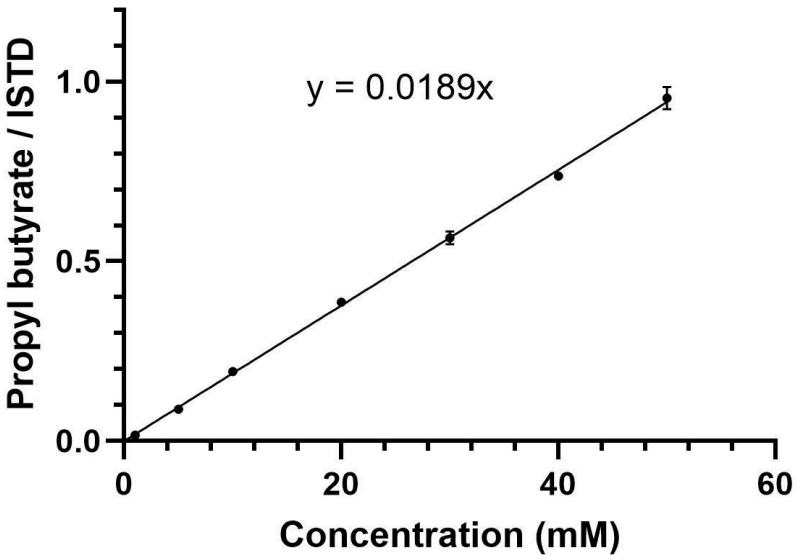

Figure A1: Standard curve of peak area versus concentration propyl butyrate obtained from gas chromatography.

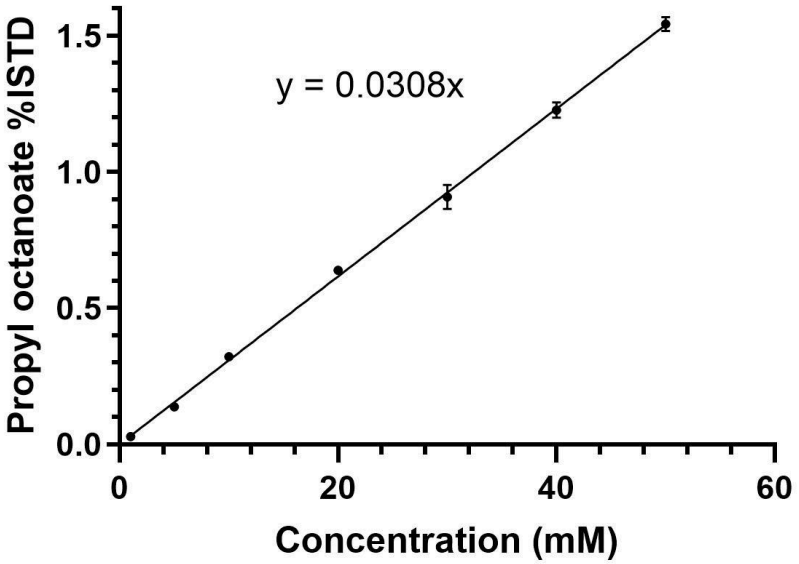

Figure A2. Standard curve of peak area versus concentration propyl octanoate obtained from gas chromatography.

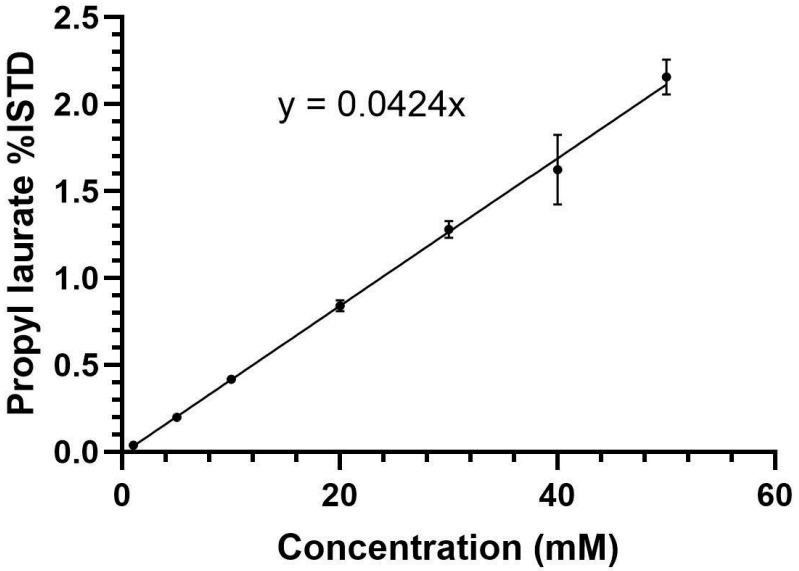

Figure A3. Standard curve of peak area versus concentration propyl laurate obtained from gas chromatography.

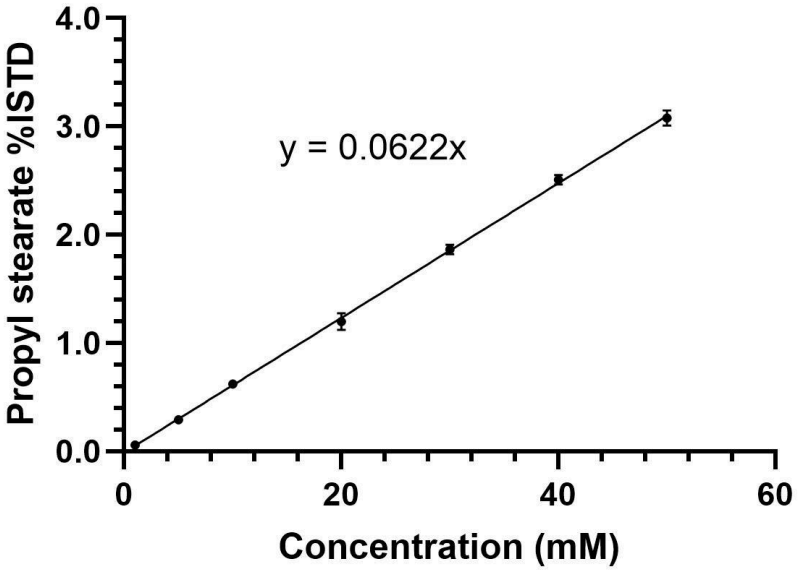

Figure A4. Standard curve of peak area versus concentration propyl stearate obtained from gas chromatography.

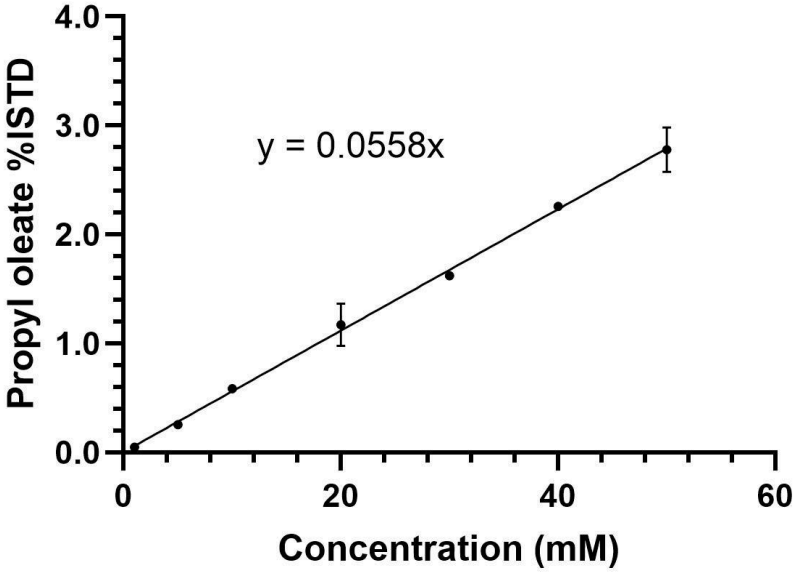

Figure A5. Standard curve of peak area versus concentration propyl oleate obtained from gas chromatography.

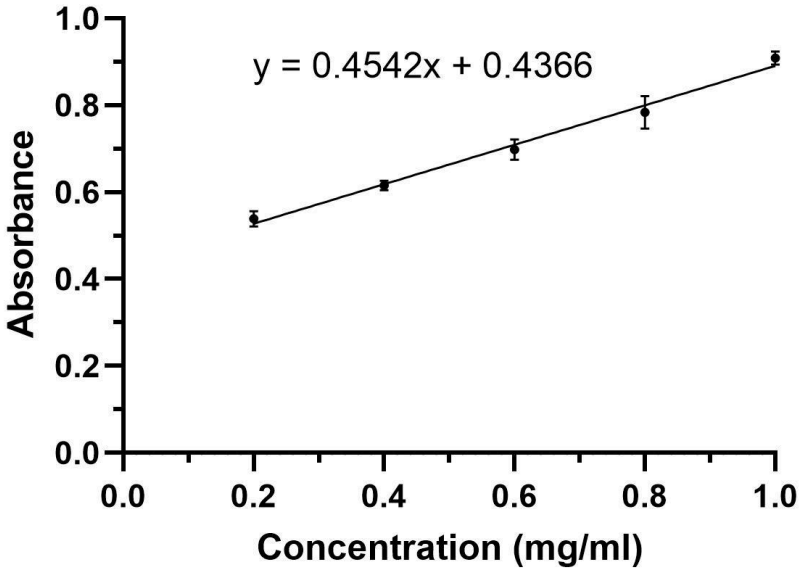

Figure A6: BSA calibration curve for the Bradford method, concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml was used. Error bars showing the distribution of the results as well as the equation for the linear regression are present.