Lipase-Based Fat Splitting of High Erucic Acid Rapeseed Oil for Industrial Applications

2021

Robert Berchtold

Supervisors:

Dr. Tuck Seng Wong (The University of Sheffield)

Dr. Kim Carmichael (CRODA Int.)

The University Of Sheffield.

CRODA

Summary

Industrial fat splitting, specifically of high erucic acid rapeseed oil is an economically valuable process, which CRODA employs to produce thousands of tons of erucic acid and other fatty acids and glycerol. This project explores enzymatic methods for HEAR oil splitting, and their potential industrial applications.

Within this work, several different lipases are compared for their activity on HEAR oil under different conditions.

Initial work was focussed on determining the activity of lipases on HEAR oil. Lipases used were from mammalian and fungal sources. Comparisons between these lipases focussed on their activity on HEAR oil measured in acid value as well as their regio-selectivity.

Both, to determine the specificity of these enzymes on HEAR oil and to gain insights into the final, and intermediate products of the splitting reaction we used thin layer chromatography (TLC).

TLC allowed us to determine intermediates of the HEAR oil splitting reaction, specifically, it allowed us to distinguish between different diacylglycerols, which let us classify lipases of unknown specificity into sn-1,3 specific and non-specific.

Thermomyces lanuginosus lipase (TLL) variants as well as *Candida rugosa* lipase and *Candida antartica* lipase A were identified as ideal lipases for HEAR oil hydrolysis.

Conditions for TLL based hydrolysis were optimised using a number of different factors including temperature, oil:water ratio, lipase amount, stirring speed, as well as buffer strength and initial pH.

Combinations of lipases were also used to fully split HEAR oil within 24 hours.

Partial HEAR oil hydrolysis for industrial use was also explored. Pre-splitting, in particular, was successfully shown to be potentially beneficial to an industrial pressure splitting process, allowing for a reduction in overall reaction time, or temperature and pressure while producing equal yields.

Contents

Summary 3
Contents 4
List of Figures 7
List of Tables 9
List of Abbreviations 10
1 Introduction 12
2 Literature Review: Enzymatic Fat Splitting 17
2.1 HEAR Oil 17
2.1.1 Oil Degumming 18
2.2 Lipases – Fat Splitting Enzymes 19
2.2.1 Lipase Use 19
2.2.2 Production of Lipases 20
2.2.3 Lipase Structure and Catalytic Mechanism 21
2.2.4 Lipase Kinetics 23
2.3 Reactor Types for Enzymatic Fat Splitting 27
2.4 Previous Enzymatic Fat Splitting Review 29
2.4.1 Analytical Techniques 29
2.4.2 Commercial Lipases used for Fat and Oil hydrolysis 31
2.4.4 Operating Conditions of lipase-based hydrolysis 37
2.5 Enzyme Immobilisation 40
2.5.1 Matrices/Supports for Immobilisation 41
2.5.2 Immobilisation techniques and methods 43
2.6 Literature Review Conclusions 45
2.7 Aims and Outline of Work 46
3 Materials and Methods 48
3.1 Materials 48
3.2 Methods 50
3.2.1 Fat Splitting Reactions 50
3.2.2 Phase separation 50
3.2.3 Acid value 51

3.2.4 Saponification value 52
3.2.5 Thin Layer Chromatography 53
3.2.6 High Performance Liquid Chromatography 54
3.2.7 Gel Permeation Chromatography 55
3.2.8 Gas Chromatography 55
3.2.9 Enzyme immobilisation 56
3.2.10 Protein Concentration Measurements (Bradford Assay) 57
4 Results – Initial Considerations 58
5 Enzyme Selection 61
5.1 Porcine Pancreatic Lipase 63
5.2 *Candida rugosa* Lipase 71
5.3 Lipozyme CalB / Novozyme 435 73
5.4 Other Novozyme Enzymes 75
5.5 *Thermomyces lanuginosus* Lipase 77
5.5.1 Initial results 77
5.5.2 GPC, and HPLC – Other Analytical Methods Used 79
5.5.3 Different TLL variants (Novozyme TL/NS40119/Sigma) 84
5.5.6 TLL Comparison with Positionally Non-Specific Lipases 85
5.6 Enzyme comparison summary 87
6 Fat Splitting - Full Split 92
6.1 Design of Experiment – TLL Optimisation 92
6.2 Using Inductively Coupled Plasma Mass Spectrometry to detect ions in enzymatic HEAR oil hydrolysate 96
6.3 Non – Degummed Oil 99
6.4 Different Reactor Types for HEAR Oil Hydrolysis 101
6.5 Enzyme Combinations 104
7 Enzyme Immobilisation 106
7.1 TLL Immobilisation 106
7.2 Reusing TLL on Epoxy Methacrylate (ECR8215) 109
7.3 Reactor Design for Immobilised TLL 111
7.4 Immobilisation of TLL variant (NS40119) and CAL-A (NS40020) on Epoxy Methacrylate Support 112
8 Pre Split Results 114
8.1 Pre-Splitting using TLL 114
8.2 Pressure Splitting of Pre-Split HEAR oil 118
8.3 Pre-Splitting using NS40020 122

9 Discussion.....123
10 Acknowledgments.....127
11 References.....128
12 Appendix.....135

List of Figures

Figure 1: Outcome of a chemical (heat + pressure) based fat splitting process compared to an enzymatic fat splitting process. 13
Figure 2: Candida rugosa lipase in the closed (gray) and open (blue) configuration. Figure adapted from Casas-Godoy et al., 2018 21
Figure 3: Diagram adapted from De Simone, 2016. Illustrated as described above the catalytic mechanism of the Ser-His-Asp catalytic triad in lipases. 23
Figure 4: Mixing in OBR 27
Figure 5: Enzyme immobilisation methods described in this chapter. y. 43
Figure 6: Reactor setups for enzymatic fat splitting. 49
Figure 7: Acid value comparison of a TLL based HEAR oil hydrolysis. 58
Figure 8: Comparisons of pH over the course of a reaction. 60
Figure 9: Standard concentration curve for enzyme solutions. Protein concentration measured in µg/ml 62
Figure 10: Comparisons of buffers as shown in table 5. 67
Figure 11: Other conditions kept constant across experiments: 68
Figure 12: Comparison of higher buffering capacities with PPL. 70
Figure 13: Candida rugosa lipase buffering capacity comparisons. 71
Figure 14: Comparing free Lipozyme CALB to PPL. 73
Figure 15: Comparison of a number of Novozyme 435 based hydrolysis reactions. 74
Figure 16: Comparison between Novozyme™ enzymes. 75
Figure 17: Acid value comparisons of TLL based splitting at a higher temperature and with more concentrated glycine/NaOH buffer. 78
Figure 18: Comparison of TLL based HEAR oil hydrolysis at 50°C, 60°C and 70°C, over 24 hours. 79
Figure 19: Gel permeation chromatography (GPC) of TLL based HEAR oil hydrolysis samples. 81
Figure 20: TLL Hydrolysis HPLC results 83
Figure 21: Comparison of different TLL variants for HEAR oil fat splitting. 84
Figure 22: Comparison between TLL and nonspecific enzymes, CRL and NS40020. 86
Figure 23: Comparison of enzymes used for HEAR oil splitting. 89
Figure 24: TLC comparison showing differences in the TLC profile of 1,3-specific lipases and non-specific lipases. 90
Figure 25: Complete design of experiment results. 94

Figure 26: Results from the highest acid value producing experiments of the DoE, see table 3. 95
Figure 27: Results of Inductively Coupled Plasma Mass Spectrometry of partially split HEAR oil samples 98
Figure 28: Experiments hydrolysing degummed and non-degummed (NDG) oil 100
Figure 29: Oscillatory baffled reactor (OBR) for oil hydrolysis. 101
Figure 30: HEAR oil splitting reaction in an ultrasound mixing reactor 102
Figure 31: Comparison between Novozyme™ enzymes and combination 104
Figure 32: Using combinations of 1,3 specific (TLL) and nonspecific (CRL and NS40020) lipases for HEAR oil splitting. 105
Figure 33: TLL immobilisation summary 107
Figure 34: TLC comparison of TLL on 6 immobilisation resins 108
Figure 35: Attempting to reuse TLL immobilised on ECR8215 in a batch tank reactor. 109
Figure 36: Immobilised TLL, reused after filtering out from a reaction mixture after 24h. 110
Figure 37: HEAR oil hydrolysis using commercially available immobilised TLL in a fixed bed reactor 111
Figure 38: Comparison of free NS40119 (TLL variant) with NS40119 immobilised on ECR8215 for HEAR oil splitting. 112
Figure 39: Combination of 1,3 specific enzyme (NS40119) and nonspecific enzyme (NS40020) immobilised on ECR8215 compared to free enzyme combinations. 113
Figure 40: 6 Pre-split HEAR oil hydrolysis reaction using 100 ppm TLL at 50C. 115
Figure 41: Comparison (a) between 100 and 200 ppm TLL using 10% w/w water and (b) 5% w/w water at 50 and 70°C 116
Figure 42: TLC slide from 5% w/w water and 1% w/w water corresponding to an acid value of 60AV and 25AV respectively. 117
Figure 43: Using HEAR oil as well as 20, 60 and 100 AV pre-split HERO for pressure splitting. 120
Figure 44: Pre-splitting using NS40020 (CAL-A), compared to TLL pre-split 122

List of Tables

Table 1: Expected acid value and sample intake 51
Table 2: Solvent systems for separating MAGs, DAGs, TAGs and FFAs using TLC. 53
Table 3: Purolite Immobilisation Resins, the code used to refer to them and their chemical properties. 56
Table 4: BSA standard concentrations 57
Table 5: Lipases previously used for fat splitting.. 61
Table 6: Enzyme concentrations as measured using Bradofrd assay 63
Table 7: 6 Buffers used for HEAR oil splitting. Buffers are numbered 1 – 6. 65
Table 8: Design of Experiment variables... 93
Table 9: Results of organic phase and middle phase ICP-MS testing. 97
Table 10: Results of pre-split material used for chemical pressure splitting recorded as acid value . 119

List of Abbreviations

a	Specific free interfacial area (m^{-1})
a_t	Total specific interfacial area (m^{-1})
AV	Acid value
CALB	*Candida antarctica* Lipase B
CRL	*Candida rugosa* Lipase
DAG	Diacylglycerol
[E]	Free enzyme concentration (mol m^{-3})
EA	Erucic acid
$[E]_t$	Total active enzyme concentration (mol m^{-3})
[ES]	Enzyme substrate complex concentration (mol m^{-3})
FFA	Free fatty acids
GC	Gas chromatography
GPC	Gel permeation chromatography
HEAR	High erucic acid rapeseed
HPLC	High performance lipid chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
LU	Lipase unit (µmol min^{-1})
k_d	Desorption constant (min^{-1})
K_m	Apparent Michaelis constant (mol m^{-3})
k_a	Adsorption constant (m^2 min^{-1})
K_e	Equilibrium constant of ES (mol m^3)
K_i	Product inhibition constant (mol m^{-3})
k_1	Reaction rate constant (m^3 mol^{-1} min^{-1})
k_{-1}	Reaction rate constant (min^{-1})
MAG	Monoacylglycerol
NDG	Non-degummed oil
[P]	Product concentration (mol m^{-3})

PPL	Porcine pancreatic lipase
[S]	Substrate concentration (mol m^{-3})
Sn	Stereospecific numbering
TAG	Triacylglycerol
TLC	Thin layer chromatography
TLL	*Thermomyces lanugionosus* lipase
υ	Reaction rate (mol m^{-3} min^{-1})

1 Introduction

Fat splitting, the production of fatty acids through hydrolysis of organically derived oils and fats is an industrially important and valuable process. The production and sale of fatty acids as well as fatty acid derivatives such as fatty acid amides or esters feeds a global market …

The global market for natural fatty acids will grow from nearly $13.5 billion in 2018 to $17.5 billion by 2023, with a compound annual growth rate (CAGR) of 5.4% for the period of 2018-2023. (BCC, 2019)

The fats and oils used in this process are naturally produced and renewable raw materials, such as oils from corn, sunflower, rapeseed, olive, coconut, palm, and others as well as various animal fats such as tallow. Globally more than $2x10^6$ tons of fatty acids are being produced from hydrolysis of oils and fats.

CRODA, a globally operating Yorkshire based company and our industrial partner, is one of the largest producers of erucic acid, which it mostly uses to produce erucamide, the fatty acid amide of erucic acid, which has numerous uses, as a slip agent or plastic additive. The global market size for fatty acid amides alone was estimated at around 310 million US$ in 2016.

Fatty Amides Market size is forecast to reach $443.76 million by 2025, after growing at a CAGR of 5.2% during 2020-2025. (ARC, 2018)

Erucic acid is produced from High erucic acid rapeseed (HEAR) oil. Erucamide, also known as Crodamide™ is used as a lubricant as well as a rubber and plastic additive. It is used from shopping bags and plastic bottles to lubrication for continuous steel casting (Nieschlag and Wolff, 1971).

Oils and fats consist by majority of compounds known as triglycerides. These are fatty esters of glycerol and three fatty acids bound through ester bonds. Hydrolysis reactions of these triglycerides involves reactions with water to split ester bonds and produce free fatty acids and glycerol. The fatty acids attached to the glycerol backbone can be of varying size. In HEAR oil, the fatty acids are generally in the range of 14 – 22 carbon atoms long (C14 – C22), the most common triglyceride contains two erucic acid (C22) and one oleic acid (C18) fatty acid, as shown in figure 1.

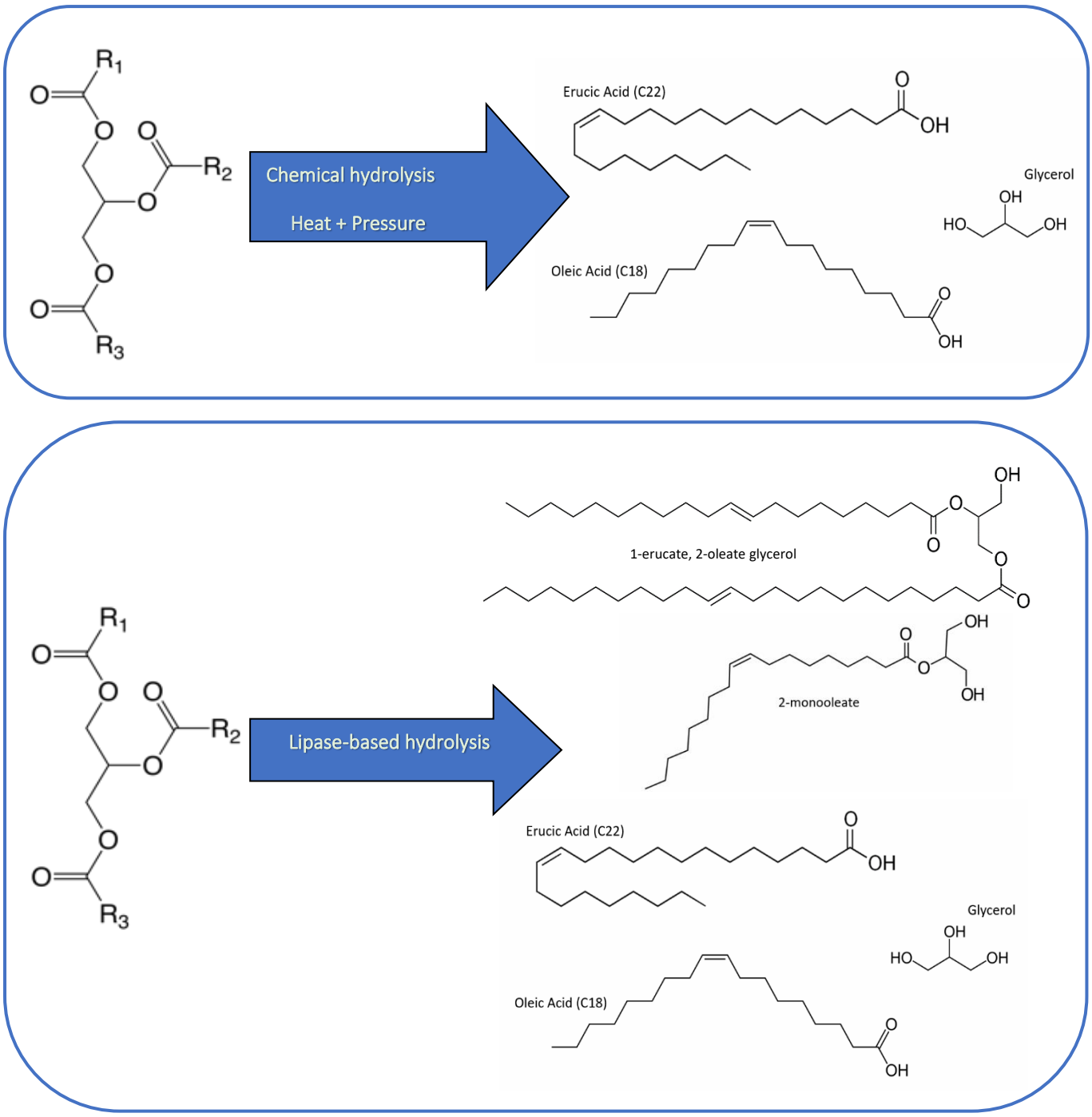


Figure 1: This figure shows the differences in outcome of a chemical (heat + pressure) based fat splitting process compared to an enzymatic fat splitting process.
R1, R2 and R3 are fatty acids attached to a glycerol backbone, forming the tryglyceride of the HEAR oil. Chemical splitting is complete, producing fatty acids (in HEAR oil, the most common fatty acids are erucic and oleic acid) and glycerol. An enzymatic process splits fatty acids one at a time and produces intermediates such as partial glycerides (mono- and di-glycerides, common species in HEAR oil shown in figure) as well as fatty acids and glycerol as final products.
Both cis and trans fatty acids occur in HEAR oil and figures are drawn so that they may fit on the page rather than indicating only one type is present.

Over the last few years and decades as biological engineering and chemical engineering have advanced more, many processes that are traditionally purely chemical and physical in nature are being replaced by enzymatic processes.

Technical enzymes are applied in detergents, pulp and paper, textile manufacturing, leather goods, fuel production and to pharmaceuticals and chiral substances. Technical enzymes are typically manufactured and used in bulk, compared to other applications.

In food, enzymes play a critical role in converting starch for ingredients in food products. Food enzymes are mainly used in the baking, fruit juice manufacturing, wine making, brewing and cheese manufacturing. (Adrie J.J. Straathof, 2000)

Animal nutrition is an important and growing area of enzyme application, particularly for pig and poultry nutrition. Feed enzymes help degrade specific feed components that are otherwise either harmful or provide no value to livestock. Enzymes are also used for animal and aquaculture nutrition. And enzymes such as amylase, nuclease, peroxidase, proteinase, glucose oxidase, superoxide dismutase and urease are applied in cosmetic products.

In the medical industry, enzymes are used as digestive aids, for wound cleaning, for lysis in vein thromboses, in acute therapy for myocardial infarction and as support in the therapy of certain types of leukaemia. (Greenberg, 2021)

These can typically do the same reaction with less energy input needed. Traditionally fermentations, baking and brewing were done using whole microorganisms, which produce several enzymes often catalysing several reactions. For example, brewing is a multi-step enzymatic process, where starch is turned into ethanol through several enzymatically catalysed reactions. Starch is broken down into sugars, and these sugars are lysed and fermented to produce alcohol. In total up to 12 separate enzymes are involved in this process. For long multi-step processes which follow metabolic pathways, such as brewing, often whole microorganisms have the advantage over purified enzymes.

Individual purified enzymes are more suitable for single step reactions, where using a whole microorganism would lead to side reactions and contamination of the end product. (Bracco, 2014; Schallmey, Domínguez de María and Bracco, 2013)

In any case enzymes such as amylases, proteases and lipases are used in various pharmaceutical, food and beverage, detergent, and biofuel industries. In addition, there is increased usage of enzyme catalysis for fine chemical production as well as processes such as fat splitting. (Bornscheuer and Buchholz, 2005)

Although there is some limited application of enzymes (lipases) for fat splitting and specifically soap production in Japan and South Korea, these are usually on a smaller scale, and lipase-based solutions for fat splitting are not commonly used due to a number of drawbacks. For example, the splitting time for a single batch can take up to 2-3 days, and enzyme prices are high and add extra cost to every batch being produced. (Gandhi, 1997)

Current industrially relevant processes for the hydrolysis of oils and fats on a large scale, therefore, mostly do not use enzymes but instead use high pressures and temperatures. These conditions lead to the complete split of fatty acids from the triglyerol backbone. The most commonly used of these industrial processes is called the Colgate-Emery Process. Typically, an operating temperature of 240 - 250°C and pressures of up to 50 bar are used in this process. These temperatures and pressures are maintained in an up to 30-meter-tall metal column, through which the reaction mass flows continuously. Fats or oils to be split are introduced from the bottom of the column, whereas water is introduced at the top of the column. As the water descends through the splitting column the oil ascends, and the hydrolysis of the oil or fat occurs where the oil and water meet (Barnebey and Brown, 1948). This reaction produces free fatty acids, which are extracted from the top of the column as well as glycerol containing wastewater (sweetwater), extracted from the bottom of the column. Both the fatty acid mixture as well as the sweetwater need to undergo further purification steps before the product can be collected. Fatty acids are concentrated, and waste products as well as impurities are removed.

Similarly glycerol, a much less valuable by-product needs to be purified and distilled to be able to be sold (Lascaray, 1952).

Often times high temperatures and pressures cause discolouration and other undesirable characteristics in the final fatty acid product, and costly protection and deprotection steps must be taken to avoid this. Protection steps are done to form a protective group by reversibly reacting a functional group, which decreases the overall reactivity of that functional group under the physical and chemical conditions the molecule will be subjected to.

The protective group is then removed after the reaction. This is referred to as a deprotection step.

Despite the high efficiency and yield of the Colgate-Emery process, the very high temperature and pressure requirements have made attempts at developing enzymatic processes for this reaction very attractive. In addition, Lipases, which are the class of enzymes capable of catalysing fat splitting reactions are a group of relatively well studied enzymes.

A lipase-based process has a number of potential advantages over current industrial processes. One of these advantages is a 20° – 70°C operating temperature. Most enzymes work best in the 20° – 40°C range, however more recently some extremophile fungus produced lipases have been shown to be capable of withstanding temperatures up to 80° - 90°C. (Bharathi and Rajalakshmi, 2019; Mehta, Bodh and Gupta, 2017)

This is important for our intended process, in particular as many of the heavier fatty acids and partial glycerides produced have melting points between 20° - 30°C.

Comparing the mode of splitting of chemical and lipase-based processes, lipase based splitting processes are more complicated. In a chemical-based fat splitting reaction, a high temperature and pressure is created, sometimes in the presence of a metal catalyst. The energy from this is used as activation energy to break the ester bonds holding triglycerides together. In this process, all ester bonds (R1, R2, R3 in figure 1) are broken.

In an enzymatic process on the other hand, each ester bond is broken individually through an interaction with the lipase at the oil/water interface. In addition to this, some lipases are sn-1, 3-specific, meaning these lipases prefer to break ester bonds in the 1 and 3 position on the glycerol. Sn refers to the stereospecific numbering system, each carbon atom in glycerol is assigned a number, with a fatty acid attached to each of them (R1, R2 and R3 in figure 1). The other class of lipases are non-specific, meaning they have no preference in which ester bond to break, although they still only break a single bond with each interaction.

In high erucic acid rapeseed (HEAR) oil, also known as HERO, specific fatty acids are always in the same position on the glycerol backbone. Erucic acid, which makes up around 50 – 60% of fatty acids in HEAR oil is concentrated in the sn-1 and 3 position of the triglyceride. Smaller C18 fatty acids such as oleic, linoleic, and linolenic acid which make up most of the remaining fatty acids in HEAR oil are concentrated in the sn-2 position. Figure 1 shows the main expected products of enzymatic hydrolysis of HEAR oil. This Enzyme selectivity could be used to target production of only erucic acid via 1,3 specific hydrolysis, leaving a valuable side product in 2-oleate glycerol also known as monooleate. Whether this targeted production is possible remains to be seen in experimentation.

Over the course of this project, we will develop lipase based HEAR oil hydrolysis processes and evaluate these for industrial viability. To do this we will be comparing them to current industrial processes as described above as well as comparing them to each other as well as learning from previous attempts at fat and oil splitting which will be described in the following section.

2 Literature Review: Enzymatic Fat Splitting

In the last 20 to 30 years enzymatic reactions have been increasingly investigated for their viability for industrial purposes. Reactions, which are usually and traditionally carried out using high pressure, high temperature, and metal catalysts, can often be carried out using enzymes such as proteases, lipases, and amylases, to catalyse reactions such as the hydrolysis of cellulose, or breakdown of protein, fat, and sugar contents for various purposes (Kirk, Borchert and Fuglsang, 2002; Gotor-Fernández, Busto and Gotor, 2006).

Fat splitting is carried out using a group of enzymes called lipases. Increased attention to concepts such as green chemistry and energy efficiency has led to an increased use of lipases for many different purposes, as they act as catalysts allowing for a lower energy input needed for the same reactions. This increased use of enzymes has also led to more lipases with different properties such as heat, pH or other tolerances being discovered and categorised. In addition to this there has been a lot of innovation when it comes to processes and methods to optimise lipase-based hydrolysis reactions.

2.1 HEAR Oil

For the purpose of this thesis, High erucic acid rapeseed (HEAR) oil will be split using lipases. HEAR oil is a high viscosity oil extracted from the seeds of the plant *Brassicus napus*, commonly known as rapeseed. HEAR oil consists mostly of a mixture of triglycerides, with small amounts of other glycerides and fatty acids. There are other types of rapeseed oils such as low erucic acid rapeseed (LEAR) oils, which are produced for food and industrial purposes. The difference between HEAR and LEAR oil is the amount of erucic acid present in the triglycerides which make up the oil. In HEAR oil erucic acid makes up 40 – 60% of the fatty acids bound in triglycerides. Erucic acid is concentrated in the sn-1 and 3 positions of the triglyceride backbone, as rapeseed lipases which build these triglycerides lack the ability to esterify long chain fatty acids in the sn-2 position of the glycerol backbone (Przybylski and Mag, 2011).

Besides its use in fat splitting, HEAR oil and other vegetable oils rich in erucic acid such as crambe oil have long been used in industrial and commercial applications. HEAR oil has been used as a lubricant in steel casting, although this is less common today. The hydrogenated triglyceride itself as well as

wax esters prepared from the long chain fatty acids have properties comparable to commercial wax. Various chemical transformations of the triglycerides of high erucic oils such as oxidative cleavage produce valuable products (Nieschlag and Wolff, 1971).

2.1.1 Oil Degumming

Degumming of oils is a process whereby impurities are eliminated from crude vegetable oils. Especially phospholipids, the titular gums, but also calcium, magnesium and iron ions need to be removed before the oil can undergo high temperature and pressure processes. These impurities are responsible for loss of material during industrial processes due to their emulsifying properties (Deffense, 2009).

There are several different methods of degumming vegetable oils, including water degumming, acid treatment, TOP degumming (combination of both). TOP stands for Totaal Ontslijmings Process, a dutch term for a complete degumming or deliming process. (Ohlson, 1992)

HEAR oil is degummed by a version of the TOP process.

Water degumming is done by heating an oil to around 80C and adding 5-10% v/v of water then stirring for 10 – 20 mins before centrifuging. Acid degumming involves heating an oil again to 80C before adding a 2% v/v of a concentrated aqueous acid. For top degumming first a water degumming step is performed before the mixture undergoes an acid degumming step and is neutralised using low concentrations of an aqueous alkali. Different degumming processes have different uses. For very high initial metal content TOP degumming is less efficient, however, for removing phospholipids TOP is the most efficient process (Zufarov *et al.*, 2009).

There are also lipase based degumming processes being developed, although these are not in large scale industrial use yet (Yang, Wang and Yang, 2006).

However, for an enzymatic process, non-degummed oil may be beneficial. Several of the mineral ions present in non-degummed oil have been reported to increase enzyme activity. In addition to this, emulsifying properties may help with mixing organic and aqueous phases of the reaction (Bisht, Yadav and Darmwal, 2013).

2.2 Lipases – Fat Splitting Enzymes

This section will be considering the production and properties of lipases as well as previous uses of lipases for the splitting of oils and fats both in industry and academia.

Throughout biotechnology, including organic synthesis, lipases are one of the most commonly used classes of enzymes.(Chandra *et al.*, 2020)

Lipases are an example of class III enzymes also known as hydrolases. This class of enzymes catalyse hydrolysis reactions. Lipases specifically catalyse the hydrolysis of ester bonds in lipids, although they are also capable of forming ester bonds; the reverse of the hydrolysis reaction. (Saha, Jordan and Bothast, 2009)

Lipases have been available for industrial purposes longer than most other enzymes, and as a result they have been used for longer. The mode of action of lipases in between the water and oil interphase has led to it evolving a resistance to denaturing effects, which would destroy many other types of enzymes such as proteases and amylases. Many lipases are even resistant to the denaturing effects of organic solvents as well as often being able to tolerate high pH variances (Schmid and Verger, 1998).

2.2.1 Lipase Use

Lipases are extremely versatile and in recent years have become an ever more important group of enzymes, due to their wide variety of uses and relatively easy mass production. Lipases can be used directly in different industries or indirectly for production and processing of raw materials. (Schmid and Verger, 1998)

Lipases are used directly in the textile industry for removal of lubricants as well as modification of polyester fibres (Hasan, Shah and Hameed, 2006). Their most important direct use is likely in the detergent industry, where addition of lipases reduces the overall environmental impact of detergent products, as well as allow for detergents to be used at lower temperature. Lipases are used for removal of oil and fat stains from fabrics as part of detergent mixtures. Fungal lipases specifically are preferred for this application (Takamoto *et al.*, 2001).

Lipases have also been used in pulp and paper industries, to break down oleaginous waste and degrade triglycerides present in paper pulp slurry (Reetz and Jaeger, 1998). Similarly, lipases have

been used to aid in the degradation of biodegradable polymers. Since lipases catalyse reversible reactions there is also research being done into the possibility of lipase-based synthesis of polyesters.

Lipases have of course also been used for numerous other applications, including medical, cosmetics, and various other uses. The most relevant for our work are their use in oleochemical industry, where lipases are used in the production of biodiesels as well as the synthesis and degradation of ester bonds (Singh and Mukhopadhyay, 2011; Ribeiro *et al.*, 2011).

Although lipase-based processes are not currently employed for large scale fat splitting, using enzymes for pre-splitting has been suggested for a while now. Pre-splitting involves producing a partially split mixture of glycerides, which, when used for pressure splitting has preferable properties to untreated HEAR oil (Anderson and Wenzel, 1996).

2.2.2 Production of Lipases

In recent years interest in microbial lipases, specifically fungal lipases, has led to increased production of fungal lipases and new methods of producing fungal lipases. Fungal lipases are preferred over bacterial or other microbial lipases for their ease of production and wider variety of applications and specificities. (Chandra *et al.*, 2020)

Of particular interest are lipases from thermophilic fungi, which are the only eukaryotic organisms capable of growth at temperatures above 45°C, many are capable of growth of temperatures up to 70°C or more, implying enzymes which are also capable of functioning at these temperatures (Maheshwari, Bharadwaj and Bhat, 2000).

Most fungal lipases are produced using solid state fermentation (SSF), which is a process of cultivating microbial organisms in solid substrates. It is usually carried with no or little moisture. Filamentous fungi such as *Aspergillus* sp. are particularly suitable for this and often lipases from other fungi are expressed in these filamentous fungi. Lipases produced using SSF often have greater thermal stability compared with lipases produced using submerged fermentation. (Kumar and Kanwar, 2012; Gutarra *et al.*, 2005)

The second major way microbial lipases are produced is submerged fermentation (SMF), which is cultivation of microbial organisms in liquid medium. The advantages of this method are better control over variables such as temperature and pH as well as a higher culture homogeneity.

Both methods are commonly used in the production of lipases for both research and industry (Geoffry and Achur, 2018).

Fermentation processes can be operated in batch, continuous as well as fed-batch reactors. Batch reactors of various types have been used for SMF, whereas typically packed-bed reactors are used for SSF. Fed-batch processes are variations of this, where extra nutrients are added during the fermentation process to better control the metabolism of the microorganism. Continuous processes are usually easier to scale up, due to continuous input of reagents and output of product. Using a continuous culture increased lipase production by 50% compared to batch fermentation (Singh and Mukhopadhyay, 2011).

2.2.3 Lipase Structure and Catalytic Mechanism

Lipases as mentioned are biological catalysts, and as such they have evolved and changed under various natural conditions, providing them with slightly different properties, natural inhibitors, or ideal temperatures. Lipases, along with all other types of enzymes are soluble in water, however, the main natural substrate for lipases are glycerides, which are largely insoluble in water. The enzyme

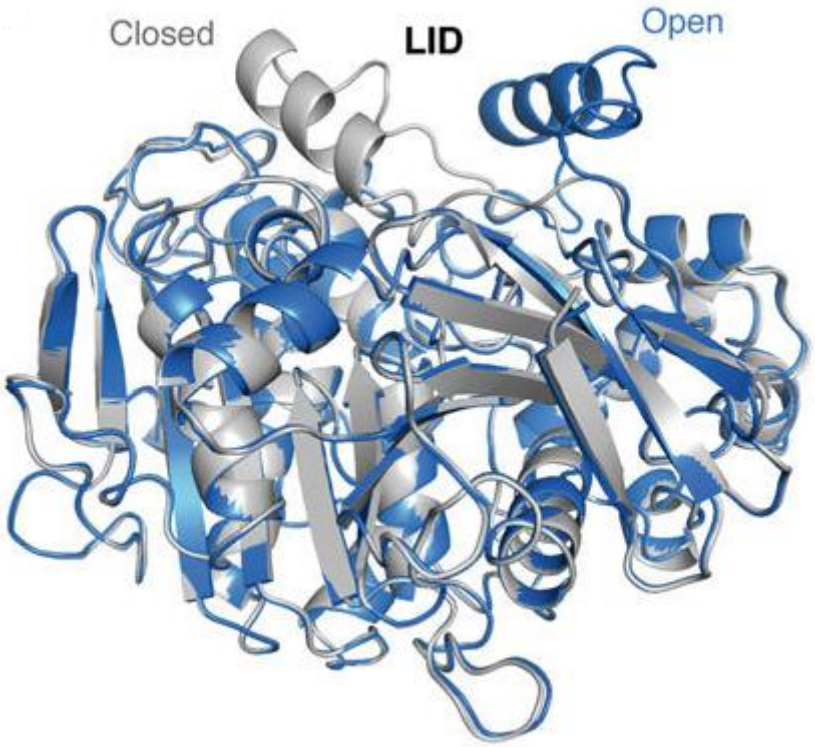


Figure 2: Candida rugosa lipase in the closed (gray) and open (blue) configuration. The lid is indicated at the top of the image. Figure adapted from Casas-Godoy et al., 2018

therefore acts through interfacial activation. Lipases are adsorbed onto the surface of very hydrophobic glyceride droplets (oils and fats) and can then act on the interfacial area between the water and oil. This adsorption mechanism is dependent on a large hydrophobic pocket surrounding the active site of the enzyme. To keep the lipase stable and still soluble in water, this hydrophobic pocket is protected by a lid region, which is a sequence of hydrophilic polypeptides. In its "closed" form this lid region has a hydrophobic inside, and a hydrophilic outside. The lid structure is mobile and upon adsorption onto the hydrophobic glyceride droplet the lid moves by rotating around two hinge regions. This exposes the hydrophobic active site region to the outside, resulting in the "open" form of the lipase, see figure 2 (Zhao *et al.*, 2017; Casas-Godoy *et al.*, 2018).

Before the lid moves, the lipase undergoes some changes upon encountering a glyceride at the oil-water interphase. More hydrophobic loops are displayed on the lipases surface, allowing the initiation of triglyceride to bind to the active site. This allows the lid to open and the more hydrophobic environment inside the lipase is revealed, which subsequently allows for interactions between the active site and a glyceride molecule within the glyceride droplet. The active site consists of a catalytic triad which is mostly conserved in various lipases. This catalytic triad functions in the same way as many others like it found in nature (Brzozowski *et al.*, 1991; Kapoor and Gupta, 2012).

To explain the mechanism of the hydrolysis reaction the example of porcine pancreatic lipase (PPL) on a triglyceride will be used. In PPL, the catalytic triad consists of Ser 153, His 264 and Asp 177. When a reaction is initiated, the aspartic acid forms a hydrogen bond with His 264, which increases the pKa of the histidine molecule, specifically of the other nitrogen. This allows His 264 to act as a powerful base, causing it to deprotonate Ser 153. This deprotonated serine can then act as a nucleophile, allowing it to attack the ester bonds of fatty acids, specifically the ester bond on the 1 and 3 carbons of the triglyceride backbone – shown in part 1 of figure 3. This leads to the formation of a negatively charged tetrahedral intermediate, the triglyceride bound to the lipase by serine, which is stabilised in the oxyanion hole. Next, the carbonyl reforms, attached to serine, with the glycerol backbone (diglycerol) leaving – shown in part 2 of figure 3. To detach the fatty acid carbonyl from serine, a water molecule donates a proton to His 264, forming a hydroxyl group (HO^-). This hydroxyl group reacts with the serine-fatty acid carbonyl, forming a second negatively charged tetrahedral intermediate. This second intermediate again reforms into a free carbonyl (the free fatty acid) and both His 264 and Ser 153 are "reset" – final state shown in part 3 of figure 3 (Widmann, Juhl and Pleiss, 2010; De Caro *et al.*, 1981; Verger, 1997; De Simone, 2016)

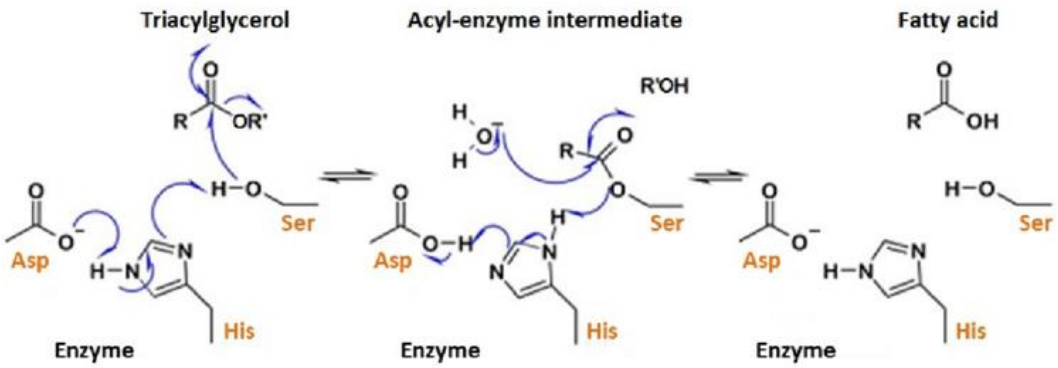


Figure 3: Diagram adapted from De Simone, 2016. Illustrated as described above the catalytic mechanism of the Ser-His-Asp catalytic triad in lipases.

The connection between lipase structure and lipase specificity, however, is poorly understood. The lid region seems to indicate lipase specificity, in terms of substrate molecular mass and size as well as positional specificity. Mutations in the hinge region near the lid also impact the chain-length selectivity as well as thermal stability of lipases, further indicating this region's importance to specificity. (Albayati *et al.*, 2020; Li *et al.*, 2015; Mohamed *et al.*, 2018)

2.2.4 Lipase Kinetics

Lipases generally have complex mechanisms, especially when it comes to the hydrolysis of triglycerides in oil and fat splitting.

Essentially, 3 different reactions are occurring at any one point. Triacylglycerides (TAG) are hydrolysed into Diacylglycerides (DAG) and a fatty acid, DAG are hydrolysed into Monoacylglycerides (MAG) and a fatty acid, and MAG are hydrolysed into glycerol and a fatty acid. Each of these individual reactions can be written as

$$[E] + [S] \rightleftharpoons [ES] \rightleftharpoons [E] + [P]$$

Equation 1

where the substrate [S] refers to TAGs, DAGs or MAGs, and the product [P] refers to DAGs, MAGs or glycerol respectively.

Different lipases have different rates for each of these reactions, and different specificities for chain length or positional specificity in TAGs alone. In addition to this there are different types of DAGs

and MAGs, which the lipases could act differently on. In the case of HEAR oil fatty acid chain lengths are C22 (erucic acid) and C18 (oleic, linoleic and linolenic acid) (McNeill, Shimizu and Yamane, 1991).

These reactions can be simplified and summarised, as each TAG being hydrolysed into three fatty acid molecules and glycerol.

This hydrolysis reaction occurs at the interface between the enzyme containing aqueous phase and the oil phase. Therefore, the main method of enzymatic fat splitting is to directly bring the aqueous phase containing the enzyme in contact with the organic oil phase. The interfacial area, however, does not remain constant, irrespective of agitation or proportion of oil to water. Therefore to accurately model the kinetics of this reaction, agitation as well as substrate concentration need to be taken into account (Sulaiman, 2006).

To accurately describe the lipase catalysed reaction, we can rewrite the above equations as this set of equations (equation 2).

$$[E] \underset{}{\overset{k_a}{\rightleftharpoons}} [E^*]$$

$$[E^*] + [S] \underset{}{\overset{k_1}{\rightleftharpoons}} [E^*S]$$

$$[E^*S] \xrightarrow{k_{cat}} [E^*] + [P^*]$$

Equation 2

The first step describes the adsorption of the enzyme into the interfacial area. This process is dependent the adsorption (k_a) and desorption (k_d) constants as well as the concentration of free enzyme (E) and interfacial enzyme (E^*).

At the interface, the interfacial enzyme and the substrate (S) form an interfacial enzyme substrate complex (E^*S). The rate of formation is dependent on the concentration of enzyme at the interface, the concentration of substrate and rate constants for the formation (k_1) and breakdown (k_{-1}) of the complex.

The final step describes the formation of interfacial product from the enzyme substrate complex, which depends on the concentration of the interfacial enzyme substrate complex as well as the catalytic rate constant of the enzyme (k_{cat}). (Jamie *et al.*, 2017; Tsai, Wu and Chiang, 1991)

The adsorbed enzyme as well as the enzyme substrate complex concentrations are assumed to be constant in a "quasi-steady state". It is also assumed that the interfacial product concentration (P*) is low and therefore occupies a negligible fraction of the interfacial area. It is also assumed that P* is proportional to the concentration of free product (Jamie *et al.*, 2017; Sulaiman, 2006; Al-Zuhair, Hasan and Ramachandran, 2003).

In addition to this it has been shown that at low enzyme concentrations, the area takes up by enzymes which have penetrated the interface, is negligible compared to the total interfacial area. These assumptions allow us to use this simplified rate equation (equation 3) for lipase based hydrolysis reactions (Sulaiman, 2006; Jamie *et al.*, 2017).

$$\upsilon = \frac{k^*_{cat}[E]_t[S]}{K_c\left(\frac{k_d}{k_p a_t^2} + 1\right) + [S]}$$

Equation 3

where $K_c = (k_{cat} + k_{-1}) / k_1$ and k^*_{cat} is equal to k_{cat}/ C*. (C* is a constant relating to proportionality of product at the interface and free product).

Comparing equation 3 to the Michaelis-Menten equation (equation 4) shows that the K_m equivalent in the above equation is not a constant and in fact changes depending on the total specific interfacial area (a_t).

$$\upsilon = \frac{k^*_{cat}[E]_t[S]}{K_m + [S]}$$

Equation 4

This is one of the explanations for why pure Michaelis-Menten kinetics cannot predict or accurately model the hydrolysis rate of oil by lipases in most mechanically agitated systems. The total interfacial area changes depending on operating conditions such as stirring speed, substrate concentration as well as temperature or even the shape of the reaction vessel. There are however conditions under which a Michaelis-Menten model could be successfully applied. For example oil-water micro-emulsions, with emulsification reagents are stable emulsions, which have a much larger, and more importantly a constant interfacial area (Knezevic, Siler-Marinkovic and Mojovic, 1998).

There are other models, which attempt to explain the rate of reaction in these micro-emulsion systems.

A model using product inhibition dependent rate equations was developed on the basis of a Ping-Pong-Bi-Bi mechanism, which is where products are formed prior to all of the substrate's binding. In addition production of an initial product changes the enzyme temporarily, allowing it to produce a secondary product (Malcata, Hill and Amundson, 1992).

The following equation is the rate equation showing a Michaelis-Menten equation (equation 5) accounting for product inhibition.

$$v = \frac{k^*_{cat}[E]_t[S]}{K_m\left(1 + \frac{[P]}{K_i}\right) + [S]}$$

Equation 5

If we consider the initial rate of reaction, the concentration of the product [P] is likely to be low enough to be negligible in comparison to the initial substrate concentration [S] or the K_m value. This equation can therefore be simplified to the original Michaelis-Menten equation as shown above.

To conclude, lipase kinetics are complex, and difficult to model. Although broadly following Michaelis-Menten kinetics several other factors also affect the rate of reaction. In a perfectly mixed system, where water and substrate are continuously replaced and the interfacial area is not subject to large changes in size, the Michaelis-Menten equation can model the rate of reaction.

2.3 Reactor Types for Enzymatic Fat Splitting

There are many different types of reactors which can be used for enzymatic fat splitting. The most common type of bioreactor is a simple stirred batch reactor, from its simplest form, an open stirred beaker to jacketed reactors, for temperature control or even pressurised reactors, where both temperature and pressure can be controlled. These types of reactors are generally easy to set up and operate, and are ideal for using free enzyme as well as immobilised enzyme – more about enzyme immobilisation below (Kang and Rhee, 1989).

In continuous processes other reactor types such as oscillatory baffled reactors (OBR) are commonly used. OBRs create mixing by moving baffles (or moving liquid through baffles) in a repeating reciprocating motion. These reactors are usually tubular although this mixing system can also be applied to a tube-shaped batch reactor. This motion creates vortices during the "forward" motion (figure 4 a) in the inter baffle-zone which are then moved into the main flow during the "backwards" motion (figure 4b).

This creates several zones where mixing between oil and water occurs allowing interfacial enzymes such as lipases to work at very high efficiency. OBRs are more usually used with free enzyme. (Abbott *et al.*, 2013)

For immobilised enzymes specifically the most commonly used type of reactor is a packed bed or fixed bed reactor. The biocatalyst is fixed in place and reagents are pumped through the immobilised enzyme mass. These reactors can be used both in recirculating batch experiments and in continuous reactions (Yang and Rhee, 1992b).

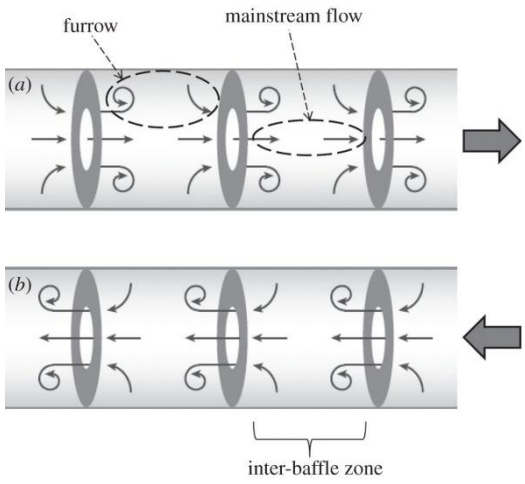


Figure 4: Mixing in OBR

Vortexes formed are forced into main flow– adapted from Abbot et al. 2013

Fixed bed reactors have previously been used for the hydrolysis of fats using lipases immobilised on a hydrophobic porous support. (Brady *et al.*, 1988)

More exotic variations of fixed bed reactors have also been used for lipase based oleochemical reaction. A SpinChem rotating bed reactor was used with immobilised lipase, and good reusability of the enzyme was reported (Pithani *et al.*, 2019).

Similarly, to fixed bed reactors, another reactor type used with immobilised enzymes are membrane reactors. These reactors have a diaphragm or membrane on which the enzyme is immobilised. No major mass transfer occurs through the membrane, instead the phases remain on each side of the membrane (Pronk *et al.*, 1988).

2.4 Previous Enzymatic Fat Splitting Review

Enzymatic fat splitting for industrial purposes is not a new concept. There are many reports of enzymatic fat splitting on different oils and fats, some with industrial processes in mind, and some for academia.

A number of different patents and process descriptions have been published, however for the industrial production of erucic acid from HEAR oil none of the described processes would be suitable. Current processes are much more cost efficient, and hundreds of tons of erucic acid are produced in continuous processes, where yield losses from 99% splitting efficiency to 97% splitting efficiency can cost hundreds of thousands of pounds in profits.

In this section I will describe previous processes for enzymatic catalysis of fat and oil splitting. Although my own process will focus on the hydrolysis of oil to produce fatty acids, there are many previous processes which focus on the production of biodiesels or other esters, which still have valuable insight into conditions used for optimal enzyme function or interesting analytical techniques.

2.4.1 Analytical Techniques

This section will detail some of the analytical techniques used for the analysis of fat splitting reactions.

There are several common products of enzymatic fat splitting reactions, and several different chemical reactions within the overall fat splitting process as described in section 2.2.3 and 2.2.4. As a result, several different analytical methods will need to be used to understand the enzymatic fat splitting process and to fully describe the rate of reaction. (Jurado *et al.*, 2008)

2.4.1.1 Acid value

Acid value titration is a common technique for determining the fatty acid content and therefore fatty acid production from oils and fats. It is used by Croda for analysis of degree of oil hydrolysis in their industrial full scale fat splitting process. It is also used in Croda laboratories at a smaller, bench-top scale for determining fatty acid content of samples.

The acid value refers to the number of milligrams of potassium hydroxide which are used to neutralise free fatty acids in a sample of one gram. This number increases as more fatty acids are present in the sample. (*Japanese Pharmacopoeia XIV, Chapter 17: Fats and Fatty Oils Tests*, 2001)

2.4.1.2 High Performance Liquid Chromatography

High performance liquid chromatography, HPLC, is another, more detailed technique for analysis of fatty acid content as well as partial glycerides. HPLC uses columns holding packing material as a stationary phase, and liquid into which the dissolved sample is injected as a mobile phase. (Malviya *et al.*, 2010)

Both normal phase and reverse phase HPLC are used for the analysis of lipids. HPLC is a very common method for analysing fatty acids and fat splitting reactions. However, HPLC requires standards, which may or may not be commercially available for several of the partial glyceride products. In addition to this many of the common detectors used for HPLC such as UV detectors cannot distinguish lipids from background signal. (Moreau, 2006)

2.4.1.3 Thin Layer Chromatography

Thin layer chromatography (TLC) is a widely used, cheap and versatile method for separation. TLC also has the advantage of requiring relatively little sample preparation and a low time of separation. (Wall, 2005)

In a 2001 study the regional specificity of bacterial lipases was determined using TLC (Lanser, Manthey and Hou, 2002). TLC is a useful method for this as it is quick and allows for visual determination. In most oils there are at least two separate diacylglycerides and their separated appearance on TLC slides can be used to indicate specificity of the lipases which produced them. (Fuchs *et al.*, 2011)

2.4.2 Commercial Lipases used for Fat and Oil hydrolysis

In 2007 a study was carried out where three different lipases were compared for their ability to hydrolyse soybean oil to extract essential fatty acids. Two microbial lipases, *Thermomyces lanuginosus* lipase (TLL) and *Candida rugosa* lipase (CRL) were compared to an animal cell derived lipase, Porcine pancreatic lipase (PPL). The microbial lipases showed much higher activity and overall yield. A 70% and a 53% degree of split was reached with CRL and TLL respectively compared to 23% degree of split using PPL. However the study found that PPL preferentially split essential fatty acids, fatty acids which the human body cannot produce, making it more suitable for the intended application. (Freitas *et al.*, 2007; Kaur, Chugh and Gupta, 2012)

Similar operating conditions were utilized for the hydrolysis of safflower oil, specifically for liberation of linoleic acid (LA) which is concentrated in safflower oil. LA is a C18 fatty acid, which is used in the food industry for synthesis of flavours. Again, the activity of PPL, TLL and CRL was compared using equivalent amounts of enzyme by weight. Effects of temperature, reaction as well as enzyme and substrate concentrations were measured and compared. While TLL showed remarkable heat resistance and PPL showed consistent activity through varied pH and substrate concentrations, CRL proved to be the ideal enzyme for safflower oil splitting, likely due to the smaller fatty acid sizes, which CRL targets preferentially. (Aziz, Husson and Kermasha, 2015).

Candida rugosa lipase (CRL) as well as several other lipases including *Thermomyces* lanuginosus lipase (TLL), Porcine pancreatic lipase (PPL), and more fungal lipases were used as biocatalysts for the hydrolysis of Moringa oleifera Lam oil. Hydrolysis reactions were carried out without the use of buffers of emulsifiers, and instead only water was used. Initial results indicated CRL having the highest activity on Moringa oleifera Lam (MOL) oil. After a design of experiment optimisation process using CRL, optimal conditions for the hydrolysis reaction were established. The reaction progress was measured after 3 hours, and the percentage of hydrolysis was determined using acid/base titration. Molecular docking analysis also showed how CRL displays stable binding to C18:1 fatty acid (oleic acid), which is the most common fatty acid in MOL oil (Barbosa *et al.*, 2019).

For our purposes, CRL, TLL as well as PPL are enzymes of interest. These three enzymes have a lot of literature and characterisation studies available for further information. Especially CRL and PPL have a long history of being used in industrial applications. As a result, we took a more in depth look at each one and their previous use in fat splitting. In addition, we will use these three enzymes as well as lipases from *Candida antarctica* for our fat splitting experiments.

2.4.2.1 *Candida Rugosa* Lipase

Candida rugosa lipase has a long history of use in fat and oil hydrolysis reactions. In 1984 CRL was used to split different fats. Its rate of hydrolysis was compared on olive oil, coconut oil and tallow (beef fat). After 72 hours of reaction time, all three substrates were hydrolysed to 90% degree of split. Olive oil was more rapidly hydrolysed than other fatty acids. CRL was also compared to a lipase from *Rhizopus arrhizus* and a lipase form *Aspergillus niger*. *R. arrhizus* lipase showed a lower rate of reaction due to its sn-1,3 specificity and lack of ability to hydrolyse the long fatty acids concentrated in those positions. These enzymes were also evaluated for their ability to catalyse the reverse esterification reaction although no yields above 70% were observed (Linfield *et al.*, 1984).

In another series of experiments, both free and immobilised *Candida rugosa* lipase (CRL) was compared for its hydrolysing capabilities of several different oils. CRL was immobilised on membranes using several immobilisation techniques such as covalent and adsorption methods as described in more detail in the following section 2.5. For comparison, CRL's activity for hydrolysis of olive oil, palm oil, as well as castor oil was observed. The different TAG compositions of these oils lead to CRL displaying different rates of hydrolysis. Castor oil for example, was hydrolysed at a far slower rate than the other two oils it was compared to, by both immobilised as well as free lipase. Castor oil primarily consists of ricinoleic acid, which is suspected of not fitting the active site of CRL as well as other fatty acids (Gupta *et al.*, 2011).

Similarly in a packed bed reactor CRL was immobilised on a resin and used for continuous hydrolysis of olive oil in isooctane. (Han and Rhee, 1986) The olive oil dissolved in isooctane was then fed through the immobilised lipase at equal rates to the process water containing a 25 mM triethanolamine buffer at pH 7. The activity of the lipase was halved after 220 hours at 30°C, showing high stability. For the initial 80 hours activity remains mostly constant. Follow up experiments also used n-hexane as the organic solvent and used supplemented glycerol to stabilise the enzyme further (Yang and Rhee, 1992a). This use of glycerol, which is a product of the reaction may slow the overall rate, as it shifts the equilibrium of the reaction away from the products.

Between 2008 and 2013, a more traditional water based buffer-oil emulsion, and hydrolysis catalysed by *Candida rugosa* lipase was examined by an Indian team (Goswami, Basu and De, 2009),. They chose *Candida rugosa* lipase due to reports of its activity on castor oil, the team went through a number of process optimisation steps where in turn several conditions of the hydrolysis reaction such as stirring speed, temperature, pH and buffer to oil ratio were varied (Goswami, Basu and De,

2013). After several process iterations, a conversion rate of 60% degree of split after 6 hours was achieved. The percentage of split here is calculated by using the measured acid value and dividing it by the measured saponification value of the oil.

When enzyme from the initial hydrolysis reaction was reused, it was found that the activity of the reused enzyme had dropped to around 80% output of the initial hydrolysis reaction. Reused enzyme could only achieve a conversion rate of 48% degree of split after 6 hours. Throughout their experiments very high agitation (1100 rpm stirring) and high water to oil ratios are used. The traditional enzymatic fat splitting reaction is more like an oil in water emulsion rather than truly a 1:1 ratio. This guarantees that water is always in excess and available for hydrolysis as well as diluting the glycerol product. This allows for a longer reaction time without the reaction equilibrium shifting backwards (Goswami, Basu and De, 2009).

In the review paper in 2013, 4 years later, the researchers summarise a large number of lipases which had been used for the hydrolysis of castor oil previously. Several additional factors compared to the 2009 paper are also listed, such as the use of metal ions such as magnesium and calcium to enhance enzyme function. Additionally, the specific activity of some lipases towards different triglycerides, such as fatty acid chain length and positional specificity is also mentioned as a potential factor of impact on the rate of the hydrolysis reaction (Goswami, Basu and De, 2013).

For an example of lipase size or substrate specificity, a hyper-thermostable *Pseudomonas sp.* Lipase showed a large increase in activity towards castor oil compared to olive oil (268% compared to 100%). Throughout literature, there are many other enzymes, which have similar differences in activity towards different oils, depending on positional or chain length specificity (Rathi *et al.*, 2000).

The kinetics of the CRL catalysed hydrolysis of palm oil were also studied in 2003. A kinetic model was developed from experimental results using CRL to hydrolyse palm oil. The researchers determined the relationship between the rate of reaction and operational conditions such as stirring speed and ratios of oil to water. In addition, their experiments recorded the relative amounts of palmitic acid and oleic acid being produced over time and showed that these are mostly consistent with the ratio these fatty acids occur naturally in palm oil. (Al-Zuhair, Hasan and Ramachandran, 2003). This confirms the non-specific activity of CRL throughout the reaction, as all fatty acids from the triglyceride backbone are hydrolysed. In a sn1,3 specific lipase, certain fatty acids would accumulate initially.

2.4.2.2 Porcine Pancreatic Lipase

Porcine pancreatic lipase (PPL) is an animal derived lipase, which has been used for the hydrolysis of oils and fats for a long time. Its partial structure, the N-terminal half including the active site, was first described in 1979, and it was one of the first descriptions of lipase structure (Bianchetta *et al.*, 1979). It is one of the cheapest and most readily accessible commercially available lipases. Compared to commercially available microbial lipases 20 – 40 years ago, PPL had a broad selectivity, relatively high activity, and thermal stability as well as the ability to tolerate organic solvents as well as low water concentrations. With the availability of more types of fungal lipases in recent years this is somewhat lessened, however PPL still has many attractive qualities for industrial processes (Mendes, Oliveira and de Castro, 2012; Hertweck and Boland, 1997).

A number of metal ions such as Na^{+}, $Mg^{2+}$and Ca^{2+} have also reportedly led to an increase the rate of PPL catalysed oil hdyrolysis. Although the mechanisms aren't known exactly it is presumed that these ions increase the pH at the oil/water interface, where the enzyme is concentrated. As PPL works best between pH 7-9, this likely increases activity, especially as more fatty acids are produced and the overall pH of the reaction mixture is lowered. Ca^{2+} as well as Mg^{2+} also form insoluble fatty acid salts, which prevents enzyme inhibition from excessive products in the reaction mixture pushing the equilibrium of the reaction backwards (Goswami, Basu and De, 2011; Goswami, De and Basu, 2012; Mendes, Oliveira and de Castro, 2012).

PPL has often been used in organic solvent-based hydrolysis systems. In a 1991 paper, the activity of PPL on tallow, vegetable oil and fish oil was tested. Complete hydrolysis of the vegetable oil was achieved after 20 hrs at 45°C with the oil and process water dissolved in an organic solvent system (Bilyk *et al.*, 1991).

Porcine pancreatic lipase also has a history of use in green chemistry and has been used to break down waste cooking oil including used sunflower and palm oils (Arroyo *et al.*, 1996).

2.4.2.3 *Thermomyces Lanuginosus* Lipase

Other lipases such as *Thermomyces lanuginosus* lipase (TLL) have also been used for enzymatic fat and oil splitting. Relatively high rates of hydrolysis reaction have been observed for TLL based hydrolysis of palm oil, soybean oil and sunflower oil hydrolysis.

Specifically, in 2010 researchers observed the progress of a TLL based hydrolysis reaction of an oil. TLL, a sn-1, 3 specific enzyme, had strong non-specific activity on diacylglycerols in a second stage of an oil hydrolysis reaction. Especially when this was compared to the very strongly sn-1, 3 specific first step of the reaction on triacylglycerols (Fernandez-Lafuente, 2010).

TLL specificity was also able to be modulated using different immobilisation supports. In an ethanolysis of high oleic sunflower oil, TLL immobilised on divinylbenzene resin produced primarily a single diacylglyceride, indicating a high degree of sn-1,3 specificity of the biocatalyst. TLL immobilised on Purolite C18 resin showed almost perfect sn-1,3 specificity as well as significantly increased activity compared to commercially available immobilised TLL (IM TL). However, TLL immobilised on an octadecyl support produced two different diacylglycerides during the hydrolysis reaction, indicating non-specific biocatalysis (Abreu Silveira *et al.*, 2017).

Different immobilization techniques and supports can have wide ranging differences in specificity and activity of immobilized enzyme. In one example from this paper, temperature, and pH differences during immobilization on C18 purolite produce very different properties. 30°C and pH 8.5 during the immobilization process produces a very sn-1, 3 specific TLL with 10 times higher activity than commercial free TLL enzyme, whereas pH 7.0 and 25°C produce a positionally non-specific immobilized enzyme (Abreu Silveira *et al.*, 2019).

It is likely that these specificity modulating experiments worked due to the overall relatively low rate of reaction as well as the lower temperatures at which these experiments were carried out (25C – 30C). At higher temperatures acyl migration can lead to fatty acids initially in the 2-position on the glycerol backbone moving to the 1 position allowing the hydrolysis of these fatty acids by 1,3 specific lipases (Li *et al.*, 2010).

Free, rather than immobilised TLL was also used in the production of biodiesels. Biodiesels are esters of fatty acids with short chain alcohols such as methanol or ethanol. Using TLL at 2.3% v/v with a 1:1 soybean oil to water ratio at 60°C a degree of split of 89% was reached after 48 hours. Using free enzyme, a relatively small amount can be used due to the higher rate of reaction of free enzymes. No buffers were used in this reaction, and as a result a relatively long reaction time is required (Cavalcanti-Oliveira *et al.*, 2010).

2.4.2.4 *Candida Antartica* Lipases

One of the most common lipases used for general oleochemistry as well as specifically fat and oil splitting and esterification reactions is *Candida antartica* lipase B (CAL-B). (Gotor-Fernández, Busto and Gotor, 2006) CAL-B is a 1,3 specific lipase with a small lid which does not fully occlude the active centre. Despite this it remains an interfacial lipase, working at the oil-water interface. This may in fact make it's handling easier, as it has a lower tendency to form aggregates and clump like some other lipases (Martinelle, Holmquist and Hult, 1995).

Novozym 435 is the commercial name of the immobilised version of CALB, created by Novozymes. It was first created in 1992 and has been used in a large number of industrial applications due to its temperature stability as well as ease of use (Ortiz *et al.*, 2019).

In a 2013 study, the lipase based ethanolysis of crambe oil using a recirculated packed bed reactor was tested. Novozyme 435 was used to concentrate erucic acid from crambe oil. An excess of ethanol instead of water was used, making this an ethanolysis reaction. Optimal residence time, temperature and oil to ethanol ratios were determined. The recirculating packed bed reactor allowed for close approximation of continuous systems in a more controllable batch environment. The progress of the reaction was measured using gas chromatography of the fatty acid ethyl esters produced by the reaction (No *et al.*, 2013).

Despite its many unique properties, *Candida antarctica* lipase A (CAL-A) has received significantly less interest and attention than CAL-B. It possesses a remarkable heat resistance (up to 90°C) and tolerance of low pH. There is no homology between the N-terminal sequences of CAL-A and CAL-B, in the case of CAL-A there is no similarity in sequence with any other lipase. CAL-A is also the only reported lipase with a sn-2 preference, rather than the 1,3 specific or nonspecific properties of other lipases. (Anderson, Larsson and Kirk, 1998) This selectivity however is not prominent enough to allow splitting of only sn-2 fatty acids, and CAL-A is therefore usually classified as nonspecific for fat splitting and esterification purposes (Domínguez de María *et al.*, 2005; Rogalska *et al.*, 1993).

2.4.3 Operating Conditions of lipase-based hydrolysis

In 2003, three inventors from Germany filed a patent (Brunner, Frische and Kilian, 2002) for an enzymatic fat splitting process, that could counteract many of the limitations of lipase based fat splitting such as long reaction times and low yields. This process was developed based on a previous process, where an initial fat splitting reaction is allowed to progress until it the rate of the hydrolysis reaction slows down. When this reduction in the rate of fat splitting is detected, the partially split reaction mixture undergoes a phase separation process, where an organic phase containing unreacted oil and free fatty acids, and an aqueous phase containing water, glycerol, and enzyme are produced. Subsequently, vacuum distillation or other separation methods are used to concentrate free fatty acids out of the partially split reaction mass. The rest of the unreacted mass, which consists of mostly triglycerides with a few di- and monoglycerides, is fed back into a second oil hydrolysis reaction. No single reaction mass is allowed to reach completion. The reaction is always stopped once the rate of reaction slows down, which means that on average a maximum percentage of split of 80 – 90% degree of split is reached, compared to the 99% degree of split which current chemically based industrial processes can reach.

This method allows for relatively short reaction times, especially when compared to many other enzymatic processes, where these relatively short reaction times of a few hours would be considered impossible.

As an approach to enzymatic fat splitting this is possibly the most industrially viable way.

Certain aspects of this process can potentially be used not just for fat splitting but also for production of fine chemicals through fat hydrolysis. Using sn-1, 3 specific splitting and separating out a monoglycerol or other partial glyceride side product may become an economically viable process.

In a similar invention from 2013, a two-step enzymatic fat splitting process was created. In an initial step an emulsion of the fat, a polar organic solvent (t-butanol) as well as water was created, which was then subjected to lipase-based hydrolysis in a stirred tank. After an initial hydrolysis step, the reaction mixture now contained partially split oil containing diacylglycerols (DAG), monoacylglycerols (MAG), fatty acids and glycerol and was processed using an ion exchange resin. The processed reaction mixture was then hydrolysed using a lipase again. The result was a 99% triglyceride conversion, where up to 88% of the produced product was free fatty acid. However relatively high amounts of lipase were used for this result. 1 gram of immobilised enzyme per 10 grams of oil is a very high catalyst to reaction mass ratio, and reusability of lipases is required for this process to be economical (Lali, 2013).

In an example of a single step process in the enzymatic fat splitting in industry, lipases were used to replace previous industrial processes to produce polyunsaturated fatty acids as well as soaps. Miyoshi Oil & Fat Co., Japan, used a lipase from *Candida cylindracae* to split oils and fats in a reaction with glycerol to produce monoacylglycerols (MAG), diacylglycerols (DAG) as well as new triacylglycerols (TAG). Rather than fat splitting, this reaction could be more accurately described as fat modification. In particular in this process, the reaction was optimised to maximise production of MAG through adjusting both the reaction temperature and fat type (McNeill, Shimizu and Yamane, 1991) .

This process, however, does not produce free fatty acids, as the TAGs are reacted with glycerol rather than water. As such, production of free fatty acids is not the goal, instead direct maximisation of the production of MAGs as well as other partial glycerides and full TAGs, in a single-step reaction is achieved.

Another approach to fat splitting is not for production of fatty acids but to produce partial glycerides such as diacylglycerides (DAGs) and monoacylglycerides (MAGs). In 1997 a team from Hyderabad, used *Candida cylindracea* lipase for the hydrolysis of rapeseed and mustard oil, both oils rich in erucic acid and other long chain fatty acids (C20 and C24). By limiting the amount of water available for the hydrolysis reaction, very little of the erucic acid, which is concentrated in the sn-1 and 3 positions of the oil's triglyceride was split. The shorter chain fatty acids concentrated in the sn-2 position of the triglyceride were hydrolysed first, as they are more susceptible to the lipase used. The resulting product is dierucin, which can be esterified to produce trierucin, which cannot be produced naturally by rapeseed or mustard seed plants (Kaimal, Prasad and Rao, 1993).

Another process developed for the complete hydrolysis of castor oil used 1,3 specific lipases for complete splitting. Here the oil is dissolved in tert-butanol solvent with 1g of oil per 5ml of solvent. 0.05 ml of water was added along with 0.2 g of immobilised lipase. The mutual solubility allowed for a single-phase reaction mixture, allowing for the low amount of process water and enzyme. In a regular two-phase immiscible system, lipase needs to interact at the oil-water interphase, whereas here the interphase area is not limited by mixing, as all reagents are within a single mixed phase. Acyl migration, the movement of fatty acids on the 2-position of the triglyceride to a 1 position on the glycerol backbone is a necessary part of this reaction as purely 1,3 specific lipase is used. Non-polar solvents mediate acyl migration, allowing for up to 90 % yield of free fatty acids (Odaneth *et al.*, 2016).

The effects of varying operating conditions on hydrolysis rate were tested by Noor et all in 2003. Lipase-SP398 was supplied by Novozyme™ and used to split palm oil in a stirred batch tank reactor.

Four different factors were varied and their effect on the rate of hydrolysis was observed and compared to a set of standard conditions. Agitation, surfactant concentration, substrate concentration and enzyme concentration were varied and their effect on the initial hydrolysis rate was measured using acid-base titration. Surfactant concentrations of up to 35 g/litre were found to be beneficial. Faster agitation speed, as well as dispersion stirring was found to improve the initial hydrolysis rate compared to slower agitation speed and marine propellor based stirring. An increase in both substrate and enzyme concentration was also found to increase the initial speed of hydrolysis (Noor, Hasan and Ramachandran, 2003).

Eventually enzyme concentration as well as substrate concentration will only increase the initial rate up to a point. Raising the enzyme concentration too far will lead to solubility problems and precipitation of enzymes from the solution, although crowding and interference between enzymes would reduce the rate of reaction even before this.

Lipase catalysed hydrolysis of soy oil was also carried out using an ultrasonic bath to mix the organic and aqueous phases of the reaction. This was compared to a shaking flask type reactor. Ultrasound mixing was reported to increase the thermal stability of the lipase used (*Candida lipolytica* lipase), as well as allow for higher enzyme loading and less necessary process water. Although the pH stability of the enzyme was unchanged in both processes, overall, the rate of reaction in an ultrasound mixed system was doubled compared to a shaking reactor (Liu *et al.*, 2008).

Other reactor and process innovations were also tested by a team from Slovenia in 2000 (Primožič, Habulin and Knez, 2003). In this paper, the effects of high-pressure on a Lipolase 100T (*Aspergillus niger* lipase) catalysed fat splitting reaction was tested. The innovative part of this process was the use of supercritical CO_2 as a solvent, allowing for very easy extraction of free fatty acid as well as unreacted triacylglycerides, and mono- or diacylglycerides. While this process simplifies downstream processing, some of the features which make enzyme-based methods so attractive are discarded. Up to 200 bar pressure is used, much more than even in a chemical splitting process. This makes the process more dangerous and requires more expensive reactor types, compared to classical enzymatic splitting processes, which happen at atmospheric pressure in cheap reactors. In addition, this process does not solve any of the traditional problems of enzymatic processes such as very long reaction times and relatively low yields; after 48 hours only 92% of fatty acids were split from the glycerol backbone.

2.5 Enzyme Immobilisation

An often-described problem with the application of enzymes in industrial processes is the relatively high price of purified enzymes. Amongst the many ways of addressing this problem, enzyme immobilisation, allowing for reusing and regenerating of enzymes, is a common one.

In the examples of lipase use for fat splitting described above both free and immobilised enzymes are described.

There are of course, many potential benefits to enzyme immobilisation. Using an ideal immobilisation material and technique, can allow for enhanced enzyme specificity, reduced product inhibition, and increases to the overall stability of the enzyme in extreme temperatures, pH as well as mechanical stress (Datta, Christena and Rajaram, 2013).

Immobilisation of enzymes has a long history in both academia as well as industry. Enzymes were first immobilised in 1953, with the first industrial application of an immobilised enzyme in 1969. (Bornscheuer and Buchholz, 2005)

Generally, enzymes are immobilised for a few main reasons. Immobilised enzymes can be recovered after a reaction and reused, which is significantly more cost effective than free enzymes which cannot be reused. Usually, immobilised enzymes also display a higher thermal stability, chemical stability and mechanical force resistance than free enzymes, which allows for more extreme reaction conditions, such as lower or higher pH, higher temperature and better mixing, than using free enzymes. Additionally immobilised enzymes have a longer operational stability, meaning they can be used for a longer time or more repeated processes before denaturing or otherwise losing their catalytic efficiency. Therefore, processes involving immobilised lipases could allow for the development of an efficient, continuous large scale industrial process, due to higher returns, and less ongoing operating costs than smaller scale batch processes using free lipases (Cynthia Spahn and Shelley, 2008).

Immobilised lipases also have the advantage of potential decreased contamination of the end product through residual lipases, which simplifies or removes the need for further downstream treatment to remove free soluble lipases.

Immobilisation can not only enhance enzyme specificity but can even change it to suit the needs of a process better. This is true of other enzyme properties, for example in a strongly mixed system a immobilisation resin with smaller pores may be able to protect the enzyme better than a resin with larger pores which are more exposed to outside stress (Rodrigues *et al.*, 2013).

Similarly smaller pores exclude larger substrate molecules, and certain support materials are extremely hydrophobic, such as divinylbenzene, or extremely hydrophilic such as polyethyleneimine, which creates micro-environments around the enzyme, where only the intended substrate will reach the enzyme (Rodrigues *et al.*, 2013).

Generally, these advantages of immobilising enzymes, come with the disadvantage of a reduction in biocatalyst activity, sometimes unintentional changes in specificity and selectivity, and possible leakage from the immobilisation support. These changes are due to distortions of the enzyme due to interactions with the solid support. The enzyme has less space and options for movement. This can also be used to improve enzyme properties, for example by immobilising a hyperactive form of the enzyme (Garcia-Galan *et al.*, 2011).

When choosing to immobilise enzymes there are many factors to consider. In 2017 a project immobilising TLL used different concentrations of enzyme solutions during immobilisation. Higher concentrations leading to more crowded immobilised lipase, which led to much lower stability of the enzyme. Immobilised TLL prepared using lower lipase concentrations was significantly more stable at lower pH levels. This crowding effect is a result of enzymes ending up very close together on the support material, which causes interference. The resulting reduction in stability is shown in this project through measuring the residual activity during thermal inactivation. This crowding effect seems to be varying in different conditions. For example, when the stability is tested at pH 5.0, the effect appears to be reversed, and immobilized enzymes prepared using a higher enzyme concentration solution are more stable. This is likely due to different states of folding and unfolding at different pH levels (Zaak *et al.*, 2017).

2.5.1 Matrices/Supports for Immobilisation

Immobilisation support materials must ideally be inert, possess mechanical durability and even the ability to regenerate. In many cases these materials can even increase specificity and activity as well as reduce product inhibition of the immobilised enzyme.

There are many different natural polymers that can be used as supports or matrices for enzyme immobilisation. Examples of these are cellulose, collagen, pectin, sepharose, alginate, chitin or Starches. There are many ways these natural polymers are used, however the most common use is for increased stability and longevity of the immobilised enzyme. Stability is especially important for processes which take place under extreme pH, higher temperatures, high salt concentrations, or

other enzyme denaturing conditions. In some cases, even the activity of the immobilised enzymes can be enhanced.

There are also other inorganic materials which can be used for enzyme immobilisation. Various ceramics, silica, glass, or more specialised materials such as zeolites and celite can be used for immobilisation. Zeolite for example is a microporous crystalline solid, also known as a “molecular sieve”; due to its heterogeneous surface with multiple possible adsorption sites, it is well suited for modulating enzyme properties such as specificity and other interactions.

Of course, these inorganic support materials are also used to increase enzyme stability under extreme pH and temperature (Garcia-Galan *et al.*, 2011; Katchalski-Katzir, 1993).

Also commonly used are synthetic polymers, such as polyethylene glycol, styrenic polymers, polyaniline and methacrylamide, or methacrylated epoxy. In recent times epoxy-based support materials have proven to be especially useful in industrial applications. All synthetic polymer-based support materials are highly modifiable, allowing for different binding methods to the same support material through modification of side groups. This flexibility allows for much easier modulation of enzyme specificity, and activity. In addition, enzymes immobilised on synthetic polymers are often more heat stable and can withstand more extreme pH values (Murty, Bhat and Muniswaran, 2002).

2.5.2 Immobilisation techniques and methods

Enzymes can be immobilised in many different ways. For industrial considerations and the purpose of this work, there are three industrially relevant methods for immobilising enzymes, adsorption, covalent immobilisation, and entrapment.

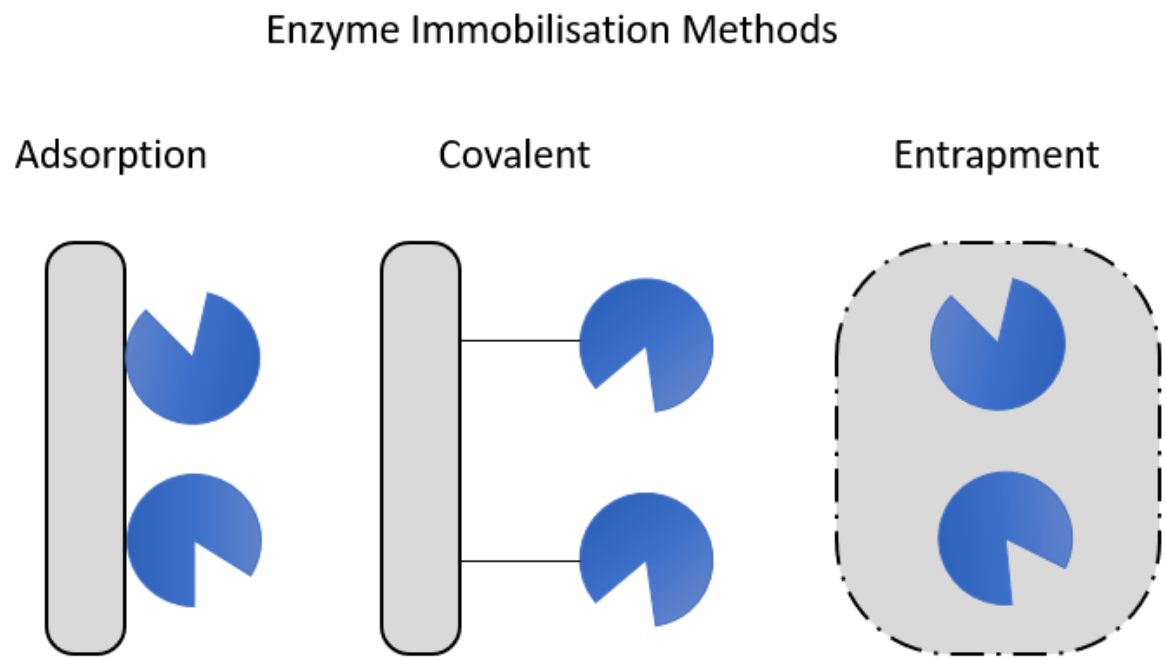


Figure 5: Enzyme immobilisation methods described in this chapter. The enzyme is shown in blue, the support material in grey.

2.5.2.1 Adsorption Immobilisation

Adsorption refers to the binding of enzymes to different types of supports generally through hydrophobic, non-covalent interactions as well as ionic, salt linkages. Enzymes which are adsorbed onto supports are protected from aggregation and proteolysis, which especially in living systems is a concern (Cynthia Spahn and Shelley, 2008). Although individual bonds between the enzyme and the support structure are often weaker, adsorption typically works through a large number of weaker hydrogen bonds and van der Waals interactions (Díaz and Balkus, 1996). This multipoint attachment generally increases the stability of the immobilised enzyme.

Adsorption immobilised enzymes are however more susceptible to leaking from the support and often have reduced activity. This is due to the inability of this method to guarantee enzyme orientation on the support material as shown in figure 5 (Nguyen and Kim, 2017)

2.5.2.2 Covalent Immobilisation

Covalent immobilisation uses covalent bonds between the lipase and a solid support structure. The most common form of covalent bonding involves amine sidechains on the enzyme forming amides with an appropriate solid support resin. Newer methods use epoxy resins which more readily form strong covalent bonds with different side chains on the enzyme (Cynthia Spahn and Shelley, 2008).

Often spacers such as glutaraldehyde are used to allow for additional thermal stability. This also allows the enzyme to always be the same distance from and orientation towards the support material to reduce activity reduction as shown in figure 5 (Díaz and Balkus, 1996).

Amino acids like arginine, aspartic acid or histidine are typically used to attach the enzyme. (Datta, Christena and Rajaram, 2013).

Covalently immobilised enzymes are generally highly reusable, with long-term operational stability and longevity. In addition, there is often a lower amount of enzyme leaking than with other immobilisation methods. This makes them ideal for industrial applications. (Nguyen and Kim, 2017)

2.5.2.3 Entrapment Immobilisation

Entrapment refers to encasing enzymes within gels or fibrous supports by encapsulating them. This can also include covalent or non-covalent bonds to hold enzymes in place, although on the inside instead of the outside of a support structure. Usually however the enzyme is not bound to the support material. (Kumar, Singh and Singh, 2008).

Generally, this method uses porous materials, which allow for substrates and products to access the enzyme within but does not allow for the enzyme to move to the substrate. This protects the enzyme from environmental conditions and improves mechanical stability. In some cases, this can even increase enzyme activity and efficiency (Shen *et al.*, 2011).

Entrapment immobilisation is usually conducted through the polymerisation of the support material in a mixed solution with the enzyme.

This method is particularly appropriate for enzymes with narrow optimal conditions, as the microenvironment of the enzyme can be directly controlled through the pH and polarity of the

encapsulating material. However, there is again a risk of leakage with larger pore sizes, making this technique more suited to smaller enzyme substrates. (Nguyen and Kim, 2017)

2.6 Literature Review Conclusions

Most prior studies concerning enzymatic fat and oil splitting focus on two different goals. The splitting process is either carried out to completely split all fatty acids from the triglyceride, producing only fatty acids and glycerol in the reaction. The other option is to only partially split the triglyceride to produce other valuable products such as monoacylglycerides or diacylglycerides, as well as ethanolysis or methanolysis for the production of biofuels.

When the goal is to produce partially split oil, detection of neutral products such as diacylglycerides and monoacylglycerides or fatty acid methyl/ethyl esters is necessary. As a result, studies with this goal usually use TLC, HPLC or GC to detect the product of the reaction (Farias, Torres and Canela, 1997).

Most previous projects use HPLC for detection, TLC however has a lot of advantages over these more expensive methods. Commercially available pre-coated plates, a low analysis time, inexpensive equipment, and a lower solvent use than HPLC make TLC a convenient process for secondary analysis of our reaction. TLC detection limits are as low as 50 ng for neutral lipids and fatty acids (Fuchs *et al.*, 2011).

Many different reactor types were used. For most applications of lipase-based fat and oil splitting, a variation of a batch stirred reactor was used. When immobilised lipases were used, often a fixed bed reactor or variation of a fixed bed reactor such as a rotary bed reactor was used. In a few cases, other types of reactors such as OBR, shaken and ultrasound mixed reactors were used.

2.7 Aims and Objectives

The main objective of this thesis was an exploration of enzymatic methods for HEAR oil hydrolysis as well as finding potential industrial applications for an enzymatic fat splitting process.

The initial objective of this work was to evaluate the use of buffers for the enzymatic hydrolysis of HEAR oil. Buffers are commonly used, however rarely in industry. Buffers usually add substances that would in current industrial processes be filtered out of the initial reagents of oil and water, such as sodium, phosphate, or chloride ions – see section 2.1.1 on oil degumming. In addition, the presence of charged ions in the products might complicate downstream processing such as vacuum distillation or other separation and clean-up processes in an industrial setting.

Another major objective was comparing currently commercially available lipases for HEAR oil splitting and selecting a subset of these for further optimisation. Different lipases have different activities on HEAR oil, and different optimal conditions which may be more or less suited to an industrial process. The main criteria for this enzyme evaluation was activity as measured by acid value.

Different types of HEAR oil were also used for enzymatic hydrolysis, specifically degummed HEAR oil was compared to non-degummed HEAR oil as there are some reports of lipases benefiting from mineral ions and other charged particles in the oil (see chapter 2.1.1).

Enzyme immobilisation was also explored as a way of increasing the stability and allowing for the potential reusability of enzymes. Immobilised enzymes were compared to non-immobilised enzymes for their ability to split HEAR oil.

Partial enzymatic HEAR oil hydrolysis reactions were also evaluated for industrial use. Partial hydrolysis reactions can produce more useful compounds and potentially more optimal starting materials for further use in current industrial HEAR oil processes. Industrial production of erucic acid at CRODA uses a continuous process as larger scale industrial processes tend towards. Continuous processes are often times more suitable for larger scale processes, as they reduce waste, improve quality and reduce running energy consumption, although initial energy consumption when starting the reaction may be higher. Continuous processes are continuously fed reagents and raw materials into a reaction chamber and products are continuously removed. In batch processes on the other hand a total amount of reagents is introduced into a closed reaction chamber and after a reaction time a total amount of product is removed. In a lab context however, using batch processes to test and compare enzymatic oil hydrolysis reactions with varying conditions is more appropriate, as they

are more suitable for smaller scale processes, and adjusting conditions during a continuous process is more difficult. For HEAR oil, a 1,3 specific splitting reaction would lead to the liberation of erucic acid initially and stopping the hydrolysis reaction here could lead to an accumulation of desirable partial glyceride or confer other desirable properties onto the material.

Different types of batch style small scale processes were used to give indications for future, larger scale industrial trials. These larger continuous industrial processes will be out of the scope of this thesis. Results from batch reactors will still give indications of what kind of conditions should be used for continuous processes within the limitations of our experimental batch processes.

3 Materials and Methods

3.1 Materials

Lipases: Porcine pancreatic lipase (powder, 125 U/mg), *Thermomyces lanuginosus* lipase (solution 100,000 U/g), as well *Candida rugose* lipase (powder 998 U/mg) were obtained from Sigma-Aldrich (UK). Another version of *Thermomyces lanuginosus* lipase (solution, no further information) Lipozyme CalB (solution, no further information), Novozyme 435 (immobilised, 5,000 U/g), Lipozyme TL IM (immobilised, no further information), as well as experimental lipases NS 40020 and NS 40119 (solution, no further information) were obtained from Novozymes A/S (Denmark). High erucic acid rapeseed (HEAR) oil, as well as non-degummed HEAR oil and a sample of fully split HEAR oil from the Hull fat splitting plant were generously provided by Croda International Plc. (UK).

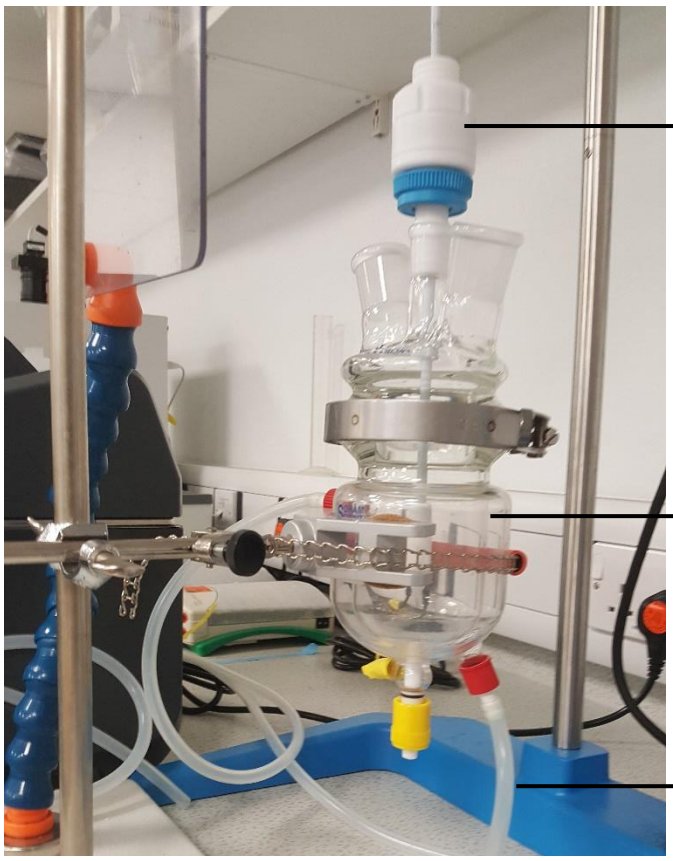
The auto titrator used was a TitroLine 7000 made by Xylem Analytics (UK). Manual and auto titrators used in CRODA laboratories were of Metrohm Titrando make.

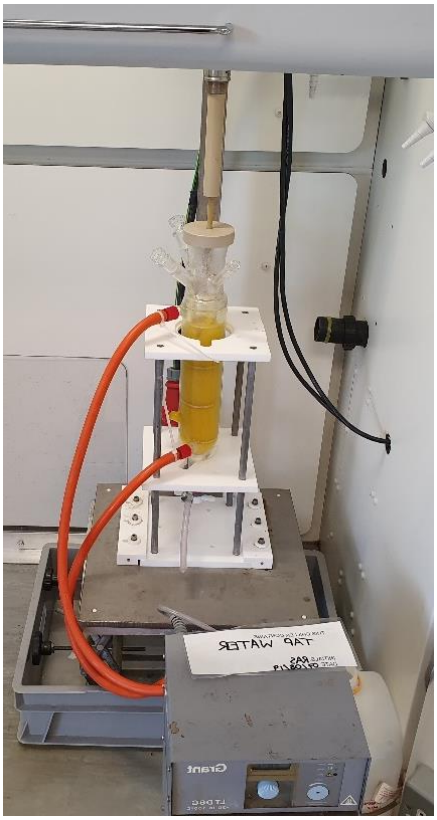
The hydrolysis reactors used consisted of a jacketed glass flask of 250 ml and 500 ml capacity with a lid and a rubber ring fitted tightly to the flask. A two-bladed paddle impeller (4 cm in diameter) was submerged in the reaction mixture through a stirrer guide in a hole in the lid. The jacket of the flask was then connected to a water cooler/heater set to 0.5 – 1°C above the reaction temperature.

The oscillatory baffled reactor (OBR) used was a 500 ml capacity jacketed glass reactor with plastic baffles being raised and lowered using an external motor. This reactor was heated to target temperature using a silicon oil heater connected to the reactor jacket.

The fixed bed reactor was operated using a 250 ml beaker connected to the jacketed glass fixed bed reactor with rubber tubes. The reaction mass was circulated through the system using a peristaltic pump. Enzyme was loaded into the 20 ml capacity bed of the reactor. The flask was heated using a bench-top hot plate, while the reactor was kept at temperature using a silicon oil heater connected to the reactor jacket.

All three reactor setups are shown in figure 6 below.





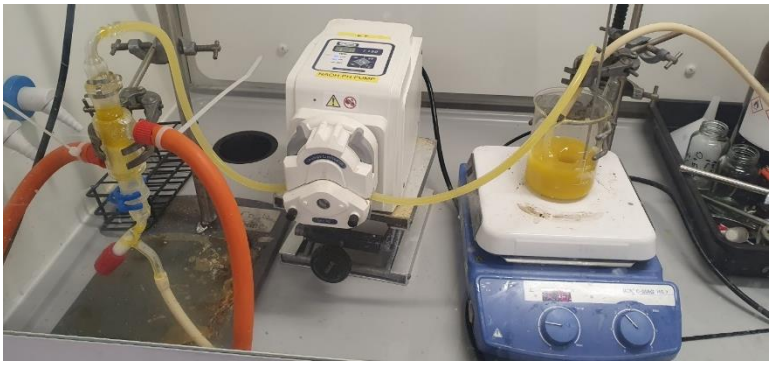


Figure 6: Reactor setups for enzymatic fat splitting. Top left shows the jacketed batch reactor. Bottom left shows the oscillatory baffled reactor, bottom right shows the fixed bed reactor setup.

3.2 Methods

3.2.1 Fat Splitting Reactions

Around 70-100 ml of HEAR oil was weighed and added to a 250 ml capacity jacketed reactor. This oil was heated up to target temperature in the reactor for 20 minutes. The buffer/water was weighed out in a specified ratio (from 1:1 – 0.5:1) and heated up to target temperature. The buffer/water as well as enzyme (either dry or from stock solutions) were added to the reactor. Enzyme amounts are given in ratio to the amount of oil used (1% w/w for dry enzymes, and 0.01% w/w for enzymes in solution). The reaction mixture was mechanically stirred using an overhead stirrer through a stirrer guide. A stirrer speed of 400 rpm was used.

Samples of around one to three grams, determined by the expected acid value, were removed regularly (~1 sample per hour) for the first two - six hours of the experiment and again after 24 hours. Samples were taken by opening the reactor and extracting a mixed sample using a serological pipette while the stirrer was still running.

All values were scaled up for bigger reactors used (300-400 ml of HEAR oil for a 1L capacity jacketed reactor). Ratios for other reactors are specified in the results section, setup for most reactors is otherwise identical.

3.2.2 Phase separation

The reaction mixture was transferred from the reaction vessel to a 500 ml separating funnel. The aqueous phase was removed initially and stored for any further experiments. To remove the remaining water from the organic phase a concentrated brine solution (>5 M NaCl) as well as heptane were added, and the separating process repeated. The remaining organic phase was then dried using sodium anhydride overnight. The remaining heptane was removed from the organic phase using a rotary evaporator. The remaining organic phase was then dry enough for GPC and GC analysis.

3.2.3 Acid value

Acid value was measured through the titration of an oil solution in an organic solvent (diethyl ether, propanol, ethanol, acetone, or mixtures of these) with a solution in alcohol of sodium or potassium hydroxide. The acid value was expressed as the amount in milligrams of potassium hydroxide required to neutralise the free fatty acids in 1 g of oil. (*Japanese Pharmacopoeia XIV, Chapter 17: Fats and Fatty Oils Tests*, 2001)

The acid value can then be used to calculate the percentage of split or the percentage of free fatty acid (FFA). The acid value can be multiplied with the molecular weight of the fatty acid, in our case a mixture of erucic and oleic acid and divided by the molecular weight of the titrant multiplied by 10, 56.1*10 for KOH. The acid value is expressed in mg/g, which is why the multiplication by 10 is necessary. For FFA% expressed as erucic acid, mol wt = 338 g/mol. For FFA% expressed as oleic acid, mol wt = 282.4 g/mol. The factor for erucic acid is 0.6025, the factor for oleic acid is 0.5034.

Table 1: Expected acid value and sample intake

Expected AV (mgKOH/g)	***Sample intake (g) of organic matter***
1.5-10	10
10-25	5
25-75	3
75-125	2
125-175	1
175-275	0.5

$$AV = \frac{M_{KOH} * V * c}{m}$$

Where:

AV = acid value in mg KOH/g
M_{KOH} = molecular mass of potassium hydroxide, 56.1 g/mol
V = the volume of 0.1M KOH titrant used in ml
c = the concentration of the KOH solution, 0.1 mol/L
m = the mass of sample used in grams

For the acid value, a sample (amount as in table 1) of the well mixed oil/water emulsion (reaction mass) was taken and dissolved in 50 ml of ethanol/acetone. The ratio of aqueous phase to organic phase was assumed to remain constant. For a 1:1 ratio of initial water to oil, half of the sample was assumed to be organic matter. The acid value of the sample was determined using the auto titrator

(TitroLine 7000) filled with the 0.1 M KOH solution in ethanol to titrate. The volume of the KOH (V) solution used was then used to determine the acid value of the sample using the formula above.

3.2.4 Saponification value

The saponification value represents the number of milligrams of KOH required to neutralise the free fatty acids as well as saponify the esters produced or remaining in 1 g of oil phase.

For the saponification value, a 1-2 g sample of the well mixed oil/water emulsion is taken and dissolved in 50 ml of ethanol/acetone. 25 ml of the 0.1 M KOH solution in ethanol is added to the sample and heated under reflux for 40 mins to 1 hour. The sample is then titrated while the solution is still warm using the auto titrator filled with the 0. 5 M HCl in ethanol to produce a result (a) – the amount of HCl solution in ml used. This process is repeated without the oil/water emulsion to determine a blank result (b) – the amount of HCl solution used. The saponification value is then calculated from these two values by the following formula.

$$SV = \frac{(a-b) * M * M_{KOH}}{m}$$

Where:

SV = acid value in mg KOH/g
(a-b) = the difference in volume used for the blank test run and the tested sample in mL
M = the Molarity of the HCl solution in mol/L
M_{KOH} = molecular mass of potassium hydroxide, 56.1 g/mol
m = the mass of sample used in grams

The saponification value represents the maximum amount of fatty acids bound which can be liberated. Therefore, the percentage of split can then be calculated by dividing the measured acid value of a sample with the saponification value measured for that batch of HEAR oil.

The saponification value for each batch of HEAR oil is shown in acid value graphs with the dotted line annotated with a number and “SV”. The saponification value varies slightly for each batch of HEAR oil used.

3.2.5 Thin Layer Chromatography

Thin layer chromatography (TLC) is used to separate and analyse different products of the HEAR oil hydrolysis reaction. TLC silica plates, the solid phase of the TLC experiment, were obtained from "CAMAG", Switzerland.

Samples of 20 mg and 50 mg are taken from a HEAR oil hydrolysis reaction and dissolved in 1 ml of ethanol. These samples are stored at -20°C to inactivate the enzyme (Kanwar *et al.*, 2005; Goswami, Basu and De, 2009) and stop any remaining hydrolysis or other enzyme activity. Storing the sample in ethanol allows us to use it immediately from storage without any preparation steps.

Several different solvent systems (table 2), the mobile phase of the TLC experiment, were used to separate free fatty acids (FFA), from monoacylglycerides (MAG), diaclyglycerides (DAG), and triacylgylcerides (TAG). Different solvent systems produce different separation between the various products of the HEAR oil splitting reaction. Using solvent system 3 allowed us to distinguish between different types of diacylglycerols (DAGs), which are produced by lipase based HEAR oil splitting.

Table 2: Solvent systems for separating MAGs, DAGs, TAGs and FFAs using TLC.

	Solvent Systems	**Ratio (v:v)**
1	Toluene : Chloroform : Methanol	85 : 15 : 5
2	Toluene : Chloroform : Acetone	7 : 2 : 1
3	Hexane : Diethyl ether : Acetic acid	70 : 30 : 1
4	Diethyl ether : Hexane : Methanol	65 : 35 : 3
5	Hexane : Ethyl ether : Ethyl acetate : Acetic acid	80 : 10 : 10 : 1

Solvent system 3 is used throughout the rest of this thesis, as it allows us to see different types of DAGs, specifically, it allows for differentiation of sn-1,3 DAG and sn-1,2 DAG. The presence of these different DAGs has important implications for the sn-1,3 specificity of the lipases used for hydrolysis.

2 µl of the dissolved sample in ethanol are spotted onto a 10 cm long silica coated TLC plate. This plate is placed in a development chamber where the solvent mixture reaches up to 1 cm from the bottom of the plate. The solvent is allowed to rise until it reaches around 1 cm from the top of the plate. The plate is then removed, dried, and the solvent front is marked.

Initially three different methods were used to develop and detect spots on the TLC plates.

1. Placing the plate into a container filled with iodine vapour followed by dipping the plate in 2,7-dichlorofluorescein and immediately drying the plate with a hot air gun for 3 – 5 minutes. Spots can be detected under UV light.
2. Dipping the plate into 1% p-anisaldehyde in methanol-sulphuric acid (9:1, v/v), and immediately drying the plate with a hot air gun for 3-5 minutes or until spots are clearly visible.
3. Dipping the plate into potassium permanganate solution (0.5% w/v $KMnO_4$ in 1N NaOH) and immediately drying briefly with a hot air gun.

Eventually a modified method was developed; the plate is placed into a container filled with iodine vapour and placed into a 50°C oven for 15-20 minutes. The plate is then retrieved and scanned ~5 minutes later. Iodine-stained spots start to fade after around 10 - 30 minutes; however, this adapted method is significantly faster and does not require use of a toxic counterstaining solution. Since a digital picture of the stained TLC plate is taken, no toxic chemicals or staining or de-staining solution needs to be used.

3.2.6 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) specifications: 250 mm x 4.6 mm, 100 Å pore size C18 column, Acetonitrile, THF, Methanol, prepared solvents (filtered), Column heater set to 40°C

A 20 µl sample of the pure organic phase taken from a splitting reaction after separation is dissolved in 10 ml of methanol, then filtered through a 0.2 µm filter into a HPLC vial. The HPLC column was pre-equilibrated with a solvent mixture of Acetonitrile:Methanol:THF (40:40:20) for 30 mins to an hour at a flowrate of 1 ml/min.

The initial solvent mixture for 10 minutes was a 90:10 ratio of Acetonitrile:0.1% w/w Acetic acid in water. The sample was eluted through a 10 minute gradient elution back to a 40:40:20 Acetonitrile:Methanol:THF solvent mixture. This solvent mixture was used for 20 minutes.

Samples were detected using UV at both wavelengths of 204 nm and 238 nm.

Samples were compared to standards (oleic acid, erucic acid, glycerol monooleate, hear oil, and dioleoyl glycerol) ran under the same conditions. Unfortunately, no pure di-erucyl glycerol or mixed erucic acid and oleic acid diacylglycerides could be sourced; di-oleyl glycerol is used as a stand in for other DAGs and should allow us to determine peaks and positions of DAGs produced by HEAR oil.

3.2.7 Gel Permeation Chromatography

The gel permeation chromatography (GPC) machine used for these experiments was the Agilent 1260 instrument. A PLgel guard and 2 x PLgel 3µm 100Å columns 300 x 7.5 mm were used. Tetrahydrofuran was used as the solvent (mobile phase). 0.1 g of the dried (water removed) organic phase is dissolved in 10 ml of tetrahydrofuran (THF) and well mixed. 2 ml of this sample/THF mixture are filtered through a 0.4 um filter and placed into the autosample taker of the GPC machine. This takes a 50 µl injection volume at 40°C. The flow rate of the GPC machine is set at 0.1 ml/s for 30 minutes. A refractive index detection method is used to determine when and at what intensity the sample components exit the column.

3.2.8 Gas Chromatography

The column specifically for triglyceride analysis is the CP TAP Triglyceride column – 25 m x 0.25mm x 0.1µm. The GC that was used was a Thermo Trace 1300 with split/splitless injector and a flame ionisation detector (FID) for detection.

We also used a ZB-1HT inferno 30m x 0.25mm x 0.25µm for high temperature work and for the analysis of FFA, MAG, DAG, and TAG. CRODA standard analytical protocol was used for GC sample preparation.

3.2.9 Enzyme immobilisation

Six different resins for enzyme immobilisation were provided by Purolite. These were from the Lifetech™ ECR Enzyme Immobilization Resin range. Three resins were used for covalent binding, the remaining three were used for adsorption binding (see table 3)

Table 3: Purolite Immobilisation Resins, the code used to refer to them and their chemical properties.

Code	Chemical Name	Active group	Pore Size (Å)	Binding Type
ECR8215	Epoxy methacrylate	Epoxy	1200-1800	Covalent
ECR8285	Epoxy/butyl methacrylate	Epoxy	400-600	Covalent
ECR8315	Amino C2 methacrylate	NH_2 (short spacer)	1200-1800	Covalent
ECR1090	Macroporous styrene	None	900-1100	Adsorption
ECR1030	DVB/methacrylate	None	200-300	Adsorption
ECR8806	Octadecyl methacrylate	None	500-700	Adsorption

Immobilization was done over three hours instead of the 40 hours described in the manual. Protein concentration in the supernatant was measured every half hour initially, then every hour, however it was found to be constant after three hours. To determine the protein concentration a Bradford assay kit was used, and samples measured in triplicate.

Around 1.5 g of resin were weighed out and washed using 1 M glycine/NaOH buffer (pH 9) for epoxy binding resins and 0.05 M glycine/NaOH (pH 9) buffer for adsorption and amino binding resins.

100 mg of enzyme were used per gram of resin and dissolved in a buffer to resin ratio of 4:1 (v/w). The enzyme solution was then added to the resin, and the resulting slurry gently mixed in room temperature a shaker for 3 hours. Samples for protein concentration were taken every hour.

At 3 hours the resin was filtered, and the remaining protein concentration of the liquid determined as described below. The resin was then washed using 5 ml of water and filtered again for subsequent storage at 4^{o}C until use. Protein concentration in the final supernatant as well as the wash was measured. Using initial protein concentration these measurements were used to estimate the amount of enzyme bound to the resin.

3.2.10 Protein Concentration Measurements (Bradford Assay)

The Bradford Assay which was used to measure protein concentration in this work is a Coomassie dye-binding colorimetric assay which responds to different concentrations of protein in a solution. When Coomassie binds protein in acidic solution an absorption shift from 465 nm to 595 nm occurs, which can be detected in a microplate format assay.

The "Better Bradford Protein Assay Kit" by Bio Basic Inc. was used for all protein concentration determinations. First, Bovine serum albumin (BSA) is diluted in a series according to the table below.

Table 4: BSA standard concentrations

BSA concentration (µg/ml)	0	5	10	15	20	25	30	100	150	200	250	300

This forms the standard curve against which other protein samples are compared to determine their concentration. The sample is then diluted in a series to attempt to place its absorption within the standard curve as shown in table 4. The sample and standard curve BSA dilutions are then loaded onto a microwell plate according to the Kit instructions. The absorbance at 595nm is then measured and the average absorbance of the diluted protein sample is then compared to the standard curve. If the sample does not have an absorbance in the standard curve it is diluted until an absorbance within the standard curve is registered.

4 Results – Initial Considerations

Throughout the results sections following this chapter, samples for acid value determination were taken from the reaction mixture, which is a biphasic material. Both the aqueous as well as organic phase was taken to determine the acid value, however the acid value method considers only the organic phase. In this chapter we show that a mixed phase sample when only the weight of the organic phase is considered will return almost identical acid values as a phase separated organic phase sample. To do this we assumed that the ratio of the weight of the organic phase and the weight of the aqueous phase towards one another does not change from initial reaction conditions as described in the methods section 3.2.3.

The reason this way of sampling could be inaccurate is that as the fatty acids are split off the glycerol backbone, the glycerol moves from the organic phase into the aqueous phase. At the same time weight is moving from the aqueous phase into the organic phase, as each ester bond from the glycerol backbone to the fatty acid requires a water molecule to be broken. This could potentially change the ratio of the organic phase to the aqueous phase during the course of the reaction. Some fatty acids however are also likely to end up in the aqueous phase as they are partially soluble. Therefore, to determine the amount of fatty acid produced from a given amount of water and HEAR oil without a le is difficult. The amount of fatty acids in the organic phase can be determined accurately however, using phase separation techniques, and then using the organic phase in the acid value calculations. This approach, however, misses out any fatty acid that might be present in the aqueous phase or accumulate between the phases. Generally, for all mixed phase measurements we assume the ratio does not change from the starting conditions. This approach has its own drawbacks, especially when using buffered systems, as some buffer is present in the sample during

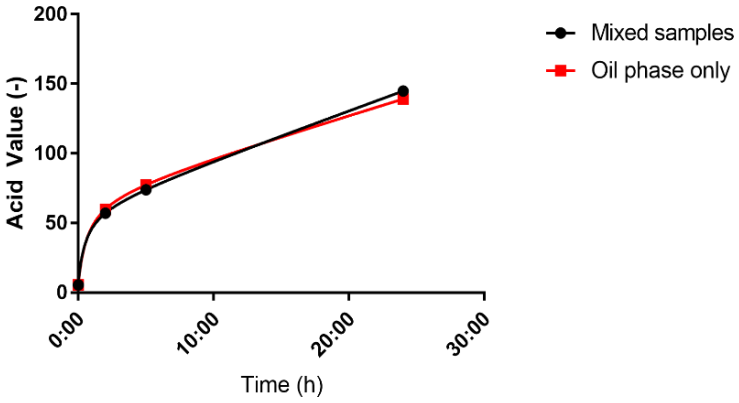


Figure 7: Acid value comparison of a TLL based HEAR oil hydrolysis.

Samples were processed for AV measurements by separating them into an organic and aqueous phase before measurements, compared to equivalent samples which were not separated.

acid value measurements. The presence of buffers stabilises pH, which interferes with the titration using potassium hydroxide.

Samples from buffered reaction show inconsistencies in acid value, which were not considered at the time these reactions were carried out and samples were taken and analysed. For example in some sections (section 6.5 and 6.6) there are acid values which exceed the theoretical value of fully split HEAR oil. Especially higher acid values seem to be inconsistent and potentially unreliable.

This is another reason why work completed at a later point does not use buffers and instead uses water only.

As can be seen in figure 7, the acid value whether measured using mixed samples or separated samples shows only very small differences when not using buffers. After 2 hours the recorded acid values were 57.0 for the mixed sample compared to 59.8 for the separated oil phase sample. In the 24-hour mixed sample, the recorded acid value was 144.87 compared to 139.15 for the separated oil phase sample. For the purposes of comparison between different enzymes these error ranges proved to be more than sufficient. Both mixed sampling as well as separated oil phase samples are used throughout, although this should not have any significant impact on results. Only pre-split samples are universally oil phase only, see pre-split section below for details.

Acid value (AV) measures the milligrams of potassium hydroxide required to neutralize the FFA in one gram of fat. It is unrelated to molecular weight and, by coincidence, is around twice the percentage of FFAs when this is expressed on an oleic-acid only basis. Details of how the AV can be used further are described in the methods section.

Some assumptions about the conditions of the reaction mixture were also tested. We assume that the pH of the mixture decreases over the course of the reaction, as fatty acids are created. We also tested the pH decrease with buffers instead of just water and found that, as assumed when creating our method, this will maintain a higher pH over the course of the reaction. The results of these experiments, using *Thermomyces lanuginosus* lipase (TLL) and 400 mM glycine / NaOH buffer set to pH 9, is shown in figure 8.

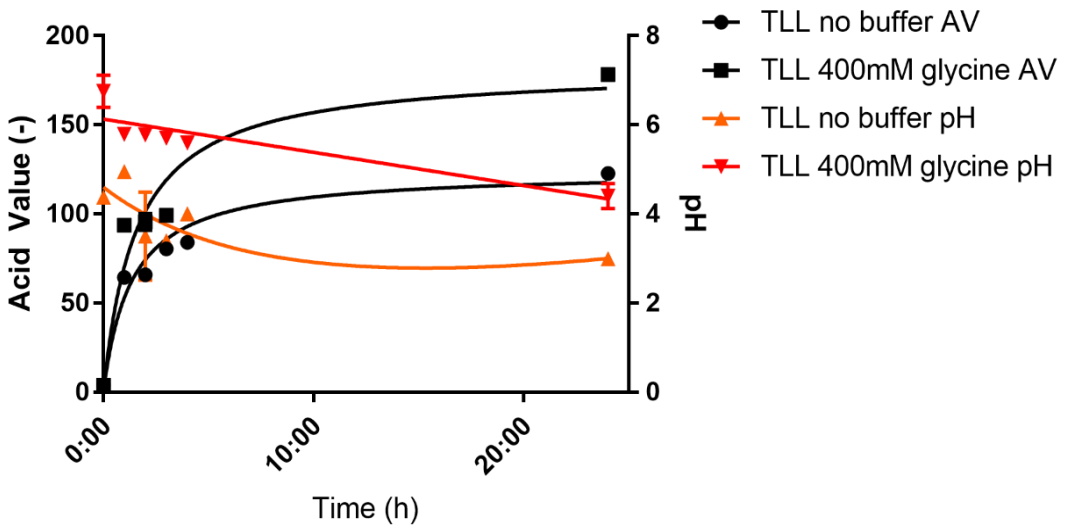


Figure 8: Comparisons of pH over the course of a reaction.

With acid value over the course of the reaction. Both pH and AV are compared between a no-buffer reaction and a 400 mM glycine/NaOH, pH 9 buffer reaction.

For all curve fitting over 24 hours, we use the following formula (One site total – Graph Pad)

$$Y = Bmax(X)/(Kd + X) + NS * X + Background$$

This equation is arbitrary, and simply produces a best fit line for most lipase oil hydrolysis reactions over 24 hours.

5 Enzyme Selection

In this section we intend to compare the suitability of five commercially available enzymes as well as two unknown enzymes from Novozyme for HEAR oil splitting. To do this we will compare their activity for HEAR oil splitting using mainly acid value measurements.

To compare between different enzymes first we must define how we will measure the amount of enzyme used in each reaction. Typically for these types of industrial applications enzyme amounts are measured in LU (lipase units) as shown in table 5. These are defined by the amount of enzyme (lipase) which can liberate 1 µmol of butyric acid per minute (from tributyrate under pH 7 and 30°C). The problem with LU is that lipases have different rates of catalysis depending on the specific triglyceride used. Long chain fatty acids or branched fatty acids will produce substantially different rates of reaction than a standardised test with a tributyrin substrate. In addition to this, standard conditions for LU determination as described above are different from conditions in the HEAR oil splitting reaction.

Table 5: Lipases previously used for fat splitting. Lipase units (U) and price sourced from manufacturer, (Novozyme, Sigma Aldrich).

Lipase	**Efficiency**	**Specificity**	**US$ / Lipase Unit (2018)**
***Candida rugosa* Lipase (CRL)**	700 U/mg	Short chain (<C16) Non-specific	$1x\ 10^{-3}$
Porcine Pancreatic Lipase (PPL)	100-400 U/mg	1,3 specific	1.1×10^{-5}
***Thermomyces lanugionosus* Lipase (TLL)**	100 U/mg	1,3 specific	6.6×10^{-3}
Lipozyme CALB	4-10 U/mg	Non-specific	6.8×10^{-3}
Novozyme435	5 U/mg	1,3 specific	0.04

While initially we used LU for comparison (for PPL and CRL) we later switched to just comparing weights of enzyme added. This allowed for a more accurate comparison for the liquid enzymes, and especially NS40119 and NS40020 where the LU was unknown. In addition to that LU is determined using tributyrin, whereas we are splitting much larger triglycerides with longer fatty acid chains. Different enzymes have different rates of reaction when splitting different sized fatty acids.

Therefore, to give a quick indication of the amounts of enzyme in the liquid preparations of lipases, we measured their protein concentrations. We assumed no other proteins or inactive enzymes were present in these preparations. All enzymes that were available as liquid preparations are compared in this way (TLL – all varieties, NS40020, NS40119, Lipozyme CALB). Novozyme 435, a commonly used immobilised enzyme in commercial applications was also compared in this way. The amount of enzyme bound to the immobilisation resin was determined as described in the methods section on immobilisation, and same amounts of enzyme as in liquid lipase preparations were used.

Comparing enzyme activity this way allows us to determine which enzyme is better at splitting specifically HEAR oil triglycerides per amount of enzyme mass. Lipase units could not be used for experimental enzymes such as NS40119 and NS 40020 as they were not available, and conducting our own lipase unit determinations would have been an additional step on top of determining their concentration.

Determining the Concentration of Liquid Lipase Preparations

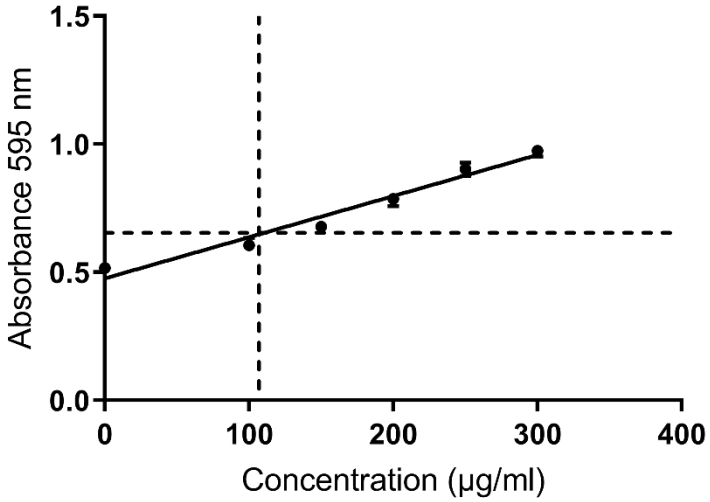


Figure 9: Standard concentration curve for enzyme solutions. Protein concentration measured in µg/ml

*Dashed lines show absorbance of the diluted TLL solution. Y = 0,001607*X + 0,4763,*

In figure 9, the dashed line shows the absorbance of a 1/100 diluted TLL solution. This together with a standardised concentration gradient, allow us to calculate the protein concentration of the original TLL solution. The average absorbance of 1% v/v TLL solution is 0.6548. This lets us calculate a 1/100

TLL concentration of 106.9 µg/ml. This means that the original TLL solution concentration was 10.69 mg/ml.

The absorbance of other dilutions of the TLL solution fall outside the range of the calibration curve and therefore cannot be used to accurately determine the protein concentration.

The concentration of other liquid lipases was determined in the same way to allow for easy comparisons between different enzymes based on weight of enzyme added. Lipase concentrations of other commercial lipases available as solutions are shown in table 6 below.

Table 6: Enzyme concentrations as measured using Bradofrd assay

ENZYME	CONCENTRATION
TLL – FROM SIGMA ALDRICH	10 mg/ml
TLL – FROM NOVOZYME	15 mg/ml
CALB	7.5 mg/ml
NS40119	40 mg/ml
NS40020	75 mg/ml

5.1 Porcine Pancreatic Lipase

To test our initial experimental fat splitting reaction setup, and calibrate and refine the overall method, porcine pancreatic lipase (PPL) was chosen. The activity of PPL on HEAR oil was determined using acid value.

PPL is a sn-1,3 specific lipase of mammalian (pig) origin, which has previously been used for fat splitting on comparable oils to HEAR oil. Its main advantage is that, in comparison to other enzymes, it is relatively cheap. In addition, it is widely available and easy to source compared most other commercially available lipases. Although it is cheap enough that immobilisation and reusability are likely not worth the effort compared to using the free enzyme, there are issues with it when it comes to use of this lipase for the food and cosmetics industries. As the name **porcine** pancreatic lipase implies, anything produced using PPL will not be kosher or halal due to the source of PPL being the pancreas of pigs. This is an important factor when it comes to marketability of any products derived from PPL based reactions.

There are, in addition to its price and wide availability, some other advantages when it comes to PPL.

PPL is also available as a powder rather than in solution, which allows us to determine a base amount of enzyme to use for our HEAR oil splitting reaction and makes it easier to work with and easier to compare to other powdered/solid enzymes.

As the fat splitting reaction progresses, fatty acids are produced, which causes the pH of the reaction's mixture drops, which in turn reduces the activity of lipases. Buffered systems are often the first approach to making enzymatic reactions viable in industry.

Traditionally enzymatic processes suffer from slow reaction times and low yields due to relatively low reaction rates of enzymes compared to chemical fat splitting using 250°C and 40 bar. These already slower rates are further lowered by the lowering in pH over the course of the reaction, as fatty acids are produced. Buffers may be a solution to these problems.

Initial experiments were set up to compare the effect of 6 different buffers and additive combinations on the amount of fatty acid produced (measured using acid value) using a set of standard conditions (35°C, 400 rpm stirring, 1 g of enzyme per 100 g of oil). These were then compared to the acid value results of a hydrolysis reaction using only oil, water, and enzyme at the same standard conditions.

These reactions should allow us to determine if buffers are valuable or viable for fat splitting as well as establish base conditions for fat splitting, such as stirring speed, amount of enzyme relative to our reagents and oil to water ratios.

Initial buffer tests were carried out using a glycine/NaOH based buffer as well as an ethanolamine-based buffer. In addition to this several previously reported additives to the buffers such as $MgCl_2$ as well as surfactants (Tween80 and Span80) were tested based on previous results (Goswami, Basu and De, 2011). Values for 100 ml of each buffer are shown in table 7.

Initially, pH 9 buffers were used, as the production of a large amounts of fatty acids would likely reduce the overall pH of the reaction mixture. Therefore, to maintain the pH of the reaction mixture at around pH 7-8, the optimal pH for PPL, a slightly higher initial pH was chosen.

In addition, $MgCl_2$, or more specifically magnesium ions in solution, had previously shown stabilising effects on lipases, allowing for a wider optimal temperature and pH range. Surfactants were also used in previous work, and could also fulfil a different role, acting as emulsifiers allowing for more thorough mixing of the oil and water phases, and therefore allowing for easier access to the oil for the water-soluble interfacial activated lipases. (Goswami, Basu and De, 2011)

1 g of enzyme per 100 g of oil was chosen as similar amounts were referenced in previous work using PPL as well as other powder form lipases. We also started with an oil:water ratio of 1:1, and 400 rpm stirring, which seemed sufficient for mixing from visual inspection. With our initial setup this was also close to the maximum stirring speed possible. Using these conditions as standards for follow up experiments allows us to compare different enzymes and buffers before we varied each of these conditions one by one as well.

Table 7: 6 Buffers used for HEAR oil splitting. Buffers are numbered 1 – 6. Results and comparisons with HEAR oil splitting without buffer are shown in the figure below. All buffers were adjusted to pH 9 using HCl and NaOH as needed. The final concentration of the glycine buffer is 50 mM glycine

Buffers	**0.2 M Glycine (ml)**	**0.2 M NaOH (ml)**	**100 mM ethanolamine (ml)**	**100 mM $MgCl_2$ (ml)**	**1.167 M Span80 (µl)**	**0.41 M Tween80 (µl)**	**Water (ml)**
1	25	4.4					70.6
2			50				50
3	25	4.4		10			60.6
4			50	10			40
5	25	4.4		10	428	366	59.809
6			50	10	428	366	39.206

For our initial buffer tests, the results represented in figure 9, show that generally the glycine/NaOH based buffers seem more suited to the HEAR oil hydrolysis reaction. Use of glycine-based buffers led to higher acid values. This might also be due to the presence of sodium hydroxide, which is used to adjust the pH of the buffer. Sodium ions had also been reported to have a stabilising effect on lipases. It may also simply be that the glycine buffer has a naturally higher buffering capacity compared to ethanolamine-based buffers leading to a more sustained activity over the reaction time.

As can also be seen in figure 9, the addition of $MgCl_2$ appears to lead to an increased amount of fatty acid being produced, especially when using the glycine buffer. Despite some evidence in literature that suggests a positive effect of surfactants on fat hydrolysis reactions (Goswami, Basu and De, 2011), it appears that surfactants Tween80 and Span80 do not impact the reaction, or even affect it negatively.

Across all different experiments, it is clear that the initial part of the reaction occurs relatively quickly, as a lot of fatty acid is produced within two hours. The reaction appears to slow down following this initial reaction. There are several possible causes of this.

It is possible that porcine pancreatic lipase (PPL) is not strictly 1, 3 specific, and possibly the initially visible fast rate of reaction corresponds to a sn-1,3 specific hydrolysis reaction, which occurs faster, while the slower overall rate of reaction visible in figure 10, is non-specific and occurs in the background throughout the reaction. It is also possible that PPL could preferentially and more rapidly split triglycerides over other partial glycerides such as di-, and monoacylglycerides. This would also lead to an initially fast production of fatty acids, which slow down once triglyceride concentrations are reduced.

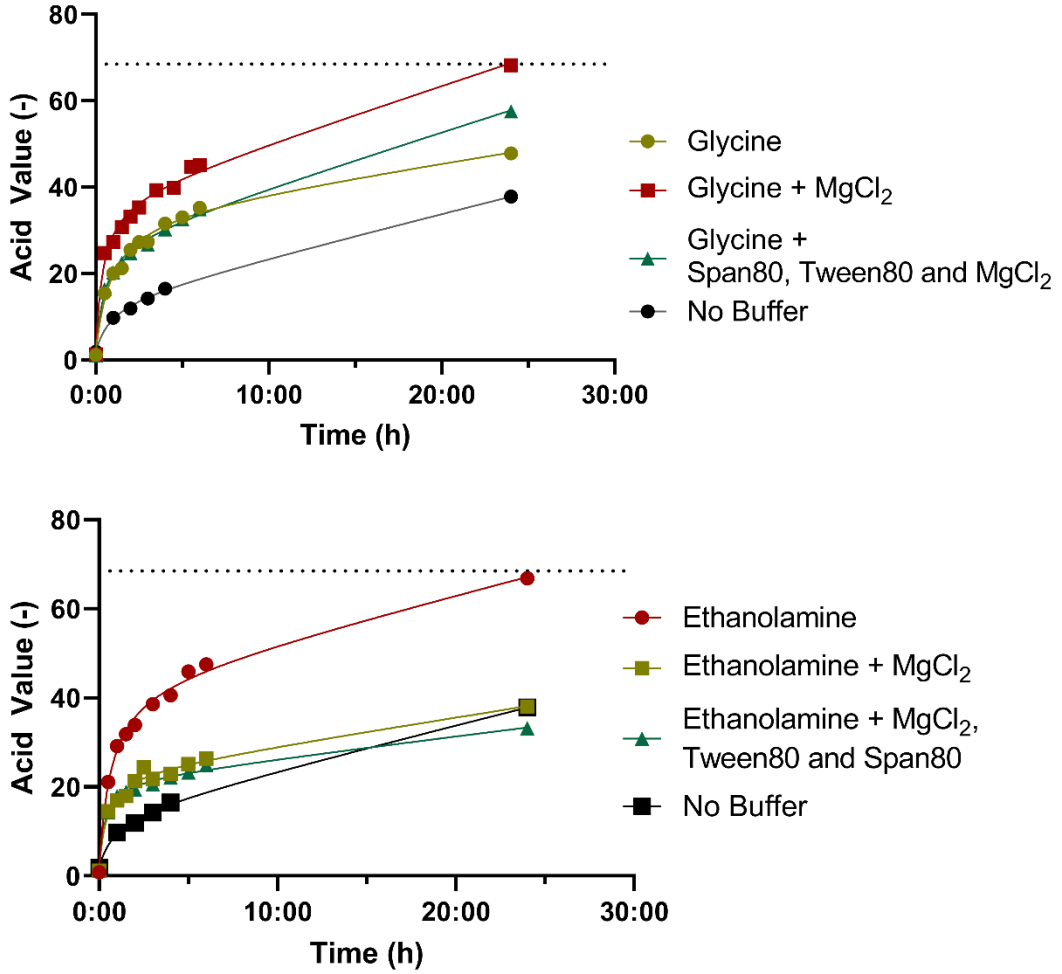


Figure 10: Comparisons of buffers as shown in table 5.

Used for HEAR oil splitting. Progress of the reaction is measured in acid value (AV) over 24 hours. Highest AV achieved after 24 hours is marked on the graph as a dotted line (68.5). Full results in appendix.

Another possibility is that as fatty acid is being produced, the pH of the reaction mixture drops quickly below the optimal pH of PPL, inhibiting the enzyme and slowing down the overall rate of the reaction. Although this can be somewhat mitigated by buffers as shown in figure 10 above, in buffered reactions show improved overall production of fatty acids and resulting higher acid values at all time points. In addition, due to the overall reversible nature of the reaction it is possible that as more fatty acids are being produced the equilibrium of the reaction shifts and the rate of the hydrolysis reaction decreases.

Production of glycerol and the resulting equilibrium shift towards the reagents from the products is another possible reason for the slowdown of the rate of reaction over the course of the reaction. Most likely all of these reasons for the slowing of the reaction apply.

To compare the acid value to the total percentage of split, we measured the saponification value, representing all of the potentially hydrolysable material in the oil. Specifically, this number can be used to calculate the percentage of split by dividing the recorded acid value with the saponification value. The saponification value of this batch of HEAR oil was around 180.

It should be noted however, that assuming PPL is very strictly 1, 3-specific that the maximum percentage of split would be 66%, rather than 100% split. As such, the maximum acid value would be around 120-125. Of course, no enzyme or biological process works like this. Usually there is some leakage of non-specific activity, as well as a slow natural process, where fatty acids migrate from the sn-2 position to the sn-1 position on the glycerol backbone (acyl migration), leading to all fatty acids being split even by 1,3 specific lipase action. (Li *et al.*, 2010; Laszlo, Compton and Vermillion, 2008)

For future PPL experiments we chose the glycine buffer with $MgCl_2$ added (buffer 3 in table 6), as this setup produced the highest acid value, and therefore the highest splitting efficiency and the largest amounts of fatty acids produced. For future experiments this is the buffer referred to simply as “glycine buffer”.

The relatively low acid values recorded in figure 10 compared to a full split acid value which would be around 180 AV led us to increase the amount of enzyme used, as well as vary buffering strengths as shown in the remainder of this section.

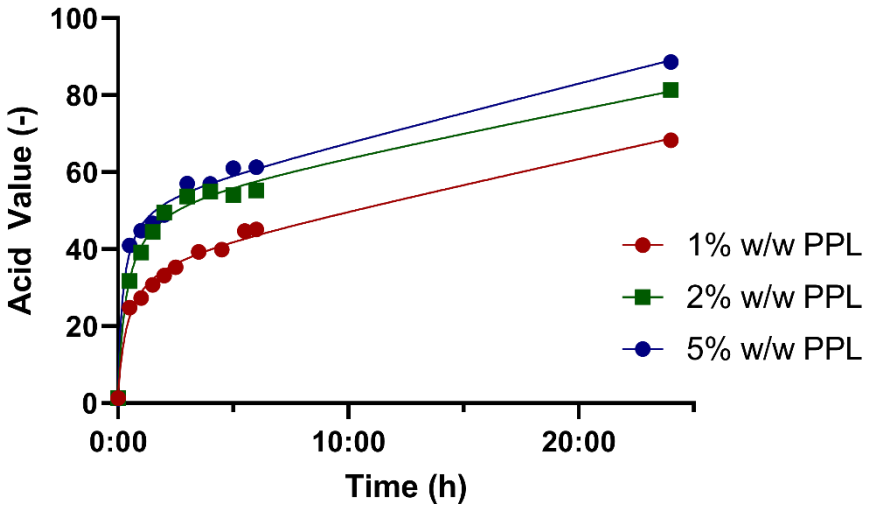


Figure 11: Other conditions kept constant across experiments:

Buffer: 50 mM glycine, 10 mM MgCl2, NaOH to pH 9. 400 rpm stirring, 30°C, 1:1 weight ratio of oil and buffer.

More enzyme used leads to a higher acid value. However, the effect of additional enzyme diminishes between 2% w/w and 5% w/w enzyme.

Initially we used double the concentration of PPL (2 g of enzyme per 100 g of oil), as well as five times the concentration (5 g of enzyme per 100 g of oil) as shown in figure 11. Otherwise, conditions were kept identical to initial experiments to allow for comparison (30°C, 400 rpm, 1:1 oil:water). Increasing the concentration of PPL leads to a clear increase in recorded acid value, which corresponds to an increase in the amount of fatty acid produced.

The acid value increased at all time points of the reaction when the enzyme concentration is increased. In addition, the overall pH of the reaction mixture dropped significantly, especially with the higher enzyme concentration.

This is likely due to the fast production of fatty acids lowering the pH quickly, which leads to denaturation of enzymes and therefore less available enzyme for the hydrolysis reaction, slowing the overall progress of the reaction.

Another step we took to increase the amount of fatty acid being produced in the reaction was to increase the buffering capacity of our buffer. We theorised that a reduction in pH of the reaction mixture over the course of the reaction would lead to a decrease in the activity of the lipase. Increasing the buffering capacity would therefore lead to the pH remaining more stable further into the reaction and allow the enzyme to be more active at the resulting higher pH.

As figure 12 below shows, increasing the buffering capacity increases the rate of reaction and acid value over time. The buffering capacity is increased through increasing the concentration of glycine in the glycine/NaOH buffer, meaning more acids can be produced during the reaction without the pH changing significantly. This provides additional evidence, that the production of fatty acids lowers the pH of the reaction mixture, and that this can be prevented or at least reduced by using buffers.

A potential problem with using buffers is the creation of fatty acid salts when using ions as well as impacts on acid value measurements due to the presence of ions used to adjust the pH of buffers. As acid value is a pH-based titration measurement, buffers and their pH stabilising effects can affect results as discussed above in section 4 Results – Initial Considerations.

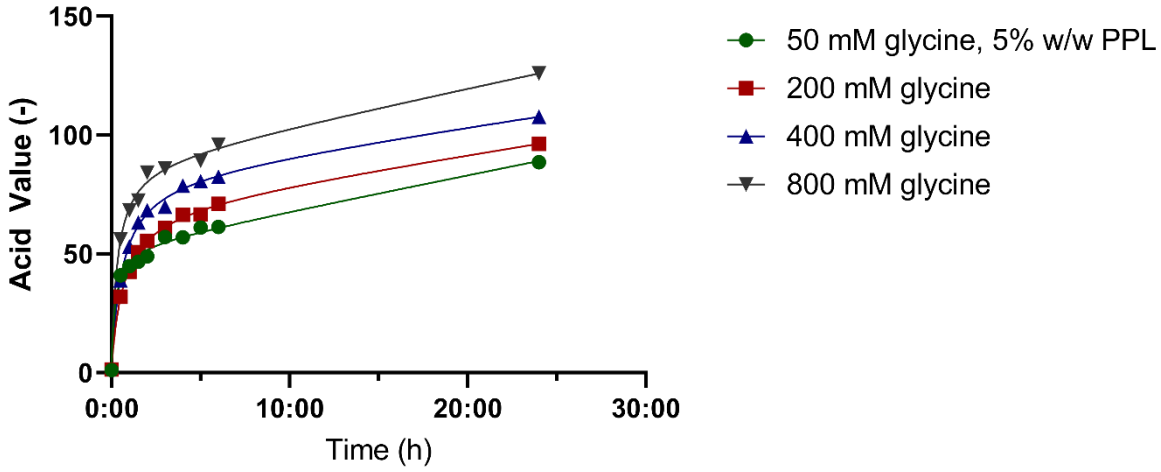


Figure 12: Comparison of higher buffering capacities with PPL.

Other conditions kept constant across experiments: 5% w/w PPL, 10 mM MgCl2, NaOH to pH 9. 400 rpm stirring, 30°C, 1:1 ratio of oil and buffer. A higher buffering capacity, as indicated by glycine concentration, leads to a higher acid value.

5.2 *Candida rugosa* Lipase

Another interesting fungal lipase for fat splitting purposes is the lipase from *Candida rugosa* (CRL), as described in the literature review section above. Although CRL is a positionally non-specific lipase, it has a very high reported activity, as well as previously reported use in industrial and academic applications of fat splitting (Al-Zuhair, Hasan and Ramachandran, 2003; Gupta *et al.*, 2011). CRL also reportedly prefers shorter chain fatty acids for hydrolysis compared to the longer chain fatty acids present in HEAR oil.

However, CRL is a very well-studied lipase, with previously reported, detailed kinetic models for its hydrolysis of triglycerides.

For the purpose of completely splitting HEAR oil in a process mimicking current chemical processes, CRL might be a very interesting enzyme. Especially when looking at using it in combination with a 1, 3 specific enzymes, which will be explored in a process optimisation section below.

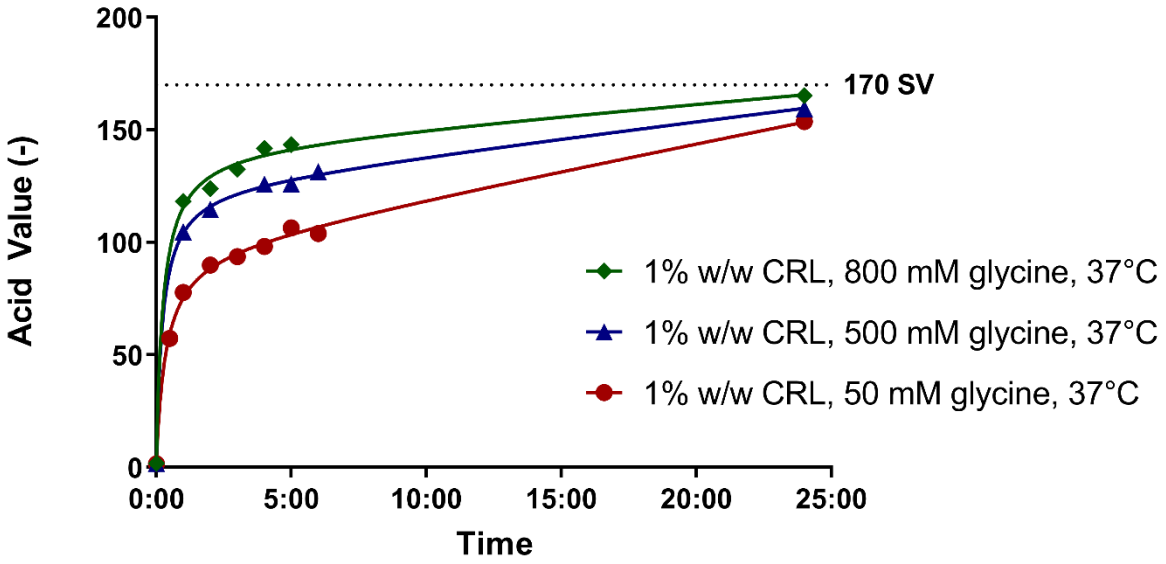


Figure 13: Candida rugosa lipase buffering capacity comparisons.

*More glycine allows for a higher buffering capacity. This directly affects the initial rate of reaction, although the overall amount of fatty acids produced after 24 hours remains similar. The dotted line at 170 represents the Saponification value of the batch of HEAR oil used. AV/SV * 100 results in a percentage of split.*

Overall, CRL shows much more activity than PPL at similar amounts used. As shown in figure 13, after 24 hours an acid value of 160 is reached when using 800 mM glycine/NaOH buffer. There is a bigger difference in the initial reaction between different buffering strengths. When using a 50 mM

glycine buffer, an acid value of 77 is reached after 1 hour of reaction time, with increased buffering strength this initial reaction activity increases. When using 500 mM glycine buffer an acid value of 104 is reached after one hour of reaction time, and with an 800 mM glycine buffer and acid value of 118 is reached after one hour of reaction time.

Interestingly, in the initial reaction increasing the buffering capacity has a significant effect on the acid value produced, whereas for the later part of the reaction the increased buffering capacity seems to have less of an effect. This is possibly due to CRLs reported pH stability, where a decrease in pH affects the lipase less. All three different buffering strengths used lead to a degree of split of above 90% after 24 hours. Using 800 mM glycine we can observe a degree of split of 84% after 5 hours.

5.3 Lipozyme CalB / Novozyme 435

Lipozyme CalB, is the trademark name of the B lipase from *Candida Antarctica*. Like TLL, this enzyme is temperature stable, and retains activity even at temperatures where other enzymes are denatured and inactive.

In its immobilised form, bound on a poly-(methyl methacrylate) resin, the enzyme is known as Novozyme 435. This immobilised form of CAL-B is one of, if not the most used lipase in industry, employed in a number of different uses from esterification reactions to bio diesel production.

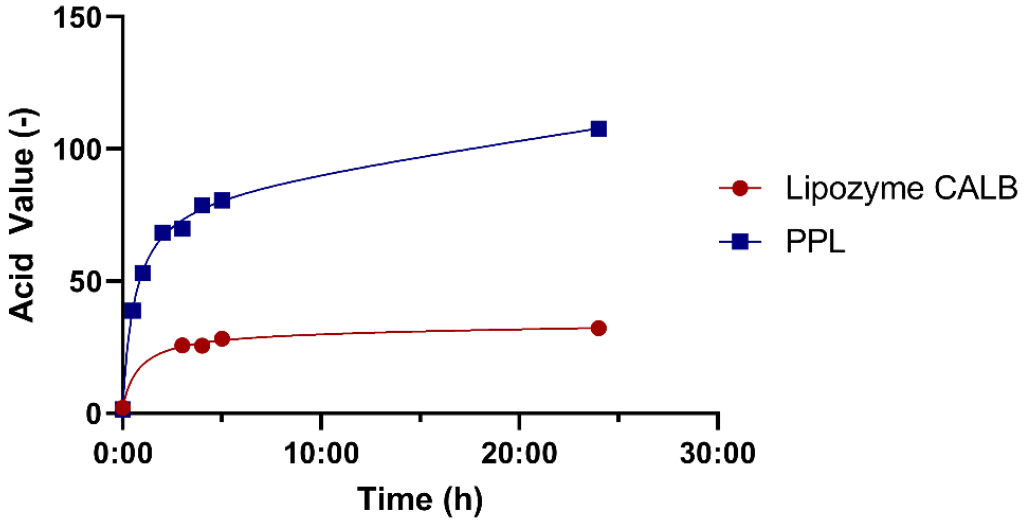


Figure 14: Comparing free Lipozyme CALB to PPL. Equivalent amounts of enzyme used, Overall, a much lower rate of reaction compared to PPL. Buffer used is 400 mM glycine, pH9 with NaOH. The reaction for Lipozyme CALB was carried out at 36°C, for PPL at 30°C.

Using Lipozyme CAL-B is not ideal for HEAR oil splitting. Equivalent amounts of CAL-B produced a much lower amount of fatty acid than PPL see figure 14.

Splitting HEAR oil for 1 hour using PPL produces a higher acid value than splitting HEAR oil for 24 hours using Lipozyme CALB. The acid value stalls at around 30 AV after 4 hours and no more splitting occurs. This is possibly due to the enzyme not being capable of splitting the higher molecular weight fatty acids such as erucic acid, which make up the majority of fatty acids in HEAR oil. (Anderson, Larsson and Kirk, 1998)

Lipozyme CALB with 400 mM glycine/NaOH buffer (ph 9), only manages to reach an acid value of 34, and CALB without buffer only reaches an acid value of 7-8 after 24 hours.

Despite the relatively low amount of fatty acids produced by the free enzyme Lipozyme CALB, we decided to test some additional conditions using its immobilised form Novozyme 435.

Throughout the experiments using Novozyme 435, again, the enzyme shows a slower initial rate of reaction compared to PPL based hydrolysis, and a steadier, and slower increase in acid value over the course of the reaction (see figure 15). The Novozyme 435 used in this experiment was Lipozyme CALB immobilised on silica beads, which made the lipase able to resist higher temperatures as per the manufacturer's specifications. This allowed us to increase the temperature up to 60°C. However, even with this increase in temperature to 60°C, and with a higher concentration of enzyme (5g of Novozyme 435 compared to 1g of PPL), Novozyme 435 based hydrolysis still cannot compete with other lipases-based hydrolysis. As a result, we decided to focus on other enzymes for this reaction.

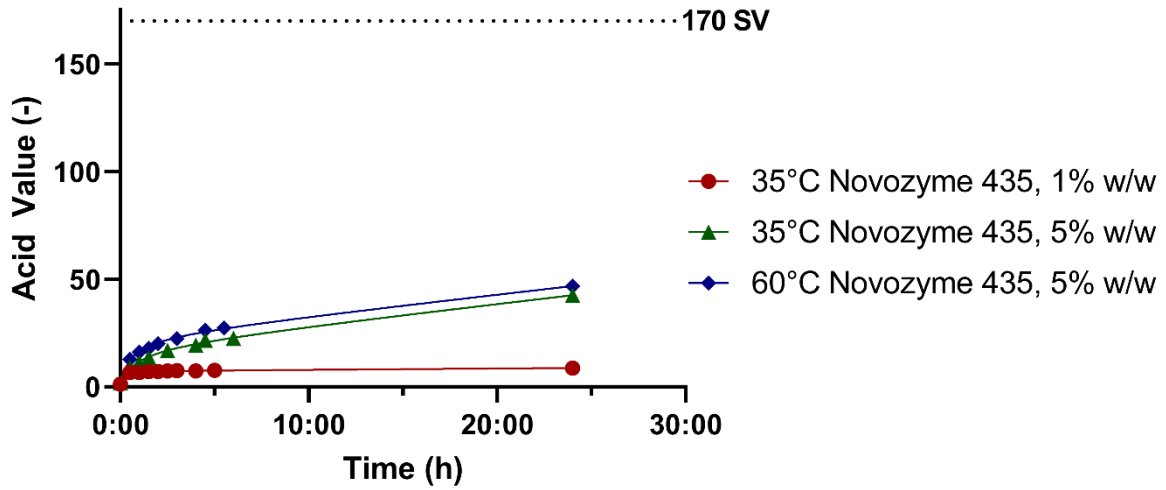


Figure 15: Comparison of a number of Novozyme 435 based hydrolysis reactions.

Generally, Novozyme 435 based hydrolysis shows a lower initial rate as well as an overall lower rate of reaction throughout all experiments. For Novozyme 435 w/w percentages include the resin as well as enzyme, as there is no information on how much enzyme is bound from the manufacturer.

Neither Novozyme 435, nor the free enzyme version CAL-B are suitable for producing a large amount of fatty acids from HEAR oil. Very little free fatty acid is produced, and not many partial glycerides are produced either (see TLC slide in Appendix)

5.4 Other Novozyme Enzymes

In addition to more commonly used fungal lipases, I was able to use two unknown lipases, not currently available for commercial use. One was described as a sn-1, 3 specific lipase (NS40119), identified as a TLL variant, which was later confirmed by Novozymes. The second unidentified enzyme was a nonspecific lipase (NS40020), identified as *Candida antarctica* lipase A through the following results and confirmation from literature. Both are stable up to 90°C according to information from Novozymes, who provided these lipases to the lab.

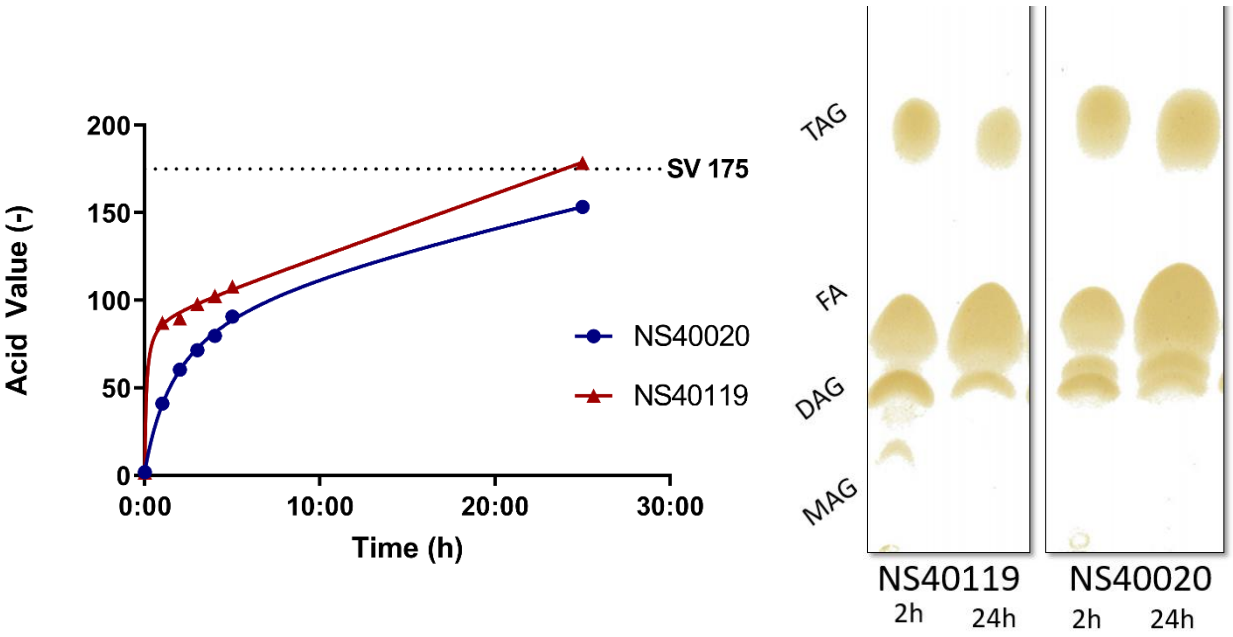


Figure 16: Comparison between Novozyme™ enzymes.

Comparison of NS40119 and NS40020 using 0.01% w/w of enzyme/oil each at 50C. NS40119 shows a faster initial reaction. TLC experiments provide evidence for the non-specificity of NS40020 by the presence of two different DAGs in roughly equal amounts. NS40119 only shows production of a single DAG suggesting it is 1, 3- specific.

As shown in figure 16, NS40119 has a higher activity, producing very similar acid value results as TLL. The initial activity is very high as with other 1,3 specific enzymes, whereas NS40020 appears to be less active and produce fewer fatty acids than NS40119 when used in equivalent amounts. Generally fatty acids are being produced more consistently over time rather than in an initial burst.

NS40119 reached close to 100% split after 24 hours of reaction time. NS40020 reaches up to 90% degree of split after 24 hours.

Looking at our TLC results in figure 16, NS40119 shows a typical pattern of a 1,3-specific lipase, meaning only one DAG can be clearly seen. Although some activity is likely splitting DAGs non-specifically similarly to other 1,3 specific lipases, which is why a faint band for a second DAG can also be seen on the TLC slide.

For NS40020, initially the lower DAG is more pronounced after 2 hours of reaction time, whereas in other non-specific lipases such as CRL the upper DAG is more pronounced. After 24 hours however, both DAGs are equally present. The results from this suggest that NS40020 is a sn-2 specific lipase. The only lipase mentioned in previous literature in the context of sn-2 specificity is CAL-A. Based on this as well as later conversation with Novozyme, NS40020 was confirmed to be a CAL-A variant.

In addition to this for both NS40119 and NS40020 after 24 hours there is only a small amount or no observable amount of MAG left. This suggests either preferential splitting of MAGs or spots for MAG being diluted by glycerol due to the overall high degree of split, making it hard to detect on TLC slides with high degrees of split.

5.5 *Thermomyces lanuginosus* Lipase

Thermomyces lanuginosus lipase (TLL) was identified as an enzyme of interest for splitting HEAR oil. Particularly interesting is TLL's temperature stability; its ability to withstand high temperatures without denaturing, allowing for higher rates of reaction than other enzymes. Some previous literature (see literature review) suggests TLL could be stable up to 90°C. To further study the reaction of TLL catalysing the hydrolysis of HEAR oil and to study the process of erucic acid production several experiments were set up. As was done initially with PPL, enzyme amounts and buffering strength as well as some other factors were altered, and acid values were taken to compare the reaction progresses.

In addition to this several experiments were conducted to determine the composition of the reaction mixture as well as products produced over the course of the reaction. Additionally, we tested for the presence of inorganic contaminants in the reaction mixture.

Experiments using TLL were to test a complete split for industrial purposes, and as a result we were interested in maximising the produced acid value and reducing the reaction time needed to produce a high acid value. A percentage of split of higher than 95% was targeted as a minimum splitting yield for industrial viability.

5.5.1 Initial results

Our initial interest in TLL was sparked by its remarkable temperature related properties. So as for our initial experiments we started at 30°C, to allow for comparison with PPL and other enzymes. We then ran experiments with an increased temperature of 50°C and later at higher temperatures up to 70°C as well. 50°C was used as a standard temperature after initial testing for TLL and other heat stable lipases such as NS40020 and NS40229. In addition to this we tested increasing buffering capacity, comparable to initial experiments on PPL. The much-increased production of fatty acids compared to PPL and other industry standard enzymes likely lowered the enzyme efficiency of TLL in our initial testing using a 50 mM glycine/NaOH buffer, so increasing the buffering capacity to 400 mM glycine was also evaluated.

As shown in figure 17, both increase in temperature to 50°C as well as increases in buffering capacity showed significant proportional increases in acid value produced over time. At 50°C and with a 400

mM pH9 glycine/NaOH buffer an acid value of 173 was reached after 24 which is close to a complete split, meaning all or most of the fatty acids bound in triglycerides in HEAR oil are released as free fatty acids. Using a weaker buffer of 50 mM glycine at 50°C, a lower acid value of 154 is reached after 24 hours and using TLL at 30°C only results in an acid value of 119 after 24 hours. Clearly, both the buffering capacity but more so the temperature has a great effect on TLL based HEAR oil hydrolysis.

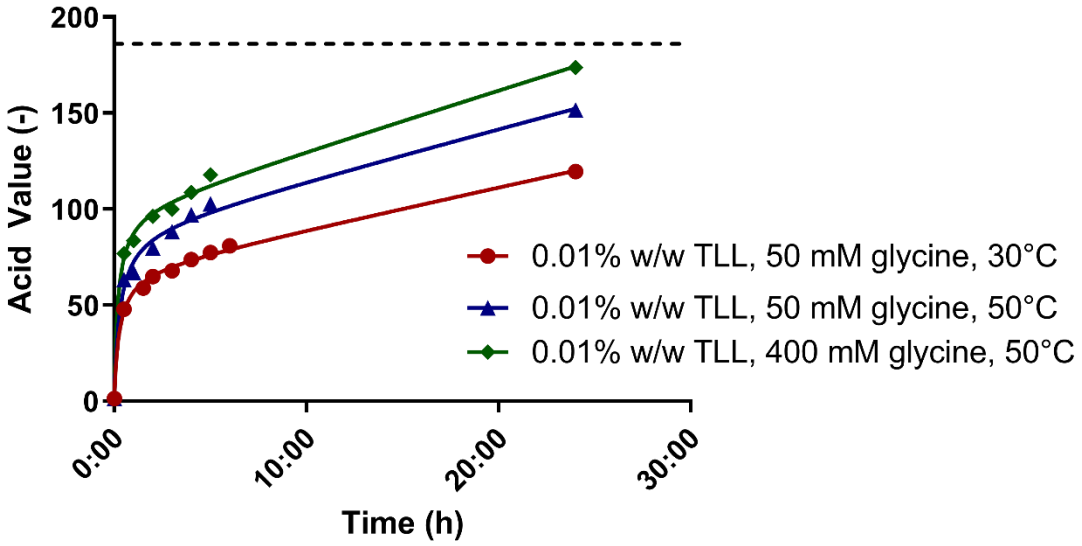


Figure 17: Acid value comparisons of TLL based splitting at a higher temperature and with more concentrated glycine/NaOH buffer.

Clearly results from figure 17 show that using TLL at higher temperatures is actually beneficial to its catalytic activity. As TLL is a very thermostable enzyme, to further increase the acid value produced and find the limits of TLL heat resistance, the temperature was increased from an initial 50°C, to 60°C and 70°C. Industrial reactions involving fatty acids and oils are often at slightly higher temperatures due to the viscosity of the oils and fatty acids involved. Higher temperatures allow for easier movement of the oil. For this reason, having an enzyme capable of performing and remaining active at higher temperatures (60 – 90°C) is important. Additionally, as buffers are generally not used in industry for many different reasons, we decided to test higher temperatures in a non-buffered system.

As can be seen in figure 18, for HEAR oil splitting, activity of TLL peaks between 50°C and 60°C, with a significant decrease in activity at 70°C as can be seen in figure 18. After 5 hours, the acid value produced at 70°C is almost half that produced at 60°C; 50AV compared to 90AV. After 24 hours, the acid value at 70°C only reaches 77 - 87 AV.

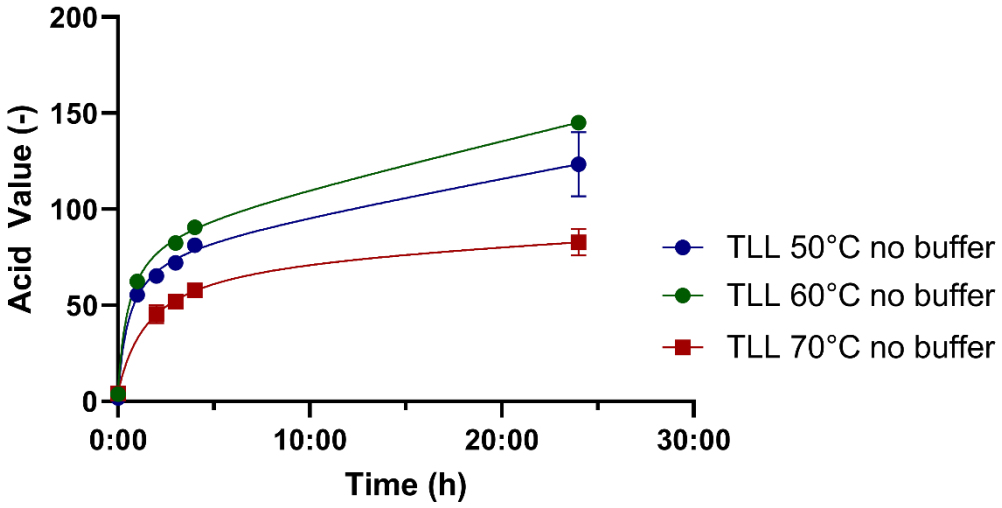


Figure 18: Comparison of TLL based HEAR oil hydrolysis at 50°C, 60°C and 70°C, over 24 hours.

5.5.2 GPC, and HPLC – Other Analytical Methods Used

In order to confirm results obtained using thin layer chromatography (TLC) we used additional analysis methods. TLC proved to be a reliable and fast system for split HEAR oil analysis and allowed us to process a lot of samples quickly with little method development. However, to add extra data and allow for more certainty when interpreting TLC results, we decided to also analyse samples from TLL based HEAR oil splitting using Gel Permeation Chromatography (GPC) as well as High Performance Liquid Chromatography (HPLC). We additionally used Gas Chromatography to analyse the original fatty acid content of HEAR oil – results for this are shown be in the appendix.

The techniques shown in this section were still in initial stages and done with Croda as part of an internship in their laboratories. As a result, these techniques were only applied to a limited number of samples and could not be continued due to COVID19. A lot of the data was only available in the form of cropped screenshots and as such is incomplete.

Gel Permeation Chromatography

In addition to TLC, the products of TLL were analysed using gel permeation chromatography (GPC) (figure 19). At the flowrate used (0.1ml/min), the main monoacylglyceride by-product, monooleate, is indistinguishable from erucic acid. This is due to the separation almost purely by molecular weight

in GPC. Monooleate is a C18 fatty acid with a glycerol (three carbons) attached and a molecular weight of 358.57 g/mol, and erucic acid, the main fatty acid produced contains 22 carbon atoms (C22) with a molecular weight of 338.57 g/mol. Their molecular weight is close enough that the GPC at this flow rate with the columns used cannot be used to separate them.

The two main fatty acids produced are erucic and oleic acid. Some triglyceride remains even after 24h. The 24h sample used for GPC analysis was 83.5% split.

In addition, samples collected after 2h and 5h were analysed. These samples were very similar. More fatty acids appear to have been produced and the reaction at this point has likely slowed down, as confirmed by acid value readings between 2 and 5 hours.

Shown in figure 19, we can clearly see 4 distinct peaks corresponding to triglyceride on the far left, followed by diglycerols, the smaller and broader peak. The peaks on the right correspond to fatty acids as well as glycerol monooleate. The taller peak in the fully split sample shows monooleate and erucic acid, and the smaller peak represents C18 fatty acids.

Glycerol monooleate cannot be distinguished from erucic acid, and assuming 1,3 specific splitting these two should be the most common products.

All peaks were assigned using CRODA internal standards for analysis of HEAR oil, partially split HEAR oil and HEAR oil hydrolysis products.

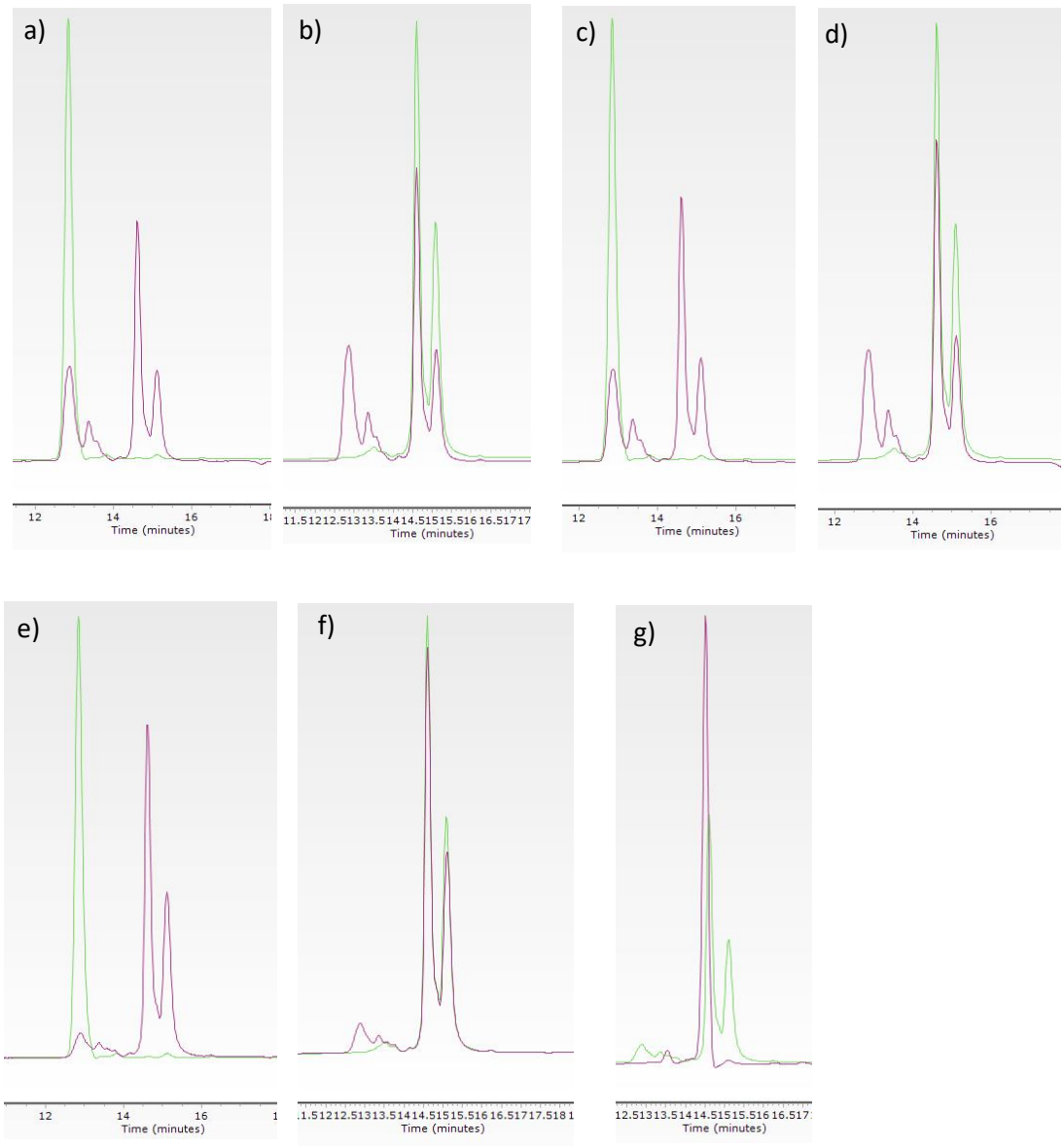


Figure 19: Gel permeation chromatography (GPC) of TLL based HEAR oil hydrolysis samples.

Taken at 2h, 5h and 24h of reaction time. The 2h sample (purple) is compared with HEAR oil (a) and fully split HEAR oil (b) (green lines). The 5h sample is compared with HEAR oil (c) and full split (d). The 24h sample is compared with HEAR oil (e) and full split (f). Fully split HEAR oil is also compared with monooleate (g).

High Performance Liquid Chromatogrphy

HPLC is a standard technique for analysis of lipids in oleochemistry. However, to analyse many of the samples of partially split HEAR oil standards are needed. Fatty acid and monooleate standards were easily obtainable, however standards for DAGs such as 1-erucate,2-oleate glycerol were harder to obtain.

In figure 20 a) below, adjusted standards for HPLC analysis can be seen. We can very clearly see a split peak for TAGs, indicating at least two main different types of TAG, shown in blue in figure 20 a. There are also several peaks for C18 fatty acids shown in figure 20 in pink. Glyceryl monooleate can be seen in brown with two peaks likely indicating different C18 fatty acid such as linoleic or linolenic acid. Erucic acid can be seen in green with a single peak.

The green signal in figure 20 b) is using partially split HEAR oil from the 4 h point of a TLL-based enzymatic hydrolysis reaction. Unsplit HEAR oil was also run on this, but not shown on this graph but was used to determine the position of unsplit triglycerides. Only DOG and Erucic acid were analytical standard levels of purity. GMO used was from Croda product Citherol, and Oleic acid was only ~90% purity. Both are sufficient to determine positions of various components of split HEAR oil.

For figure b, GMO was run at the wrong flowrate, which is why the red graph is shifted to the right significantly. Still, this allows us to confirm the presence of Erucic acid, oleic acid as well as presence of triacylglycerides (unsplit HEAR oil) and diacylglycerides in the sample. We can also observe a peak where GMO should be in figure 20 b, which allows us to infer the presence of a monoacylglyceride. This confirms the results of our TLC experiments. The same products of the enzymatic HEAR oil splitting reaction can be detected using HPLC.

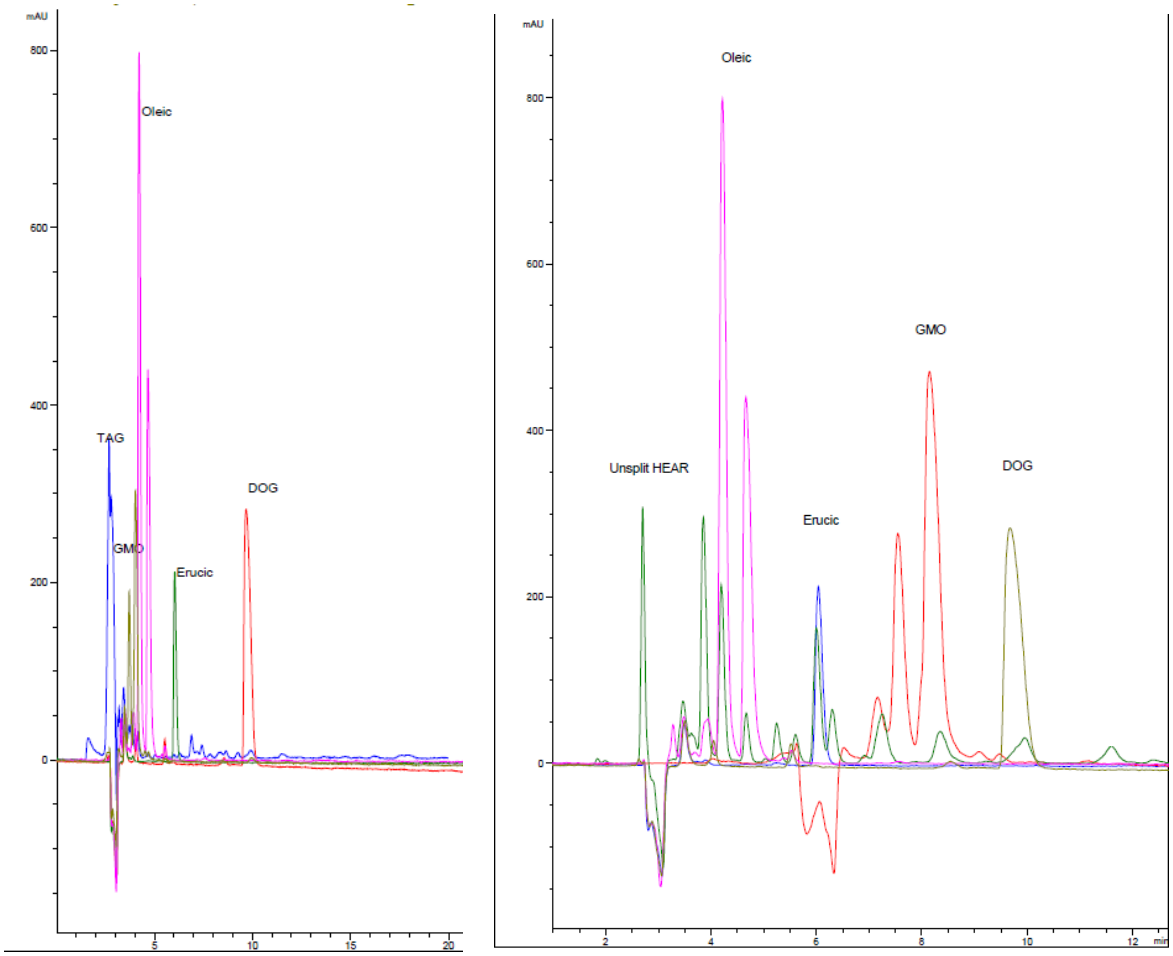


Figure 20: HPLC results

On the left, (a) shows the standards used, TAG = triacylglycerol (Unsplit HEAR oil), GMO = glycerol monooleate, Oleic = oleic acid, Erucic = erucic acid, DOG = dioleoylglycerol. On the right (b), the green line is a sample of enzymatically split HEAR oil (4hr reaction time).

5.5.3 Different TLL variants (Novozyme TL/NS40119/Sigma)

Over the course of experiments different versions of TLL were available or stopped becoming available. To determine if these versions had any major differences for HEAR oil splitting, all three variants we had access to were compared. Conditions used were identical. Amounts of enzyme between the different TLL variants were normalised through measuring the protein concentration of the different lipase solutions. Equal amounts of lipase were then used as determined through protein concentration in Bradford assays.

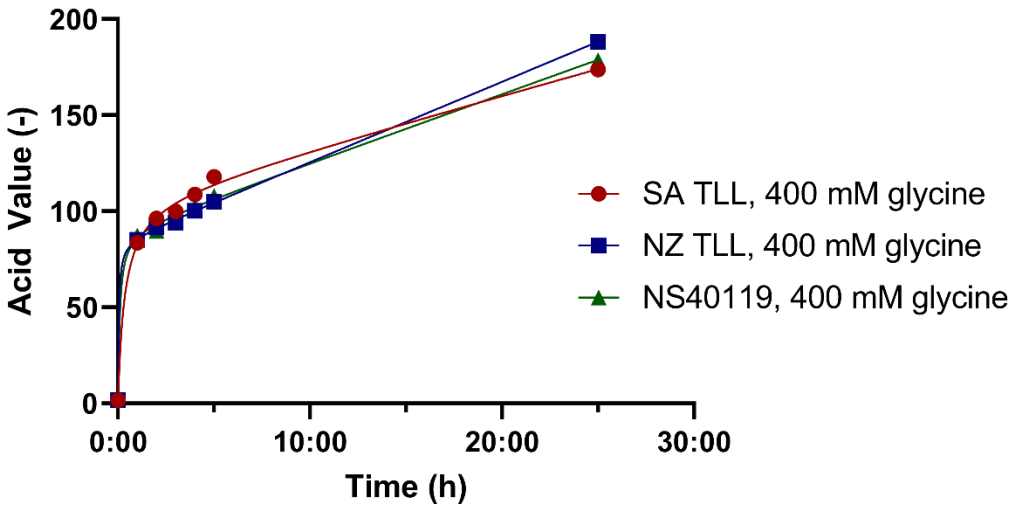


Figure 21: Comparison of different TLL variants for HEAR oil fat splitting.

SA – Sigma Aldritch, NZ – Novozyme, NS40119 – Novozyme TLL variant with 3 amino acid changes. No significant differences can be observed.

Different variants of TLL produce very similar amounts of FFAs as can be seen from the acid values shown in figure 21. No major differences in acid value can be seen. TLL sourced from Sigma seems to produce slightly higher acid values in the initial reaction, whereas Novozyme variants proved more stable over the course of the reaction. However, compared to other lipases used, these results are virtually indistinguishable. All variants of TLL are equally viable for HEAR oil hydrolysis.

5.5.4 TLL Comparison with Positionally Non-Specific Lipases

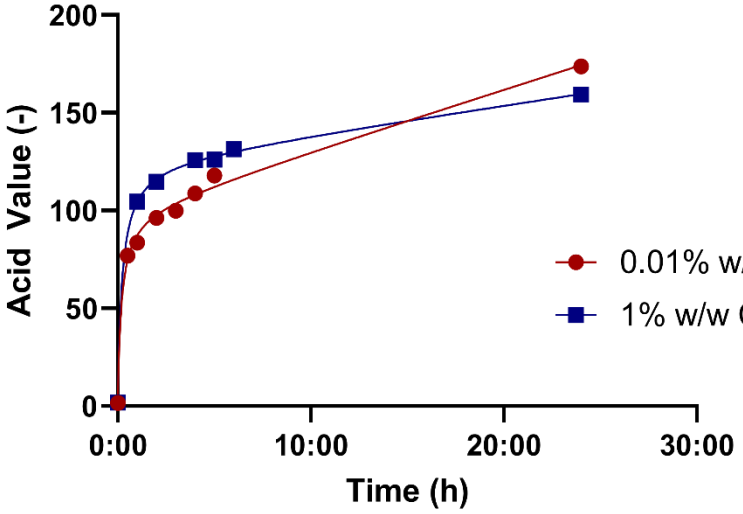
Non-specific lipases are enzymes which have no positional preference for which fatty acid bound to the triglyceride backbone will be hydrolysed. As a result, we expect non-specific lipases to have a more consistent overall rate of reaction, as only a single type of reaction should occur. Of course, factors such as product accumulation and a decrease in pH will still decrease the reaction rate over time.

The activity of non-specific enzymes will be compared to the activity of TLL as well as each other. TLL and CRL are difficult to compare, as they have different optimal temperatures, 50C and 37C respectively. In addition, CRL was only available in solid form, whereas TLL as well as NS40020 is available in solution. NS40020 and TLL are also both tolerant of higher temperatures and can be compared at the same temperature.

When using CRL to hydrolyse HEAR oil, we need to use more enzyme due to the form of the enzyme available, making direct comparisons less useful. The high initial rate of CRL may simply be due to the much larger enzyme concentration. On the other hand, the comparison of TLL and NS40020 is more appropriate as both are available in solution form. This comparison is closer to what we expected initially, with a slightly slower initial rate, but overall, more consistent rate, as can be seen in the second part of figure 22.

Comparable acid values are produced when we use 1% w/w CRL at 37C and compare the resulting acid value to using 0.01% w/w TLL at 50C. CRL requires 1000 times the amount of lipase to produce comparable results. During the initial reaction, CRL produces a high acid value, although this is likely due to the much larger amount of enzyme than TLL. After 24 hours the two enzymes produce comparable results, with CRL producing slightly less acid value overall. This is likely due to TLL's better stability at lower pH, allowing the reaction to progress at a faster rate for longer as more fatty acids are produced.

Comparing TLL and NS40020 is easier, both are available in liquid form, and both are stable at 50C. This allows us to compare equivalent enzyme amounts (0.01% w/w) at the same temperature (50C). NS40020 shows a slower increase in acid value at least initially within the first 3 hours. After 5 hours the rate of reaction becomes comparable between TLL and NS40020 and suggests similar pH stability.



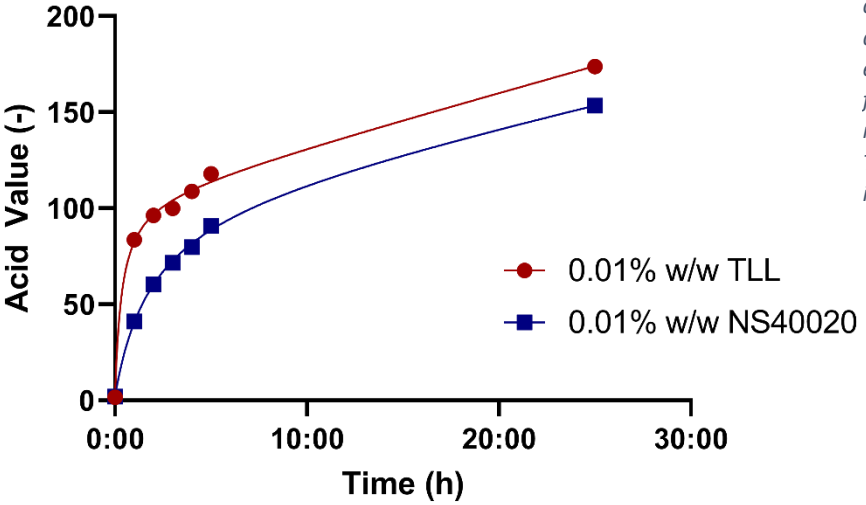


Figure 22: Comparison between TLL and nonspecific enzymes, CRL and NS40020.

CRL has different optimal conditions from TLL and was used at 37C for initial comparison. CRL was also only available as a powdered enzyme rather than in liquid form, meaning significantly more enzyme was used. Both TLL and NS40020 was available in liquid form.

5.6 Enzyme comparison summary

Results from previous sections were combined and used to evaluate and compare different enzymes for their ability to hydrolyse HEAR oil. Comparisons are made based on the acid value and identifiable products using TLC. Overall, 6 commercially available lipases as well as two experimental lipases were tested (see figure 23).

Lipozyme CAL-B is the free enzyme version of the immobilized Novozyme 435. Both were similarly unsuitable for splitting HEAR oil, producing very low acid values even after 24 hours of reaction, only reaching 30 and 50 AV respectively, which corresponds to a percentage of split of 16 – 28%. These enzymes are commonly used in industry today, however likely their activity is higher on shorter chain triglycerides and other applications.

Two different commercially available versions of TLL were tested, one sourced from Sigma Aldrich, one from Novozymes. Both produced similar results. NS40119 was also identified as a TLL variant based on its acid value results and TLC comparisons as shown in figure 23 below (and TLL section above).

Nonspecific enzymes that may also prove suitable for HEAR oil splitting were CRL and NS40020. Compared to TLL, CRL appears to have a more rapid initial reaction, reaching up to 130 AV after 5 hours, but a less active later part of the reaction, only reaching around 150 AV after 24 hours.

NS40020 on the other hand shows a less active initial reaction, only reaching less than 100 AV after 5 hours. However, its overall reaction reaches an equivalent AV to CRL after 24 hours indicating higher stability over the course of the reaction such as potential resistance to low pH from fatty acid production or glycerol tolerance.

CRL was only commercially available as a solid rather than the other high performing lipases which are available as enzyme solutions. For this reason, a larger amount of CRL had to be used than TLL or NS40119/NS40020 in these comparisons.

Positionally specific (1,3 specific) enzymes which will be used for experiments going forward are all available TLL variants, including NS40119, as these lipases showed the highest activity as measured by acid value after 24 hours.

Nonspecific enzymes for further use are CRL and NS40020. Both nonspecific enzymes produce less acid value after 24 hours of reaction, but in combination with 1,3 specific enzymes could be useful

for HEAR oil splitting. CRL was only available as a powder and is significantly cheaper than the other fungal enzymes.

The positional specificity of these lipases was determined using TLC (figure 24). A method to separate diglycerols was developed (described in the methods section), which allowed us to see the two different DAGs produced by nonspecific enzymes. Nonspecific enzymes produce equal amounts and 1, 3-specific enzymes produce a more of the 1, 2 DAGs than the 1, 3 DAGs, meaning a single DAG will be overrepresented.

For 1,3-specific lipases a single band can be seen for DAGs in figure 24. For Non-specific enzymes two separate bands can be seen for DAGs. MAGs are difficult to detect at higher acid values, as glycerol will similarly remain near the base line. Glycerol does not get stained by any of the methods used to stain glycerides and fatty acids. As a result, higher glycerol concentrations dilute spots for MAGs, making them more difficult to detect.

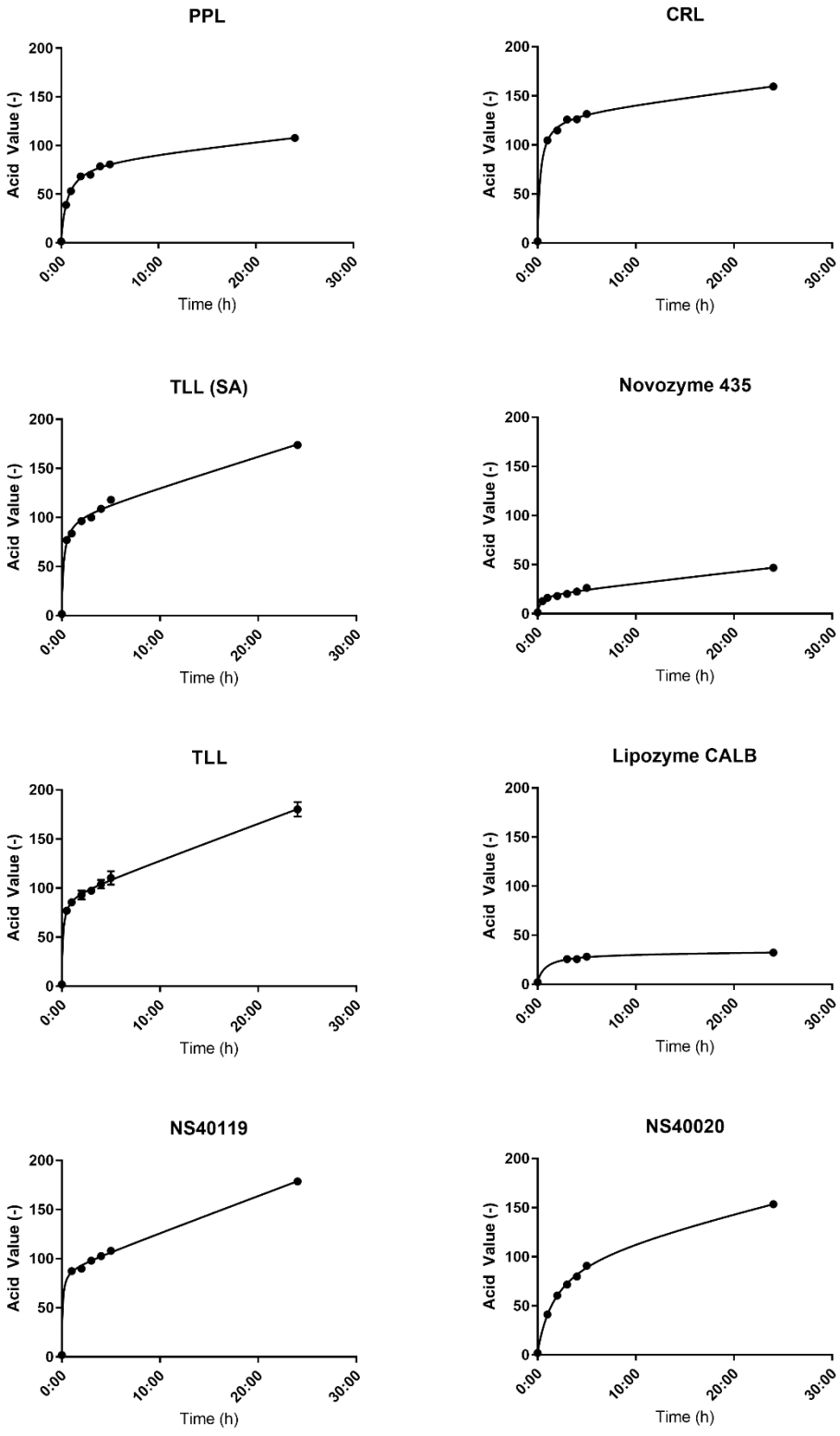


Figure 23: Comparison of enzymes used for HEAR oil splitting.

Lipases have different optimal temperatures, so where appropriate we used 30°C/50°C. Stirring 400 rpm, 1:1 oil to buffer ratio. 400 mM glycine, NaOH to ph 9. Two variants of TLL – Sigma Aldrich (SA) and Novozyme (NZ). All lipases are free enzymes except for Novozyme 435 which is immobilised, and a standard in industry which is why it is used as a comparison.

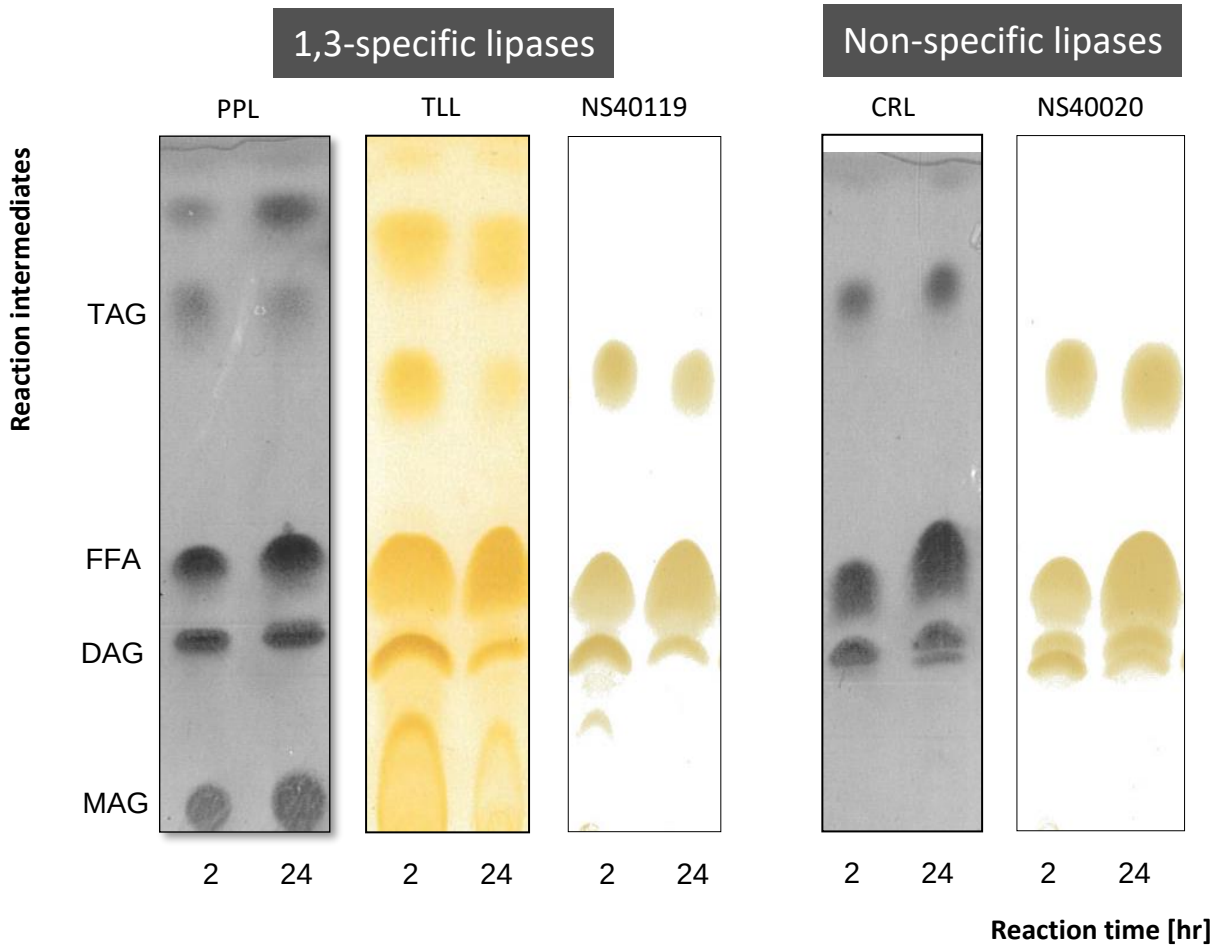


Figure 24: TLC comparison showing differences in the TLC profile of 1,3-specific lipases and non-specific lipases.

Differences are apparent in the diacylglycerol (DAG) band, which for non-specific lipases is split in two. Also visible are triacylglycerols (TAG) and free fatty acids (FFA). Monoacylglycerols (MAG) are only faintly visible in samples for NS40119, CRL and NS40020. The presence of glycerol, which remains at the same place as MAGs but is not stained by any used detection method dilutes the MAG spot.

As can be seen in figure 23, PPL produces a much lower amount of acid value overall, which is why MAG spots can be seen clearly and non-diluted in the PPL section of figure 24. Only a single DAG band is visible as expected. The FFA spot increases in size and intensity of colour from 2h to 24h. TAG are faint in both 2h and 24h spots. At the top of the TLC slide near the solvent front we can also see two spots that are unidentified but likely correspond to either fatty acid dimers or fatty acid methyl/ethyl esters (FAME/FAEE). (Gupta *et al.*, 2013)

These spots could be artifacts generated due to storage of the sample in ethanol, or through residual enzyme activity of lipases, which are known to produce FAME or FAEE in biodiesel production. (Ribeiro *et al.*, 2011; Chandra *et al.*, 2020)

TLL and NS40119 show a similar pattern, with an initially diverse sample at 2h showing presence of a single DAG band, an MAG band slightly diluted by the presence of glycerol and FFA and TAG bands. As glycerol does not get stained by our detection method, this could cause the diluted MAG to appear invisible. At 24 hours the FFA spot increase in intensity, while all other spots decrease in intensity. This is due to the sample approaching 100% split and a large majority of sample being FFA. A small amount of TAG and DAG remains even after 24 hours, however.

Candida rugosa lipase (CRL) also shows a very interesting pattern. AT 2 hours, only a single DAG spot can be seen clearly. No MAGs seem to be produced after either 2 or 24 hours. After 24 hours however, two separate DAGs can be clearly identified.

Although both NS40020 and CRL are non-specific lipases, for NS40020, initially the lower DAG appears more intensely on the TLC slide after 2 hours of reaction time. After 24 hours however, both DAGs are equally present in samples from NS40020 catalysed HEAR oil hydrolysis. These results helped us identify NS40020 as a variant of CAL-A, as a slightly sn-2 regiospecific lipase, which our TLC method is sensitive enough to detect.

6 Further TLL Investigation

TLL was the best performing enzyme out of all enzymes tested. All three variants of TLL, obtained from Sigma, Novozyme as well as the protein engineered variant NS40119 outperformed other enzymes. NS40020, (*Candida antarctica* lipase A) was the only enzyme which performed similarly with equivalently small amounts of enzyme used. As a result, for many of the optimisation steps taken in the following chapter TLL, or TLL in combination with another enzyme is used.

6.1 Design of Experiment – TLL Optimisation

Design of experiment (DoE) is an algorithmic way of generating a representative set of experiments from a large set of experiments with many variables with +1, 0, -1 values. In our set of experiments 7 different variables influencing the fat splitting reaction were chosen, Temperature, mixing speed, the oil to water ratio, the enzyme amount, and 3 factors relating to the buffer, the glycine amount, the NaOH amount and the $MgCl_2$ amount. Next, a +1, 0 and -1 value is assigned to each variable, and out of the more than 80 experiments 17 were selected as shown in table 8.

The 0 values are set as shown in italics in table 9, +1 values are shown in bold, and -1 values are regular text. These values were tested over 4-hour oil hydrolysis reactions.

As a result of covid we were not able to fully analyse these results using DoE software. As such a full justification for why these specific values of each variable were chosen can also not be given. These results will however still give an overview of the effect of the chosen variables on the a TLL based HEAR oil reaction.

Results displayed in figure 25 show that experiments which used 0.005% w/w of enzyme showed universally lower acid values throughout the 4-hour reaction. DoE 1, DoE 4, DoE 5, DoE 8, DoE 10 and DoE 12 show very low acid values and low rates of reaction; less than 100 AV is reached after 4 hours. Something went wrong with DoE 11, it is likely that the small amount of enzyme was not pipetted properly into the reaction mixture and no enzyme reached the reaction. Alternatively, it is possible that the sodium hydroxide added to bring the reaction up to pH 9 formed soaps with all the fatty acids formed.

DoE 2, DoE 3, DoE 9, DoE 13 and DoE17 all reached between 100 and 110 AV after 4 hours, which puts them in the middle of the list of acid values produced after 4 hours.

Table 8: Design of Experiment variables. +1 values in bold, 0 values in italics, -1 values normal. Highlighted in green are conditions that were favourable for the reaction and results for these are shown in figure 12.

DoE	Temp C	Mixing Speed	Organic/ Aqueous phase ratio	Enzyme Amount	Glycine Amount	NaOH added up to	MgCl2 Amount
1	40	**800 rpm**	**50%**	0.005%	0 mM	ph 7	**20 mM**
2	**60**	300 rpm	30%	0.005%	**800 mM**	ph 7	**20 mM**
3	40	300 rpm	30%	**0.05%**	0 mM	**ph 9**	**20 mM**
4	40	300 rpm	30%	0.005%	0 mM	ph 7	0 mM
5	40	300 rpm	**50%**	0.005%	**800 mM**	**ph 9**	**20 mM**
6	**60**	300 rpm	30%	**0.05%**	**800 mM**	**ph 9**	0 mM
7	40	300 rpm	**50%**	**0.05%**	**800 mM**	ph 7	0 mM
8	**60**	**800 rpm**	**50%**	**0.05%**	**800 mM**	**ph 9**	**20 mM**
9	**60**	**800 rpm**	30%	0.005%	0 mM	**ph 9**	**20 mM**
10	40	**800 rpm**	**50%**	**0.05%**	0 mM	**ph 9**	0 mM
11	**60**	300 rpm	**50%**	0.005%	0 mM	**ph 9**	0 mM
12	40	**800 rpm**	30%	0.005%	**800 mM**	**ph 9**	0 mM
13	**60**	300 rpm	**50%**	**0.05%**	0 mM	ph 7	**20 mM**
14	**60**	**800 rpm**	30%	**0.05%**	0 mM	ph 7	0 mM
15	*50*	*500 rpm*	*40%*	*0.01%*	*400 mM*	*ph 8*	*10 mM*
16	40	**800 rpm**	30%	**0.05%**	**800 mM**	ph 7	**20 mM**
17	**60**	**800 rpm**	**50%**	0.005%	**800 mM**	ph 7	0 mM

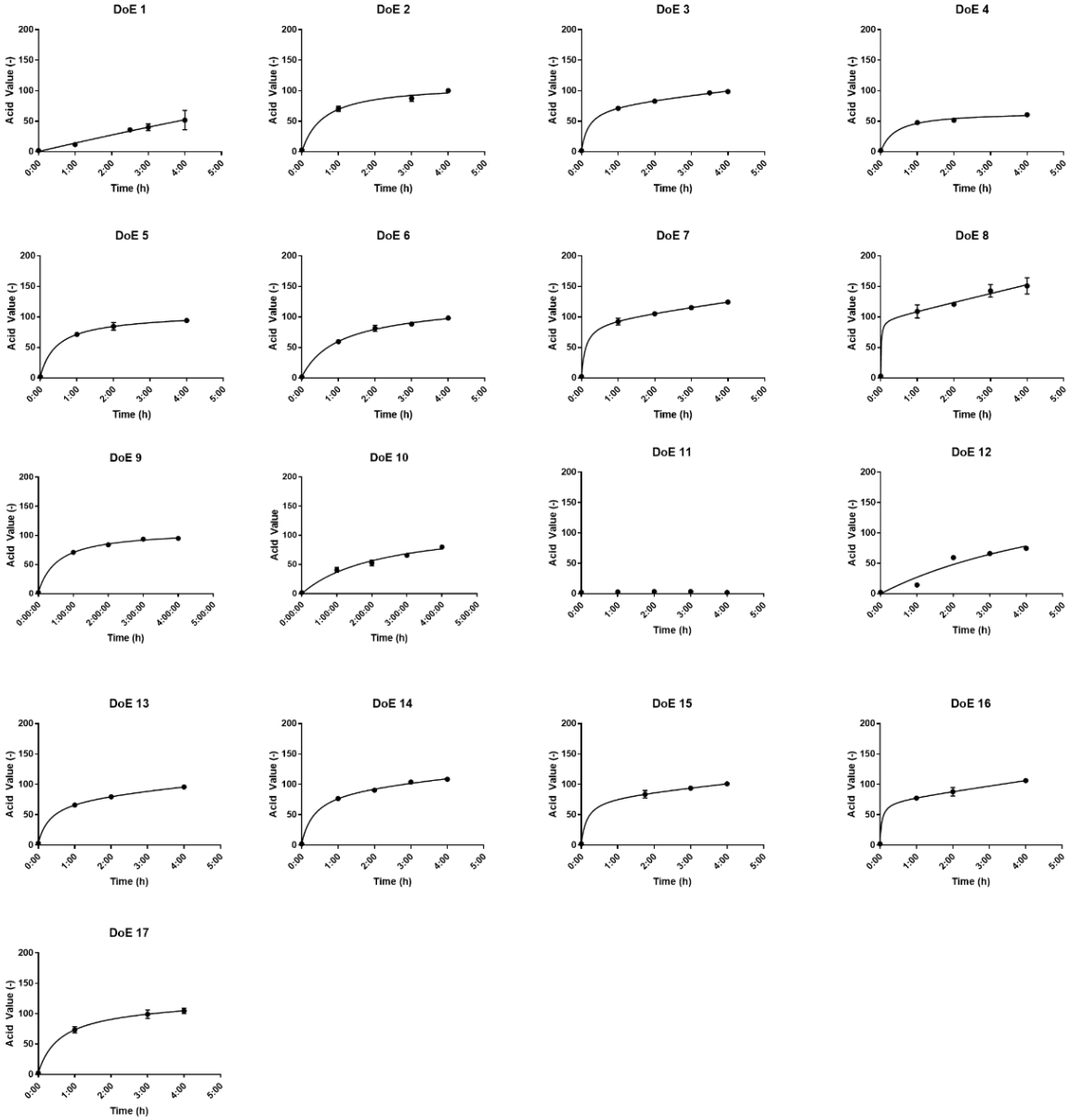


Figure 25: Complete design of experiment results.

All DoE results numbered as in table 6. Results are shown over 4 hours. 4 Experiments with the highest acid value are shown in more detail in figure 22 below.

Design of experiment number 8 turned out to be the highest AV producing conditions after 4 hours. Follow up tests over 24 hours showed that the reaction approached 100% of split with very few TAGs or partial glycerides left as shown in figure 26.

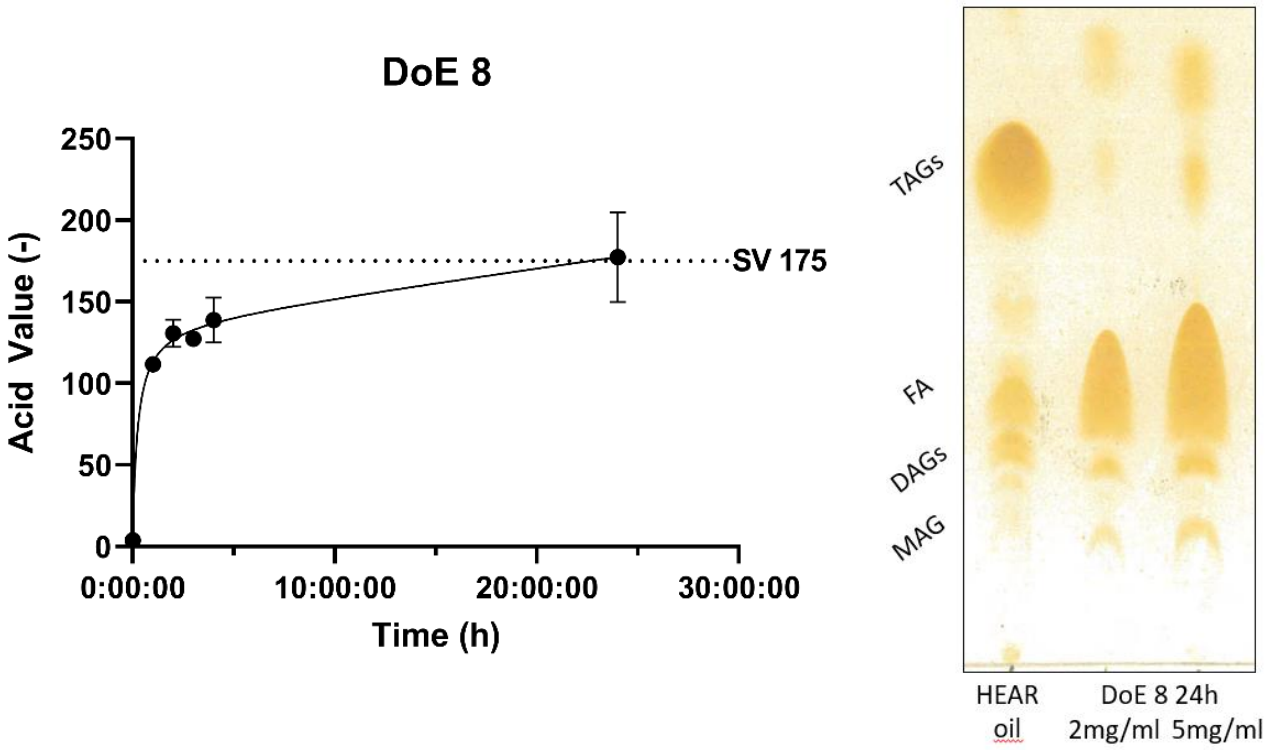


Figure 26: Results from the highest acid value producing experiments of the DoE, see table 3.

DoE 8 produced the highest acid value after 4 hours, and after 24 hours leads to full split (180-190 AV), with very few triglycerides or other partial glycerides left see TLC slide. The 24 h sample shows mostly free fatty acids, as expected from a near complete split.

6.2 Using Inductively Coupled Plasma Mass Spectrometry to detect ions in enzymatic HEAR oil hydrolysate

We used Inductively coupled plasma mass spectrometry (ICP-MS) to determine the concentration of metal ions in the organic as well as "middle" (non-dissolvable in heptane or water) phase of the enzymatic HEAR oil reaction. This was done to assess the impact of using buffers and how their use may change the end product.

Inductively coupled plasma mass spectrometry (ICP – MS) allows for extremely precise measurements of ion concentration in both organic and aqueous phase samples.

When separating the phases of HEAR oil hydrolysis reactions with buffer, we observe an aqueous, an organic and a smaller "middle" phase, which is where smaller, partially water-soluble organic molecules as well as minerals such as Magnesium, Calcium and others accumulate (as shown in figure 27).

The HEAR oil splitting reaction sample was separated using heptane and brine solution in a separating funnel to separate the organic and aqueous phase.

When separating the organic from the aqueous phase, a middle phase is formed, neither dissolvable in heptane nor the aqueous phase. This middle phase as seen in the table 9 and figure 27 below contains some amounts of Magnesium ions as well as Sodium ions.

The organic and middle phases were collected after the separation process, and Heptane was removed through rotary evaporation. Samples were then sent off-site for ICP-MS testing.

It is likely that these insoluble solids (the middle phase), which accumulate between the aqueous and organic phase are fatty acid salts/soaps with magnesium, as well as possibly Calcium and other metal ions. The large sodium concentration (highlighted in red in table 9 below) is likely due to the presence of some of the aqueous phase in the sample. The insoluble solids of the "middle phase" were difficult to separate from both the organic as well as the aqueous phase.

It also looks like the amount of metal ions in the middle phase decreases between 2h and 24h. This may be due to changing pH in the reaction mixture or an unknown enzyme related process.

Sodium also accumulates, in much larger amounts than shown in the figure 27, up to several thousand parts per million as highlighted in red in table 9. This is due to the use of Sodium hydroxide to adjust the pH of the buffer. The relatively large amounts of Magnesium are also due to the use of $MgCl_2$ in our buffer system.

Table 9: Results of organic phase and middle phase ICP-MS testing. Number corresponds to ppm in sample.

Elements (ppm)	Ca	Fe	K	Mg	Mn	Na
2h organic phase	8.845	1.120	2.492	5.728	0.0008	191.8
5h organic phase	6.695	0.7815	-0.9980	4.932	0.1347	252.4
24h organic phase (sample overheated)	10.45	1.230	-0.4138	6.970	0.1202	148.0
24 h organic phase	0.9397	1.350	-0.3925	1.002	0.058	187.7
24h Middle phase	32.45	5.581	9.983	65.70	0.257	71.44
2h Middle phase	252.4	18.88	20.45	636.8	0.7097	32170
HEAR oil	22.56	1.095	12.83	9.022	0.2203	-0.9092

Otherwise, a maximum of around 60 – 80 ppm of other mineral ions is present in the samples. Very little Manganese or iron is present in samples, these are ions which are filtered out of the HEAR oil in the degumming process. The presence of Mg and Calcium can however be necessary for enzyme function, and in any case a small presence of magnesium or calcium ions can enhance the activity of lipases.

The presence of these ions in a hard to separate middle phase are likely to interfere with currently established downstream processing techniques, which require the initial removal of many of these ions such as calcium and phosphates through degumming (see chapter 2.1.1).

Ions which accumulate in this middle phase are likely added through the glycine/NaOh with $MgCl_2$ buffer used, which contains magnesium and sodium.

As downstream processes are needed to produce a pure product, accumulation of ions which make downstream processing more difficult may be unwanted by industry. Additionally current methods produce no accumulated ions in the products as no buffers or additional ions from buffers or other additives are used.

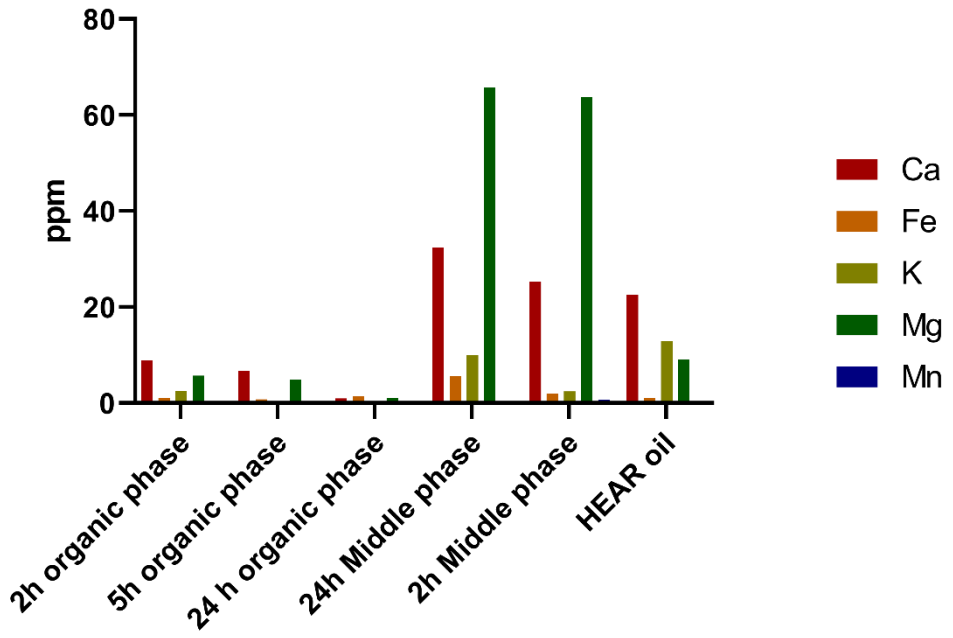


Figure 27: Results of Inductively Coupled Plasma Mass Spectrometry of partially split HEAR oil samples

Measure ion concentration in phase separated samples of HEAR oil splitting using TLL with glycing/NaOH buffer with MgCl2. Organic phases as well as a "middle phase" were separated. High concentration of Mg ions in this "middle phase" may indicate accumulation of Mg ions.

6.3 Non – Degummed Oil

In addition to using degummed, processed HEAR oil, as it is used in current industrial production, we had the opportunity to use a more crude, non-degummed (NDG) form of HEAR oil. The purpose of these experiments was to determine if there are any major differences in the rate of hydrolysis of NDG oil using PPL compared to degummed oil. For industrial considerations, non-degummed oil needs to be degummed before undergoing pressure splitting. Presence of metal ions or phosphates leads to discolouration and can damage to the end-product under pressure splitting, however enzymatic fat splitting occurs at a lower temperature and non-degummed oil could therefore be used for this process.

Inclusion of phospholipids as well as metal ions could also help enzyme function through emulsifying the reaction mixture. Several common metal ions could also help enzyme activity.

Using non-degummed HEAR oil can also help with mixing the organic and aqueous phases, as phosphates and minerals in the non-degummed oil may act as emulsifiers.

To determine if this emulsifying effect has a significant impact on the rate of hydrolysis, an oil hydrolysis reaction using non-degummed oil, both with and without buffer, and using both PPL as well as TLL was carried out. 2g of PPL/100g of non-degummed oil and 0.01g of TLL/100g of non-degummed oil were used. Experiments were carried out both with a glycine/NaOH buffer as well as without buffer.

Results, as shown in figure 28 suggest that there is no major difference between using NDG oil or degummed HEAR oil for enzymatic fat splitting, with two different commercially available enzymes, porcine pancreatic lipase (PPL) and *Thermomyces lanuginosus* lipase (TLL). There are minor qualitative differences which are not recorded in data, such as a slightly slower separation of oil and water phases after the reaction or during sample taking. In one reaction, a semi-stable emulsion forms, which needed to be separated using hexane and brine solution, even after the reaction contents are no longer stirred. In addition, non-degummed HEAR oil has a slightly lower pH, and a comparable acid value, 1.8 – 2 for non-degummed oil compared to 1.3-4 for various batches of HEAR oil.

No major difference between degummed and non-degummed HEAR oil could prove to be a major advantage for the development of an industrial process. The ability to skip degumming processes has the potential to save costs, energy, and equipment. Of course, there are other potential

problems such as downstream processing still requiring higher temperatures and pressures potentially.

Degumming oils allows them to be subjected to higher temperatures and other harsher conditions without discolouring or other damages to the product. As such, if NDG oil is used, it is likely that follow up processes such as fractional distillation and other separation processes would need to be altered.

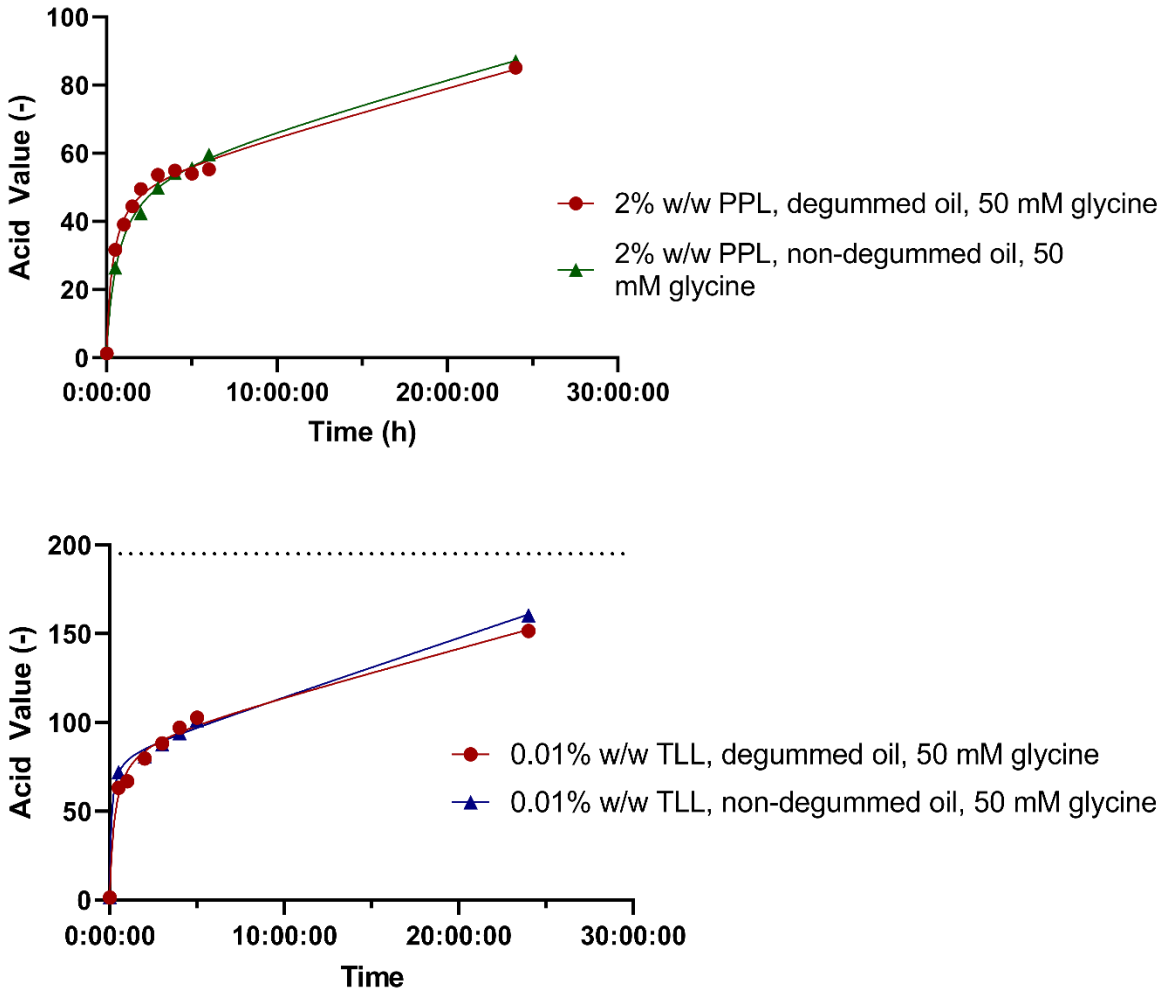


Figure 28: Experiments hydrolysing degummed and non-degummed (NDG) oil .

With 2% w/w PPL, and 50 mM glycine/NaOH buffer there are no major differences in how degummed and non-degummed oil reacts under these conditions.

With 0.01% w/w TLL and 50 mM glycine/NaOH buffer, again no major differences between degummed and non-degummed oil can be observed for the lipase catalysed HEAR oil hydrolysis reaction.

6.4 Different Reactor Types for HEAR Oil Hydrolysis

Oscillatory baffled reactors (OBR) are a special type of reactor that can provide improved mixing and therefore improve the rate of reaction. Most commonly they are found in the form of continuous oscillatory baffled reactors (COBR), although for the purposes of this reaction we will be using a batch version. Typically, they are tube shaped reactors with ring shaped baffles creating eddies in the liquid. These eddies are responsible for mixing the reaction mixture in the inter-baffle zone, as shown in figure 4 in chapter 2.3.

Each inter-baffle zone acts as its own batch reactor, leading to overall better mixing than a single stirred batch reactor. Although most commonly these types of reactors are used in continuous systems, for our purposes to test out a HEAR oil hydrolysis reaction a batch version of OBR is used.

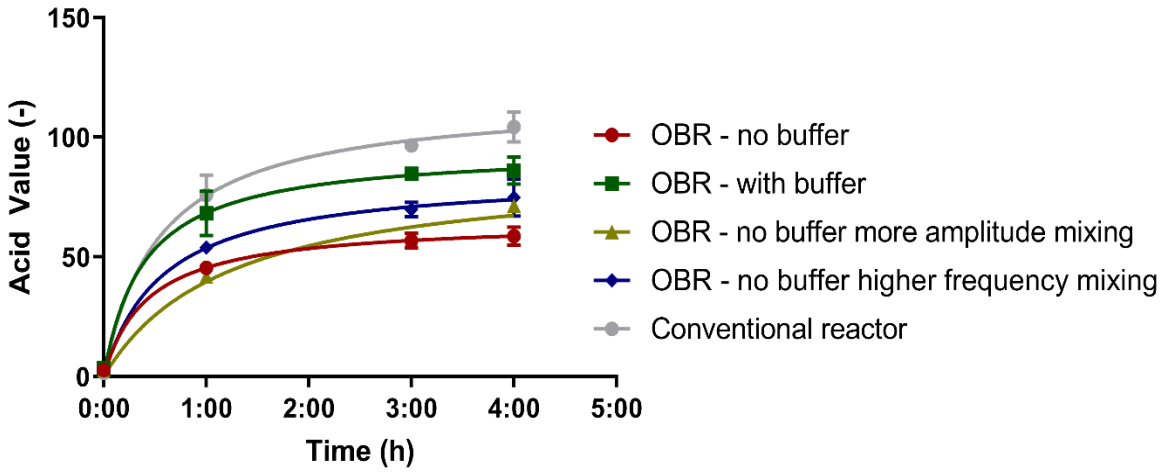


Figure 29: Oscillatory baffled reactor (OBR) for oil hydrolysis.

HEAR oil splitting reaction using TLL in an OBR over 4 hours. Reaction Conditions: 0.01% w/w TLL, 50°C, 1:1 water to oil ratio, the buffer used in the "with buffer" experiment was a 400 mM glycine buffer. Amplitude had a low and high setting which were varied. Frequency of mixing was also doubled for the "higher frequency mixing" experiment

As shown in figure 29, over 4 hours of reaction time, a batch OBR was used to hydrolyse HEAR oil with TLL as the enzyme. The progress of the reaction, measured by AV was comparable to the AV produced in a standard batch reactor after 4 hours. Both, buffer, and no buffer as well as three different mixing settings were used, to investigate if better mixing can have a similar effect on the acid value as a buffer.

Ultrasound mixing is a method where high frequency ultrasound waves are used to create an alternating high/low pressure cycle, which creates vacuum bubbles. These bubbles release shockwaves into the mixture when they collapse or merge, which produces the mixing effect. There are several previous reports on ultrasound mixing being beneficial for enzymatic mixed phase systems.

Otherwise, the reaction setup is a closed loop, where the reaction mixture is pumped through an ultrasound mixing chamber/vessel. For the ultrasound mixing vessel used, a flow rate of 100 – 400 g/min is recommended. We used a peristaltic pump at 60 rpm to pump our reaction mixture through, which resulted in a flow rate of 240 g/min. In addition, we used the 20% power setting on the ultrasound mixing device (see results figure 29).

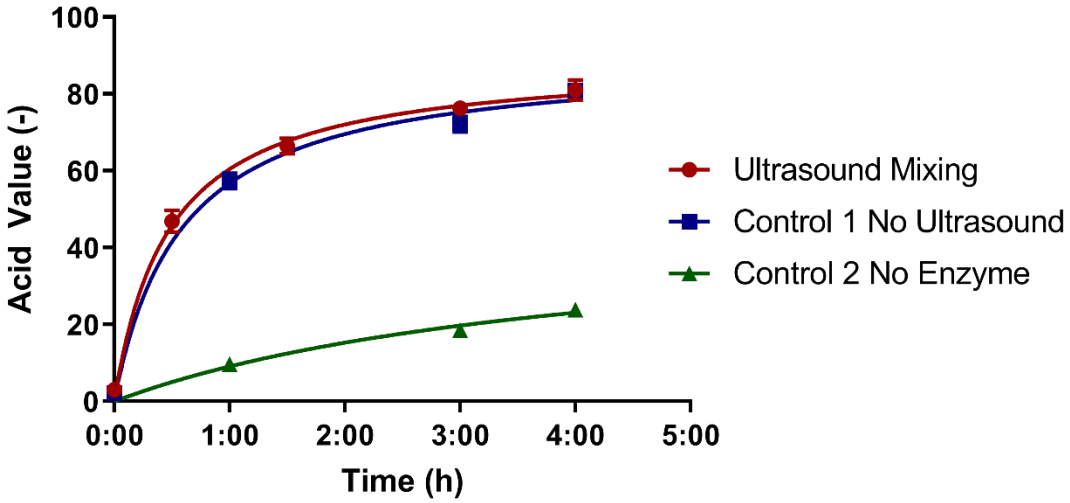


Figure 30: HEAR oil splitting reaction in an ultrasound mixing reactor

AV is compared over 4 hours, ultrasound mixing is unsuitable for this reaction, and appears to have no effect on the reaction.

Ultrasound mixing, at 20% power, appears to have no effect positive or negative on HEAR oil splitting using TLL (see figure 30). A negative control returned some acid value over 4 hours, and the formation of an insoluble white material, likely some fatty acids or partial glycerides, along with a

foul smell was also observed. Since this did not appear in the control 1 result, it is not likely to be uncleaned deposits from previous use within the reaction chamber. Instead, there is a possibility of peroxide formation when using ultrasound mixing involving a large aqueous phase, and this is a possible explanation for some activity when no enzyme is present. In any case ultrasound mixing does not appear to have a significant effect on enzyme based HEAR oil splitting. Possibly the amounts of enzyme used in our process, especially when using TLL and other solution-based enzymes, is simply too low to see any difference between stirred mixing and ultrasound mixing.

6.5 Enzyme Combinations

In addition to using single enzymes for the catalysis of HEAR oil hydrolysis, a combination of 1,3 specific and nonspecific lipase was used to increase the overall rate of hydrolysis of the reaction. It is clear by now that nonspecific and 1,3 specific lipases have different rates and ideal conditions. However, NS40020 (CAL-A) and NS40119 (TLL) both have very high reported temperature stability, and as such higher temperatures can be used in a reaction where both lipases are used in combination. In addition, the unique, slightly sn-2 specific mode of reaction of NS40020 could help improve the overall reaction rate of TLL, which preferentially cleaves DAGs.

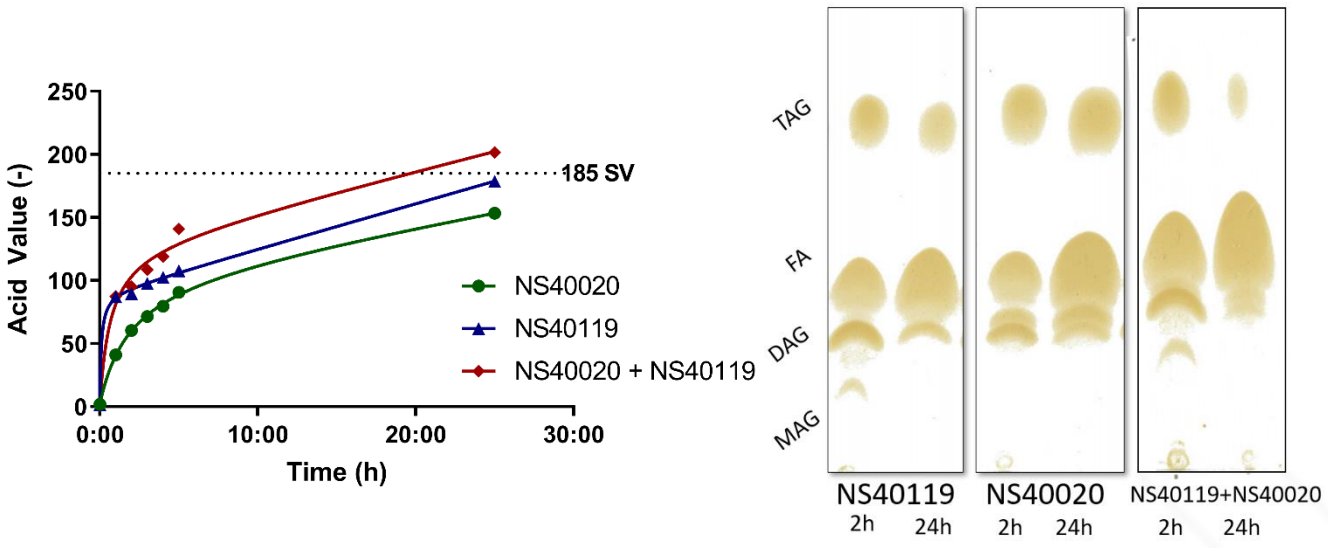


Figure 31: Comparison between Novozyme™ enzymes and combination

NS40020 and NS40119, as well as a combination 3:1 ratio NS40119 and NS40020. The buffer used for this reaction is 400 mM glycine/NaOH buffer pH 9 with 10 mM $MgCl_2$.

NS40019 + NS40020 initial splitting is 1, 3 specific – 1 single DAG seen. At 24 hours mostly FFA, some nonspecific splitting as two DAGs are present.

Equivalent amounts of enzyme were used both for individual enzymes as well as enzyme combinations. A 3:1 ratio of NS40119 to NS40020 was used in the reaction shown in figure 31. An overall acid value of 198 was reached after 24 hours. This corresponds to more than 100% split, likely this value is a slight outlier, although the TLC slide also shows only a very faint TAG spot remains after 24 hours, and the majority of the sample is free fatty acid.

TLL and CRL were also combined and compared. Although CRL does not have the sn-2 specific mode of reaction that NS40020 possesses, it is a nonspecific lipase, and production of different types of

DAGs could be beneficial for the overall rate of reaction. The problem with combining TLL and CRL was that both have different optimal temperatures. CRL was also available as a solid powdered enzyme compared to TLL which is available as an enzyme solution. As a result, dosing the lipases is trickier than with comparing two solution-based lipases as in figure 31. So, for combination of TLL and CRL, 0.007% w/w of TLL are used, and 0.3% w/w of CRL are used. Results were compared to 0.01 % w/w TLL used for HEAR oil splitting and 1% w/w of CRL used for HEAR oil splitting.

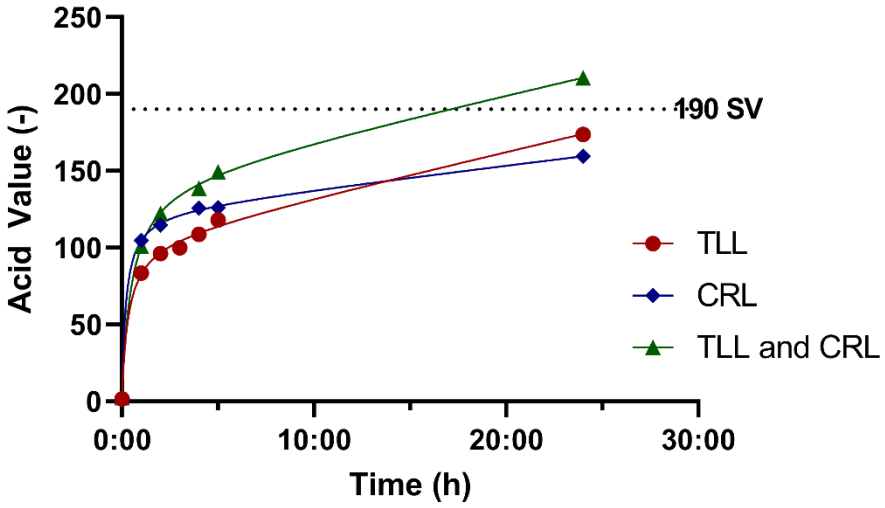


Figure 32: Using combinations of 1,3 specific (TLL) and nonspecific (CRL and NS40020) lipases for HEAR oil splitting.

As shown in figure 32, combinations of TLL and CRL reach more than 100% split, suggesting some errors in measurements. Similar results were also shown in figure 30. Combinations of TLL and CRL have comparable initial results but produces significantly more acid value after 24 hours. Up to 210 AV is reached. After 5 hours an acid value of 149 is produced by the combined use of TLL and CRL. This corresponds to a degree of split of 79%. This is likely due to interference of the buffer with acid value measurements as described in section 4.

7 Enzyme Immobilisation

7.1 TLL Immobilisation

TLL was chosen as the model enzyme for immobilisation. The enzyme was immobilised on 6 different resins as described in the methods section (3.2.9) and tested for its ability to split HEAR oil under equivalent conditions to the free enzyme using acid value measurements.

Six different resins for enzyme immobilisation were provided by Purolite. These were from the Lifetech™ ECR Enzyme Immobilization Resin range. Three resins were used for covalent binding, the remaining three were used for adsorption binding (see table 3 in the methods section)

During the immobilisation procedure, the protein concentration was measured using a Bradford Assay to determine the amount of free enzyme, and therefore the amount of enzyme bound to the resin. This was initially done over 24 hours, later it was determined that most of the enzyme was bound after just 3 hours, as shown in the initial section of figure 33.

The protein concentration of the remaining protein solution was measured, to allow us to use equivalent amounts of immobilised enzyme and free TLL for HEAR oil hydrolysis. This allows for direct comparisons between free TLL and immobilised TLL activity.

Reactions were carried out in triplicate and the reaction progress was measured in the same way as free enzyme using acid value over time. Acid values were then compared between each of the different enzyme-resin complexes and free enzyme.

Epoxy – methacrylate (ECR8215) shows the highest activity, comparable to that of the free enzyme. Specifically, unsuitable for the purpose of this reaction is ECR1030, which shows the lowest activity of all the immobilisation resins compared.

Similarly, TLL on ECR8285 has a lower activity than ECR8215, despite both being made from similar materials. In general, covalently bound TLL appears to perform better than adsorption immobilised TLL. Additionally, a pattern can be seen where resins with larger pore sizes, such as ECR8215, perform significantly better for HEAR oil hydrolysis than resins with smaller pore sizes such as ECR8285. Very small pore sizes such as ECR1030 are particularly unsuitable. This is likely due to the relatively large size of the average triglyceride molecule in HEAR oil, with two C22 and a C18 fatty acid bound to its glycerol backbone.

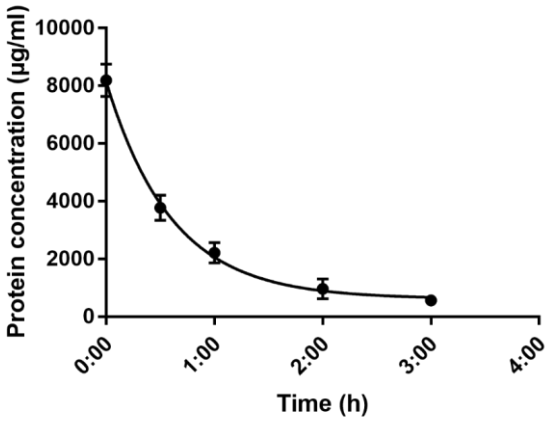


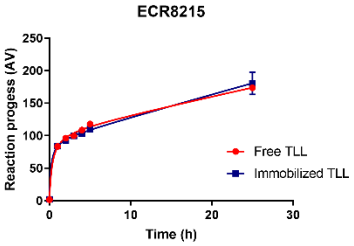
Figure 33: TLL immobilisation summary

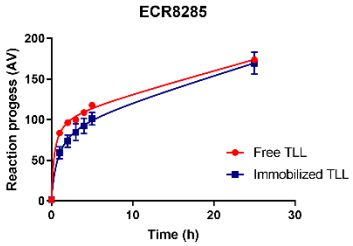
To the left is an example of the protein concentration in the supernatant over 3 hours of an immobilisation reaction with the enzyme. The amount of enzyme present levels out over around 3 hours.

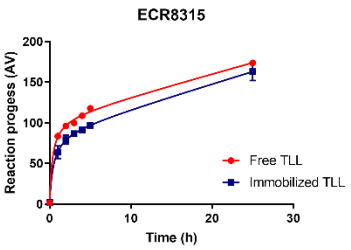
Below are comparisons of the activity of 6 different resin/TLL complexes during HEAR oil hydrolysis with free TLL. Covalently and Non-covalently bound TLL shows different activity.

Full average values available in appendix.

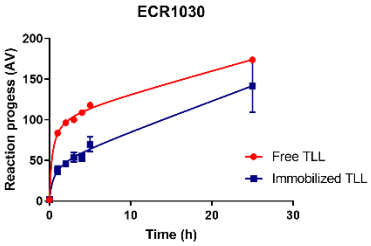
Covalent Resin

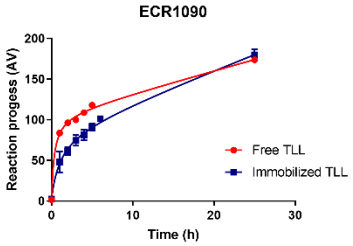


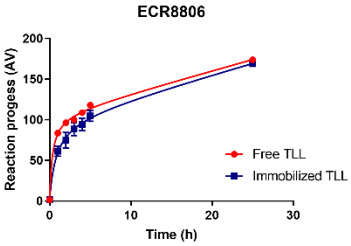




Non-covalent resin







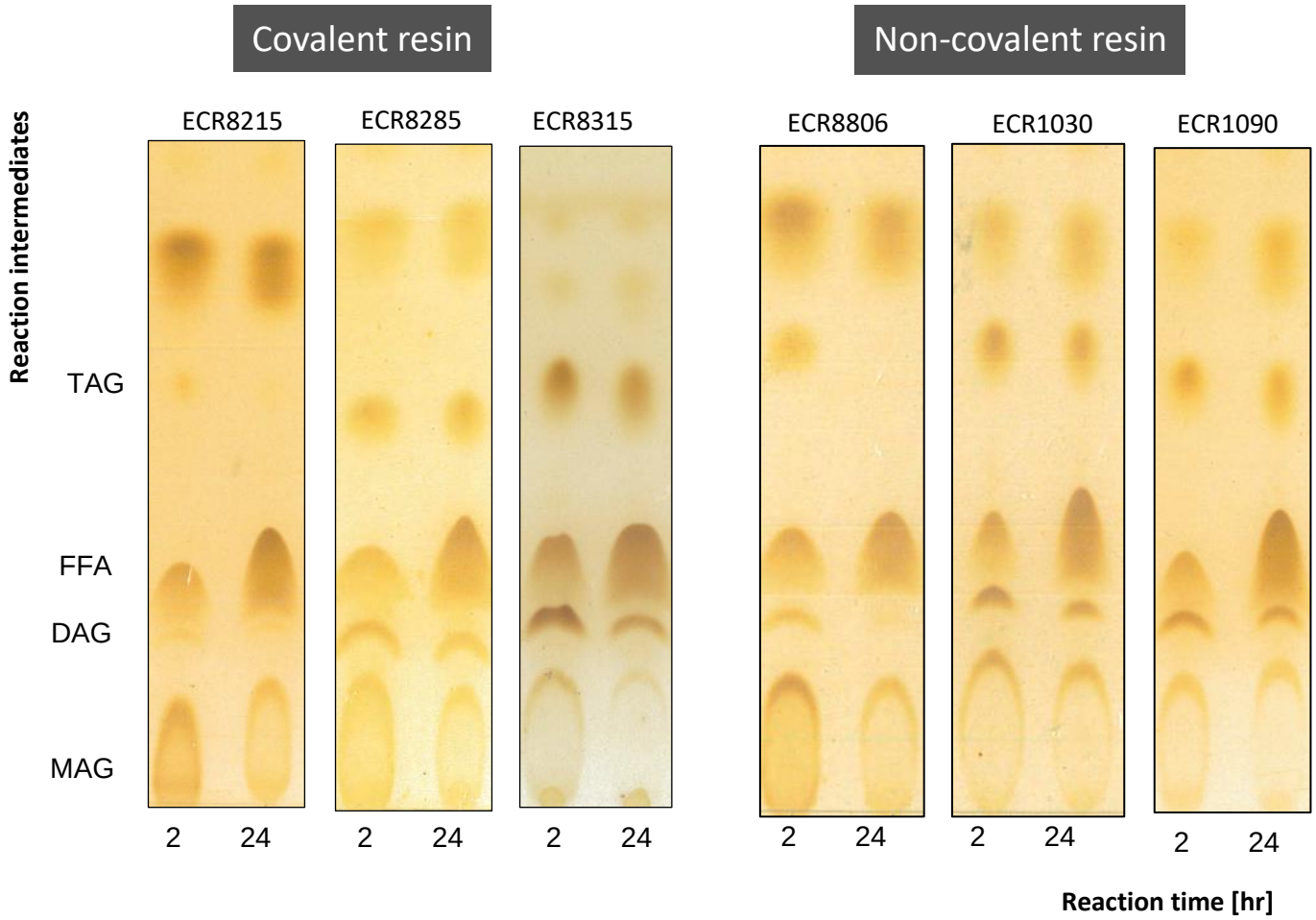


Figure 34: TLC comparison of TLL on 6 immobilisation resins.

TLL immobilised on 3 covalent and 3 non-covalent resins and used for HEAR oil hydrolysis. TLC gives some indication to level of split as well as amounts of partial glycerides.

The specificity of TLL also seems to change slightly depending on which resin is used for immobilisation as can be seen in figure 34. There are very few DAGs present in especially the 24-hour samples of ECR8215-TLL and ECR8806-TLL based HEAR oil splitting, suggesting a preference for cleaving DAGs. ECR8315-TLL in contrast seems to produce a lot of DAGs in especially the 2-hour sample, suggesting a preference for TAGs at least during the initial reaction. There are no major differences in the TLC profile of ECR8285-TLL, ECR1030-TLL, ECR1090-TLL and free TLL which are worth mentioning.

7.2 Reusing TLL on Epoxy Methacrylate (ECR8215)

One of the goals of enzyme immobilisation is the reusing of enzymes. Although the stirred jacketed reactor is not an ideal system for reusing enzymes it is possible to filter out immobilised enzyme from the reaction mixture.

However, even immobilised enzymes with their increased stability will lose some activity, and some enzyme will likely be washed off the resin during the reaction. Unfortunately, no data on how much enzyme was lost to this effect was gathered. As a result

To determine the possibility of reusing ECR8215-TLL a reaction with this enzyme-resin complex was set up using the equivalent of 0.01% w/w free TLL in ECR8215-TLL. After this initial reaction, for separation of enzyme resin from the reaction mass, the reaction mass was split up and spun down at relatively low rpms (1000 – 2000 rpm) to protect the immobilised TLL. The ECR8215-TLL enzyme resin was then be washed using n-hexane and filtered out through standard filter paper, to remove any fatty acids or acyl glycerides remaining on the resin.

The resin was then be washed with water and filtered out to remove the remaining solvent.

A second HEAR splitting reaction was then set up using the recovered and washed ECR8215-TLL. The results are in figure 35 below.

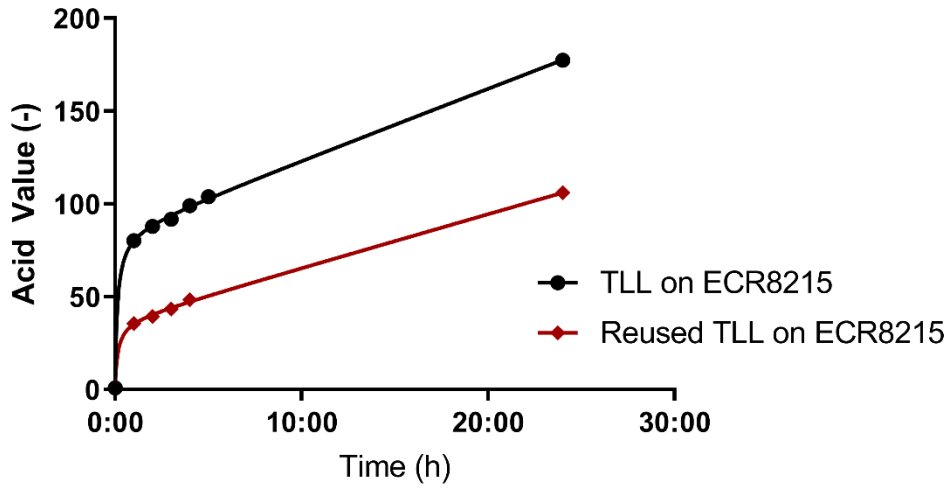


Figure 35: Attempting to reuse TLL immobilised on ECR8215 in a batch tank reactor.

Enzyme resin was filtered and washed before reuse.

Reused TLL resin appears to lose some activity, although it is likely to be a higher activity than the above graph indicates. It was not possible to recover all of the used enzyme. Of the 1g of immobilized enzyme added, 0.5g was recovered, half of the initial material.

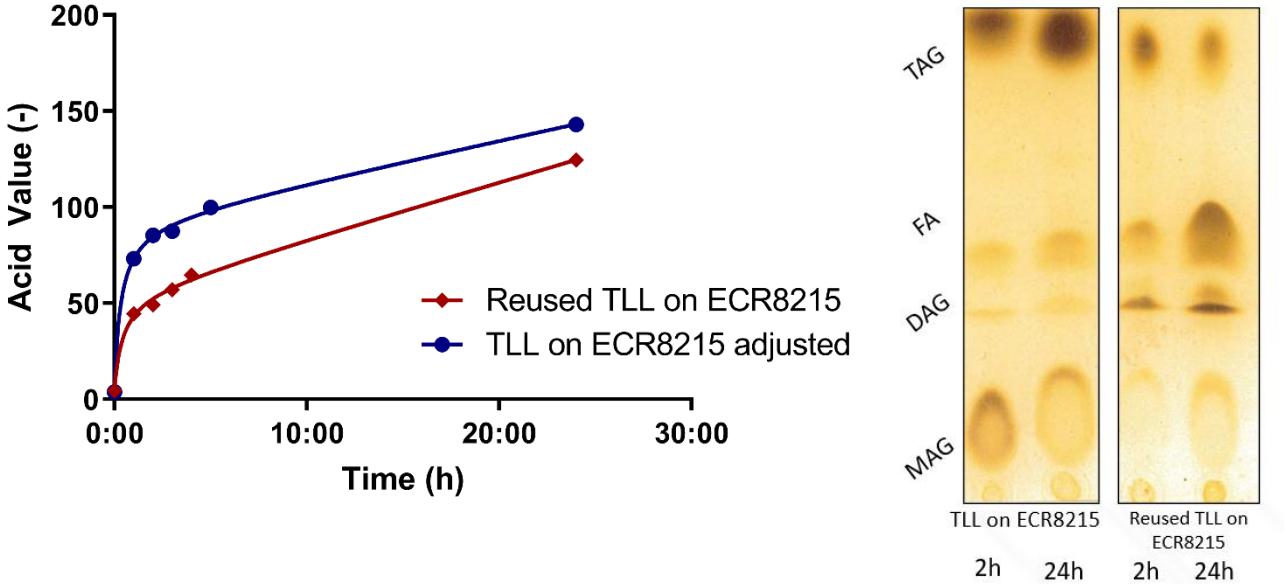


Figure 36: Immobilised TLL, reused after filtering out from a reaction mixture after 24h. Enzyme amounts were equivalent and adjusted to the amount I could recover.

For the above experiment the immobilised enzyme was filtered, then weighed and an equivalent amount of fresh immobilized enzyme was used to compare the fat splitting activity. Shown in figure 36, TLL immobilised on ECR8215 loses around 20-30% of its activity after 24 hours at 50°C.

Overall, the TLC slide (figure 36) shows that very few MAGs are being produced, instead we can see a greater concentration of DAGs in the reused TLL section. This indicates a somewhat lower activity, which is supported by the acid value tests. Top part of this TLC slide is not well resolved. TAGs and other high hydrophobicity spots likely conflicting and not separated.

7.3 Reactor Design for Immobilised TLL

To determine the reusability of immobilised TLL and work with a more appropriate reactor design for immobilised enzymes a fixed bed reactor was used during a temporary trip to a CRODA laboratory. The reaction mixture of oil and water/buffer was pumped through a fixed bed reactor at a flow-rate of 20 ml/min, holding immobilised TLL between two permeable surfaces.

For this experiment only commercially available immobilised TLL (Lipozyme TL IM) was available, rather than the in-house immobilised TLL on ECR8215. The exact method of immobilisation of this TLL preparation is not known to us. This resulted in lower acid values, and as can be seen in figure 37 below. Reusing enzymes in this setup also resulted in a very low acid value. This is possibly due to water washing the commercially available immobilised TLL of its immobilisation resin, as the immobilised enzyme is usually used in the reverse reaction, where water is removed to allow an esterification reaction to occur, and therefore water stability is not a concern.

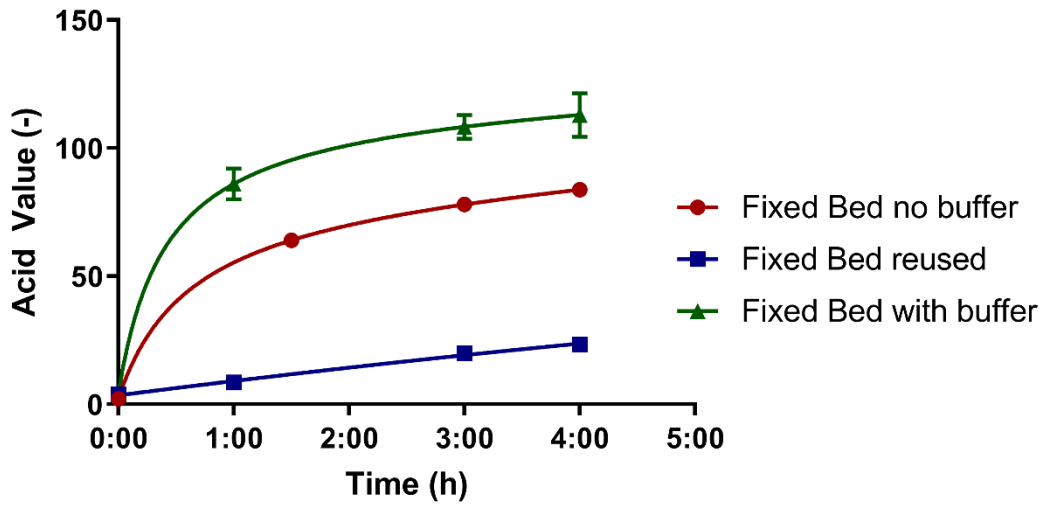


Figure 37: HEAR oil hydrolysis using commercially available immobilised TLL in a fixed bed reactor

The reaction is tested over 4 hours. Comparisons are between using buffer/no buffer as well showing reused enzyme.

As figure 37 shows, this experiment can't be used to suggest anything about the viability of reusing immobilised enzymes, although it does show that the specific type of immobilised TLL can't be reused. As the reaction mixture contains 50% w/w water, and water washes the enzyme from the resin, reuse with this type of immobilisation is impossible for the HEAR oil splitting reaction.

7.4 Immobilisation of TLL variant (NS40119) and CAL-A (NS40020) on Epoxy Methacrylate Support

ECR8215, epoxy methacrylate was also used to immobilise other versions of TLL as well as other lipases. Specifically, NS40119, a TLL variant was immobilised and compared to free enzyme. Reactions were carried out in triplicate and the reaction progress was measured the same way as free enzyme using acid value over time.

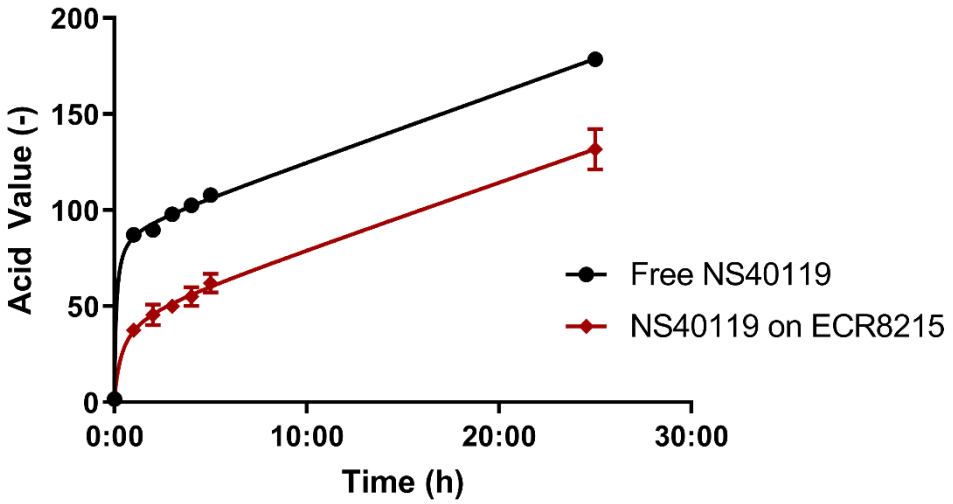


Figure 38: Comparison of free NS40119 (TLL variant) with NS40119 immobilised on ECR8215 for HEAR oil splitting.

Shown in figure 38 are triplicate result of NS40119 immobilised on ECR8215 compared to free NS40119. A clear reduction in amount of fatty acid being produced by immobilized NS40119 compared to free NS40119. This is somewhat unexpected, as TLL showed excellent activity bound on ECR8215. The mutations which enhance the activity of the free NS40119 variant may however hinder its activity when bound to a solid support.

A combination of NS40119 and NS40020 was also immobilised on ECR8215. Amounts of enzyme used were equivalent to single enzyme immobilisation, simply a 1:3 ratio of NS40020 to NS40119 was used.

The combination of free NS enzymes shows much higher activity than the immobilized combination. However even the immobilized combination shows a slightly higher activity than the immobilized NS40119 enzyme on its own, despite equivalent amounts of enzyme being used.

Immobilized NS40119 and the immobilized NS enzyme combination result in similar acid values when used for HEAR oil splitting.

Free enzyme clearly produces more fatty acids, especially in the initial reaction, reaching an acid value of over 140 within 5 hours. For the later part of the reaction (5h – 24h) both free and immobilised enzyme produce similar amounts of fatty acid (see figure 39).

There is a strong presence of both DAGs in both immobilized samples, whereas there is only very faint presence of DAGs in free enzyme sample.

An essentially complete split (99%) is observed in the 24h sample of the free combination of enzymes. The immobilized combination of NS40020 and NS40119 shows increased production of the secondary DAG, especially after at 24h.

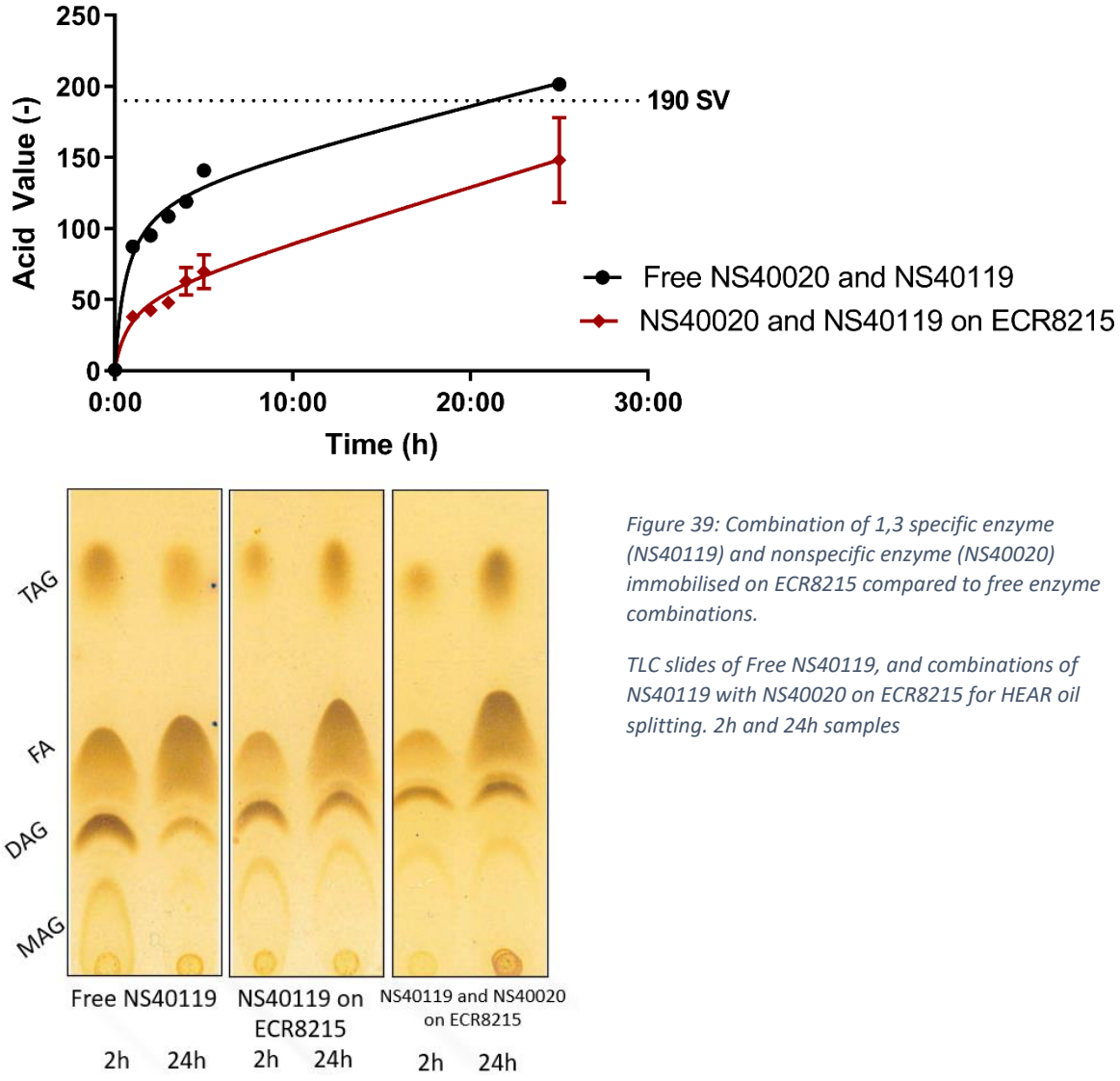


Figure 39: Combination of 1,3 specific enzyme (NS40119) and nonspecific enzyme (NS40020) immobilised on ECR8215 compared to free enzyme combinations.

TLC slides of Free NS40119, and combinations of NS40119 with NS40020 on ECR8215 for HEAR oil splitting. 2h and 24h samples

8 Pre Split Results

8.1 Pre-Splitting using TLL

Pre-splitting is an alternative method of using lipases for the HEAR oil splitting reaction. Enzymatic oil splitting reactions have significantly lower rates of reaction than currently employed industrial methods such as the Colgate-Emery method. However, even these highly efficient and fast methods have some inefficiencies. The reaction does not reach its maximum rate immediately and there is a lag period where the oil and water phase of the reaction do not mix well.

Pre-splitting produces some partial glycerides (MAGs and DAGs) from the HEAR oil, which act as emulsifiers, which could eliminate the lag period and help the oil and water phases mix from the start of the reaction. However, conventional splitting towers as they are currently employed for industrial production of free fatty acids cannot take a high amount of initial "solid" content, meaning fatty acids and partial glyceride content. So, to improve the non-enzymatic chemical splitting reaction using pre-split material, the progress of the pre-splitting reaction needs to be limited and tightly controlled.

For our initial pre-split tests, very small amounts of water (1-10% w/w of oil – see figure 40) were used to limit the rate of reaction, as well as the overall progress of the reaction. The amount of enzyme used was also very small, 100 ppm of TLL (0.01% w/w). A larger amount of enzyme can be used to speed up the initial rate of reaction and reach target acid values. For example, using 200 ppm compared to 100 ppm of enzyme will allow for an overall faster progress of partial splitting, although the overall progress of the reaction is still limited by the amount of water present.

The overall progress of reaction remains the same after 24 and 48 hours but the initial rate of reaction changes.

Limiting the amount of water allows us produce up to ~60 AV reliably within 24 hours. Using more than 4-5% w/w water means that the water may not run out in 24 hours and the reaction continues slowly. This is due to the enzyme losing some of its activity as more fatty acids are produced.

Graphs in figure 46 show comparisons of 10% w/w water and 200 ppm TLL based HEAR hydrolysis compared to the 5% water/100 ppm TLL and 10% water/100 ppm TLL reactions.

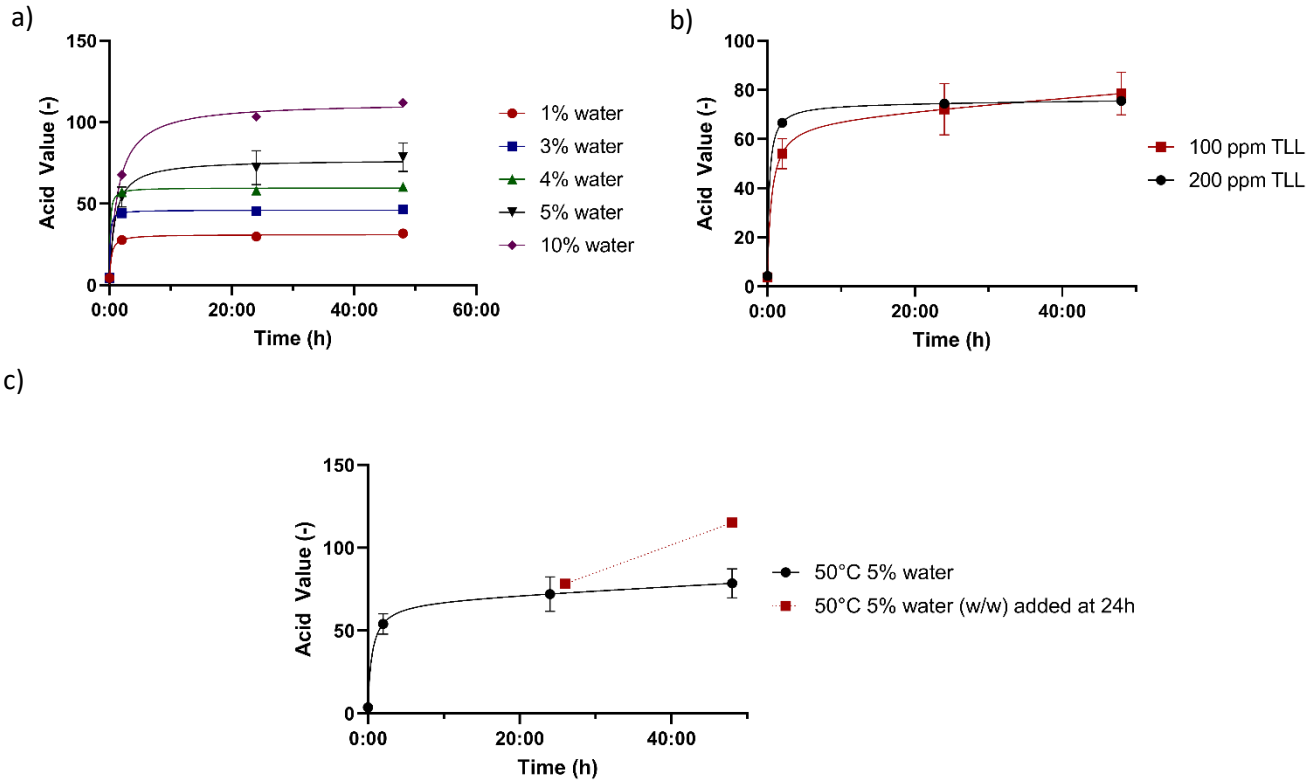


Figure 40: 6 Pre-split HEAR oil hydrolysis reaction using 100 ppm TLL at 50C.

a) 1%, 3%, 4%, 10% w/w water compared to 5% w/w water, b) 100 ppm TLL compared to 200 ppm TLL with 5% w/w water, c) 5% w/w water is used, after 24 hours the reaction is restarted with the addition of an extra 5% w/w water.

Using 5% w/w water as a baseline, different amounts of water are used to limit the progress of the HEAR oil splitting reaction. When using 1% w/w water, the reaction stops at 20-30 AV, or around 15% split. Water runs out likely 30 mins to 1 h into the reaction, and the reaction progress stops.

Using 3% w/w water, the reaction progress stops at an AV of 42. This corresponds to around 25% degree of split. Using 4% water, the reaction progress stops at an AV of 60. This corresponds to a degree of split of 36% (figure 40 a).

Attempting to use more than 4% w/w water leads to a longer reaction time before the measured AV levels out. 36% split likely represents the limit of the initial fast triglyceride hydrolysis, and all fatty acids which are easy to split from the triglyceride backbone have been split. Using 10% w/w water we can see the reaction reaches 70 AV after 2 hours but increases to around 100 AV after 24 hours and reaches 110 AV after 48 hours. Although the progress of the reaction slows down significantly it does not stop completely (figure 40 b).

Increasing the amount of enzyme used only has limited success (figure 40 c), and only the initial rate of reaction within the first two hours can be increased. This is expected and provides some evidence that water rather than enzyme concentration limits the overall progress of the reaction.

Figure 39 f) shows that it is indeed water which is limiting the progress of the reaction. We added an additional 5% w/w water to a reaction with an initial 5% w/w water after 24 hours of reaction time. The acid value was then measured at 2 hours and 24 hours after addition of the extra 5% w/w water. The acid value increased from 65 to 105 in the experiment where 5% w/w water was added at 24 hours, whereas it only increased to an AV of 67 in the experiment where no extra water was added. This shows that the progress of the reaction is limited by water.

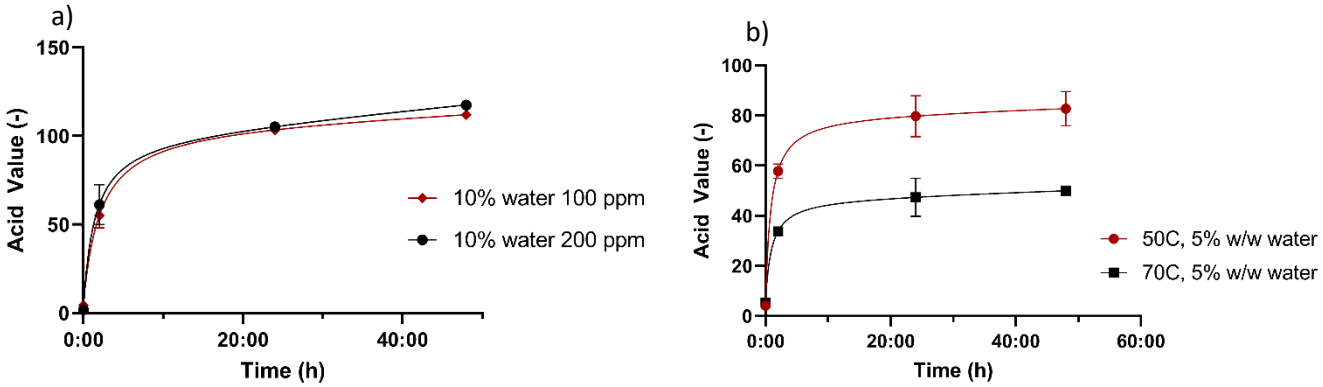


Figure 41: Comparison (a) between 100 and 200 ppm TLL using 10% w/w water and (b) 5% w/w water at 50 and 70°C

Using 10% water with 100 and 200 ppm TLL seemingly shows little difference in total acid value produced after 24 or 48 hours (see figure 41 a). This suggests that other factors than enzyme availability influence the progress of the reaction. The main factor is still availability of water; however, this cannot be the only limiting factor or the acid value would not consistently increase between 24 and 48 hours.

I suspect a combination of lack of available water, which leads to a decrease in glycosidic droplet surface area, as well as acidic conditions inhibiting enzyme function and accumulation of products slow the progress of the reaction. However rather than with 4% or 5% w/w water, where water runs out before the other factors become apparent with 10% w/w water, the reaction slows down before all water is used up. This has some implications for pre-splitting in an industrial context. To achieve an acid value of 100 during pre-split, oil will need to be reacted for a far longer time to ensure no water remains present. Alternatively, larger amounts of enzyme will need to be used for higher acid values.

Using a higher temperature is also possible as shown in figure 41 b. Using 70°C a lower acid value was produced using 5% w/w water than at 50°C. Reaction time and process water will need to be adjusted but pre-splitting can also be carried out at this higher temperature.

In any case, we have shown that specific acid values can be reached in a reproducible way. TLC slides (shown in figure 42) also confirm presence of various partial glycerides, some fatty acids and unreacted HEAR oil in the pre-split material.

Specifically, the presence of a single diacylglycerides, monoacylglycerides and some free fatty acids can be confirmed. Limiting the amount of water present seems to somewhat modify the specificity of TLL, as two separate diacylglycerides are clearly produced in the 1% w/w water samples, as well as in the 48h 5% w/w water sample. At 48 hours into the pre-splitting reaction, water has been depleted.

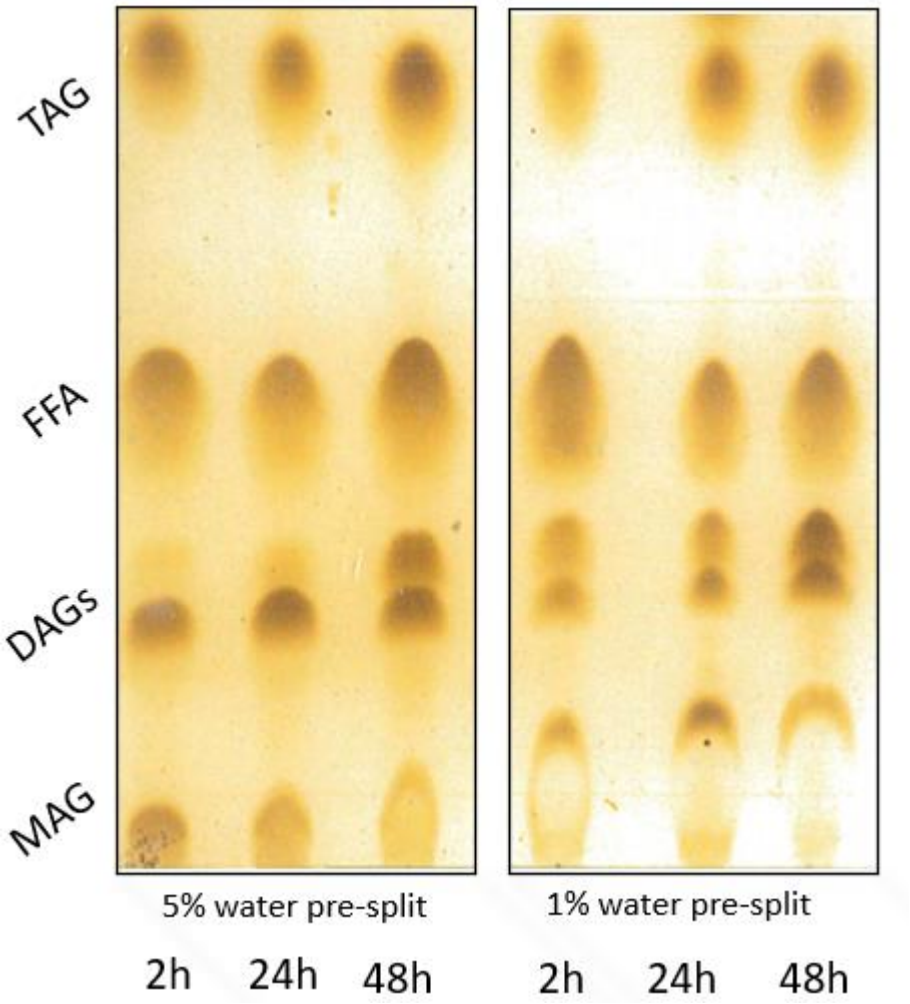


Figure 42: TLC slide from 5% w/w water and 1% w/w water corresponding to an acid value of 60AV and 25AV respectively.

8.2 Pressure Splitting of Pre-Split HEAR oil

In order to test the effect of pre-split material at set acid values on a chemical pressure and temperature-based split, I spent three weeks in an industrial laboratory with access to a pressurised autoclave, an HP60, RC1mx reactor by Mettler Toledo.

Pre-split material at three different acid values (20, 60, 100 AV) was selected to be tested and compared to a standard HEAR oil splitting process. The autoclave splitting process usually takes place at 250°C, in the enclosed reactor, which reaches up to 42 bar pressure during the reaction. To test the pre-split material and compare it to HEAR oil experiments were planned as can be seen in table 10 below.

Specifically, we wanted to answer the question if using pre-split material instead of HEAR oil can reduce necessary energy (temperature/pressure) or time this energy is applied (reaction time) to achieve comparable final acid values.

Initially we attempted to split untreated HEAR oil using pressure and temperature both being varied between 150°C, 200°C and 250°C, with 20, 30 and 40 bar pressures. 250°C and 40 bar pressure was the previous standard for completely splitting HEAR oil. In a usual splitting reaction, the reaction would be stopped after a time, the organic phase separated from the aqueous phase and reused for a second splitting reaction. In order to create a useful comparison, we only employed a single splitting reaction, and compared pre-split material by using different conditions, specifically temperature and pressure as well as varying reaction times.

When using the pressure splitting reactor, lower pressures (20 and 30 bar) could only be created by releasing steam from the reactor, which would change the oil to water ratio and make the reactions more difficult to compare directly. At 250°C all the water within the reactor is in the form of steam, which increases the pressure to above 40 bar. We therefore decided to simply vary the temperature and reaction time for comparisons, as this should allow us to get enough of an indication of what effect if any using pre-split material for pressure splitting has.

Table 10: Results of pre-split material used for chemical pressure splitting recorded as acid value

Experiment No.	Initial Acid Value of HEAR oil/Pre-split material	Reaction Time (mins)	Temperature (°C)/Pressure (Bar)	Final Acid Value (Average)
1	1.2 (HERO)	90	200/16	9.9
2	1.2 (HERO)	60	250/40	125.96
3	1.2 (HERO)	120	250/40	161.7
4	1.2 (HERO)	90	250/40	156.18
5	60	120	250/40	171.5
6	60	120	200/16	127.89
7	20	120	250/40	163.5
8	20	90	200/16	72.64
9	100	90	200/16	127.64
10	100	60	250/40	162.97
11	100	120	200/16	144.65
12	20	120	200/16	83.01
13	60	90	200/16	116.89
14	100	120	250/40	163.32
15	60	60	250/40	160.84
16	20	60	250/40	154.84

From the results shown in figure 43 and table 10, we can see untreated HEAR oil splitting using a temperature of 200°C and 16 bar pressure does not yield a very high amount of fatty acids produced after a 90-minute reaction time. The acid value of 9.9 suggests only very minimal reaction happening. However, using pre-split material results in a significant amount of fatty acid production at 200°C and 16 bar. Using 20, 60 and 100AV pre-split material results in a splitting reaction occurring. This suggests that using pre-split material could allow for a lowering of the energy requirements, meaning the temperature and pressure input for the reaction.

The standard conditions for a complete split are 2hrs of reaction time at 250°C with 40 bar pressure. This results in a close to complete split. Using a lower reaction time results in slightly less acid value being produced. However, when using pre-split material for a reaction (all three types of pre-split material, 20 AV, 60 AV and 100 AV) at 250°C and 40 bar pressure results in a high acid value (complete split) after only half the reaction time.

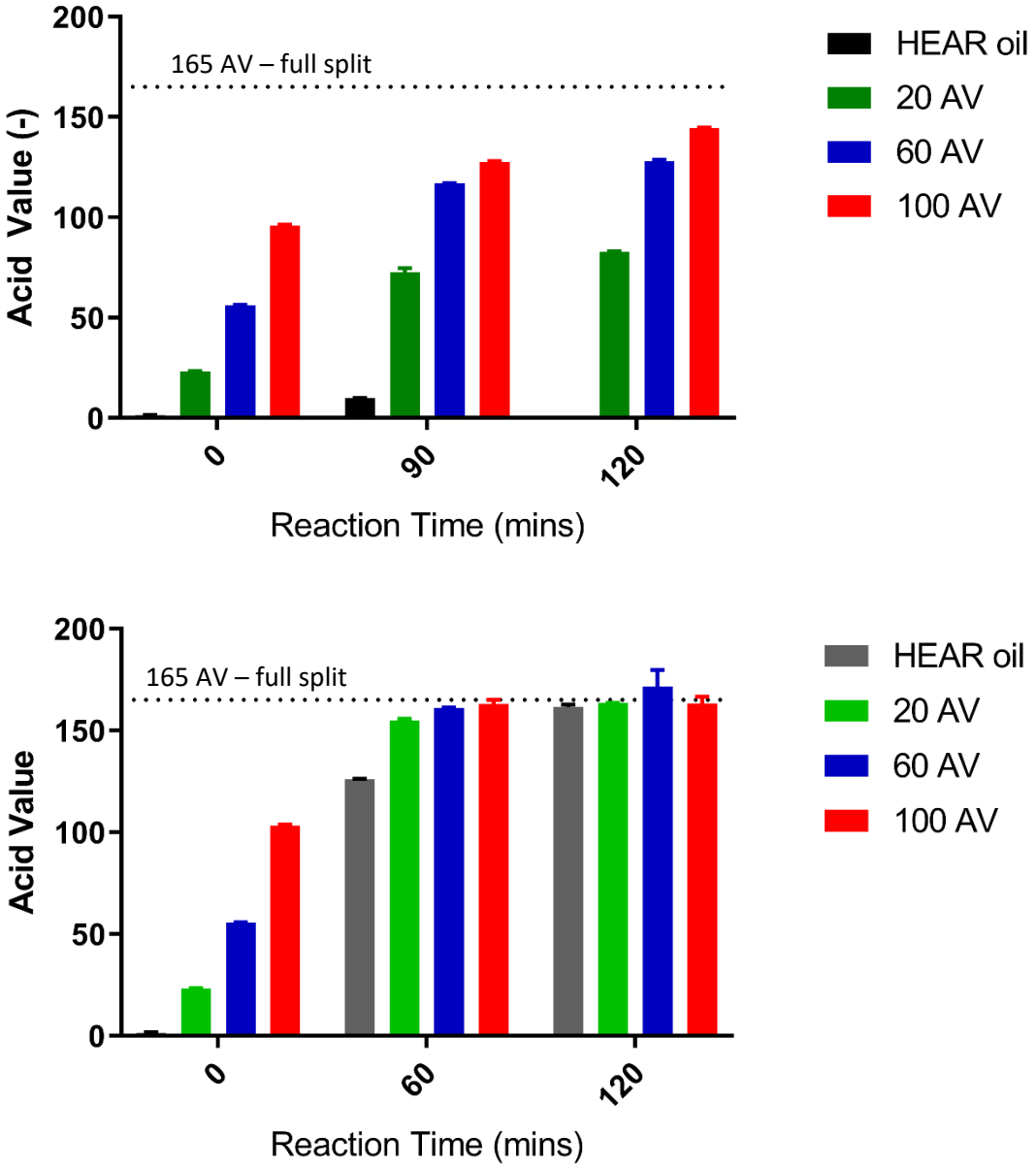


Figure 43: Using HEAR oil as well as 20, 60 and 100 AV pre-split HERO for pressure splitting. (a) Conditions, 200C, 16bar. (b) Conditions: 250C, 40 bar.

For the set of experiments at 200°C, pressure splitting reaction times of 90 mins and 120 mins were used for pre-split material. While untreated HEAR oil only reaches an acid value of around 10 after 90 minutes of pressure splitting, using pre-split material shows significant splitting reactions occurring. 20 AV pre-split material reaches acid values of 72 AV after 90 mins and 83 AV after 120 mins of reaction time. 60 AV pre-split material reaches acid values of 116 AV after 90 mins and 127 AV after 120 mins, and 100 AV pre-split material reaches acid values of 127 AV and 144 AV. There are some diminishing returns of using higher acid value pre-split material. 100 AV pre-split material only reached a final AV of 144 after 120 mins of reaction time, an increase of 44 AV. Using 20 AV pre-

split material however, we see an increase of 60 AV after 120 mins and using 60 AV pre-split material we see an increase of 67 AV after pressure splitting.

In any case, using pre-split material allowed us to pressure split HEAR oil at a significantly lower temperature than standard operation. Untreated HEAR oil at 200°C and 16 bar pressure shows little to no hydrolysis.

For the set of experiments at 250°C, pressure splitting times of 60 mins and 120 mins were used. Using untreated HEAR oil for pressure splitting we achieved an AV of 125 after 60 mins and almost completely split the HEAR oil with an AV of 162. The Saponification value for this batch of HEAR oil was 165, meaning 162 AV represents a 98% degree of split.

Using 20 AV pre-split material, after 60 mins of pressure splitting an acid value of 154 AV is observed. For all other pre-split material, the increase in AV between using untreated HEAR oil and pre-split HEAR oil is less than the AV of the pre-split material. Therefore, no real observations about the reaction can be drawn from these results. From the 20 AV, 60 min pressure splitting reaction however, we can see that using pre-split material can allow us to significantly lower the reaction time and produce equivalent amounts of fatty acid and reach an equivalent degree of split in a lower time.

However, even if the pre-split material has no actual effect on the reaction and only increases the initial acid value, this will still speed up the overall process of industrial fat splitting, as feeding higher acid value material allows for an increased overall production, or a lower environmental impact due to lower reaction times with equivalent production to current industrial processes.

8.3 Pre-Splitting using NS40020

Pre-splitting with enzymes other than TLL was not a focus for this pre-splitting section, but as a proof of concept, pre-splitting with a non-specific lipase, NS40020 (CAL-A) was also carried out. 100 ppm of NS40020 was used at 50°C with 5% w/w water and compared to TLL based pre-splitting.

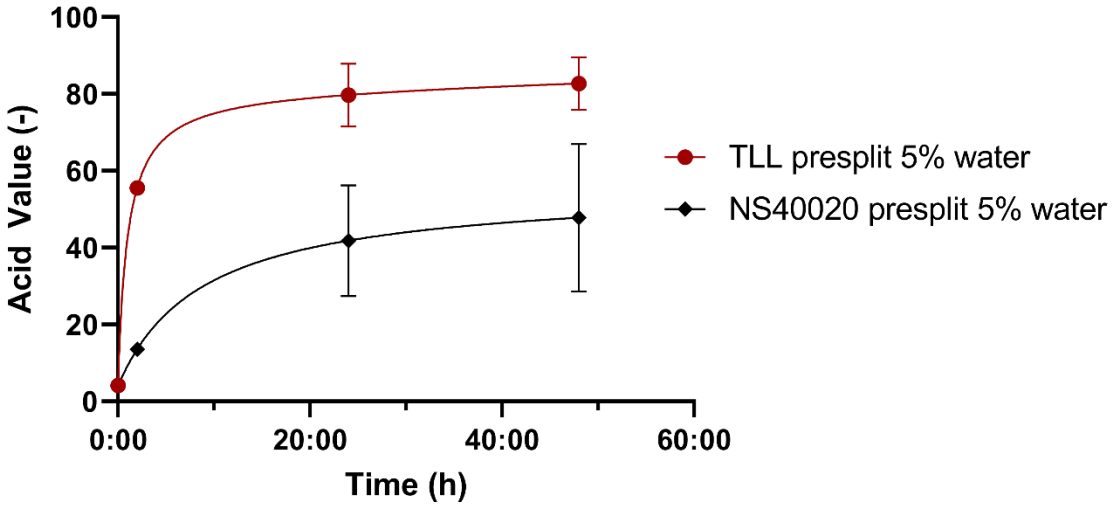


Figure 44: Pre-splitting using NS40020 (CAL-A), compared to TLL pre-split

Pre-splitting using NS40020 works, however likely longer reaction times are needed. NS40020 seems less suited to low water environments and has a lower initial rate of catalysis, as previously seen in full split attempts using NS40020. Nevertheless, pre-splitting using 5% w/w water and 100 ppm NS40020 produces a pre-split mass with an acid value of 40 – 60 AV as shown in figure 44.

9 Discussion

Enzymatic fat splitting for industrial use poses a number of unique problems. Generally, the currently employed process for at least HEAR oil splitting is simply too efficient, especially at larger scales, to be replaced.

Hundreds of tons of erucic acids are produced over a day of running the current continuous pressure splitting column used by CRODA. In the context of this scale, a decrease in yield from 99% to 97% would likely cost CRODA millions of pounds. To replace this exact process with an enzymatic process would require a multiple times larger reactor as well as almost loss free post-production processes to recover both finished products and unsplit oil.

Over the last four years we collected data for the purpose of determining if an enzymatic HEAR oil splitting process could be economically viable in an industrial context, and while a direct replacement of current processes is unrealistic, there are some solutions to industrially viable HEAR oil splitting.

To address this overall problem two distinct solutions to allow for industrially viable HEAR oil splitting were found.

One solution, which is how traditionally the problem was approached is the "**full split**" option. This means attempting to completely split all fatty acids from the triglyceride backbone, to produce exclusively FFAs and glycerol, thereby emulating the results of the current industrial process employed for fat splitting. Of course, current processes usually use high temperature and pressure to split HEAR oil, which allows for very high reaction rates and yields. Current processes are also on very large scales which for our enzymatic process are not possible to replicate. So, the processes developed for full split are looking at a smaller scale process for possible localised erucic acid production rather than to replace the international supply of erucic from the CRODA splitting plant.

A number of candidate lipases were identified for their ability to split HEAR oil. Specifically, *Thermomyces lanuginosus* lipase (TLL) was identified as a viable candidate for HEAR oil splitting. Extremely low amounts of free TLL (0.05% w/w of oil), especially compared to previous literature (Barbosa *et al.*, 2019; Lali, 2013), were able to liberate up to around 95% of fatty acids within 24 hours at 60°C. These reaction conditions were optimised using a design of experiment approach.

Another viable option using free enzymes were combinations of TLL and *Candida antartica* lipase A (CAL-A). Both enzymes are derived from thermophilic fungi and as a result these lipases are able to withstand temperatures of up to 60°C without a reduction in activity. CAL-A also has an unusual mode of action and is the only lipase which preferentially splits sn-2 fatty acids. A 3:1 ratio of TLL:CAL-A with 0.01% w/w of enzyme overall, achieved more than 95% split within 24 hours.

However, to achieve these incredibly high rates of split for enzymatic reactions, buffer systems were required. An aqueous 400 mM and 800 mM glycine / NaOH buffer (pH 9) was used instead of pure water. Presence of buffer in the aqueous phase of the reaction can lead to problems during product separation and purification processes as they are currently used in industry. Fractional distillation is often used to separate fatty acids and unreacted triglyceride. Glycerol is also usually distilled out of the wastewater of the fat splitting process. Both of these follow up processes can be disrupted by the presence of NaOH, particularly in the hard to separate “middle phase”, which forms between the organic and aqueous phases of the enzymatic reaction mixture.

An additional problem for enzymatic “full split” reactions is that in reality, there are three separate reactions occurring, a triglyceride splitting reaction, a diacylglyceride splitting reaction and a monoacylglyceride splitting reaction. When using acid value, the progress of all three reactions is measured simultaneously, making kinetics of the overall process difficult to model.

Using free enzyme also has implications for development of a continuous system. Enzymes are relatively expensive, and while lipases are amongst the cheapest enzymes in terms of bulk cost, they are still the most expensive reagent in fat splitting.

An immobilised system which allows for reuse of enzymes in combination with an OBR or fixed bed reactor is therefore also a potential option for complete split. Pumping the reaction mixture through the enzyme on a solid support, such as in a fixed bed reactor, rather than mixing the reagents with the enzyme allows for different continuous possibilities as well. Enzyme recovery and renewal is possible, provided the enzyme is immobilised on a suitable water-stable resin. The results of this work are missing some information to allow us to truly comment on industrial suitability and would likely need to be repeated for further work. Additional research into optimal reactor type would be necessary here, but enzyme supports such as epoxy-methacrylate were shown to limit the rate of reaction only very slightly.

All three of these options, optimised free TLL, lipase combinations (TLL and CAL-A) as well as immobilised TLL on epoxy-methacrylate are however only initial indications in a lab-based batch

system, and would need further work in reactor design as well as fine tuning follow up processes as well as economic evaluations before they could be truly declared economically viable.

Most previous attempts at enzymatic fat splitting reached around 40-60% degree of split within 6-8h of reaction time, and higher degrees of split required longer reaction times of up to 48 hours. (Freitas *et al.*, 2007; Gupta *et al.*, 2011; Odaneth *et al.*, 2016) This matched what we found in section 5 and 6. The yields and reaction times of these enzymatic processes are not able to compete with the current large scale industrial processes, which reach much higher yields in a much shorter time.

The second major solution to finding an economically viable enzymatic fat splitting process is **pre-splitting**.

Pre-splitting involves partially splitting HEAR oil into a monoacylglycerides, diacylglycerides and fatty acids in a mixture with unreacted triglyceride. This pre-split mixture has significantly improved reaction properties when used in a chemical pressure based splitting reaction compared to untreated HEAR oil as shown in the pre-splitting section in the results section. Partial glycerides can act as emulsifiers, promoting mixing of the aqueous and organic phase, which can lower the necessary reaction temperature and pressure as well as reduce reaction times. Relatively low amounts of free TLL (100 ppm) can be used in the pre-splitting reaction. When this material is pressure split, the lipase will denature and be destroyed, leaving only small traces of polypeptides in the final reaction mixture. These polypeptides should leave the pressure splitting reaction in the aqueous phase where they can be removed.

Pre-splitting is a relatively easy to carry out process involving only the oil, water, and a lipase, in this case TLL. In fact, it could be done with an almost unchanged setup to current oil storage on the CRODA Hull industrial site.

Currently oil is stored at 90°C prior to undergoing the Colgate-Emery splitting process for up to a week. If this setup could be changed to slightly stirred tanks at 60°C for example, all untreated HEAR oil could be processed into pre-split HEAR oil before being fed into the pressure splitting process. This allows for either a reduction in the reaction time, temperature, or pressure. This of course would allow for additional production at no extra cost or current production at a lower cost, both in terms of money as well as environmental impact. Even using pre-split material with a degree of split as low 15 – 25%, the reaction conditions could be lowered by up to 50°C with less than half the pressure. Alternatively, reaction times in our batch experiments were halved with equivalent acid value produced when using 15 or 25% pre-split HEAR oil.

As a result, I would recommend further testing in larger oil batches as well as scaled up tests for pre-split material in the current continuous pressure splitting process.

To conclude, while completely enzymatically splitting HEAR oil to directly produce a maximum amount of erucic acid is possible, it is likely not viable at current industrial scales. Although a smaller scale reactor could be set up, reaction times are high and yields lower and operating costs likely similar or higher. While this could be implemented in a smaller local production of erucic acid, as a replacement to current industrial processes it is unsuitable.

The only currently industrially viable option for lipase based HEAR oil splitting we could identify in this work is therefore pre-splitting. Pre-splitting can be carried out with minor modifications to current industrial set-up and has the potential to reduce reaction times by up to half, with potential energy savings or production increases at equivalent energy costs.

My recommendations for the further development of pre-splitting are to scale the experiment up and test if our lab-scale results hold up. After this the process could be tested through upgrading current HEAR oil storage tanks to large, agitated tanks, either through stirring or otherwise and implemented on the production site.

10 Acknowledgments

I would like to express my great appreciation to Dr. Tuck Seng Wong, my primary research supervisor for providing mentorship and guiding and supporting me through this project. I am also grateful to Kim Carmichael, my secondary industrial supervisor for the opportunity to experience and work at CRODA facilities.

I would also like to thank CRODA employees, Rachel Scullion and Kees van der Sar for their help, shared experience, and advice during my time in CRODA Ditton and CRODA Gouda laboratories.

I am additionally grateful to members of the Tuck lab group, specifically Valeriane Kaita and Melvin Mikuzi for creating an enjoyable environment and support network for each other.

Finally, I would like to thank my parents for their unconditional support of me and my academic journey.

11 References

Abbott, M. S. R., Harvey, A. P., Perez, G. V. and Theodorou, M. K. (2013) 'Biological processing in oscillatory baffled reactors: operation, advantages and potential', *Interface Focus,* 3(1), pp. 20120036-20120036.

Abreu Silveira, E., Moreno-Perez, S., Basso, A., Serban, S., Pestana Mamede, R., Tardioli, P. W., Sanchez Farinas, C., Rocha-Martin, J., Fernandez-Lorente, G. and Guisan, J. M. (2017) 'Modulation of the regioselectivity of Thermomyces lanuginosus lipase via biocatalyst engineering for the Ethanolysis of oil in fully anhydrous medium', *BMC Biotechnology,* 17, pp. 88.

Abreu Silveira, E., Moreno-Perez, S., Basso, A., Serban, S., Pestana-Mamede, R., Tardioli, P. W., Farinas, C. S., Castejon, N., Fernandez-Lorente, G., Rocha-Martin, J. and Guisan, J. M. (2019) 'Biocatalyst engineering of Thermomyces Lanuginosus lipase adsorbed on hydrophobic supports: Modulation of enzyme properties for ethanolysis of oil in solvent-free systems', *Journal of Biotechnology,* 289, pp. 126-134.

Adrie J.J. Straathof, P. A. (2000) *Applied Biocatalysis.* 2nd Edition edn.: CRC Press.

Al-Zuhair, S., Hasan, M. and Ramachandran, K. B. (2003) 'Kinetics of the enzymatic hydrolysis of palm oil by lipase', *Process Biochemistry,* 38(8), pp. 1155-1163.

Albayati, S. H., Masomian, M., Ishak, S. N., Mohamad Ali, M. S., Thean, A. L., Mohd Shariff, F. B., Muhd Noor, N. D. and Raja Abd Rahman, R. N. (2020) 'Main Structural Targets for Engineering Lipase Substrate Specificity', *Catalysts,* 10(7).

Anderson, E. M., Larsson, K. M. and Kirk, O. (1998) 'One Biocatalyst–Many Applications: The Use of Candida Antarctica B-Lipase in Organic Synthesis', *Biocatalysis and Biotransformation,* 16(3), pp. 181-204.

Anderson, K. W. and Wenzel, J. D. (1996) *Improved Fat Splitting Process*. https://patents.google.com/patent/WO1996038534A1/en. [Online].

ARC, I. 2018. Fatty Amides Market - By Product Type, By Product Form, By Function, By End-Use Industry and By Geography, Analysis- Forecast 2020 – 2025. Industry ARC.

Arroyo, R., Sanchez-Muniz, F. J., Cuesta, C., Burgillo, F. J. and Sanchez-Montero, J. M. (1996) 'Hydrolysis of used frying palm olein and sunflower oil catalyzed by porcine pancreatic lipase', *Lipids,* 31(11), pp. 1133-1139.

Aziz, M., Husson, F. and Kermasha, S. (2015) 'Optimization of the Hydrolysis of Safflower Oil for the Production of Linoleic Acid, Used as Flavor Precursor', *International journal of food science,* 2015, pp. 594238-594238.

Barbosa, M. S., Freire, C. C. C., Almeida, L. C., Freitas, L. S., Souza, R. L., Pereira, E. B., Mendes, A. A., Pereira, M. M., Lima, Á. S. and Soares, C. M. F. (2019) 'Optimization of the enzymatic hydrolysis of Moringa oleifera Lam oil using molecular docking analysis for fatty acid specificity', *Biotechnology and Applied Biochemistry,* 66(5), pp. 823-832.

Barnebey, H. L. and Brown, A. C. (1948) 'Continuous fat splitting plants using the colgate-emery process', 25(3), pp. 95-99.

BCC (2019) *Oleochemical Fatty Acids: Global Markets to 2023*: **BCC Publishing**.

Bharathi, D. and Rajalakshmi, G. (2019) 'Microbial lipases: An overview of screening, production and purification', *Biocatalysis and Agricultural Biotechnology,* 22, pp. 101368.

Bianchetta, J. D., Bidaud, J., Guidoni, A. A., Bonicel, J. J. and Rovery, M. (1979) 'Porcine pancreatic lipase. Sequence of the first 234 amino acids of the peptide chain', *Eur J Biochem,* 97(2), pp. 395-405.

Bilyk, A., Bistline, R. G., Haas, M. J. and Feairheller, S. H. (1991) 'Lipase-catalyzed triglyceride hydrolysis in organic solvent', *Journal of the American Oil Chemists' Society,* 68(5), pp. 320-323.

Bisht, D., Yadav, S. K. and Darmwal, N. S. (2013) 'An oxidant and organic solvent tolerant alkaline lipase by P. aeruginosa mutant: Downstream processing and biochemical characterization', *Braz J Microbiol,* 44(4), pp. 1305-1314.

Bornscheuer, U. T. and Buchholz, K. (2005) 'Highlights in Biocatalysis – Historical Landmarks and Current Trends', *Engineering in Life Sciences,* 5(4), pp. 309-323.

Bracco, P. (2014) *Selective steroid hydroxylation by bacterial cytochrome P450 monooxygenases.*

Brady, C., Metcalfe, L., Slaboszewski, D. and Frank, D. (1988) 'Lipase immobilized on a hydrophobic, microporous support for the hydrolysis of fats', *Journal of the American Oil Chemists' Society,* 65(6), pp. 917-921.

Brunner, K., Frische, R. and Kilian, D. (2002) *Method for Enzymatic Splitting of Oils and Fats*. [Online].

Brzozowski, A., Derewenda, U., Derewenda, Z., Dodson, G., Lawson, D., Turkenburg, J., Bjorkling, F., Huge-Jensen, B., Patkar, S. and Thim, L. (1991) 'A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex', *Nature,* 351(6326), pp. 491-494.

Casas-Godoy, L., Gasteazoro, F., Duquesne, S., Bordes, F., Marty, A. and Sandoval, G. (2018) *Lipases: An Overview.*

Cavalcanti-Oliveira, E., Rufino da Silva, P., Peçanha Ramos, A., Alexandre Gomes Aranda, D. and Freire, D. (2010) *Study of Soybean Oil Hydrolysis Catalyzed by Thermomyces lanuginosus Lipase and Its Application to Biodiesel Production via Hydroesterification.*

Chandra, P., Enespa, Singh, R. and Arora, P. K. (2020) 'Microbial lipases and their industrial applications: a comprehensive review', *Microbial Cell Factories,* 19(1), pp. 169.

Cynthia Spahn and Shelley, D. M. (2008) 'Enzyme Immobilization in Biotechnology', *Recent Patents on Engineering,* 2(3), pp. 195-200.

Datta, S., Christena, L. R. and Rajaram, Y. R. S. (2013) 'Enzyme immobilization: an overview on techniques and support materials', *3 Biotech,* 3(1), pp. 1-9.

De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P. and Rovery, M. (1981) 'Porcine pancreatic lipase. Completion of the primary structure', *Biochimica et Biophysica Acta (BBA)-Protein Structure,* 671(2), pp. 129-138.

De Simone, A. (2016) *Engineering the genetic code of Escherichia coli with methionine analogues and bioorthogonal amino acids for protein immobilization.*

Deffense, E. J. O. (2009) 'From organic chemistry to fat and oil chemistry*', 16(1), pp. 14-24.

Domínguez de María, P., Carboni-Oerlemans, C., Tuin, B., Bargeman, G., van der Meer, A. and van Gemert, R. (2005) 'Biotechnological applications of Candida antarctica lipase A: State-of-the-art', *Journal of molecular catalysis. B, Enzymatic,* 37(1), pp. 36-46.

Díaz, J. F. and Balkus, K. J. (1996) 'Enzyme immobilization in MCM-41 molecular sieve', *Journal of Molecular Catalysis B: Enzymatic,* 2(2), pp. 115-126.

Farias, R. N., Torres, M. and Canela, R. (1997) 'Spectrophotometric Determination of the Positional Specificity of Nonspecific and 1,3-Specific Lipases', *Anal Biochem,* 252(1), pp. 186-189.

Fernandez-Lafuente, R. (2010) 'Lipase from Thermomyces lanuginosus: Uses and prospects as an industrial biocatalyst', *Journal of Molecular Catalysis B: Enzymatic,* 62(3), pp. 197-212.

Freitas, L., Bueno, T., Perez, V. H., Santos, J. C. and de Castro, H. F. J. W. J. o. M. a. B. (2007) 'Enzymatic hydrolysis of soybean oil using lipase from different sources to yield concentrated of polyunsaturated fatty acids', 23(12), pp. 1725-1731.

Fuchs, B., Süß, R., Teuber, K., Eibisch, M. and Schiller, J. (2011) 'Lipid analysis by thin-layer chromatography—A review of the current state', *Journal of Chromatography A,* 1218(19), pp. 2754-2774.

Gandhi, N. N. (1997) 'Applications of lipase', *Journal of the American Oil Chemists' Society,* 74(6), pp. 621-634.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R. and Rodrigues, R. C. (2011) 'Potential of Different Enzyme Immobilization Strategies to Improve Enzyme Performance', *Advanced Synthesis & Catalysis,* 353(16), pp. 2885-2904.

Geoffry, K. and Achur, R. N. (2018) 'Screening and production of lipase from fungal organisms', *Biocatalysis and Agricultural Biotechnology,* 14, pp. 241-253.

Goswami, D., Basu, J. K. and De, S. (2013) 'Lipase applications in oil hydrolysis with a case study on castor oil: a review', *Critical Reviews in Biotechnology,* 33(1), pp. 81-96.

Goswami, D., Basu, J. K. and De, S. J. B. a. B. E. (2009) 'Optimization of process variables in castor oil hydrolysis by Candida rugosa lipase with buffer as dispersion medium', 14(2), pp. 220-224.

Goswami, D., Basu, J. K. and De, S. J. B. a. B. E. (2011) 'Erucic acid production using porcine pancreas lipase: Enhancement by mixed surfactants', 16(2), pp. 327-336.

Goswami, D., De, S. and Basu, J. (2012) *Effects of process variables and additives on mustard oil hydrolysis by porcine pancreas lipase.*

Gotor-Fernández, V., Busto, E. and Gotor, V. (2006) 'Candida antarctica Lipase B: An Ideal Biocatalyst for the Preparation of Nitrogenated Organic Compounds', *Adv. Synth. Catal,* 342(8), pp. 797-812.

Greenberg, S. (2021) 'Enzymes In Industry: Market Trends You Need To Know'.

Gupta, S., Goyal, R., Deedwania, A. and Kumar, V. (2013) *Single-Phase Transesterificatiom of Non-Edible Oil.*

Gupta, S., Ingole, P., Singh, K. and Bhattacharya, A. (2011) 'Comparative study of the hydrolysis of different oils by lipase-immobilized membranes', *Journal of Applied Polymer Science,* 124(S1), pp. E17-E26.

Gutarra, M. L. E., Cavalcanti, E. D. C., Castilho, L. R., Freire, D. M. G. and Sant'Anna, G. L. (2005) 'Lipase production by solid-state fermentation', *Applied Biochemistry and Biotechnology,* 121(1), pp. 105-116.

Han, D. and Rhee, J. S. (1986) 'Characteristics of lipase-catalyzed hydrolysis of olive oil in AOT-isooctane reversed micelles', *Biotechnol. Bioeng,* 28(8), pp. 1250-1255.

Hasan, F., Shah, A. A. and Hameed, A. (2006) 'Industrial applications of microbial lipases', *Enzyme and Microbial technology,* 39(2), pp. 235-251.

Hertweck, C. and Boland, W. (1997) 'Porcine pancreatic lipase (PPL) - a versatile biocatalyst in organic synthesis', *J. Prakt. Chem,* 339(1), pp. 200-202.

Jamie, A., S Alshami, A., A, A. and Malaibari, Z. (2017) *Development and Validation of a Kinetic Model for Enzymatic Hydrolysis Using Candida rugosa Lipase.*

Japanese Pharmacopoeia XIV, Chapter 17: Fats and Fatty Oils Tests (2001): Interpharm Pr.

Jurado, E., Camacho, F., Luzón, G., Fernández-Serrano, M. and García-Román, M. (2008) 'Kinetics of the enzymatic hydrolysis of triglycerides in o/w emulsions: Study of the initial rates and the reaction time course', *Biochemical Engineering Journal,* 40(3), pp. 473-484.

Kaimal, T. N. B., Prasad, R. B. N. and Rao, T. C. J. B. L. (1993) 'A novel lipase hydrolysis method to concentrate erucic acid glycerides in Cruciferae oils', 15(4), pp. 353-356.

Kang, S. T. and Rhee, J. S. (1989) 'Characteristics of immobilized lipase-catalyzed hydrolysis of olive oil of high concentration in reverse phase system', *Biotechnol. Bioeng,* 33(11), pp. 1469-1476.

Kanwar, S., Kumar Kaushal, R., Jawed, A., Gupta, R. and Chimni, S. (2005) *Methods for inhibition of residual lipase activity in colorimetric assay: A comparative study.*

Kapoor, M. and Gupta, M. N. (2012) 'Lipase promiscuity and its biochemical applications', *Process biochemistry (1991),* 47(4), pp. 555-569.

Katchalski-Katzir, E. (1993) 'Immobilized enzymes — learning from past successes and failures', *Trends in Biotechnology,* 11(11), pp. 471-478.

Kaur, N., Chugh, V. and Gupta, A. K. (2012) 'Essential fatty acids as functional components of foods- a review', *J Food Sci Technol,* 51(10), pp. 2289-2303.

Kirk, O., Borchert, T. V. and Fuglsang, C. C. (2002) 'Industrial enzyme applications', *Current Opinion in Biotechnology,* 13(4), pp. 345-351.

Knezevic, Z. D., Siler-Marinkovic, S. S. and Mojovic, L. V. (1998) 'Kinetics of lipase-catalyzed hydrolysis of palm oil in lecithin/izooctane reversed micelles', *Applied Microbiology and Biotechnology,* 49(3), pp. 267-271.

Kumar, A. and Kanwar, S. S. (2012) 'Lipase production in solid-state fermentation (SSF): recent developments and biotechnological applications', *Dynamic Biochemistry, Process Biotechnology and Molecular Biology,* 6(1), pp. 13-27.

Kumar, P., Singh, V. K. and Singh, D. K. (2008) 'Kinetics of enzyme inhibition by active molluscicidal agents ferulic acid, umbelliferone, eugenol and limonene in the nervous tissue of snail Lymnaea acuminata', *Phytotherapy Research,* 23(2), pp. 172-177.

Lali, A. M. (2013) *Enzymatic Process for Fat and Oil Hydrolysis*. [Online].

Lanser, A. C., Manthey, L. K. and Hou, C. T. J. C. M. (2002) 'Regioselectivity of New Bacterial Lipases Determined by Hydrolysis of Triolein', 44(5), pp. 336-340.

Lascaray, L. (1952) 'Industrial fat splitting', 29(9), pp. 362-366.

Laszlo, J. A., Compton, D. L. and Vermillion, K. E. (2008) 'Acyl Migration Kinetics of Vegetable Oil 1,2-Diacylglycerols', *Journal of the American Oil Chemists' Society,* 85(4), pp. 307-312.

Li, R., Du, W., Lu, D., Dai, L. and Liu, D. (2015) 'Study on the enzyme's 1,3-positional specificity during lipozyme TL-mediated biodiesel production', *RSC Advances,* 5(77), pp. 62460-62468.

Li, W., Du, W., Li, Q., Sun, T. and Liu, D. (2010) 'Study on acyl migration kinetics of partial glycerides: Dependence on temperature and water activity', *Journal of Molecular Catalysis B: Enzymatic,* 63(1), pp. 17-22.

Linfield, W. M., Barauskas, R. A., Sivieri, L., Serota, S. and Stevenson, R. W. (1984) 'Enzymatic fat hydrolysis and synthesis', *Journal of the American Oil Chemists' Society,* 61(2), pp. 191-195.

Liu, Y., Jin, Q., Shan, L., Liu, Y., Shen, W. and Wang, X. (2008) 'The effect of ultrasound on lipase-catalyzed hydrolysis of soy oil in solvent-free system', *Ultrasonics Sonochemistry,* 15(4), pp. 402-407.

Maheshwari, R., Bharadwaj, G. and Bhat, M. K. (2000) 'Thermophilic Fungi: Their Physiology and Enzymes', *Microbiology and Molecular Biology Reviews,* 64(3), pp. 461-488.

Malcata, F. X., Hill, C. G. and Amundson, C. H. (1992) 'Hydrolysis of butteroil by immobilized lipase using a hollow-fiber reactor: Part III. Multiresponse kinetic studies', *Biotechnology and Bioengineering,* 39(10), pp. 1002-1012.

Malviya, R., Bansal, V., Pal, O. and Sharma, P. (2010) 'High performance liquid chromatography: A short review', *Journal of Global Pharma Technology,* 2, pp. 22-26.

Martinelle, M., Holmquist, M. and Hult, K. (1995) 'On the interfacial activation of Candida antarctica lipase A and B as compared with Humicola lanuginosa lipase', *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 1258(3), pp. 272-276.

McNeill, G. P., Shimizu, S. and Yamane, T. (1991) 'High-yield enzymatic glycerolysis of fats and oils', *Journal of the American Oil Chemists' Society,* 68(1), pp. 1-5.

Mehta, A., Bodh, U. and Gupta, R. (2017) 'Fungal lipases: A review', *Journal of biotech research,* 8(1), pp. 58-77.

Mendes, A. A., Oliveira, P. C. and de Castro, H. F. (2012) 'Properties and biotechnological applications of porcine pancreatic lipase', *Journal of Molecular Catalysis B: Enzymatic,* 78, pp. 119-134.

Mohamed, R. A., Salleh, A. B., Leow, T. C., Yahaya, N. M. and Abdul Rahman, M. B. (2018) 'Site-directed mutagenesis: role of lid region for T1 lipase specificity', *Protein Engineering, Design and Selection,* 31(6), pp. 221-229.

Moreau, R. A. (2006) 'The analysis of lipids via HPLC with a charged aerosol detector', *Lipids,* 41(7), pp. 727-734.

Murty, V. R., Bhat, J. and Muniswaran, P. K. A. J. B. a. B. E. (2002) 'Hydrolysis of oils by using immobilized lipase enzyme: A review', 7(2), pp. 57-66.

Nguyen, H. H. and Kim, M. (2017) 'An overview of techniques in enzyme immobilization', *Applied Science and Convergence Technology,* 26(6), pp. 157-163.

Nieschlag, H. J. and Wolff, I. A. J. J. o. t. A. O. C. S. (1971) 'Industrial uses of high erucic oils', 48(11), pp. 723-727.

No, D. S., Zhao, T., Kim, B. H., Choi, H.-D. and Kim, I.-H. (2013) 'Enrichment of erucic acid from crambe oil in a recirculated packed bed reactor via lipase-catalyzed ethanolysis', *Journal of Molecular Catalysis B: Enzymatic,* 87, pp. 6-10.

Noor, I. M., Hasan, M. and Ramachandran, K. B. (2003) 'Effect of operating variables on the hydrolysis rate of palm oil by lipase', *Process Biochemistry,* 39(1), pp. 13-20.

Odaneth, A. A., Vadgama, R. N., Bhat, A. D. and Lali, A. M. J. A. B. a. B. (2016) 'Tuning Lipase Reaction for Production of Fatty Acids from Oil', 180(3), pp. 504-515.

Ohlson, R. (1992) 'Modern processing of rapeseed', *Journal of the American Oil Chemists' Society,* 69(3), pp. 195-198.

Ortiz, C., Ferreira, M. L., Barbosa, O., dos Santos, J. C. S., Rodrigues, R. C., Berenguer-Murcia, Á., Briand, L. E. and Fernandez-Lafuente, R. (2019) 'Novozym 435: the “perfect” lipase immobilized biocatalyst?', *Catalysis Science & Technology,* 9(10), pp. 2380-2420.

Pithani, S., Karlsson, S., Emtenäs, H. and Öberg, C. T. (2019) 'Using Spinchem Rotating Bed Reactor Technology for Immobilized Enzymatic Reactions: A Case Study', *Org. Process Res. Dev,* 23(9), pp. 1926-1931.

Primožič, M., Habulin, M. and Knez, Ž. J. J. o. t. A. O. C. S. (2003) 'Parameter optimization for the enzymatic hydrolysis of sunflower oil in high-pressure reactors', 80(7), pp. 643-646.

Pronk, W., Kerkhof, P. J. A. M., Helden, v. C. and Riet, v. t. K. (1988) 'The hydrolysis of triglycerides by immobilized lipase in a hydrophilic membrane reactor', *Biotechnol Bioeng,* 32(4), pp. 512-518.

Przybylski, R. and Mag, T. (2011) 'Canola/rapeseed oil', *Vegetable Oils in Food Technology: Composition, Properties and Uses. Blackwell Publishing Ltd., Hoboken*, pp. 107-136.

Rathi, P., Bradoo, S., Saxena, R. K. and Gupta, R. (2000) 'A hyper-thermostable, alkaline lipase from Pseudomonas sp. with the property of thermal activation', *Biotechnology Letters,* 22(6), pp. 495-498.

Reetz, M. T. and Jaeger, K.-E. (1998) 'Overexpression, immobilization and biotechnological application of Pseudomonas lipases', *Chemistry and physics of lipids,* 93(1-2), pp. 3-14.

Ribeiro, B. D., Castro, A. M. d., Coelho, M. A. Z. and Freire, D. M. G. (2011) 'Production and Use of Lipases in Bioenergy: A Review from the Feedstocks to Biodiesel Production', *Enzyme Res,* 2011(1), pp. 615803-16.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R. and Fernández-Lafuente, R. (2013) 'Modifying enzyme activity and selectivity by immobilization', *Chem Soc Rev,* 42(15), pp. 6290-6307.

Rogalska, E., Cudrey, C., Ferrato, F. and Verger, R. (1993) 'Stereoselective hydrolysis of triglycerides by animal and microbial lipases', *Chirality,* 5(1), pp. 24-30.

Saha, B. C., Jordan, D. B. and Bothast, R. J. (2009) 'Enzymes, Industrial (overview)', in Schaechter, M. (ed.) *Encyclopedia of Microbiology (Third Edition)*. Oxford: Academic Press, pp. 281-294.

Schallmey, A., Domínguez de María, P. and Bracco, P. (2013) 'Biocatalytic Asymmetric Oxidations in Stereoselective Synthesis', *Stereoselective Synthesis of Drugs and Natural Products*, pp. 1-26.

Schmid, R. D. and Verger, R. (1998) 'Lipases: Interfacial Enzymes with Attractive Applications', *Angewandte Chemie International Edition,* 37(12), pp. 1608-1633.

Shen, Q., Yang, R., Hua, X., Ye, F., Zhang, W. and Zhao, W. (2011) 'Gelatin-templated biomimetic calcification for β-galactosidase immobilization', *Process Biochemistry,* 46(8), pp. 1565-1571.

Singh, A. K. and Mukhopadhyay, M. (2011) 'Overview of Fungal Lipase: A Review', *Appl Biochem Biotechnol,* 166(2), pp. 486-520.

Sulaiman, A.-Z. (2006) *Kinetics of Hydrolysis of Tributyrin by Lipase.*

Takamoto, T., Shirasaka, H., Uyama, H. and Kobayashi, S. (2001) 'Lipase-catalyzed hydrolytic degradation of polyurethane in organic solvent', *Chemistry Letters,* 30(6), pp. 492-493.

Tsai, S.-W., Wu, G.-H. and Chiang, C.-L. (1991) 'Kinetics of enzymatic hydrolysis of olive oil in biphasic organic-aqueous systems', *Biotechnology and Bioengineering,* 38(7), pp. 761-766.

Verger, R. (1997) ''‘Interfacial activation’of lipases: facts and artifacts', *Trends in Biotechnology,* 15(1), pp. 32-38.

Wall, P. E. (2005) *Thin-layer chromatography [electronic resource] : a modern practical approach.* Cambridge: Cambridge : Royal Society of Chemistry, c2005.

Widmann, M., Juhl, P. B. and Pleiss, J. (2010) 'Structural classification by the Lipase Engineering Database: A case study of Candida antarctica lipase A', *BMC Genomics,* 11(1), pp. 123-123.

Yang, B., Wang, Y.-H. and Yang, J.-G. (2006) 'Optimization of enzymatic degumming process for rapeseed oil', *Journal of the American Oil Chemists' Society,* 83(7), pp. 653-658.

Yang, D. and Rhee, J. S. (1992a) 'Continuous hydrolysis of olive oil by immobilized lipase in organic solvent', 40(6), pp. 748-752.

Yang, D. and Rhee, J. S. (1992b) 'Continuous hydrolysis of olive oil by immobilized lipase in organic solvent', *Biotechnol. Bioeng,* 40(6), pp. 748-752.

Zaak, H., Siar, E.-H., Kornecki, J. F., Fernandez-Lopez, L., Pedrero, S. G., Virgen-Ortíz, J. J. and Fernandez-Lafuente, R. (2017) 'Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from Thermomyces lanuginosus immobilized on octyl agarose beads', *Process biochemistry (1991),* 56, pp. 117-123.

Zhao, Z., Hou, S., Lan, D., Wang, X., Liu, J., Khan, F. I. and Wang, Y. (2017) 'Crystal structure of a lipase from Streptomyces sp. strain W007 – implications for thermostability and regiospecificity', *FEBS J,* 284(20), pp. 3506-3519.

Zufarov, O., Schmidt, Š., Sekretár, S. and Cvengroš, J. (2009) 'Ethanolamines used for degumming of rapeseed and sunflower oils as diesel fuels', *Eur. J. Lipid Sci. Technol,* 111(12), pp. 985-992.

12 Appendix

Chapter 5

Chapter 5.1 PPL

PPL based HEAR oil splitting using 6 different buffers compared to no buffer. Full results as shown in figure 9.

	Acid values based on which buffer was used			
Time (h:min)	Glycine	Glycine + $MgCl_2$	Glycine + Span80, Tween80 and $MgCl_2$	No Buffer
0:00	1.16	1.28	1.08	1.876
0:30	15.44	24.77	16.37	
1:00	20.01	27.27	20.2	9.75
1:30	21.2	30.71	22.5	
2:00	25.48	33.16	24.73	11.9
2:30	27.32	35.33		
3:00	27.39		26.69	14.2
3:30		39.28		
4:00	31.55		30.24	16.5
4:30		39.85		
5:00	33.05		32.57	20.26
5:30		44.71		
6:00	35.23	45.13	34.88	
24:00	47.8	68.25	57.55	37.8

Time (h:min)	**Ethanolamine only**	**Ethanolamine with $MgCl_2$**	**Ethanolamine with $MgCl_2$, Tween80 and Span80**
0:00	0.85	1.18	1.32
0:30	21.09	14.53	14.34
1:00	29.19	16.97	17.92
1:30	31.84	17.97	18.85
2:00	33.91	21.31	19.34
3:00	38.56	21.77	20.55
4:00	40.55	22.9	22.11
5:00	45.9	25.07	23.24
6:00	47.53	26.38	24.87
24:00	66.82	38.06	33.17

Chapter 5.5 Enzyme Comparison

Gas Chromatography

Retention Time [min]	Area [%]	Likely compound
10.747	2.65	C16
13.929	11.36	C18:1
14.824	11.44	C18:2
15.991	7.35	C18:3
17.711	5.52	C20:1
17.868	1.57	C20:1 TRANS
22.559	54.17	C22:1
27.269	1.02	C24

Retention Time [min]	Area [%]	Likely compound
10.731	2.69	C16
13.923	10.64	C18:1
14.825	12.24	C18:2
15.993	8.0	C18:3
17.708	4.72	C20:1
17.867	1.63	C20:1 TRANS
22.592	53.86	C22:1
27.283	1.11	C24

As shown in tables 11 and 12, similar percentages of fatty acids are observed, both in completely split HEAR oil and the organic phase of an enzymatic splitting reaction after 24 hours. The fraction of C18s in the fully split HEAR oil is slightly lower, while the C22 fraction is slightly larger. However, all percentages are likely within error ranges or within the slight differences in HEAR oil intake. Different batches of HEAR oil have slightly different fatty acid profiles.

Interestingly we see no signal for any fatty acids below C16, although it is also possible that their signal was not strong enough for detection in the enzymatically split sample.

In conclusion however, GC is not a suitable analytic method for analysing fat splitting products. To analyse using GC all fatty acids are split from the glycerol backbone, and only fatty acid content is taken for measurement. We can therefore not detect partial glycerides; we can only determine the fatty acid proportions of the original HEAR oil batch.

Full tables of results from gas chromatography of split HEAR oil. Values shown in the shortened tables above are highlighted in green.

Retention Time [min]	Area [%]	Likely compound
10.747	2.65	C16
11.057	0.01	
11.153	0.11	
11.289	0.01	
11.671	0.03	
11.944	0.03	
12.393	0.02	
12.527	0.07	
13.413	0.8	
13.929	11.36	C18:1
14.02	0.65	C18:1 TRANS
14.824	11.44	C18:2
15.121	0.01	
15.515	0.03	
15.991	7.35	C18:3
17.075	0.67	C20:0
17.711	5.52	C20:1
17.868	1.57	C20:1 TRANS
18.809	0.43	
19.965	0.08	
20.263	0.06	
21.624	0.63	C22:0
22.559	54.17	C22:1
23.596	0.82	
24.711	0.04	
25.193	0.2	
26.468	0.19	
27.269	1.02	C24
27.977	0.03	
TOTAL	100	

Retention Time [min]	Area [%]	Likely compound
8.987	0.03	C14
9.751	0.01	
9.905	0.01	
10.423	0.01	
10.731	2.69	C16
11.039	0.02	
11.135	0.13	
11.277	0.01	
11.657	0.03	

11.924	0.03	
12.38	0.02	
12.512	0.08	
13.401	0.78	
13.923	10.64	C18:1
14.012	0.67	
14.211	0.01	
14.825	12.24	C18:2
15.112	0.01	
15.503	0.03	
15.993	8.0	C18:3
17.069	0.64	C20:0
17.708	4.72	C20:1
17.867	1.63	C20:1 TRANS
18.807	0.43	
19.967	0.07	
20.256	0.06	
21.671	0.62	
22.592	53.86	C22:1
23.611	0.94	
23.937	0.02	C22:2
27.716	0.03	
25.197	0.22	
25.907	0.01	
26.472	0.2	
27.283	1.11	C24
TOTAL	100	

Chapter 5.6

Chapter 5.6 Enzyme comparison summary based on acid value over 24 hours produced by splitting HEAR oil. Shown here are the full acid value results for enzyme comparison as shown in figure 21. For TLL averages of different TLL variants are shown here.

Time (h:min)	PPL	TLL	CRL	Lipozyme CALB	Novozyme435	NS40020
0:00	1.4	1.7	1.8	2.01	1.29	1.95
0:30	38.85	76.94			12.81	
1:00	53.12	87.12	104.65	23.52	16.12	41.07
2:00	68.28	92.82	114.74	22.37	17.85	60.49
3:00	69.84	97.27	125.83	25.64	20.06	71.55
4:00	78.63	103.87	126.03	25.62	22.37	79.79
5:00	80.57	110.23	131.37	28.15	26.3	90.76
24:00	107.69	180.15	159.47	32.24	46.67	153.34

Chapter 7

Full table of acid value results for comparison of TLL immobilised on 6 different resins for the hydrolysis of HEAR oil. All values are the average of triplicate repeats. Graph shown in figure 32

Time (h)	ECR8215	ECR8285	ECR8315	ECR1030	ECR1090	ECR8806
0	1.90	1.82	2.20	1.93	2.34	1.98
1	84.04	59.21	63.98	38.16	47.95	61.29
2	92.53	73.79	79.30	45.73	61.57	74.82
3	99.83	84.57	86.72	53.77	74.61	88.62
4	103.22	92.27	91.49	53.64	81.35	94.25
5	110.40	101.23	96.96	69.73	91.26	104.75
6	180.62	169.45	163.00	141.38	179.79	168.91

Chapter 8

Full table of acid value results from pre-splitting HEAR oil using TLL with varying conditions as described in chapter 8, figure 39.

Time (h)	5% water	1% water	3% water	10% water
0	3.70	4.4	4.45	4.4
2	54.08	27.65	44.2	67.3
24	72.11	29.86	45.43	103.37
48	78.54	31.73	46.38	111.96

Time (h)	5% water	50°C 5% water (w/w) added at 24h
0	3.70	
2	54.08	
24	72.11	
26		78.26
48	78.54	115.15

Time (h)	5% water, 200 ppm TLL
0	4.31
2	66.65
24	74.45
48	75.66