Norwegian University
of Life Sciences

Master's Thesis 2019 60 ECTS

Faculty of Science and Technology
Reaction Engineering and Catalysis

Novozyme® 435 as Bio-catalyst for Biomass Based Esters

Iver Bergh Hvidsten
Environmental Physics and Renewable Energy

Preface

This thesis concludes my education in environmental sciences. I was challenged on a new field in bio-chemical catalysis, as my education was mostly based in physics, mathematics and programming. My greatest thank goes out to my supervisor, Jorge Mario Marchetti, for guiding me and helping with practical and theoretical aspects in this thesis. No questions were stupid and effort was always made to give a sufficient answer. I would also like to thank *NMBU* and my supervisor for giving me the opportunity to try a research based master thesis.

I would like to give special thank to my sister, Kine Bergh Hvidsten, who helped me with the understanding of organic chemistry and my parents for massive support and help when the motivation was dwindling.

My final thanks goes out to my classmates, the class of *Environmental Physics and Renewable Energy, 2014* who have made my stay at *NMBU* very fun and interesting.

Ås, December 16^{th} 2019

Iver Bergh Hvidsten

Abstract

Sugar based bio-surfactants are green alternatives to synthetic surfactants which have gained increasing interest over the past 20 years. In this thesis, lauric acid and vinyl laurate has been investigated as acyl donor and galactose as acyl acceptor. The solvent of choice was acetone and the catalyst was Novozyme® 435. A new reaction was discovered from vinyl laurate to lauric acid and methyl laurate which was further investigated through response surface methodology. The chosen independent variables were reaction time and catalyst amount, the resulting equation was $Conversion = 53.168 - 4.54795 \cdot cat + 32.283 \cdot t + 0.160424 \cdot cat^2 + 0.043 \cdot cat \cdot t - 4.79922 \cdot t^2$ with an $R^2 = 0.899$. The optimum value for conversion based on this model was 88.47 % with $t = 3.46\ h$ and $cat = 22.0711$ %. The trial at the optimum confirmed the model with conversions as high as 94 % and 96 %.

Sammendrag

Sukkerbaserte biologiske surfaktanter er et alternativ til syntetiske varianter. Disse har fått økende oppmerksomhet i løpet av de siste 20 årene i vitenskapelig forskning. I denne masteroppgaven er vinyllaurat og laurinsyre undersøkt som acyldonor og galaktose som acylakseptor. Løsemiddelet som ble brukt var aceton og den biologiske katalysatoren var *Novozyme®435*, *N435*. Det ønskede produktet var en ester av galaktose og laurinsyre. Gjennom forsøk på å reprodusere litteratur, ble en bireaksjon mellom vinyllaurat og aceton oppdaget, katalysert av *N435*. Produktene som ble funnet fra denne reaksjonen var metyllaurate og laurinsyre. Denne reaksjon ble videre undersøkt med et *design av eksperimenter*, *DOE*, og med *respons overflate metodikk*, *RSM*. De to uavhengige variablene var mengde katalysator og reaksjonstid. Den optimerte likningen for omgjøring ble $Omgjoring = 53.168 - 4.54795 \cdot cat + 32.283 \cdot t + 0.160424 \cdot cat^2 + 0.043 \cdot cat \cdot t - 4.79922 \cdot t^2$ med en $R^2 = 0.899$. Den optimale verdien for omgjøring i følge modellen var 88.47 % med de uanhengig variablene t=3.46h and cat = 22.0711 %. To reaksjoner med disse initialbetingelsene ble gjennomført og resulterte i en gjennomsnittlig omgjøring på 95 %, som er innenfor standardavviket til modellen.

Nomenclature

$CALB$ Candida Antartica Lipase B

DOE Design of Experiments

ESI Emulsion Stability Index

$Ewt\%$ Enzyme Weight %

FID Flame Ionization Detector

GC Gas Chromatograph

HLB Hydrophilic Lipophilic Balance

IS Internal Standard

MS Mass Spectrometer

$N435$ Novozyme® 435

RSM Response Surface Methodology

$SFAE$ Sugar Fatty Acid Ester

$t-BuOH$ Tertiary Butanol

List of Figures

2.1 β-D-galactose, a) molecule and b) physical properties 4
2.2 Lauric acid, a) molecule and b) physical properties 6
2.3 Vinyl laurate, a) molecule and b) physical properties 7
2.4 t-BuOH, a) molecule and b) physical properties 9
2.5 Acetone, a) molecule and b) physical properties 10
2.6 Bio-molecule of the lipase *CALB* 13
2.7 Reaction schemes of a) esterification and b) trans-esterification . 15
2.8 Molecule of $\beta - D - galactose - 6 - O - laurateester$ 17
2.9 *HLB* scale 18
2.10 Scheme of a *DOE* with 2^2 factorial design with star-points 25

3.1 Picture of two reactors used for syntheses, a) *Parr batch reactor* b) *Quark glass reactor* 28
3.2 Picture of the *plate reactor* setup 29
3.3 Picture of *GC* setup, *GC* to the left and *GC-MS* to the right. 30
3.4 Picture of the *Rotavapor R-3*, with the vacuum pump to the left. . 31
3.5 Pictures of some equipment used. μ-pipette at the top, filter papers below, prep-bench at the bottom left and the scale in the bottom right. 33
3.6 *GC*-ramp used in analysis 36

4.1 Yield of Reaction 1.14 40
4.2 *GC* spectra of reaction 1.14 41
4.3 Results, in yield, of trial 2.1-2.5 43

4.4 Comparison of *GC* spectra from reaction 3.2 45
4.5 *MS* spectra of trials 3.1-3.5 46
4.6 Experimental conversion plotted against theoretical conversion . 49
4.7 Surface plot of the *RSM* as a wire frame with contours at the base 50
4.8 Estimated response surface at the top and contours of estimated response surface at the bottom. 51

5.1 *GC* spectra of sample 0 and the final sample from the first central point, reaction DOE 1. 58
5.2 GC-spectre from the final sample in reaction DOE 12. 59
5.3 Picture of icing of t-BuOH in the cooling column inside the rotary evaporator 61
5.4 Proposed side reaction schemes for trials with acetone and vinyl laurate 62
5.5 Picture of enzyme pre- and post use. 63
5.6 *GC*-ramp development 1 66
5.7 *GC*-ramp development 2 67

List of Tables

2.1 A collection of sugars used for *SFAE* synthesis 4

2.2 Collection of fatty acid and vinyl esters used for *SFAE* synthesis. . 8

2.3 Collection of organic solvents used for *SFAE* synthesis. 11

2.4 Compilation of prior research in *SFAE* synthesis. 21

2.5 Statistical formulas used in the *RSM* analysis 26

3.1 Materials used in this work with its manufacturer and chemical purity. 32

4.1 Result in conversion and yield of 3.1-3.5 44

4.2 Results from 3.6-3.10 in yields to different products. 47

4.3 Encoded values for *DOE*, α-values are calculated with $\alpha = \pm 2^{n/4}$, where n is number of variables in the design. 48

4.4 *DOE* with 12 reactions. The independent variables, their encoding and the conversion used in the response surface analysis. . . . 48

4.5 Statistical analysis on the model. 49

5.1 Key changes made during the first batch of reactions. 54

B1 Reactions conducted before the *DOE* analysis 81

B2 Reactions conducted to build the *DOE* analysis. 82

Contents

Preface **I**

Abstract **III**

Summary **V**

Nomenclature **VII**

List of Figures **X**

List of Tables **XI**

1 Introduction **1**

2 Theory **3**

2.1 Sugar 3

2.1.1 Galactose 3

2.1.2 Sugars Used for *SFAE* Synthesis 4

2.1.3 Feedstock 5

2.2 Free Fatty Acids and Their Vinyl Esters 6

2.2.1 Lauric Acid 6

2.2.2 Vinyl Laurate 7

2.2.3 Acyl Donors Used for *SFAE* Synthesis 7

2.3 Solvent 9

2.3.1 Tertiary Butanol 9

2.3.2 Acetone . . . 10
2.3.3 Solvent Less Systems . . . 10
2.3.4 Solvents Used for *SFAE* Synthesis . . . 10
2.3.5 Solubility of Sugars in Organic Solvents . . . 11
2.4 Enzyme . . . 12
2.4.1 Anhydrous Solvent . . . 12
2.4.2 Immobilization . . . 13
2.4.3 Production . . . 13
2.4.4 Enzymes in Literature . . . 14
2.5 Reaction Principles . . . 15
2.5.1 Advantages of Enzymatic Synthesis . . . 15
2.6 Sugar Fatty Acid Esters . . . 17
2.6.1 Properties and Functions . . . 17
2.6.2 Applications . . . 19
2.6.3 Prior Research on SFAE . . . 19
2.7 Modeling and Analytical Theory . . . 22
2.7.1 Gas Chromatography . . . 22
2.7.2 Mass Spectrometry . . . 23
2.7.3 Titration . . . 23
2.7.4 Response Surface Methodology . . . 24

3 Equipment, Materials and Methodology 27
3.1 Equipment . . . 27
3.1.1 Reactors . . . 27
3.1.2 Analytical Instruments . . . 29
3.1.3 Purification Equipment . . . 31
3.1.4 Other Equipment . . . 31
3.2 Materials . . . 32
3.3 Methodology . . . 34

3.3.1 Reaction Procedure 34
3.3.2 Reaction Sampling 34
3.3.3 GC and MS Samples 35
3.3.4 GC and MS Method 36
3.3.5 Titration 36
3.3.6 Processing of Reaction Solutions 37

4 Results **39**
4.1 Batch 1, Reproduction of Literature 39
4.2 Batch 2, Optimization of Reaction 1.14 42
4.3 Batch 3, Reproducible Results 44
4.4 Batch 4, Design of Experiments 48

5 Discussion **53**
5.1 Batch 1 53
5.2 Batch 2 55
5.3 Batch 3 56
5.4 Batch 4 57
5.5 General Discussion 60

6 Conclusion **69**

Appendix A **78**
The 12 Principles of Green Chemistry 78

Appendix B **80**
Conducted Reactions 80

Chapter 1

Introduction

In recent years with global temperature increase, the need for greener alternatives are becoming more evident. In the field of green chemistry, 12 principles were proposed in 1998 by *Anastas* and *Warner* [1] as guidelines to shift the chemical industry into a more environmentally friendly direction. The principles are listed in Appendix A. Among these principles, the utilization of renewable feedstocks are specifically addressed to make the chemical industry greener. In addition to be renewable, the origin of the reaction components should not be in competition with food production, but rather attempt to utilize waste products from different industries and densely populated areas when possible. Many chemical processes utilize dangerous and hazardous components and effort should be made to minimize the overall toxicity of chemical processes and to prevent spillage. A major factor to accomplish the 12 principles lies in the shift from a petrochemical based to a bio-based economy, in addition to further develop the industrial production by environmentally friendly standards.

The synthesis of different *Sugar Fatty Acid Esters*, **SFAEs**, has been subject to attention in the scientific community due to bio-biodegradability, non-toxicity and low environmental impact [2]. A key aspect that makes this synthesis possible is the shift in equilibrium towards the synthesis of esters when free or immobilized lipases are employed in anhydrous reaction media, e.g. organic solvents, ionic liquids and super critical fluids [3]. More over, a single step reaction helps to reduced the overall energy consumption in the process along with relatively low reaction temperatures and ambient pressures. These esters are usually synthesised by esterification where a free fatty acid and a sugar are dissolved or suspended in an anhydrous solvent in the presence of the selected bio-catalyst. Trans-esterification with vinyl esters is also a possible route to ob-

tain such esters. Based on their favourable properties of being non-toxic and an antimicrobial agent [4], they can be used as an emulsion agent in the food industry and for various creams. For these uses the *hydrophilic liphophilic balance*, **HLB**, which is based on the size of the polar sugar and non-polar fatty acid, synthesised in the ester. In other words the desired *HLB* can be engineered through substrate selection. Under the trademark name *RYOTO™ SUGAR ESTER*, *Mitsubishi Chemical Corporation* has done just this by offering varieties of *SFAE* based on sucrose and different fatty acids to cover most of the *HLB*-scale. This process however is operating in the absence of lipase and it's of interest to investigate the possibility to synthesise these with a bio-catalyst.

In this thesis galactose has been investigated for the production of *SFAE* with both lauric acid and its vinyl ester. The solvents employed included t-BuOH and acetone with *Novozyme®435*, **N435**, as the bio-catalyst. During trials to reproduce the results from *Universidad Complutense Madrid* by *Monreal* [5] and *Alonso* [6], a reaction to methyl laurate catalyzed by *N435* were discovered. The reactions of the sugar esters described by the authors from Spain were not successfully reproduced. The methodology developed at *Norwegian University of Life Sciences*, **NMBU**, let us investigate this side reaction that involved vinyl laurate and acetone catalyzed by *N435* solved in acetone. With the parameters catalyst amount and reaction time, the side reaction was investigated in a 2^2 factorial *design of experiments*, **DOE**, with star points and four central points. Each reaction at the desired encoded variables, 12 in total, were measured on the conversion of vinyl laurate and used in the *response surface methodology*, **RSM**, to produce a quadratic model of optimal conversion. The optimum from the model were at 88.57%, and at the optimal conditions two reactions were conducted and resulted in an average of ~ 95% which is within the standard deviation on the model.

This thesis first present the theory on *SFAE* in Chapter 2, where the different components in the reaction are presented with a literature search of prior research in the field. The reaction principles and theory on modeling and analytical equipment are also presented in the second chapter. In Chapter 3, equipment, materials and methodology used and/or developed in this thesis are presented. In Chapter 4, the important results are presented with corresponding discussion in Chapter 5. Finally, in Chapter 6 the conclusions from this thesis are listed, with suggestions for further work on *SFAE* syntheses at *NMBU*.

Chapter 2

Theory

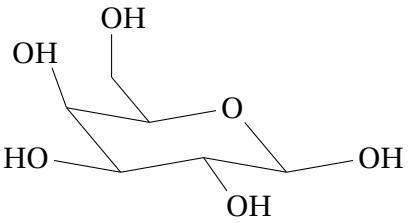
The theory part of this thesis gives a basis for the synthesis of *sugar fatty acid esters*, **SFAEs**, with references to prior work in the field. The raw materials are presented in Chapter 2.1 and 2.2. Then the solvents are presented in Section 2.3 and the enzyme in Section 2.4. Furthermore, Section 2.5 gives a basis for the chemical reactions in question and in Section 2.6, the proposed product is presented with properties and applications. In the final part of the theory, Section 2.7 gives a basic understanding of the analytical tools used in this work.

2.1 Sugar

Carbohydrates is a vast group of organic molecules and the most important source of energy for animal metabolism. The carbohydrate group governs four major sub-groups: mono-, di-, poly-, and oligo-saccharides. Sugars are simple carbohydrates of mono- or di-saccharides. In this work a simple sugar, galactose, were investigated for *SFAE* synthesis.

2.1.1 Galactose

Galactose is a mono-saccharide which can be found in most living organism and is displayed in figure 2.1a with some physical properties in table 2.1b. Although the abundance of galactose, it is rarely found on its own, but rather bonded in longer carbohydrate chains. For instance di-saccharides, lactose, and polysaccharides, galactans and chitosan. Galactose can also be found in combination with other molecules for instance lipids also called galactolipids.



(a) β-D-Galactose molecule

(b) Physical properties of galactose

Property	Value
Molecular Weight $[g/mol]$	180.16
Flash Point $[\,^{\circ}C\,]$	410.8
Melting Temperature $[\,^{\circ}C\,]$	167
Relative Density	1.732

Figure 2.1: β-D-galactose, a) molecule and b) physical properties

2.1.2 Sugars Used for *SFAE* Synthesis

Prior to this thesis a wide variety of sugars have been tested to produce biosurfactants. An overview is presented in table 2.1. From this table the sugars that have been investigated extensively are glucose, fructose and sucrose. Sucrose esters are already been commercialized by *Mitsubishi Chemical Corporation* with a patent to produce *SFAE*, under the trademark name *RYOTO*™ *SUGAR ESTER*. This process however is without a bio-catalyst.

Table 2.1: A collection of sugars used for *SFAE* synthesis, with the addition of longer chained saccharides.

	Sugar	ref
Monosaccharides	Galactose	[5]–[8]
	Glucose	[4], [9]–[15]
	Xylitol	[12], [16]
	Fructose	[11], [12], [17], [18]
Disaccharides	Lactose	[3], [19], [20]
	Sucrose	[4], [11], [12], [17], [21]
	Sorbitol	[12]
	Maltose	[4]
Polysaccharides	Fructans	[22]
	Chitosan	[23]
Oligosaccharides	Oligofructose	[24]

2.1.3 Feedstock

Promising feedstocks for the production of galactose are macro- and micro-algea. Amongst the micro-algea *C. Vulgaris* contains high content of galactose, bound in polysaccharides and galactolipids . The production of this strain has an annual yield of about 2000 tonnes (2004). In the family of macro-algeas, or seeweeds, the red strain in particular show high yields of sugars. This strain contains high amounts of carrageenan and agar which both are built up of galactose. The production of macro-algea outcompetes micro-algae by 4 orders of magnitude. Sea-based production of algae is a promising feedstock for carbohydrates and in particular galactose. [25]–[27]

Some major benefits of algae is as mentioned that they need an hydrous environment to grow and there is no shortage of water-based environments on earth. The sea-based plants can grow in numerous environments for instance sewage, fresh water, saline water, acidic/basic to mention some. Another advantage is rapid growth and how well the plants utilize cultivation area compared to land based crops. Algae can grow in numerous environments and can grow relatively fast. [28]

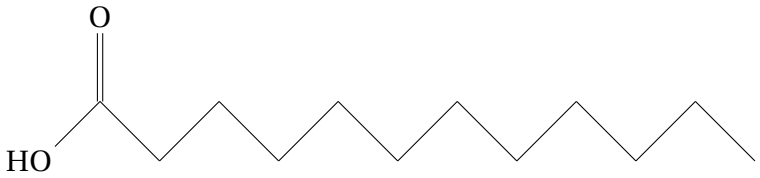
An overly simplified method to extract simple sugars from algae is by drying the algae and then extract the oil contents. After the oil has been extracted, a dry carbohydrate mass is left which then can be hydrolysed to obtain shorter sugar strains and finally simple sugars. [29]

2.2 Free Fatty Acids and Their Vinyl Esters

The organic group of oils consists of mono-, di- and tri-glycerides, where one, two and three fatty acid chains are linked to a glycerol molecule respectively. The configuration of the fatty acids is dependent on the type of oil in addition to the specific fatty acid chain that is attached. When the glyceride is subject to cleavage at the ester bond, free fatty acids will separate from glycerol. The free organic acids are further divided into three subgroups: saturated-, unsaturated- and monounsaturated fatty acids. In this work lauric acid, a free saturated fatty acid with 12 carbons, have been investigated for *SFAE* synthesis. The vinyl ester of lauric acid, vinyl laurate, have also been investigated for the same purpose.

2.2.1 Lauric Acid

Lauric acid is a free saturated fatty acid with 12 carbons, see figure 2.2a and table 2.2b for molecular structure and key information. This fatty acid has favourable properties for the use in especially food-industry as it has been shown to inhibit bacterial growth both in its natural form [30], [31] and when the fatty acid is synthesised into different sugar esters [32]. This fatty acid is mainly produced from palm kernel- and coconut oil [33] by hydrolysis of the triglyceride to separate the free fatty acid from the ester bond. Lauric acid can also be found in other plant oils such as *arecanut* oil and *mandarin* oil [34].



(a) Lauric acid molecule

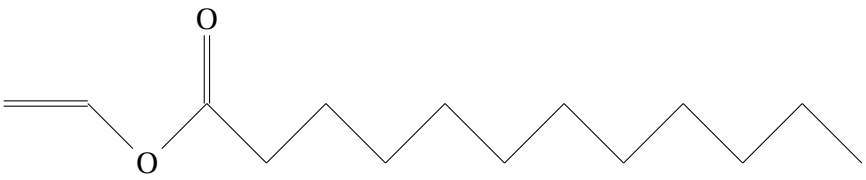
(b) Physical properties of lauric acid

Property	Value
Molecular Weight [g/mol]	200.322
Flash Point [$^{\circ}C$]	112.8
Melting Temeprature [$^{\circ}C$]	43
Relative Density	0.883

Figure 2.2: Lauric acid, a) molecule and b) physical properties

2.2.2 Vinyl Laurate

Vinyl laurate is the vinyl ester of lauric acid, more precisely a double bonded carbon-carbon molecule as the second organic group in the ester-bond. The molecule is drawn in figure 2.3a with the addition of some physical properties in Table 2.3b.



(a) Vinyl laurate molecule

(b) Physical properties of vinyl laurate

Property	Value
Molecular Weight $[g/mol]$	226.35
Flash Point $[\,^{\circ}C\,]$	125
Melting Temperature $[\,^{\circ}C\,]$	7.22
Relative Density	0.873

Figure 2.3: Vinyl laurate, a) molecule and b) physical properties

2.2.3 Acyl Donors Used for *SFAE* Synthesis

A good variety of fatty acids and some vinyl esters have been tested before to produce *SFAE* and some are presented in Table 2.2. From the Table, saturated fatty acids have been tested extensively as acyl donors for the esterification-reaction with carbon-chain length ranging from 4 to 18. Within this group lauric- and palmitic acid are the most tested. In addition to the saturated fatty acids, a mono-unsaturated fatty acid, oleic acid, have been tested as acyl donor.

When the raw material is changed for a vinyl ester, the reaction is changed to a trans-esterification. Unlike the saturated fatty acids in this collection, the vinyl esters have not been tested in such extent as only two different vinyl esters are in this compilation. Among the two, vinyl laurate have gained more interest in the scientific community than vinyl palmitate.

Table 2.2: Collection of fatty acid and vinyl esters used for *SFAE* synthesis.

	Name	**Carbons**	**Ref.**
SFA	Butyric acid	4	[16]
	Hexanoic acid	6	[13], [15]
	Caprylic acid	8	[3], [5], [13], [24]
	Decanoic acid	10	[13]
	Lauric acid	12	[3], [11], [13]–[17], [19], [20], [24], [35], [36]
	Myristic acid	14	[13]
	Palmitic acid	16	[3], [10], [13], [15], [17], [18], [24]
	Stearic acid	18	[24]
MUFA	Oleic acid	18, cis-9	[6], [12], [16], [23], [37]
VE	Vinyl laurate	12	[4], [11], [21], [22]
	Vinyl palmitate	16	[4], [9], [21]
Other	FAC	10,12,14,16	[8]

SFA - Saturated Fatty Acids, **MUFA** - Mono-Unsaturated Fatty Acids,
VE - Vinyl esters, **FAC** - Fatty Acyl Chlorides

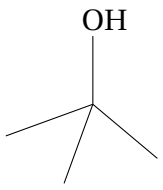
2.3 Solvent

The solvents used in these trials were acetone and tertiary butanol with the addition of some solvent less systems. Organic solvents are characterized by having at least one carbon and one hydrogen atom and being able to solve other compounds. Other than these, organic solvents comes with a wide variety in functional groups and molecular shapes. Acetone is the simplest ketone and tert butanol is the simplest tertiary alcohol. Interest in such solvents has increased the last 20 years in the field of biochemical synthesis.

There lies an important decision in choosing the reaction medium, as organic solvents are generally hazardous and in many cases hostile to the environment. A survey of solvent selection guides has been published where a collection of solvent guides are considered and concluded with a ranking of selected solvents [38]. The final results has ranked solvents from *"recommended"* to *"highly hazardous"* based on considerations of health effects, safety and impact on the environment. It is of interest to investigate greener alternatives to hazardous solvents such as pyridine, hexane and tetrahydrofuran. Acetone and tert butanol are ranked as *"recommended or problematic?"* and both can be produced through a fermentation process of biomass [39].

2.3.1 Tertiary Butanol

Tertiary Butanol, or **t-BuOH**, is the simplest tertiary alcohol and can be seen in Figure 2.4a with some key properties in Table 2.4b. This tertiary alcohol is shown to not partake in enzymatic reactions even though an hydroxy group is present [5], [8], [12], which makes t-BuOH advantageous as solvent for enzymatic reactions. More over, t-BuOH can be produced by both fermentation with the *Clostridium* bacteria and from ethanol by an aldol condensation over special metal oxide catalyst [39].



(a) t-BuOH molecule

(b) Physical properties of t-BuOH

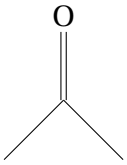
Property	Value
Molecular Weight $[g/mol]$	74.1
Flash Point $[\,^{\circ}C\,]$	11
Melting Point $[\,^{\circ}C\,]$	25
Boiling Point $[\,^{\circ}C\,]$	82
Relative Density	0.8

Figure 2.4: t-BuOH, a) molecule and b) physical properties

2.3.2 Acetone

Acetone is the simplest ketone with three carbons and a double bond to oxygen at the second carbon. The molecule and key information can be seen in Figure 2.5a and in Table 2.5b. As for t-BuOH, acetone is put in the category of *"recommended or problematic?"* as it is a volatile solvent, but to a lower degree than the ones categorized as *"problematic"* to *"highly hazardous"*.

Acetone can be produced by fermentation of biomass by the bacteria *Clostridium*. This bacteria also ferments biomass into t-BuOH, as mentioned above, and ethanol. By obtaining acetone this way, the need for a petrochemical process is eliminated and the solvent can be produced from a renewable feed stock, and hence makes the process greener.



(a) Acetone molecule

(b) Physical properties of acetone

Property	Value
Molecular weight $[g/mol]$	58.08
Flash point $[\,^{\circ}C\,]$	-18
Melting point $[\,^{\circ}C\,]$	-95
Boiling point $[\,^{\circ}C\,]$	56
Relative density	0.8

Figure 2.5: Acetone, a) molecule and b) physical properties

2.3.3 Solvent Less Systems

In solvent less systems one of the reactants acts as the solvent. Some advantages with a system like this is the deselection of possibly dangerous solvents, and in most cases less challenging to purify. On the other hand, to use raw material as solvent can become expensive as solvents are relatively cheap which is not always true for the reactants. The process has the potential to become greener, depending on the raw material selected as solvent.

2.3.4 Solvents Used for *SFAE* Synthesis

Table 2.3 presents a compilation of selected organic solvents that have been applied to synthesis for *SFAEs*. In this Table, molecular weight, melting temperature, boiling point, vapor density, *XlogP3* and their rating are denoted. The *XlogP3* is a coefficient for the octanol-water partition which characterizes the

solvents by their lipophilicity. The *XlogP3* is based on the *log3* [40]. The rating of the solvents are from *"A Survey of Solvent Selection Guides"* [38]. The rating ranges from *"recommended"* through *"recommended or problematic?"*, *"problematic"*, *"problematic or hazardous?"*, *"hazardous"* and finally *"highly hazardous"*. The solvents used in this thesis, acetone and t-BuOH, are both in the *"recommended or problematic?"* category.

Table 2.3: Collection of organic solvents used for *SFAE* synthesis.

Solvent	$M_w[\frac{g}{mol}]$	$T_m[^oC]$	$T_e[^oC]$	ρ_{vap}(air=1)	XlogP3	Rating
t-BuOH	74.1	25.4	82.3	2.55	(AA) 0.5	
Acetone	58.08	-95	56	2.0	(AA) -0.1	
EMK	72.1	-86.6	79.5	2.41	0.3	
ACN	41.05	-43.8	82	1.43	(AA) 0	
Heptan	100.205	-90.6	98.4	3.5	4.4	
Pyridine	79.102	-42	115	2.73	0.7	
THF	72.107	-108	65	2.5	0.5	
Hexane	86.18	-95.3	69	2.97	3.9	
2M2B	88.15	-9	102	1.6kpa (20C)	0.9	NA

EMK - ethyl methyl ketone, ACN - acetonitril, 2M2B - 2-methyl 2-butanol, THF - tetrahydrofuran.
- *"recommended or problematic?"*, *"problematic"*,
"problematic or hazardous?", *"hazardous"*.

2.3.5 Solubility of Sugars in Organic Solvents

The solubility of sugars in organic solvents are low due to the slight polarity of sugar-molecules that is being dissolved in a non-polar solvent. In a system with sugar suspended in an organic solvent, most of the sugar will be in the solid phase and small amounts will be dissolved into the liquid. The dissolved sugar will have access to the active site on the enzyme and when sugar is consumed in a reaction, the equilibrium of solved sugar allows the suspended phase to dissolve incrementally. The initial amount of dissolved sugar have been shown to affect the final conversion in some degree, but it is not clear if the solved sugar or the dissolution rate is governing the reaction [14].

2.4 Enzyme

In this work an immobilized form of a *Candida Antartica Lipase B*, or **CALB**, has been investigated for the synthesis of *SFAE* and the bio-molecule of CALB is shown in Figure 2.6. The commercial name of the immobilized lipase is *Novozyme 435* or **N435**, and lipases are generally extracted from yeast culture in fermentation of biomass [41]. In such an aqueous media the role of lipases are generally to hydrolyze oils and fats into free fatty acids and glycerol [42]. When this enzyme is extracted and purified it can catalyze reactions with high regio- stereo and chemoselectivity in reactions such as hydrolisis of esters, esterification and the trans-esterification [3]. Furthermore when immobilized, the system sees an increase in thermal stability and structural rigidity.

Enzymes are complex bio-molecules that catalyzes highly specific reactions. In general for enzymatic synthesis specific substrates are adsorbed onto the active site and creates an enzyme complex. This complex will then catalyze the reaction on the substrate or substrates and when done, release the product or products. The enzyme is then ready to undergo the same reaction again and again. Enzymes are known to deactivate at certain conditions especially high temperatures, usually above 60 $^{\circ}C$.

2.4.1 Anhydrous Solvent

In an aqueous reaction system, as mentioned above, lipases hydrolyzes the breakdown of oils and fats. Opposed to this, specific enzymes show catalytic activity in anhydrous solvent which drives the equilibrium in the opposite direction of hydrolysis, towards esterification and trans-esterification. In such solvents different lipases have shown to exhibit selectivity towards carbohydrates and fatty acids among others. In the field of enzymatic synthesis, there have been much attention to synthesis of bio-based chemicals solved in organic solvents. Examples of some organic solvents which have been tested with an enzymatic bio-catalyst are presented in Table 2.3 on the previous page. [41], [43]

It has been discovered that enzymes suspended in anhydrous solvents retain and even improve their catalytic behaviour. Even though the enzymes doesn't deactivate in pure anhydrous solvents, low percentages of water in the solution are necessary for "lubrication" of the enzyme. The water will help with the substrates access to the active site. Different methods to control the water content has been studied, where the most common one are the employment of molecular sieves. [44]–[46]

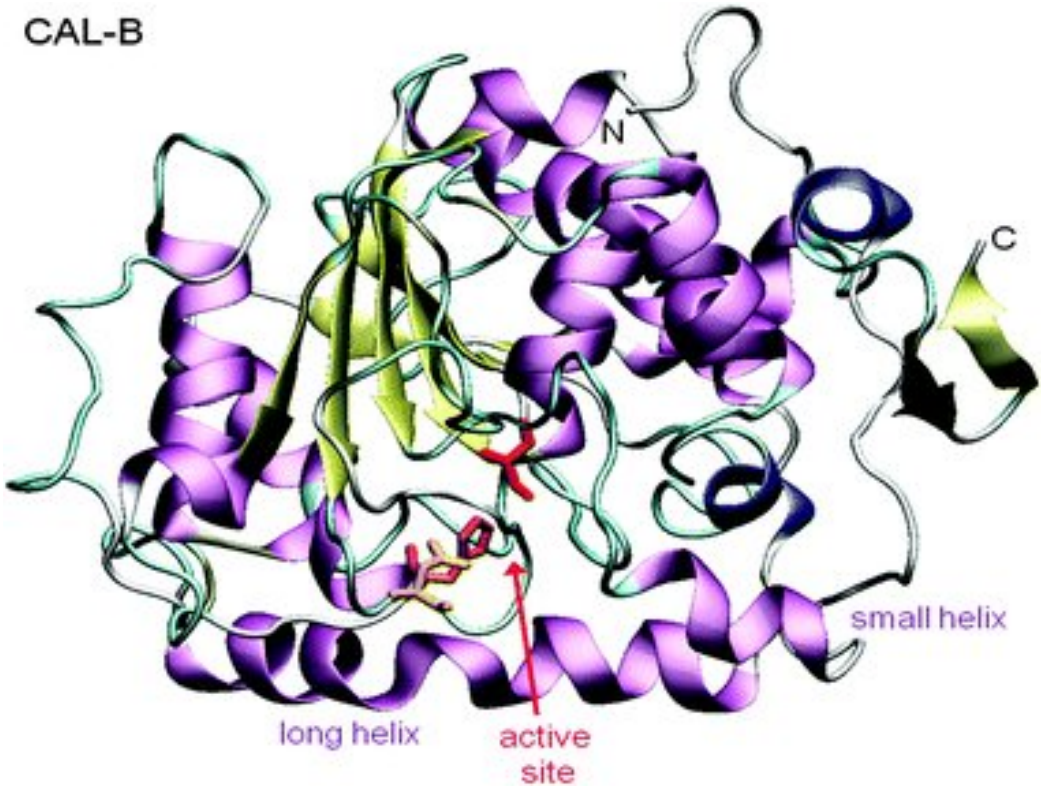


Figure 2.6: Bio-molecule of the lipase *CALB* [42]

2.4.2 Immobilization

As mentioned, an immobilized form of the lipase *CALB*, commercially known as *N435* has been chosen as the enzyme. Immobilization of the enzyme has been shown to improve the thermal- and physical stability in addition to make recovery easier after reactions [3]. *N435* is prepared by suspending *CALB* onto a hydrophobic acrylic resin carrier [47].

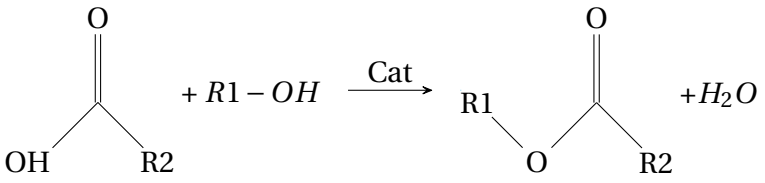
2.4.3 Production

Lipases is a natural building block of many microorganism which allows the organism to metabolize substrates i.e. break down larger molecule chains by hydrolysis. To obtain the enzymes one can grow such cultures in vast range of viable substrates, which includes by-products from different areas of food industry [41]. The two most employed methods to produce these bacteria cultures are by submerged cultures and solid state fermentation [41], [48]. *CALB*

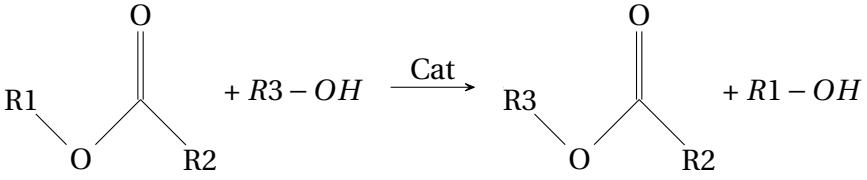
can be produced from yeast and bacteria. More over, the extraction of this enzyme is easier to do from yeast. [42]

2.4.4 Enzymes in Literature

The selection of enzyme in this thesis is based on the extensive research of an immobilized form of *CALB*, the *N435*, which have been employed for *SFAE*-synthesis in many articles with positive results [3], [4], [10]–[12], [14]–[19], [23], [24], [36], [37]. In addition, *CALB* have been tested in its free form [3], [9] and other enzymes such as *Lipozyme RM IM* [22], [37], *Lipozyme TL IM* [8], [19], [22], [37] and lipase from *Mucor meihei* [9], [19] have showed positive results as a bio-catalyst in the field of *SFAE*-synthesis.



(a) Esterification reaction



(b) Trans-esterification reaction

Figure 2.7: Reaction schemes of a) esterification and b) trans-esterification. $R1$, $R2$ and $R3$ are arbitrary organic molecules.

2.5 Reaction Principles

To synthesise an ester, two different reaction principles, an esterification and a trans-esterification, have been investigated. These reactions can be conducted through enzymatic catalyzation and also with an inorganic catalyst, such as a strong acid or a strong base.

Esterification Figure 2.7a shows a simplified esterification reaction. Here an alcohol reacts with a carboxylic acid in the presence of a catalyst to produce an ester and water as a by-product. The reactants can be categorized as acyl acceptor and acyl donor where the carboxylic acid is regarded as the acyl donor and the alcohol as the acyl acceptor.

Trans-esterification Figure 2.7b shows a simplified trans-esterification reaction. In such a reaction an alcohol-group reacts with an ester in the presence of a catalyst. The resulting products are an ester and an alcohol. The side group R1, which was bonded to the oxygen atom in the ester bond, is substituted with the R3-group.

2.5.1 Advantages of Enzymatic Synthesis

The use of enzymes in the esterification or trans-esterification of *SFAEs* have some advantages over traditional chemical routes. Firstly, the reactions with enzymes are usually conducted at lower temperatures which decreases the energy consumption of the process. The need for lower temperatures are due

to the deactivation of enzymes at higher temperatures, above 60-70 $^{\circ}C$. Secondly, elevated pressures are not needed, but can be employed to increase the yield to the desired product. If ambient pressure-systems are used, the energy consumption are further decreased. Thirdly, in chemical synthesis, protective groups of other reactive sites are usually needed due to the lack of regio-, stereo- and chemo selectivity of the selected catalyst. With the use of an enzyme which can catalyse reactions with high selectivity, the need for protective groups are minimized or eliminated altogether. Such groups can introduce impurities and possible require extra purification steps to obtain the product. By the use of an enzymatic catalyst, the reaction can be conducted at lower temperatures, ambient pressure and generally without the need for protective groups to increase the selectivity. [2]

2.6 Sugar Fatty Acid Esters

Sugar fatty acid esters, or **SFAEs**, have been given much attention in recent years based on their favorable properties as an environmentally friendly and non-toxic bio-surfactant. As mentioned in the previous chapter, these ester can be synthesised by enzymes in anhydrous solvents. Furthermore the reaction conditions are less energy intensive with a single-step reaction at relatively low temperatures and ambient pressure. In this work *galactose laurate* was the desired product and can be seen in figure 2.8.

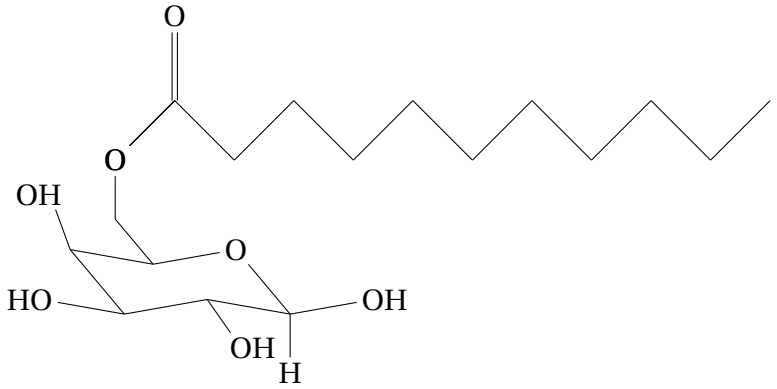


Figure 2.8: Molecule of $\beta - D - galactose - 6 - O - laurateester$

2.6.1 Properties and Functions

The properties of this class of surfactans can be characterized by the *hydrophilic lipophilic balance*, **HLB**, and the *emulsion stability index*, **ESI**. The *HLB* value is a measure of the surfactans emulsification ability in different mixtures of oil and fat, whereas the *ESI* is how well the surfactant can stay in a mixture over time.

HLB

One of the properties *SFAE* holds is not only their ability to emulsify, but also the ability to engineer the *SFAE* to have the desired emulsifying property. The *HLB* value will characterize the ability of a given agent on how well they mix different mixtures of oils in water and visa verse. The scale ranges from 0 to 20, where a low HLB value indicates solubility in oil and a high value indicates solubility in water. The values in between can be seen in 2.9 with some applications. This scale is only valid for non-ionic surfactants.

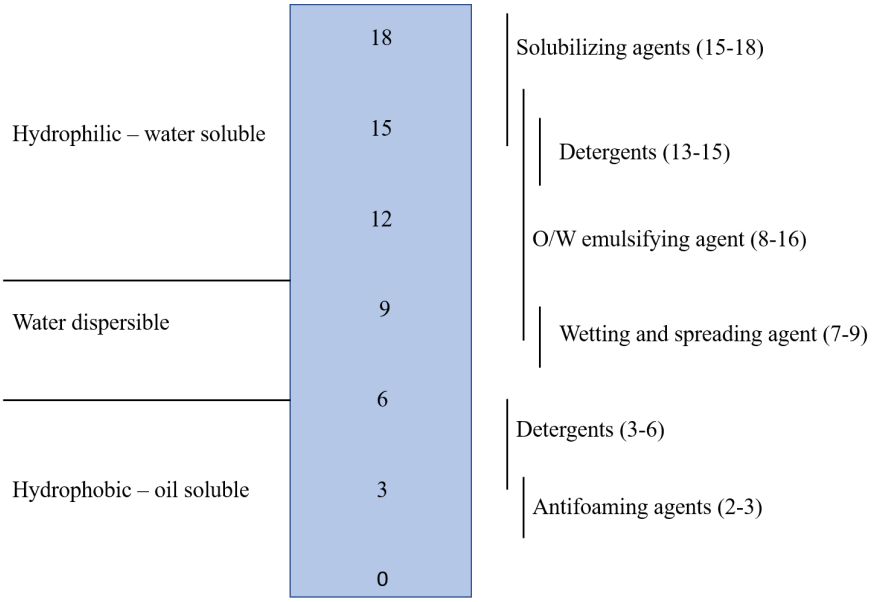


Figure 2.9: *HLB* scale

The *HLB* value for a specific emulsifier can be calculated with Griffin's method from 1954 [49].

$$HLB = 20 \cdot \frac{M_h}{M} \tag{2.1}$$

Where M_h is the molecular mass of the hydrophilic site and M is the total molecular mass. Another way of calculating it is by Davies method from 1957 [49].

$$HLB = 7 + \sum_{i}^{m} h_i - n * 0.475 \tag{2.2}$$

Where m is the total number of hydrophilic groups, h_i is the $i'th$ hydrophilic group and n is the number of lipophilic groups. The advantage of this method is that the it takes into account that the molecules can have more than one hydrophilic site.

With *HLB* determined one can make emulsions for differents uses with the right viscosity. The scale is also additive, meaning one can choose two or more emulsifiers and obtain the desired *HLB* value for a given emulsion.

ESI

Emulsion stability index, **ESI**, is a way to measure how good an emulsion holds after it is formed. There have been reported more ways to calculate the stability, here only the volumetric method is shown:

$$ESI = (1 - \frac{V_w}{V_e}) * 100 \tag{2.3}$$

Where V_e is the volume of emulsion and V_w is the volume of the separated bottom layer after a set storage time-period. This method comes with some error as the volume is read with the naked eye and can be difficult to measure the correct boundary between the phases due to blurry interfaces [50].

2.6.2 Applications

Properties of *SFAE* makes them suitable for applications across different fields such as in pharmaceutical industry, food manufacturing, cosmetics and as detergents. In common for these fields are the need for emulsifying agents that can allow mixing of insoluble phases of water and oil. The galactose laurate ester have also been shown to inhibit bacterial growth towards the *Streptococcus mutans* [32], which makes it suitable both as a emulsifying agent and preservative. In addition to being anti-bacterial, the shape of the galactose ester-molecule resembles an anti-tumor agent, the $3-O-\beta-D-galactopyranosyl-sn-glycerol$ [5].

2.6.3 Prior Research on SFAE

A selection of papers on *SFAE* synthesis is collected in table 2.4. Across these papers a wide variety of different *SFAEs* have been synthesised and the *product* in Table 2.4 are the most successful esters synthesised in each reference. In the *solvent*-column the selected reaction medias are shown and a key part of this type of synthesis is to select a solvent with favourable properties. The primary aspect in the solvent selection is to find a solvent, in which the biocatalyst doesn't deactivate. When such a solvent has been found, other aspects regarding toxicity and risk for the environment can be considered. In addition to the organic solvents, ionic liquids and solvent mixtures have also been successfully employed. In the following column, the volumes in the enzymatic reactions are denoted and across the table, quite low volumes have been tested.

It's only in later studies that this volume has been increased, and the highest in this collection are from [5] and [6] with 250 and 100 ml respectively.

Across this collection, *N435* have been employed as catalyst in the majority of the selected references. Other bio-catalysts such as *TL IM*, lipase from *Mucor meihei* and free lipase of *CALB* have showed positive results. Even the employment of non-enzymatic *zeolites* have been shown to work as an alternative route to obtain *SFAEs*.

The results obtained are generally high and results above 50 % are observed across the collection. The results are measured in conversion or yield depending on the measuring methodology selected. One draw-back in this type of synthesis of sugars and fatty acids are the seemingly long *reaction time* to obtain acceptable conversions or yields. The time spent for synthesis are usually above 1 day and in the longest cases the reaction was let to run in up to 14 $days$. In the other end of the spectre, good conversions have been accomplished in hours.

Table 2.4: Compilation of prior research in *SFAE* synthesis.

Product	**Solvent**	**Volume** $[ml]$	**Enzyme**	**Enzyme Amount**	**Time** $[h]$	**Result** [%]	**Ref**
Lactose esters	Hexane	4	N435	25 wt%	12 days	93	[3]
Galactose caprylate	t-BuOH	250	N435	~ 10 wt%	8	~ 20	[5]
Galactose oleate	2M2B	100	N435	2 wt%	18	39.8	[6]
Galactosyl fatty acyl-amino acid monoester	t-BuOH	10	TL IM	0.49g	6	95.8	[8]
Glucose palmitate	[Bmim][TfO]	5	CAL-B	0.25g	36	31.8	[9]
Glucose palmitate	Acetone	20	N435	30 wt%	4	N.A.	[10]
Xylitol oleate	t-BuOH	30	N435	0.3g	24	98	[12]
Glucose laurate	Mix IL:t-BuOH	0.3:0.2	CAL-B	5% w/v	72	59	[13]
Glucose laurate	2M2B	30	N435	0.3g	50	70	[14]
Glucose hexanoat	DMSO:2M2B	8:2	CAL	0.25g	48	113.11	[15]
Fructose palmitate	2M2B	6.36	N435	10 % w/w	24	65	[17]
Fructose palmitate	EMK	0.6	N435	22.5mg	72	82	[18]
Lactose laurate	2M2B	3	Mucor meihei	0.068g	10-14 days	27.8 mg/ml	[19]
Lactose laurate	t-BuOH	5	Aluminosilicate Zeolite	1.125 mg	10 days	92	[20]
Glucose laurate	EMK	N.A.	CCL Im	~ 60mg	72	76	[35]
Fructose di-laurate	EMK	3	N435	112.5 mg	12	N.A.	[36]
Galactose oleate	$DMSO:[Bmim][BF_4]$	50 $\mu l:1ml$	RM IM	2 % w/w	2	87	[37]

2.7 Modeling and Analytical Theory

2.7.1 Gas Chromatography

The *Gas Chromatography*, **GC**, is an analytical tool used to quantify what a given sample contains by separation based on molecular weight among other factors. The working principle is separation of chemicals through a *GC-column* with a carrier gas at a set volumetric velocity. When the given sample have drifted through the column, an electric pulse is recorded in the *flame ionization detector*, **FID**, as the chemicals are combusted. The energy released related to the quantity of the compound is graphed with its retention time in a *chromatogram*. By employing an internal standard in the samples the response factor for a given chemical can be estimated with Equation 2.4

$$R_f = \frac{A_{IS}}{A_s} \cdot \frac{m_s}{m_{IS}} \tag{2.4}$$

Where R_f is the response factor for a given chemical, A_{IS} and A_s corresponds to the area in the *GC*-spectres of internal standard and sample respectively, m_s and m_{IS} are the mass of sample and the mass of internal standard. When a retention factor has been calculated, Equation 2.5 is used to calculate the mass percentage of the chemical in an unknown sample.

$$\%m_y = \frac{1}{R_f} \cdot \frac{A_{sx}}{A_{IS}} \cdot \frac{m_{IS}}{m_{sx}} \tag{2.5}$$

Where $\%m_y$ is the mass percentage of the chemical in question, A_{sx} and A_{IS} are the areas of the sample and the internal standard respectively and the masses of sample and internal standard are denoted by m_{sx} and m_{IS}.

Conversion and Yield

The conversion is calculated based in the mass percentage of the reactant by $m = \%m_y * msx$ at given time steps. The change in mass of reactant in the sample can then be calculated with Equation 2.6.

$$Conversion = \frac{m_0 - m_i}{m_0} \cdot 100 \tag{2.6}$$

Where m_0 and m_i is the mass of reactant at the $0'th$ and $i'th$ time step. 100 is a conversion factor for percentage. In addition, the yield of the reaction is

calculated based on the areas of the peaks in the *GC*-spectres with equation 2.7.

$$Yield = \frac{A_{Desired}}{A_{Desired} + \sum A_{Others} + \sum A_{RM}} \cdot 100 \tag{2.7}$$

Where $A_{Desired}$ is the area of the desired product at a given time step, A_{Others} is the area of other materials and A_{RM} is the area of raw materials. 100 is a conversion factor. The yield to a desired product takes into account possible side products.

2.7.2 Mass Spectrometry

Mass spectrometry, **MS**, is an advanced method to classify molecules by ionization and detection of these ions. The working principle of this tool starts with separation of molecules through an *MS-column*. The next step is to ionize the separated molecules in a vacuum-chamber, into ions and radicals. Then only the ions are accelerated and concentrated before they are deflected off in a magnetic field. After deflection, the ions are detected and their *mass to charge ratio*, m/z, is calculated based on the curved path of the ions. The spectra obtained by the *MS* are the m/z versus the relative abundance of the ions. The highest peak is by convention set to 100 %, all other peaks are related to the highest peak. Each chemical is ionized differently and consequently will produce unique spectra that can be used for classification. [51]

2.7.3 Titration

Titration is an quantitatively analysis-method to investigate acidity index in a given solution. The main principle is to neutralize an acidic solution with a base or vice versa. The system consists of a titration *burette* where the *titrator* with a known concentration is contained. This titrator is then added in small volumes at a time to the unknown solution, the *analyte*, that is comprised of the unknown sample, a mixture of solvents and an indicator. This indicator changes the color of the analyte at a given pH-value and the volume used to reach this point is called the *titration volume*. When the color of the analyte changes, the acidity index in the sample can be calculated with Equation 2.8

$$A = M_w(t) \cdot 1000 \cdot C_t \cdot \frac{V_t}{w_s} \tag{2.8}$$

where A_i is the acidity index, $M_w(t)$ is the molecular weight of the titrator, 1000 is a conversion factor, C_t is the concentration of titrator , V_t is the titration volume and w_s is the weight of the sample added to the analyte solution. From this equation the change in acidity index across different solutions can be calculated based on Equation 2.9

$$Conversion = \frac{A_0 - A_i}{A_0} \tag{2.9}$$

where $Conversion$ is the change of raw material, A_0 is the initial acidity index at time zero and A_i is acidity index at each given time step.

2.7.4 Response Surface Methodology

Response surface methodology shortened as **RSM** is a method that uses statistical and mathematical tools to determine the influence of different variables on the response in a process. For simplification the process described here is an arbitrary chemical reaction investigated through a central composite design. To build such a model central points are needed to "benchmark" the reaction. The next step is to decide the other trials, and for the linear stage the number of trials needed are decided by $trials = 2^k$, where k is the number of independent variables. These trials are combinations of the high and low levels of the variables. A linear stage is usually not enough to capture all the interactions amongst the variables therefore star points are introduced, usually denoted by α, calculated with $\alpha = \pm 2^{n/4}$, where n is the number of variables in the design. The star points introduce curvature to the model. In figure 2.10 a *design of experiments*, *DOE*, with two variables and star points is shown. Here one can see the different encodings needed to investigate the variables and to build a quadratic model that takes the form of equation 2.10.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \epsilon \tag{2.10}$$

The β_0, β_i, β_{ii} and β_{ij} are regression coefficients, y is the response of the model, the independent variables are denoted with x_i and x_j and finally the deviation in the model is captured by the ϵ-term. The regression coefficients are found by solving the system of linear equations with the method of least squares, and in linear algebra denotation the equation to solve takes the form:

$$y = X\beta + \epsilon \tag{2.11}$$

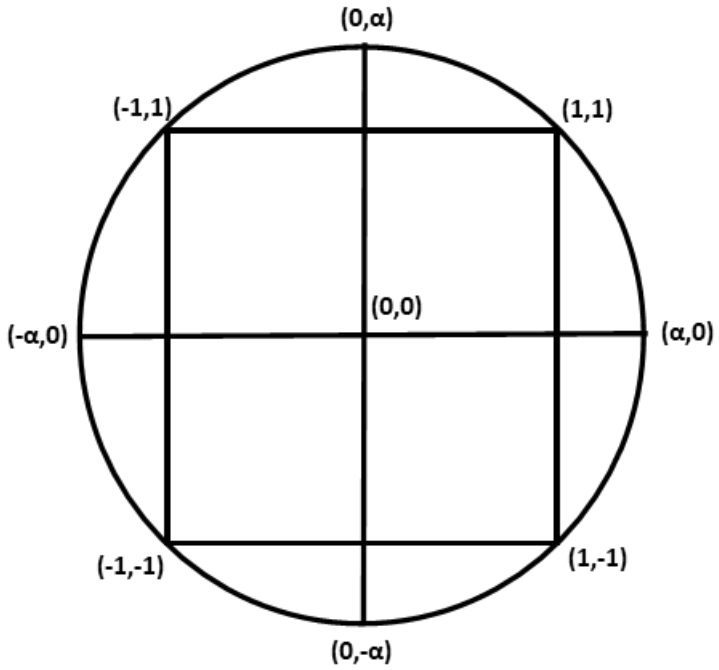


Figure 2.10: Scheme of a *DOE* with 2^2 factorial design with star-points

Here β is the vector of all the coefficients, y is the response vector, X is the matrix of all variables and ϵ is the residual-vector. Such a quadratic model can approximate the variation in a reaction in a given domain for the parameters, but with approximations, errors are introduced. The sum of squares of the errors, or SSE, denoted by ϵ can be calculated with the following equation:

$$SSE = \sum_{i=1}^{n} \epsilon_i^2 = \sum_{i}^{n} (y_i - \hat{y}_i)^2 \tag{2.12}$$

Where y_i is the true value obtained trough trial and $\hat{y}_i$ is the predicted by the model. The model should find regression coefficients that minimizes the SSE. Other statistics on the model are shown in Table 2.5. [52], [53]

Table 2.5: Statistical formulas used in the *RSM* analysis

Parameter	Formula	Description
$\hat{y}$ -	$\frac{1}{N}\sum_{i=1}^{N} y_i$	$\hat{y}$ - average of linear points, y_i - predicted response N - the total number of linear points.
I_{x_1}	$\frac{1}{2}\sum_{i=-1}^{1}(y_{1,i} - y_{-1,i})$	Variable significance in x_1, where y are the responses at the linear stage and i is an integer with the condition $i \neq 0$
I_{x_2}	$\frac{1}{2}\sum_{i=-1}^{1}(y_{i,1} - y_{i,-1})$	Variable significance in x_2, where y are the responses at the linear stage and i is an integer with the condition $i \neq 0$
$\bar{y}$	$\frac{1}{M}\sum_{j=1}^{M} y_j$	$\bar{y}$ - average of central points y_j - response of central points M - the total number of central points
s	$\sqrt{\frac{1}{M}\sum_{j=1}^{M}(y_j - \bar{y})}$	s - standard deviation of the central points
t		$Students - t$ is obtained through statistical table with a 0.025 confidence intervaland $D.f. = M - 1$.
$C.I.$	$\pm\frac{t \cdot s}{\sqrt{M}}$	$C.I.$ - confidence interval t,s and M from above.
Curvature	$\hat{y} - \bar{y}$	$\hat{y}$ and $\bar{y}$ from above. This number describes the curvature in the model.
$C.C.I.$	$\pm t \cdot s \cdot \sqrt{(\frac{1}{N} + \frac{1}{M})}$	$C.C.I.$ - the curvature confidence interval. The curvature in the model is significant if $C.I.$ is outside the confidence interval.

D.f. is degrees of freedom, **C.I** is confidence interval, **C.C.I** is the confidence curvature interval.

Chapter 3

Equipment, Materials and Methodology

3.1 Equipment

All the equipment used in this study was kindly lend to me by the *Reaction Engineering and Catalysis* group, **REC**, at the *Norwegian University of Life Sciences*, **NMBU**.

3.1.1 Reactors

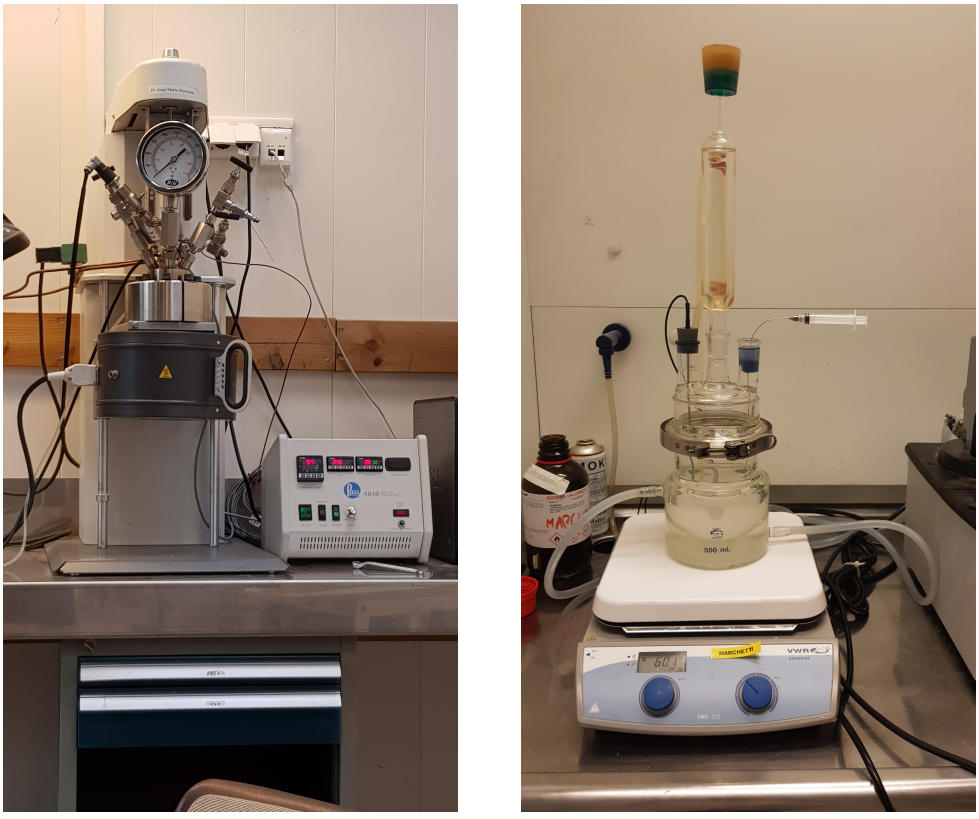
In this work three different type of reactors were used:

Parr Batch Reactor

One of the reactors used in this work was a *Parr batch reactor*, the reactor system consisted of a heavy steel vessel, a lid and side equipment, and is shown in Figure 3.1a. The vessel had a maximum volume of 500 ml. Equipped in the lid was a stirrer with two separate fins, cooling tube, sampling valve, a pressure release valve, pressure meter and thermocoupling. Connected to the reactor system was a controller that regulates the heating jacket around the reactor and the flow of cooling fluid inside the vessel. Two waterbaths were connected, one to the vessel and one to cool the pressure meter.

Quark Glass Reactor

Another reactor used in this work was the *Quark glass reactor* and is shown in Figure 3.1b. The reactor had a maximum volume of 500 ml and around the reactor, a heating jacket connected to a temperature controlled waterbath insured good temperature control. Connected to the lid was a thermocoupling, a sampling syringe and a cooling column for condensation of any solvent that evaporates. The reactor plate underneath showed the temperature of the reaction solution and was equipped with a magnetic stirrer.



(a) Parr batch reactor (b) Quark glass reactor

Figure 3.1: Picture of two reactors used for syntheses, a) *Parr batch reactor* b) *Quark glass reactor*

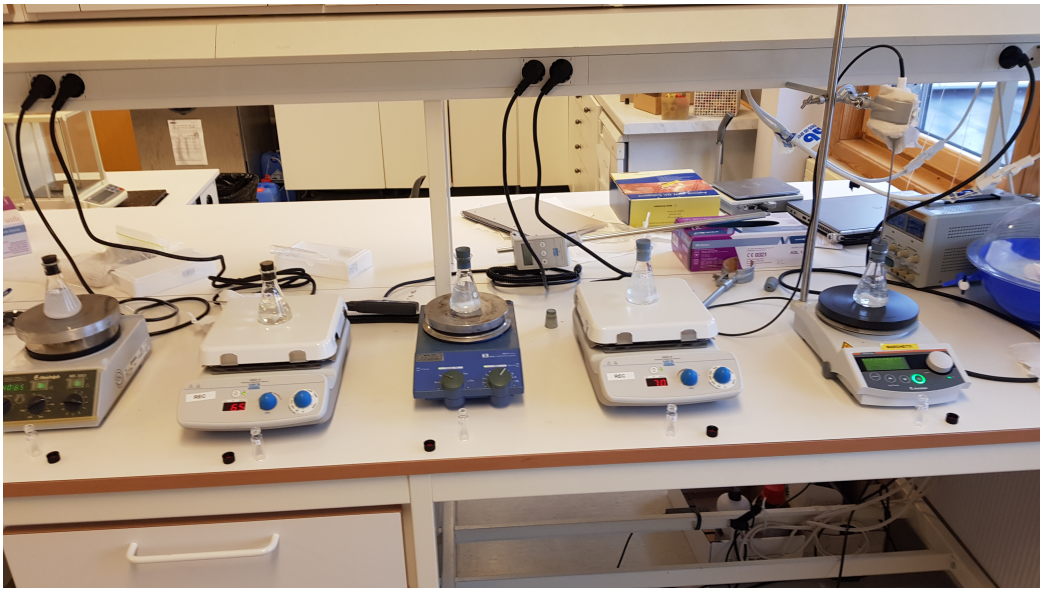


Figure 3.2: Picture of the *plate reactor* setup

Plate reactors

Five plate reactors were used in this work, namely a *Heidolph magnetic stirrer* with active temperature control, a *Heidolph MR 3003*, a *RCT basic IKA labortech* and two *AREC.X Heating Magnetic Stirrer* from *Velp Scientifica*. The reactor vessel that was chosen on top of these reactors were 50 ml *Erlenmeyer* flasks with rubber lids. The plates with the flasks can be seen side by side in Figure 3.2.

3.1.2 Analytical Instruments

Gas Chromatograph

In this work two *GC* instruments were used, they were of the type *Bruker Scion 436-GC*, equipped with an auto sampler *CP-8400*. One of them had a side module with mass spectrometer attached which was a *Scion TQ/SQ*. The systems can be seen in figure 3.3. The injection syringes were supplied by *Hamilton*. Before and after a sample, the syringe was cleaned ten times with the two different solvents acetone and methanol, five times each. In addition to the cleaning of the syringe, the plunger drew and pushed back 1 μl of sample five times before it was injected in a split/splitless regime. This regime starts with a split ratio of 1:10, then for 1 minute there is no split followed by a 1:10 split for the remainder of the run.

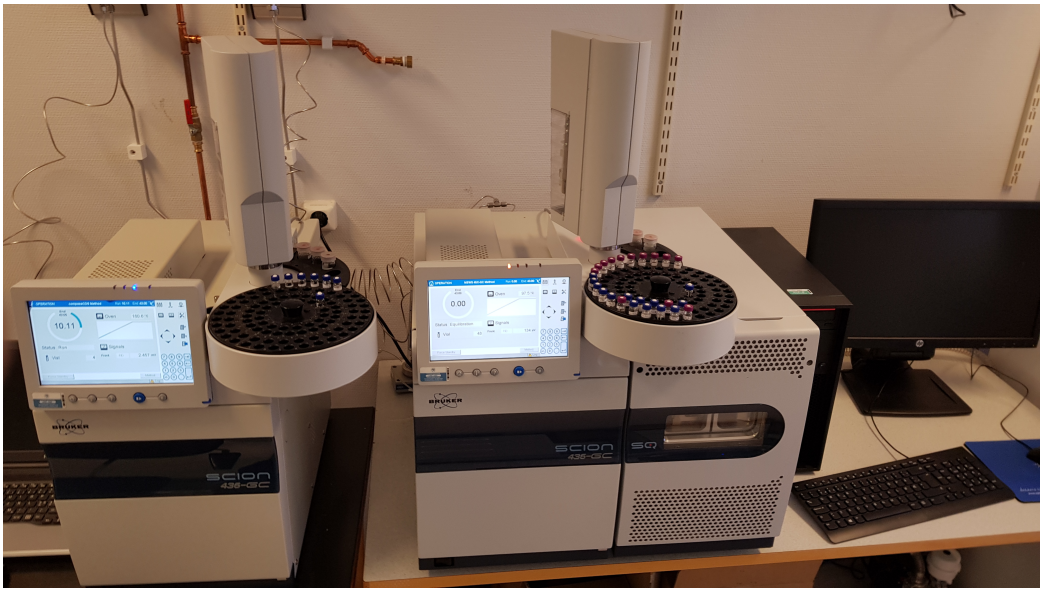


Figure 3.3: Picture of *GC* setup, *GC* to the left and *GC-MS* to the right.

GC

The column in the *GC* on the left in figure 3.3 was supplied by *Agilent technologies* and was the *DB-5HT column*. The specifications on the column were 15 m in length, 0.32 mm in diameter and 0.10 μm in film thickness. The polymer used as film in this column was 5-phenyl-methylpolysiloxane. A flow of 1 ml/min of hydrogen was used as carrier gas and combustion gas. The injector and detector temperatures were set to 275 $^{\circ}C$ and 380 $^{\circ}C$ respectively.

GC-MS

The *GC-MS* used two different columns, one for the *FID* and one for the *MS*. The *FID* column was the same as in the *GC* described above. The column on the *MS* was supplied by *Phenomenex* and was of the type *ZB-5HT Inferno*. The specifications was 15 m in length, 0.32 mm in diameter and 0.1 μm in film thickness. The film was a polyimide-coated fused silica phase. The carrier gas was helium for both pieces of equipment, but hydrogen was used for the combustion in the *FID* and vacuum pressure was used in the *MS*. The flows were 1 ml/min and 3 ml/min to the *FID* and *MS* respectively. The injector temperature was 275 $^{\circ}C$ for both the *GC* and the *MS*. The detector temperature in the *FID* was 380 $^{\circ}C$.

3.1.3 Purification Equipment

Rotary Evaporator

A *Rotavapor R-3* from *Buchi* was used for extracting solvents after the experiments. The water bath were set to a desired temperature for specific solvents and the cooling fluid in the condensation column had a constant temperature. Furthermore a vacuum pump from *Eyela* was used to reduce the pressure in the system. The setup is shown in figure 3.4 with the vacuum pump to the left, water bath in the middle and the condensations system above the waterbath.

Figure 3.4: Picture of the *Rotavapor R-3*, with the vacuum pump to the left.

Filter Paper

The filter papers were supplied by *VWR* and two sizes were used, 150 mm and 90 mm in diamater. The papers were of the type *qualitative filter paper, 410* with a particle retention of 2 μm that resulted in a slow filtration rate.

3.1.4 Other Equipment

μ-Pipette. In this work a μ-pipette from *VWR* was used and is shown in the top of Figure 3.5. The range of the pipette was from 1 to 100 μl.

Prep Bench. The preparation of samples were done in *Agilent Technologies 7696A Sample Prep Workbench* utilising a mixing program. The prep bench is shown in the bottom left of Figure 3.5.

Glassware, Plastics and Metallic Equipment. All of the other equipment, e.g. *Erlenmeyer* flasks, beakers, spoons, funnels etc. were lend by the *REC* group at *NMBU*.

Scale. Scale from *A&D* were used for *GC* samples and weighting of the raw materials. The scale had a maximum capacity of 210 g and a minimum of 1 mg. The standard deviation in the scale was 0.1 mg. The scale used in this work is shown in the bottom right in Figure 3.5.

Camera. The camera that was used to document this work was the mobile camera on a *Samsung S8*. The resolution in the pictures were 4032x2268.

3.2 Materials

The materials used in this thesis were purchased by the *REC* group at *NMBU* and are listed in Table 3.1 with manufacturer and purity. The enzyme was kindly donated by *Novozymes A/S, (Bagsværd, Denmark)*.

Table 3.1: Materials used in this work with its manufacturer and chemical purity.

	Material	Manufacturer	Purity
Reaction Components	D-(+)-Galactose	Sigma Aldrich	≥ 98%
	Lauric Acid	Sigma Aldrich	≥ 98%
	Oleic Acid	Sigma Aldrich	90%
	Vinyl Laurate	Sigma Aldrich	≥ 99.0%
Solvents	t-BuOH	Sigma Aldrich	≥ 99.0%
	Acetone	Sigma Aldrich	≥ 99.8%
Analysis	Pyridine	Sigma Aldrich	≥ 99.8%
	Hexane	Sigma Aldrich	95%
	Tetradecane	Sigma Aldrich	99%
Catalyst	Novozyme®435	Novozymes	N.A.

Figure 3.5: Pictures of some equipment used. μ-pipette at the top, filter papers below, prep-bench at the bottom left and the scale in the bottom right.

3.3 Methodology

3.3.1 Reaction Procedure

The reaction procedures were as follows: for the *Parr batch reactor* all the components were weighted and poured into the reactor with the selected solvent. Then the reactor was heated to the desired temperature and sample 0 was withdrawn. When the reaction was finished the reactor was turned off and the reaction solution was let to stay overnight in the reactor. The vessel was cleaned with hot water and detergent.

For the *Quark glass reactor* the solvent was first poured into the reactor and the temperature was set to stabilize. When the temperature was close to the desired set point, the components were weighted in plastic trays then added one at a time and re-weighted. After the components were added, sample 0 was taken followed by the addition of the enzyme and the start of the reaction. When the desired reaction time was reached, the waterbath was turned off and the reaction batch was collected. The reactor was cleaned with hot water and detergent, it was let to air dry after rinsing with alcohol.

For the *Erlenmeyer* flasks on *Plate reactors*, desired volume of solvent was measured and poured into the flasks followed by the raw materials, which were weighted in plastic trays and re-weighted. After the solution had been prepared the flasks were heated to the desired temperature and when it was reached, sample 0 was collected. The reaction started with the addition of desired amounts of enzyme and ended when the reaction time was reached and the final sample had been withdrawn.

3.3.2 Reaction Sampling

Aliquotes were withdrawn from the reactors at given time intervals and stored in an ice bath or moved to a fridge. The procedure of taking samples were a little different amongst the reactors: in the **Parr batch reactor** the sampling system relied on sufficient pressure in the reactor to push a sample out. If the system were set to run at above ambient pressure, aliquotes could be withdrawn without further actions. On the other hand, if a reaction was set to run at ambient pressure, the system had to be pressurized with air to extract the sample followed by the release of the air to the desired running pressure. For the **Quark glass reactor** a syringe was connected to the reactor, in which the samples were extracted and collected. The syringe was then cleaned with 1-3 volumes of rectified alcohol followed by a push-back of air trough the sampling syringe. Fi-

nally for the **Erlenmeyer flasks**, the sampling was done with the μ-pipette, here only sample zero and the final sample was taken.

Modified Sampling Procedure

During the trials, new methods for extracting samples in *Quark glass reactor* were developed. The need for a new method arose when particles got stuck in the sampling needle and consequently extraction of sample became an obstacle. Two new methods were developed, one involved the push-back of more air, around 3 syringe volumes. In the other method, the syringe was cleaned with acetone followed by the addition of acetone into the reactor. The volumes pushed back into the reactor were recorded.

3.3.3 GC and MS Samples

The samples from the reactions were prepared for *GC* and *MS* by weighting 0.001-0.002 g of both sample and the internal standard tetradecane. Then 1.8 ml of pyridine was added to obtain a concentration between $0.55 \cdot 10^{-3} g/ml$ and $1.11 \cdot 10^{-3} g/ml$. The samples were then mixed at ambient temperature at 3000 rpm in the *Agilent Technologies Prep Bench*. When the samples were done mixing, they were set to run on the *GC* and/or *MS* with the selected temperature ramp.

Dilution of GC and MS Samples

The dilution of *GC* samples were used to test the scales accuracy in the low range or when the areas in the spectres became too wide. The procedure consisted of making a sample with the desired amount of reaction sample and tetradecane. To this sample, 1 ml of pyridine was added. The concentration of tetradecane in the resulting solution was calculated with Equation 3.1.

$$C_{TD} = \frac{m_{TD}}{V_{pyridine}} \tag{3.1}$$

Here C_{TD} is the concentration of tetradecane in the sample, m_{TD} is the mass of tetradecane added to the vial and $V_{pyridine}$ is the volume of pyridine. From this new concentration 100 μl were extracted with the μ-pipette and moved to a new vial with the addition of 1 ml pyridine. The mass of tetradecane in the new sample is calculated with Equation 3.2.

$$m_{TD} = 100\mu l \cdot C_{TD} \tag{3.2}$$

With this new mass of tetradecane equation 3.1 was used again to get a new concentration in the sample. For repeated dilution these two equations were used to first obtain the concentration and then the mass in the new vial. Note that the volume of pyridine was 1.1 ml for all dilutions, except the first one. For simplicity the equations were based on the concentration of tetradecane rather than the sample, but both the mass and concentration of tetradecane and sample can be calculated with these equations.

3.3.4 GC and MS Method

A method for the *GC* and *MS* were developed and by using the methodology described in Section 3.3.3 **GC and MS Samples** and a quite fast ramping program, the decrease of reactant could be observed. The temperature ramp that was used is presented in Figure 3.6, which started at 90 $^{\circ}C$ and for 6.43 min increased with 7 $^{\circ}C$ /min to 135 $^{\circ}C$. After this a steep increase of 80 $^{\circ}C$ /min was initiated until the temperature was 330 $^{\circ}C$ at 8.87 min. The temperature was further increased to 360 $^{\circ}C$ at 10.87 min and plateaued until the run was completed at 17.87 min.

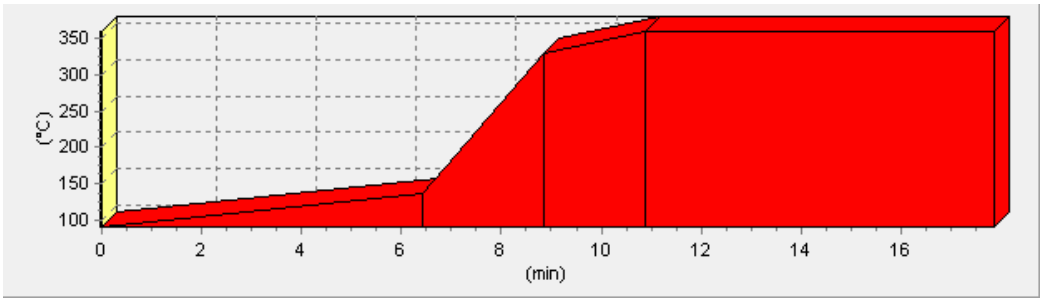


Figure 3.6: *GC*-ramp used in analysis

3.3.5 Titration

To investigate the acidity index in a solution titration was performed. Potassium hydroxide, KOH with $C_{KOH} = 0.02043M$, was used as titrator and in the analyte, differing amounts of sample was solved in a 10 ml mixture of 50:50 ethanol:diethyl ether. A few droplets of indicator solution, $\sim 0.5g$ of Phenolphthalein dissolved in rectified alcohol, was added. The titration started by slowly

adding droplets of KOH until the solution had a slight stable change to purple. The titration volumes were recorded as well as the sample weights. With Equations 2.8 and 2.9 the acidity indices and the conversions respectively could be determined.

3.3.6 Processing of Reaction Solutions

The reaction batches were filtrated with *VWR* filter papers to remove the solid particles in the suspended phase. The reactions in *Parr batch reactor* and *Quark glass reactor* were analysed separately. The solutions from the *Erlenmeyer* flasks were collected together into a bottle. The solvent was then removed and the resulting concentrate was tested on *GC* and *MS*. The solutions were stored in the fridge.

Chapter 4

Results

This chapter presents and documents the results used to understand the chemical reaction and serves as a foundation for the discussion in Chapter 5. The results are divided into four sub chapters: *Batch 1*, the attempted reproduction of literature, *Batch 2*, investigates the key result from *batch 1*, *Batch 3*, investigates reproducibility and in *Batch 4* a *DOE*-analysis was done on the discovered side-reaction with *RSM*. All reactions conducted are presented in appendix B, including failed trials.

4.1 Batch 1, Reproduction of Literature

The first batch of experiments consisted of 18 reactions and the main focus were to develop methodology with the equipment available at *NMBU*. With the developed methodology it was attempted to reproduce *Monreal* [5] and *Alonso* [6] work. The most promising result was observed in reaction 1.14.

Reactions 1.2-1.8. The concentration of raw material in the *GC* samples were too high, which resulted in wide peaks on the spectra and inconsistent conversion calculations.

Reactions 1.9-1.11. This set of reactions was to test out vinyl laurate as replacement for lauric acid in different solvents. The solvents tried were t-BuOH, acetone and a solvent less system, with vinyl laurate as both solvent and raw material. These reactions resulted in the development of new methodology, see Sections 3.3.3 and 3.3.4 for how the *GC* samples were prepared and the temperature ramp in the machine.

Reaction 1.14. The results from 1.14, a reaction between vinyl laurate and galactose solved in acetone in the presence of the enzyme, resulted in a fast reaction to an unknown product. The yield of reaction was at the most just under 90 % after 1.5 hours of reaction. The yield fluctuated for the rest of the reaction time and ended up between 70 % and 80 %. Both conversion and yield were calculated and they were similar. The yield of reaction can be seen in figure 4.1. The calculations are based on *GC* spectra, where vinyl laurate showed up at 6.84 min and the product was assumed to be the peak that showed up at 7.01 min. Selected GC-spectra from this trial are shown in Figure 4.2, with sample 0 at the top and the fourth sample at the bottom.

Reaction 1.15-1.18. These reactions did not show any noteworthy spectra, therefore 1.14 was selected as the reaction to optimize.

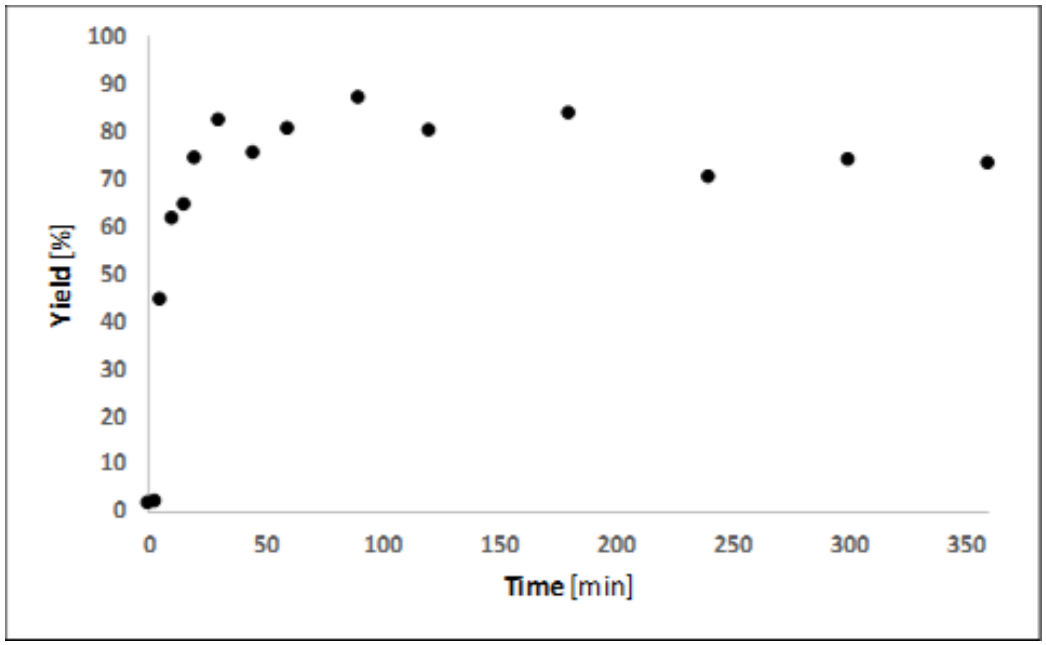


Figure 4.1: Yield of Reaction 1.14

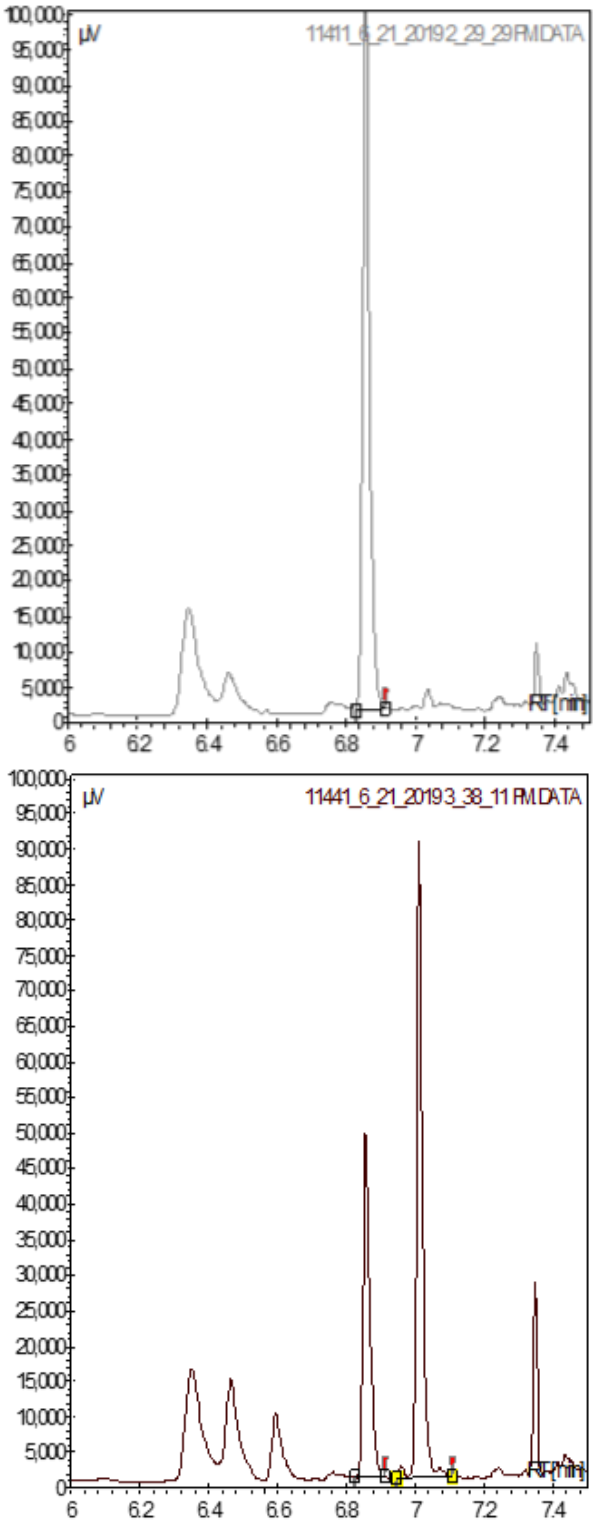


Figure 4.2: Comparison of *GC* spectra in reaction 1.14, where sample 0 is at the top and the fourth sample at the bottom.

4.2 Batch 2, Optimization of Reaction 1.14

The second batch of experiments consisted of five reactions and were conducted to reproduce the results from reaction 1.14. Reactions 2.1-2.3 investigated different molar ratios of the raw materials. These reactions yielded inconsistent results and with reactions 2.4 and 2.5 the reproducibility of trial 1.14 were investigated without success. Trial 2.3 confirmed the presence of one or more side reactions.

Reactions 2.1-2.3. The results from 1.14 were not managed to be reproduced in neither 2.1 nor 2.2. Here, the yield dropped from 80 % to 30 %, the yield of the reactions are presented in Figure 4.3. Reaction 2.3 was conducted at a later time point due to the inconsistent results from 2.1 and 2.2. The yield were higher and somewhat stable, yet contained big outliers.

Reactions 2.4 and 2.5 The reproduction of 1.14 was attempted in 2.4 and 2.5. The yield of the reactions can be seen in Figure 4.3. Although the yield increased and reached quite high values, there were outliers and the reaction was slower. The reaction solution from 2.4 was stored in the fridge and had a pale yellow shimmering. After the evaporation of solvent a yellow concentrate emerged, which was tested with *MS*. The concentrate contained methyl laurate, vinyl laurate and lauric acid.

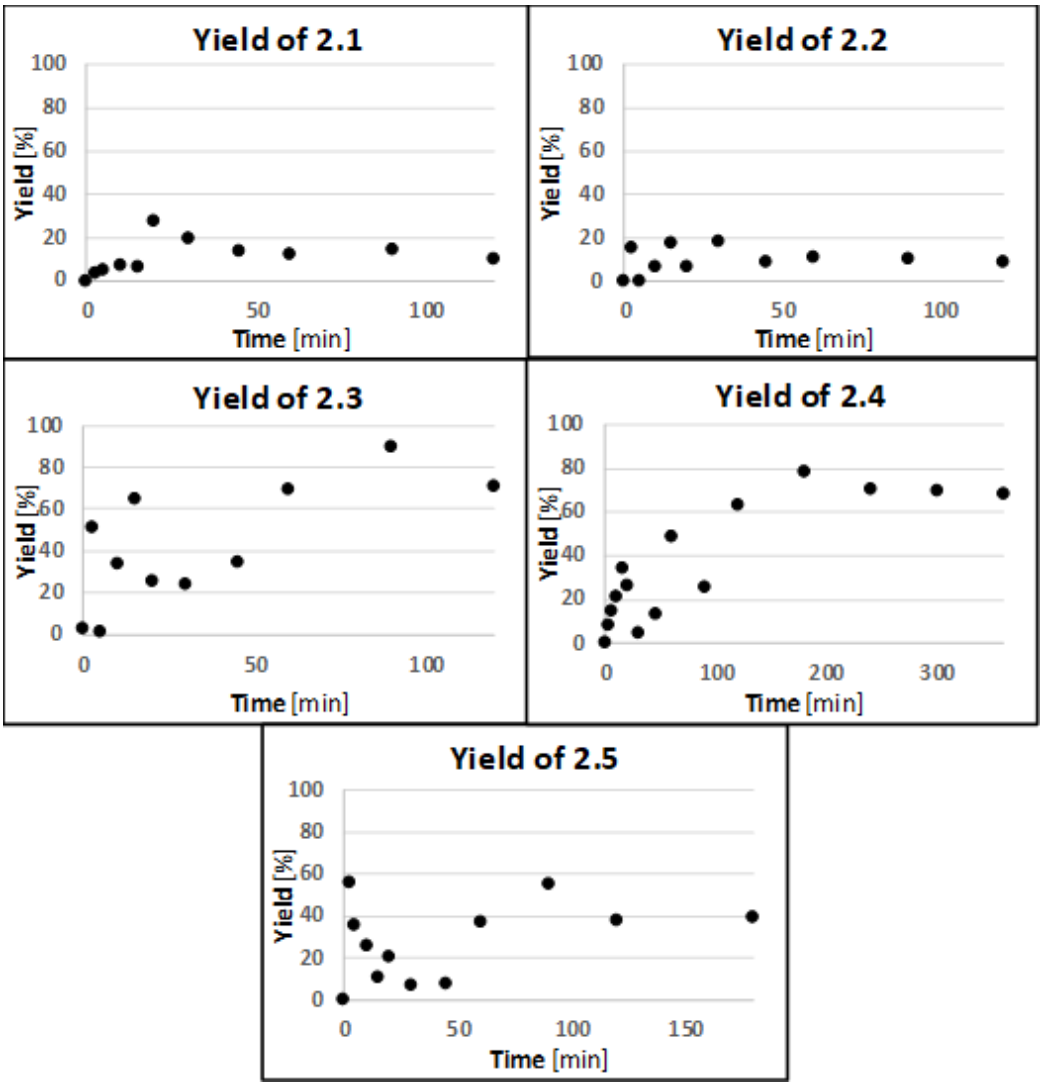


Figure 4.3: Results, in yield, of trial 2.1-2.5.

4.3 Batch 3, Reproducible Results

The third batch consisted of 10 reactions and investigated the reproducibility of the second batch. Reactions 3.1-3.5 and reactions 3.6-3.10 were conducted simultaneously in smaller volumes in an attempt to obtain consistent results. Reactions 3.1-3.5 resulted in varying conversions and yields and therefore reactions 3.6-3.10 investigated possible side reactions. The key discovery in this batch was the confirmation of a reaction of vinyl laurate and acetone catalyzed by *N435* with the resulting products: methyl laurate and lauric acid.

Reactions 3.1-3.5. These five reactions conducted simultaneously on plate reactors showed the same reaction, but with different yields and conversion, as seen in table 4.1. Similarities were observed across the *GC* spectra and in Figure 4.4 reaction 3.2 is presented. The conversions are based upon the decrease of vinyl laurate, which shows up at 6.89 min, and the yield on what was assumed as the product, at 6.6 min. After the reactions, the solutions were collected together into a glass balloon and the solvent was extracted. The remaining concentrate presented itself as a pale yellow solution, with precipitate of white flake-like particles. The solution color from 3.1-3.5 was similar to the solution in reaction 2.4. The MS spectra of the concentrate is presented in figure 4.5, with the retention time above and the three individual *MS* spectra for the components below.

Table 4.1: Result in conversion and yield of 3.1-3.5

#	Conversion [%]	Yield [%]
3.1	33.9	36.5
3.2	72.8	16.0
3.3	31.2	11.7
3.4	24.1	20.2
3.5	82.7	12.5

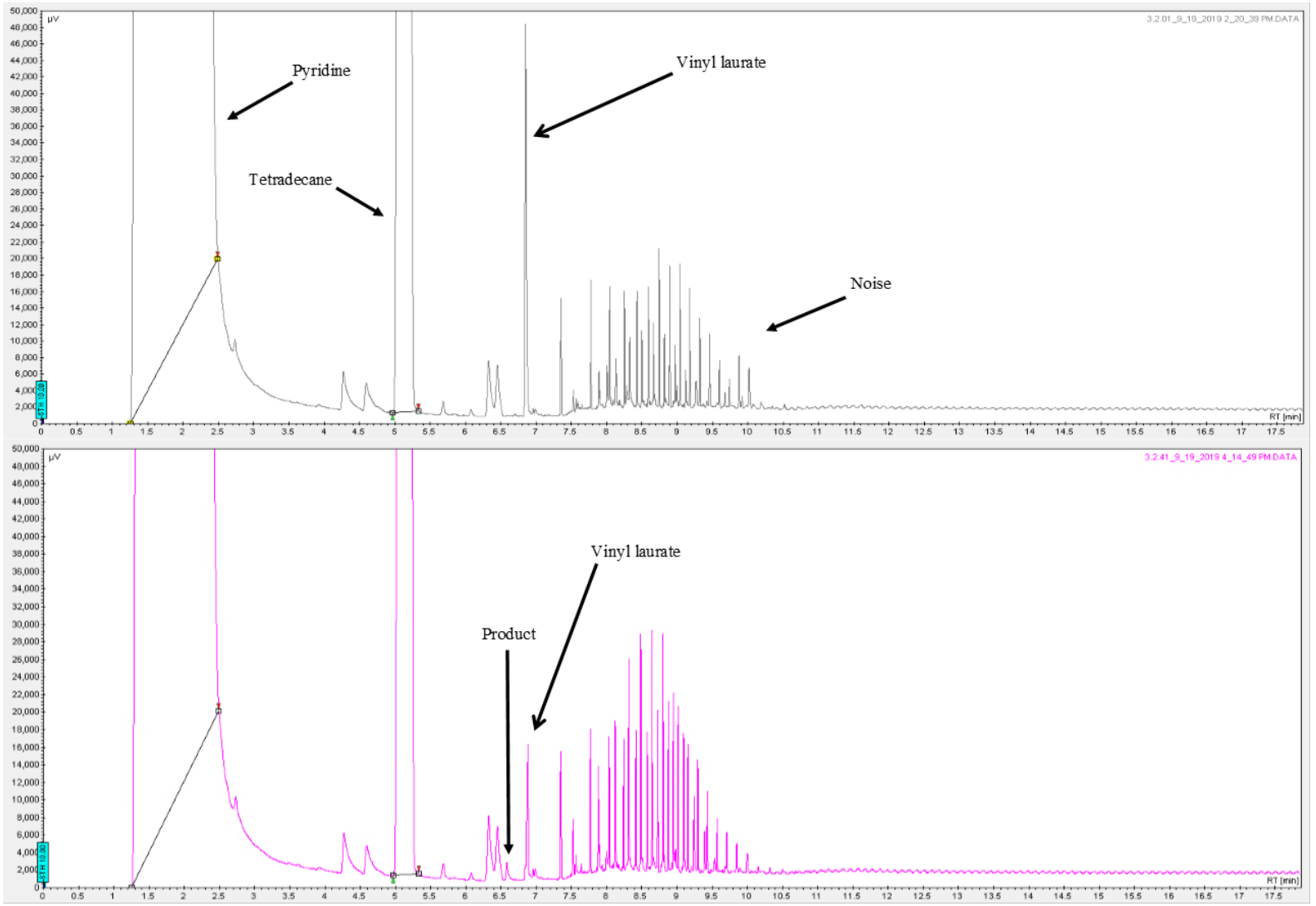


Figure 4.4: Comparison of *GC* spectra from reaction 3.2, where sample 0 at the top and the final sample at the bottom.

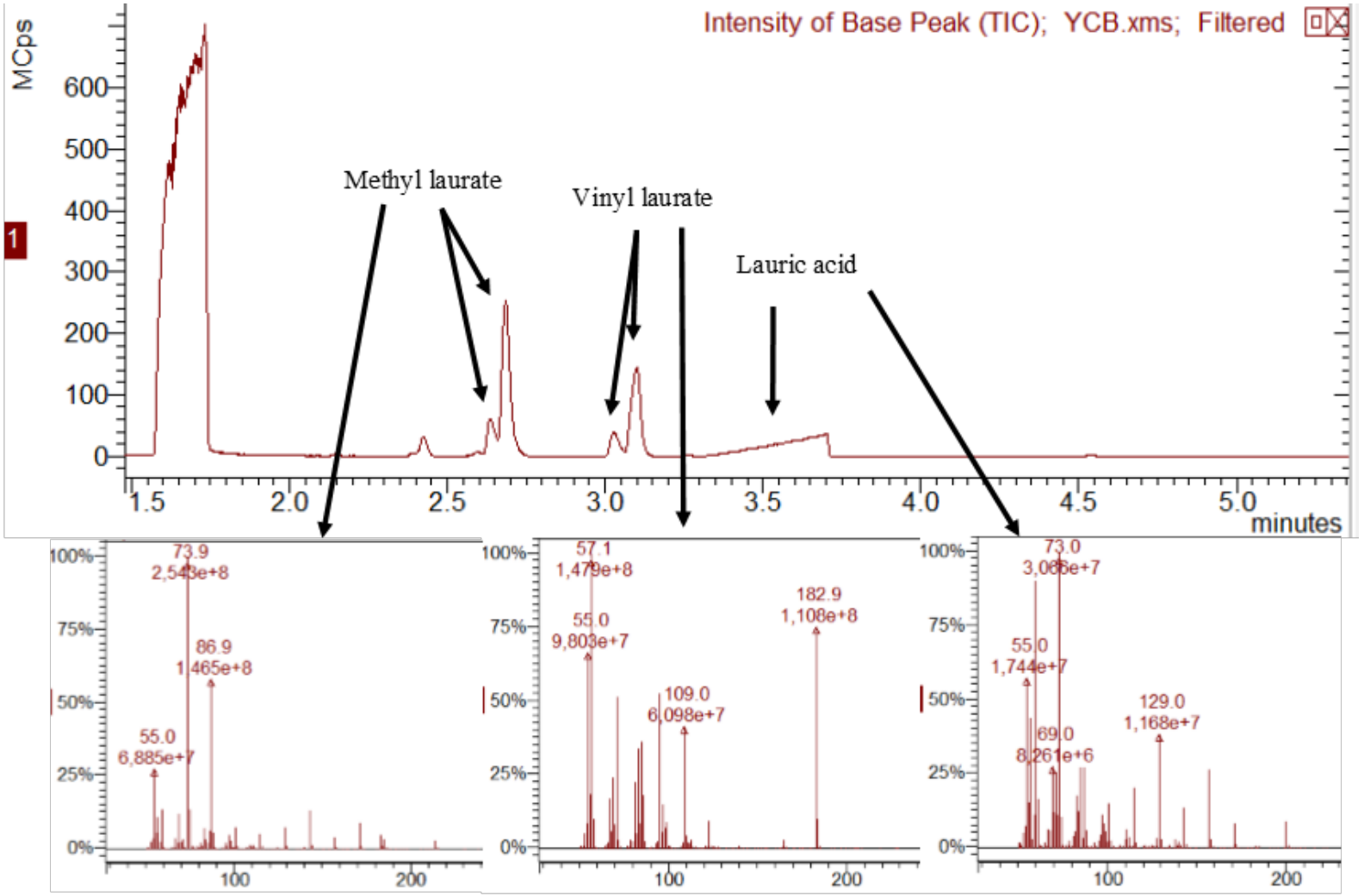


Figure 4.5: *MS* spectra of trials 3.1-3.5, with retention time above and individual *MS* spectra below.

Reactions 3.6-3.10. As mentioned, reactions 3.6-3.10 were used to investigate side reactions and resulted in the confirmation of a reaction to methyl laurate and lauric acid. The reactions measured in yield to different products are presented in Table 4.2. This table shows that the reaction to methyl laurate was observed when vinyl laurate was solved in acetone with *N435*. In reaction 3.10, methyl laurate was only observed after solvent removal.

Table 4.2: Results from 3.6-3.10 in yields to different products.

#	**Solvent**	**Raw Material**	**Product**	**Yield of ML** [%]	**Yield of LA** [%]
3.6	Acetone	VL + G	ML, LA	39.7	57.76
3.7	t-BuOH	VL + G	unknown	-	-
3.8	Acetone	G	-	-	-
3.9	Acetone	VL	ML, LA	27.8	72.2
3.10	Acetone	LA	ML	-	-

VL vinyl laurate, **ML** methyl laurate, **G** galactose. In 3.10, methyl laurate was only observed after solvent removal

4.4 Batch 4, Design of Experiments

The reaction of vinyl laurate and acetone in presence of the enzyme *Novozyme®* *435* solved in acetone was investigated. A design of experiments, **DOE**, was done with the two independent variables catalyst amount and time. The values with the corresponding encoding for the model is presented in Table 4.3. The operating conditions for temperature, stirring and molar ratio, were set to constant values of 50 $^{\circ}C$, 400 rpm and 1:1 respectively. The response surface model was done using *Statgraphics XVII* and was a central composite design with two variables, four central points and quadratic terms. The data used to build the model are presented in Table 4.4 and the statistical analysis in Table 4.5. A quadratic model is required to explain the data because the curvature in the data is outside the curvature confidence interval, as presented in the statistical Table.

Table 4.3: Encoded values for *DOE*, α-values are calculated with $\alpha = \pm 2^{n/4}$, where n is number of variables in the design.

	$-\alpha$	-1	0	1	α
Catalyst [$wt\%$]	7.9	10	15	20	22.07
Time [h]	0.17	1	3	5	5.8

Table 4.4: *DOE* with 12 reactions. The independent variables, their encoding and the conversion used in the response surface analysis.

	#	$Ewt\%$	Time [h]	Cat	t	Conversion [%]
Central points	1	15	3	0	0	79.16
	2	15	3	0	0	74.33
	3	15	3	0	0	73.96
	4	15	3	0	0	79.09
Linear points	5	10	1	-1	-1	56.73
	6	20	1	1	-1	57.36
	7	20	5	1	1	79.19
	8	10	5	-1	1	76.84
Quadratic points	9	7.9	3	$-\alpha$	0	74.06
	10	22.07	3	α	0	83.09
	11	15	0.17	0	$-\alpha$	23.61
	12	15	5.8	0	α	40.71

Table 4.5: Statistical analysis on the model.

Parameters	Response
Estimated effects and interactions	
	$\hat{y} = 61.45$ $I_t = 16.53$
Significance test: c confidence level: 95%	
Mean response (only central points)	76.63
Standard deviation	$s = 2.88$; $t = 3.18$;
Confidence interval	$\pm$ 4.58
Significant variables	time [t]
Significance of curvature	
Curvature	-9.10
Confidence curvature interval	$\pm$ 6.48
Significance	Yes

The resulting equation was 4.1 with an $R^2 = 0.899$. With this equation theoretical conversions were calculated and plotted against the experimental data and the result is presented in figure 4.6. As can be seen the fitting is accurate within the expected deviation in the model.

$$\begin{aligned} Conversion = 53.168 - 4.54795 \cdot cat + 32.283 \cdot t \\ +0.160424 \cdot cat^2 + 0.043 \cdot cat \cdot t - 4.79922 \cdot t^2 \end{aligned} \tag{4.1}$$

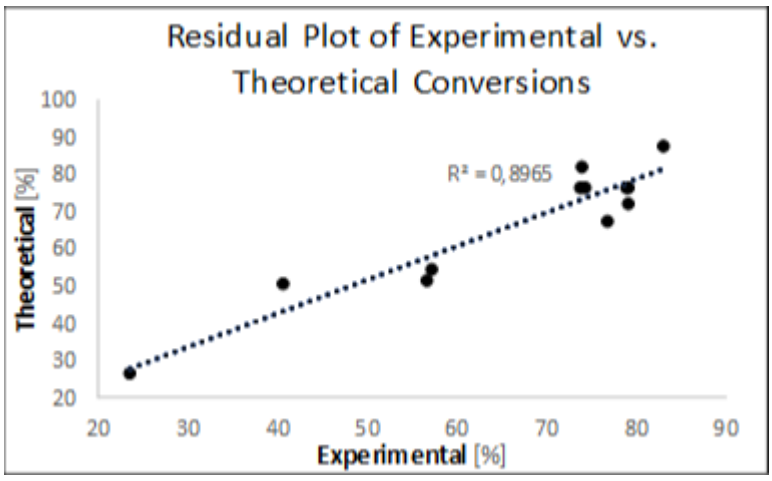


Figure 4.6: Experimental conversion plotted against theoretical conversion

The surface of the predicted model is plotted as a wire frame in Figure 4.7 with the addition of contours at the base. The shape of the model takes on a saddle like shape where time gives a negative curvature and catalyst a positive curvature. There is an increase in conversion when time increases, but only to the saddle point marked with red. After this the conversion is predicted to drop as the reaction time increases further. For the catalyst, there is initially a drop in conversion as the amount of catalyst increases until the saddle point. Beyond the saddle-point the conversion increases to its maximum at the α-value of enzyme amount.

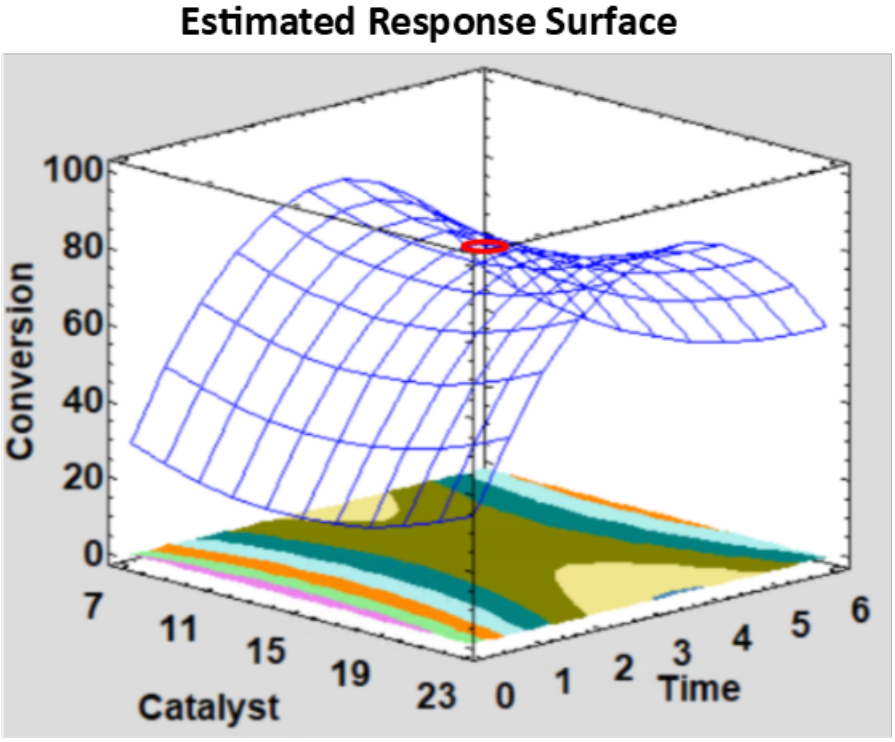


Figure 4.7: Surface plot of the *RSM* as a wire frame with contours at the base

At the top in Figure 4.8, a response surface of the model with continuous values is presented and at the bottom in this Figure, the contours of the estimated response surface are shown. This plot shows the contours with distinct levels that makes it easier to see the regions of different conversion. The optimal point is marked with a red circle. To the right of the optimal point there is a region of higher conversion, but this was not taken into account as the amount of enzyme needed for this conversion is above the amount used in the α points and hence outside the factorial design.

The optimal value for the response was 88.47 % and the optimal value for time

and catalyst amount were 3.46 h and 22.07 %. Optimal values were obtained with *Statgraphics*. Two reactions at the optimal values had a conversion of 94.87 % and 96.48 % which is above what the model predicted. The difference from 88.58 % to these results are within the expected deviation in the model.

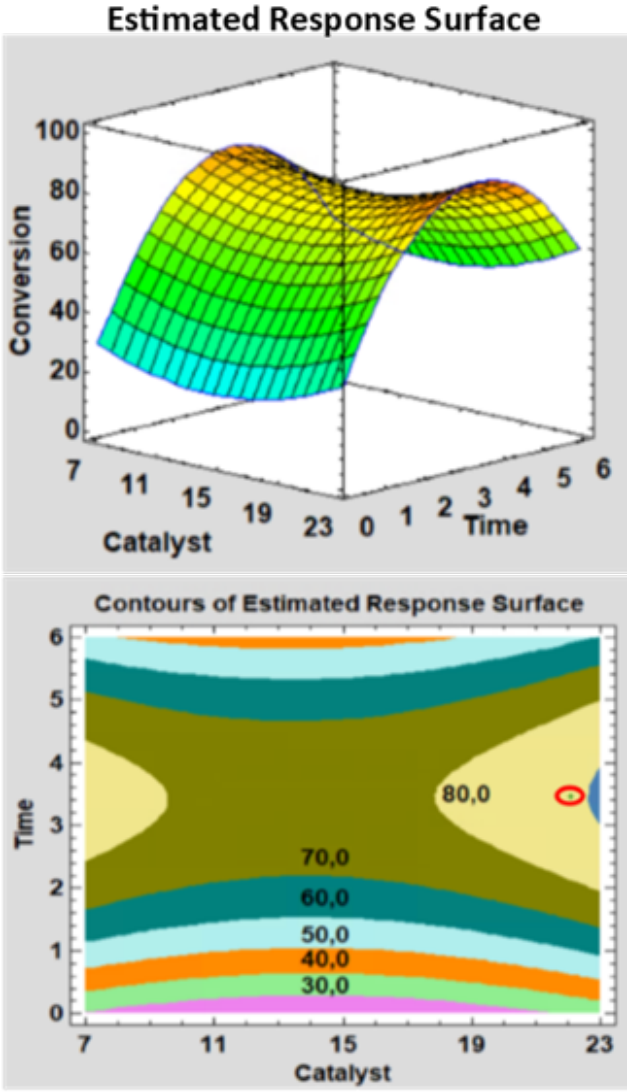


Figure 4.8: Estimated response surface at the top and contours of estimated response surface at the bottom.

Chapter 5

Discussion

The literature on the synthesis of *SFAE* describes a broad range of successful enzymatic reactions. The main challenge in this work was to find methodology, raw materials and solvents to produce a sugar based ester with the equipment available. The first challenge was to find a reactor for this kind of reaction, secondly to find a working reaction procedure and thirdly develop a consistent methodology to measure the reaction. The first part of this chapter will discuss the four reaction batches. The second part presents a general discussion regarding methodology and reaction procedure.

5.1 Batch 1

The first batch of experiments were used to develop methodology in an attempt to reproduce the literature. The methods that were developed are described in detail in Section 3.3.2 *Reaction Sampling*, 3.3.3 *GC and MS samples* and 3.3.4 *GC and MS Method*. The spectra from reaction 1.14 looked promising and similar to the spectra from Spain [5], [6]. These spectra however, were not managed to be reproduced in later reactions.

Reactions 1.1-1.13 and 1.15-1.18. The results from these reactions were mostly inconclusive. These reactions however were used to develop methodology. During the first reactions disadvantages of t-BuOH were discovered, which led to testing of other solvents. After trials with different solvents, acetone was selected as the solvent. Acetone has also been successfully employed in enzymatic synthesis of *SFAE* in the literature. The reactors used during these trials were first the *Parr batch reactor*, then in 5 ml vials and finally in the *Quark glass reactor*.

Reaction 1.14. This reaction consisted of galactose and vinyl laurate in the presence of the enzyme, *N435*, solved in acetone at 60 $^{\circ}C$ under constant stirring. In figure 4.1, a yield of around 80 % was obtained after one hour of reaction. In later trials the reaction time was shortened based on these results. The consistent results observed in 1.14 were not managed to be reproduced in later stages of this work. One explanation could be that this was a false positive where an impurity or noise was interchanged with the peak that was assumed to be the product. The peak at 7.01 min, shown below in Figure 4.2, was not observed to this extent again. This unknown peak was confirmed not to be lauric acid, based on the lauric acid standard in *GC*. Therefore the galactose ester can not be ruled out as the product in this reaction.

Table 5.1: Key changes made during the first batch of reactions.

Reaction	**Result**	**Changes on the Reaction**	**Improved Methodology**
1.1-1.5	inconclusive	-	GC
1.6-1.8	inconclusive	solvents	GC
1.9-1.11	inconclusive	solvents, raw material	GC
1.12-1.13	inconclusive	solvent, raw material	GC
1.14	promising	solvent, raw material	Quark
1.15-1.18	inconclusive	solvent, raw material	Quark

5.2 Batch 2

The second batch of experiments, in particular trial 2.3, led us to the discovery of a side reaction. In general this batch yielded quite poor results in the context of reproducibility, as can be seen in Figure 4.3. Although the results were not in accordance with each other, this batch gave the first indication that one or more side reactions were present.

Molar Ratio Investigation 2.1-2.3. The conversion calculations for 2.1 and 2.2, based on the *GC* spectra, had negative values. These negative values implied that the raw material, vinyl laurate, was produced in the reactions, however no chemicals were present in the solution that could react to vinyl laurate. With these results, 2.1 and 2.2 were regarded as inconsistent. Furthermore, reaction 2.3 suggested that one or more side reactions were present in these trials, which was based upon the decrease of vinyl laurate. In this reaction, the molar ratio were 1:5, galactose:vinyl laurate, and the spectra showed high conversion of vinyl laurate. The reaction to the galactose ester should use equimolar parts of the raw material and therefore side reactions could explain the inconsistent and non-reproducible results observed.

Titration of 2.3. In reaction 2.3 vinyl laurate was consumed in larger extent than was presumed when considering the reaction to the galactose ester. To test if vinyl laurate had reacted to lauric acid, titration was done. The acidity index in the samples were investigated without any significant change during the reaction. In the reaction solution, the concentration of vinyl laurate was 0.0628 g/ml and if lauric acid was a product, the concentration should be lower. The volumes used to titrate were low, less than 1 ml, which suggests that the base concentration, 0.02043 M of KOH, was too high. This does not necessarily mean that the solution is neutral, but may be explained by a sub optimal base concentration.

Reaction 2.4 and 2.5. For these trials a new methodology for sampling was developed which can be seen in Section 3.3.2 *Modified Sampling Procedure*. This method was developed because the sampling became nearly impossible due to insufficient pressure drop in the syringe. An explanation for this could be that particles got stuck in the sampling syringe and blocked the needle. This could compromise later samples, which was shown to not be the case, but the developed methodology helped to reduce the amount of broken syringes. The result in reaction 1.14 were neither reproduced in 2.4 nor 2.5, which could be explained by possible side reactions.

5.3 Batch 3

The third batch of experiments were separated into two parts, the first part, 3.1-3-5, was to investigate the reproducibility of the reactions and the second part, 3.6-3.10, was to investigate side-reactions. In the third batch the reactor was changed from the *Quark glass reactor* to five *Erlenmeyer* flasks that ran simultaneously. The first part showed inconsistencies, therefore the reactions 3.6-3.10 were conducted with different raw materials and solvents and an overview is shown in Table 4.2. The second part resulted in the discovery of a side reaction with methyl laurate and lauric acid as products.

Reactions 3.1-3.5. The conversions and yields of these reactions showed inconsistent results, as presented in Table 4.1. The weighting of enzyme was not especially accurate, however the differing amount added to the reactions were unlikely to explain the inconsistency observed.

Reactions 3.6-3.10. From table 4.2 it can be seen that the reactions investigated only took place when vinyl laurate and acetone reacted in the presence of the enzyme. The product from these reactions were methyl laurate and lauric acid. In general for the reactions without vinyl laurate, methyl laurate was not observed as a product. When lauric acid was the raw material there was some yield of methyl laurate, but at a much lower value. In addition, methyl laurate was not observed as a product when t-BuOH was used as solvent.

Four samples were taken from each trial. The first two samples, sample 0 and the sample before filtrate, were used to calculate conversion and yield. In addition, two more samples were taken, one after filtration and one after solvent extraction. The latter two samples could not be used for conversion calculations, but were used to investigate the reaction solution on the *MS*.

5.4 Batch 4

The *DOE* was performed on the reaction with vinyl laurate and acetone in the presence of *Novozyme 435* solved in acetone. The results are presented in Section 4.4 *Batch 4, Design of Experiments*. To summarize: the results showed a model with adequate fit based on an $R^2 = 0.899$, the quadratic terms are needed to describe the reaction because the curvature value is outside the curvature confidence interval, $\pm 6.48 > -9.10$, and the optimal values to obtain the highest response within the parameter range were 3.46 h, 22.0711 $wt\%$ and 88.46 % for time, enzyme and the conversion respectively.

For the mathematical model, the central points are crucial as the statistical properties on curvature are based on the standard deviation in the central points, as can be seen in table 2.5. The central points used in the design had a standard deviation of 2.88%. It should be mentioned that four central points were chosen out of eight points. The four central points excluded had large deviation from the average of the eight points. In Figure 5.1 the comparison of the sample 0 and the final sample from the first central point is presented. The decrease of vinyl laurate was clearly observed with a growing peak of methyl laurate at 7.2 min. During the *DOE* analysis, the spectra changed and in the later stages of the analysis, vinyl laurate was observed with a split peak. This split can be seen in Figure 5.2, where the second peak in the split was confirmed to be lauric acid. The conversion calculations were based on both of the peaks due to difficult separation of them.

In equation 4.1 the terms with time have more influence on the conversion in the model than the terms with catalyst. The t and t^2 influences the model differently as t have a positive influence on the conversion and t^2 had negative influence. These results might be due to the alpha point in time, $(0,\alpha)$, which showed significantly lower yield than expected. The reaction was run in duplicate and also run twice on the GC with two different sample methods described in Section 3.3.3 *GC and MS Samples* and *Dilution of GC and MS Samples*. The result used in the model were from the dilution method, which eliminates possible errors on the scale in the lower weight range, even though the conversion was 40.71 %. The scale was shown not to be faulty at lower weights as the results from the different methodologies showed similar conversion.

As mentioned the catalyst terms showed low significance on the conversion and the linear points was a good example for this. From the low to high values of enzyme where time is constant the conversions differentiated with 1-3 %, from Table 4.4. An explanation for these results could be that in the investigated enzyme range the solution is already saturated at the lower encoding for

enzyme amount.

The residual term, ϵ, contains the errors in the trials and the real and predicted values are plotted against each other in Figure 4.6. The certain errors to be contained in ϵ are the measurement errors from each trial, the GC-sampling and the deviation in each GC-run.

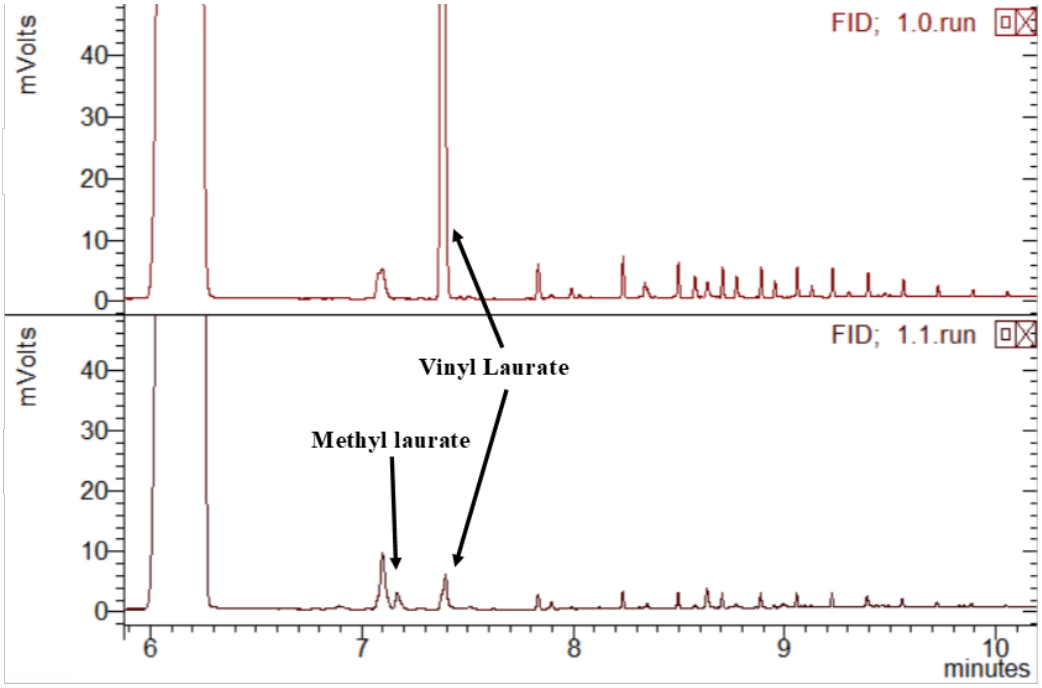


Figure 5.1: *GC* spectra of sample 0 and the final sample from the first central point, reaction DOE 1.

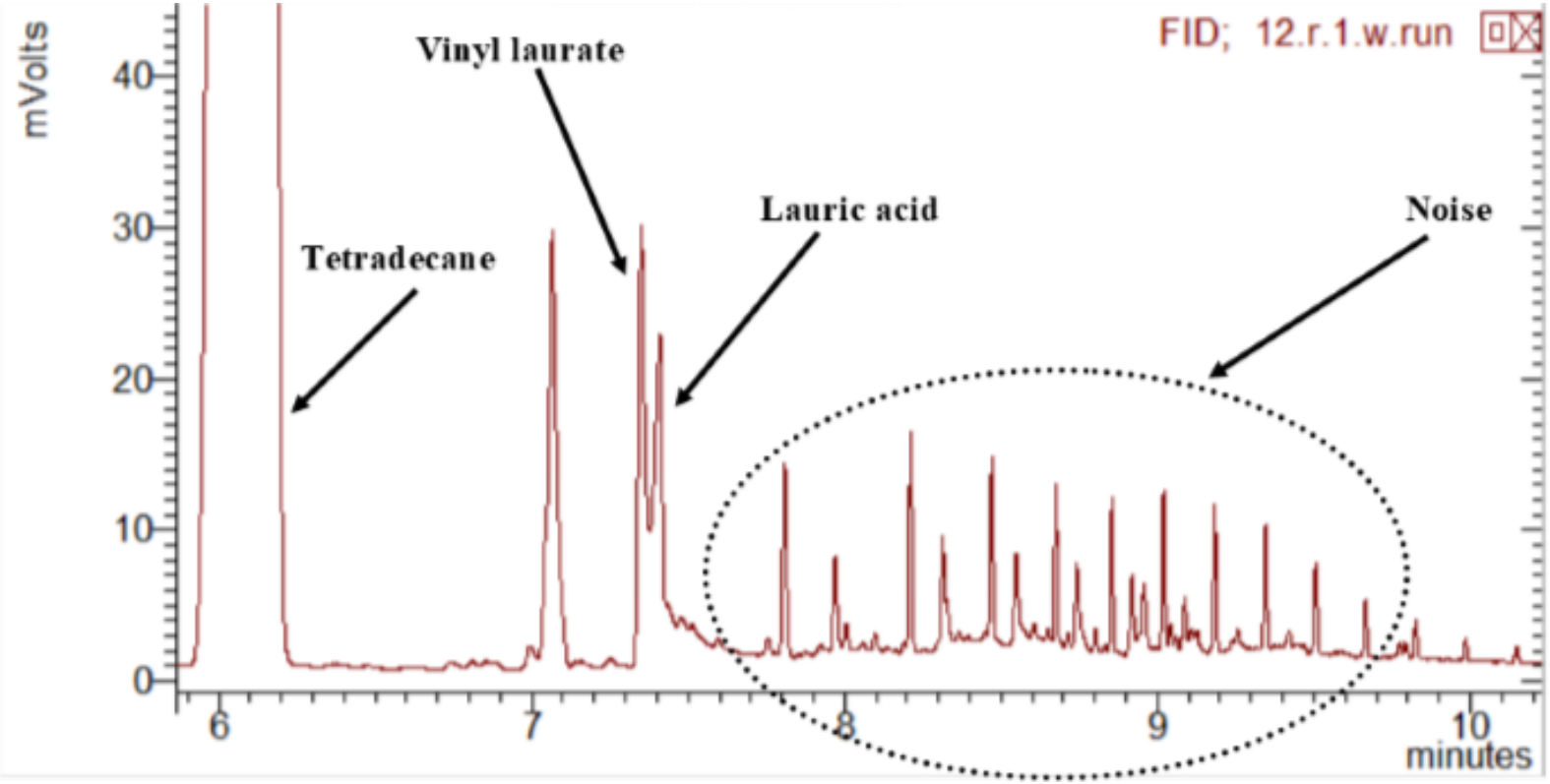


Figure 5.2: GC-spectre from the final sample in reaction DOE 12.

5.5 General Discussion

In this section aspects regarding the methodology, reaction components and side reactions are discussed.

Change of reactors

The reactions, 1.1-1.8, 1.12 and 1.13, were conducted in the *Parr batch reactor*, but due to heterogeneous reactions, the sampling was not optimal as particles could get stuck in the sampling valve. In addition the temperature control was unstable and varied too much for these enzymatic reactions. In reactions 1.9-1.11, smaller vials, 5 ml, were tested based on the volumes used in the literature, see Table 2.4. These vials were changed for the *Quark glass reactor* in reactions 1.14-1.18. Reaction 1.14 showed promising results and therefore this reactor was used in the second batch. During testing with this reactor, new methodology had to be developed due to difficulties with the sampling, where particles could get stuck in the sampling needle. The reactors used for all the remainder of the reactions, in the third batch and the *DOE*, were *Erlenmeyer* flasks on *Plate reactors*. The sampling regime was changed to sample 0 and the final sample to eliminate possible errors in the sampling. With this sampling regime, the reaction was no longer monitored and the results are based on the difference from sample 0 and the final sample.

Solvents

As mentioned earlier, one of the challenges in this work was to select an optimal solvent. The solvents tried was t-BuOH and acetone, with the addition of a solvent less system using vinyl laurate. Due to difficulties with t-BuOH, acetone was selected. Further analysis revealed that acetone played a role in the synthesis of methyl laurate, see the section discussing side reactions on the next page.

Challenges with t-BuOH Early in the initial experiments a major drawback of t-BuOH was discovered. When t-BuOH was extracted from the reaction solution, this solvent froze around the cooling column, shown in Figure 5.3. The cooling fluid used had a constant temperature below the freezing point of t-BuOH. Later t-BuOH was tried in the *Quark glass reactor* in reactions 1.16-1.18, where t-BuOH froze inside the sampling needle and consequently made sampling difficult.

Figure 5.3: Picture of icing of t-BuOH in the cooling column inside the rotary evaporator

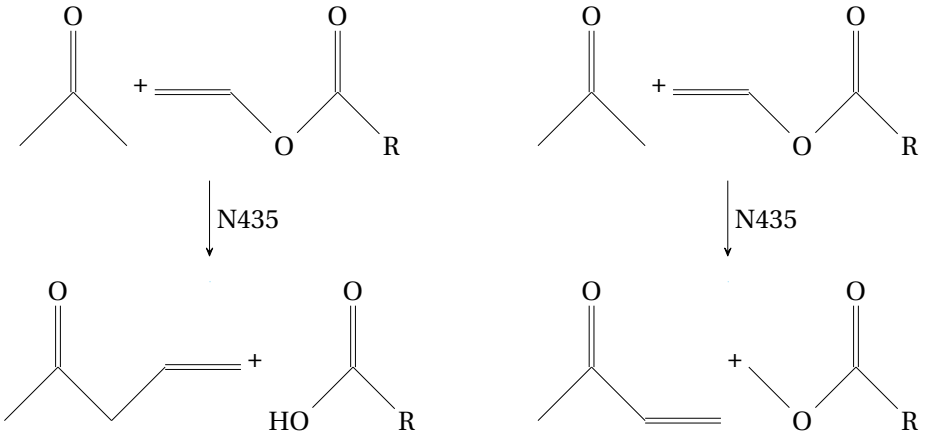
Opposed to prior arguments, the freezing temperature of t-BuOH, 25 $^{\circ}C$, is optimal for storage and to ensure no or insignificant reaction when stored even at room temperature. If t-BuOH is to be selected for further trials, measures should be made to optimize the extraction procedure for this solvent, for instance to use cooling medium at around the freezing point of t-BuOH.

Side Reactions

The side reactions were first discovered in the second batch and confirmed in the third batch. In reaction 2.3, where the molar ratio was 1:5, galactose:vinyl laurate, the conversion of vinyl laurate approached 100 %, but the yield fluctuated, as shown in Figure 4.3. This suggested that vinyl laurate was consumed in a side reaction without galactose.

In reaction 3.9, the only raw material was vinyl laurate and here it was confirmed by MS that the products were methyl laurate and lauric acid. Two proposed reactions that could explain these results are presented in Figures 5.4a and 5.4b. In these proposed reactions it was hypothesised that acetone acted as a reactant. This was confirmed in the third batch when the reaction only happened in reactions 3.6 and 3.9 when acetone was used as solvent. The confirmed products were methyl laurate and lauric acid and the acetone derivates

were proposed to be 4-pentene-2-one and 3-butene-2-one to balance the chemical equations.



(a) Reaction of acetone and vinyl laurate to 4-pentene-2-one and lauric acid

(b) Reaction of acetone and vinyl laurate to 3-butene-2-one and methyl laurate

Figure 5.4: Proposed side reaction schemes for trials with acetone and vinyl laurate

Scale

In addition to the standard deviation of the scale, it would from time to time measure different weights for the same sample. An explanation for this could be the evaporation of solvent, especially when acetone was used. The scale was shown to measure with adequate accuracy at lower weights by comparing a normal sample with a diluted sample. The two sampling methods are described in Section 3.3.3.

Enzyme

Enzymes are proteins and by incorrect handling they may deactivate or denaturate and consequently lose activity. The enzyme, *N435*, used in this work has an optimal storage temperature between 0 and 10 $^{\circ}C$. During the thesis the enzyme was stored for extended periods, 2-3 months, in room temperature at about 20 $^{\circ}C$. If the storage temperature exceeds 25 $^{\circ}C$, the enzyme should

be used within 3 months. Based on this information it is unlikely that the enzyme has been deactivated during storage. If further analysis is to be done and the same enzyme is selected, a new bottle should be purchased as each batch of enzyme lasts about 2 years. The storage information was gathered from the manufacturer, *Novozymes A/S*.

The deactivation of the enzyme was also investigated by conducting a control reaction without the catalyst. This trial showed no reaction as opposed to all the other trials where the conversion of vinyl laurate was observed in the presence of *N435*. On the other hand there is a possibility that the enzyme has changed its catalytic effect and can work on other reactions than has been shown in the literature, but this was not tested for.

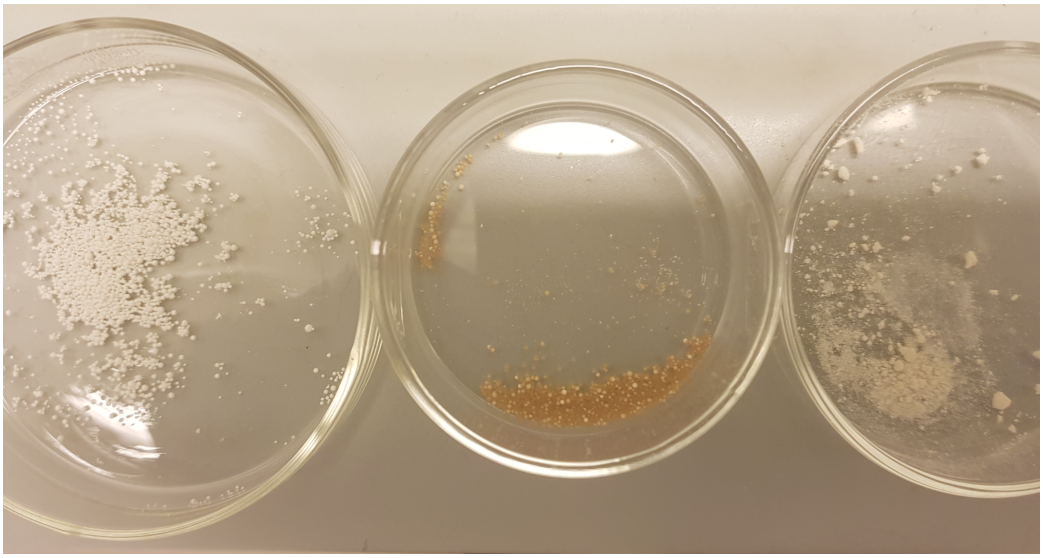


Figure 5.5: Picture of enzyme pre- and post use. From left to right: unused catalyst, used catalyst without breaking of beads and enzyme crushed to powder by stirring.

It is previously reported that the acrylic resin used in immobilization of *CALB* has a tendency to break when subjected to vigorous stirring [54]. This was also the case in this work and enzyme pre- and post-use is shown in figure 5.5. To the left in the figure are the unused beads of *N435*, in the middle is used enzyme without the beads breaking and with coloring, and finally the filtrated powder after a reaction, the powder can contain other precipitates from the reactions. The breaking of the beads is not necessarily a bad thing as the mass transfer limitation into the beads and onto the enzyme is greatly reduced when broken. Even though it doesn't affect conversion when the beads break, it is harder to re-use the bio-catalyst. To re-use the enzyme, the beads or the fine powder is

filtrated out and collected. The test for the re-use of enzyme, powder or beads, were not conducted in this work.

The weighting of the enzyme was not optimal as the beads of the immobilized enzyme had electrostatic properties which made it hard to handle. When trying to weight, the gravitational forces were exceeded by the electrostatic forces and consequently the beads had a tendency to fly out of the measuring trays. In appendix B it can be seen that the first batches for both the central- and linear points were all discarded, this was because the weight of the enzyme were not adequately measured.

GC and MS Spectra

The methodology for the *GC* and *MS* setup is described in Section 3.3.4 and for all the *DOE* reactions the *GC-MS* was used. With the developed methodology, the conversion of vinyl laurate could be measured. In Figure 5.1 this conversion can be observed with an adequate separation of components. During the trials for the *DOE* analysis, lauric acid and methyl laurate could show up in the same spectre, but this was not consistent as both of them was observed alone or not at all. Vinyl laurate was consistently observed to decrease during the reactions.

During the *DOE* reactions, vinyl laurate had little change in its retention time. In Figure 5.1 vinyl laurate appeared at 7.38 min compared to in Figure 5.2 where it showed up at 7.35 min. However, in the bottom spectre, vinyl laurate is observed with a split peak that was confirmed to be lauric acid. This split was observed more consistently in the later runs and the conversion of vinyl laurate was calculated with the assumption that both vinyl laurate and lauric acid was vinyl laurate. By assuming such, the conversions will have an extra known error with unknown quantity.

Across all spectra, noise was observed consistently, but differing in size from spectre to spectre. More over, the noise appeared between 7.8 and 10 min. For the reactant, vinyl laurate, this timing did not compromise the conversion calculations. The noise can have its origin in impurities from one or more of the sample components, for instance from the pyridine or the tetradecane.

Evolution of the GC-ramp

The development of a good temperature profile in the GC oven is of great significance for the observation of a reaction. Presented in Figure 5.6 are the first four ramps tested in chronological order. The bottom ramp, was the developed profile in which the decrease of vinyl laurate could be observed, the methodology of this setup is described in Section 3.3.4. In the other ramps the separation of components close to vinyl laurate was not adequate to measure the reaction.

During testing of samples with the method selected it was discovered that this ramp was not adequate for separation as the reaction seemed to produce lauric acid, quite close or even as a split peak to vinyl laurate, as showed in Figure 5.2. With this in mind, four new ramps presented in Figure 5.7, were also tried out, but due to time limitations they were not investigated further. The idea behind these new ramps were to start a platou when vinyl laurate was expected to show up, which in itself can take a number of iterations to get the timing correct. If further work is done, the latter ramps are a good start.

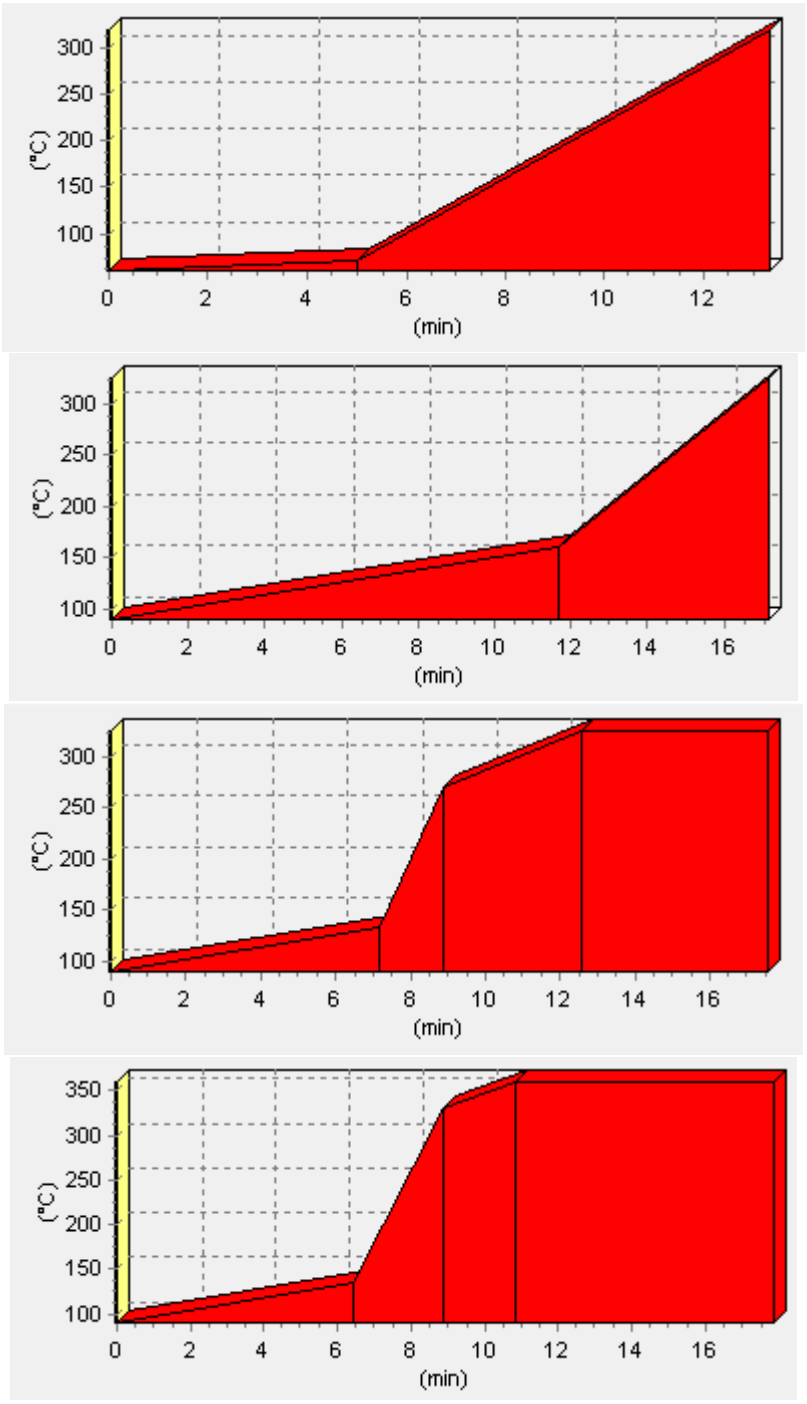


Figure 5.6: *GC*-ramp development 1, where the top ramp is the first profile tested and so on.

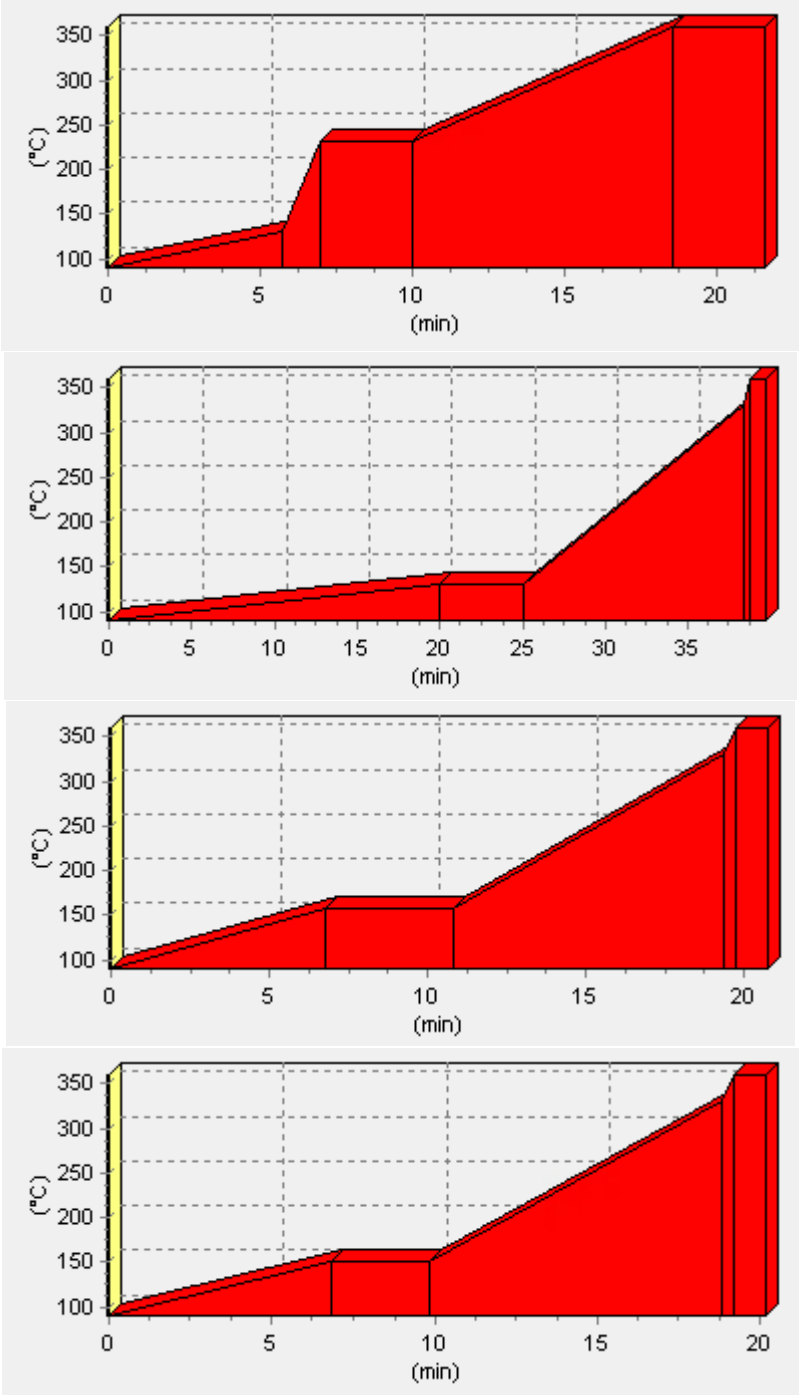


Figure 5.7: *GC*-ramp development 2, where the top profile is the fifth ramp tested and so on.

Chapter 6

Conclusion

The most important conclusions from this thesis on bio-catalytic synthesis were as follows:

- The enzyme *Novozyme 435* was discovered to catalyze the reaction of vinyl laurate and acetone to methyl laurate and lauric acid as the products.
- A *DOE* was performed on this reaction, the selected independent variables for the 2^2-factorial design were reaction time and catalyst amount.
- Based on the *DOE*, a *RSM* was performed that resulted in an $R^2 = 0.899$ between the experimental and predicted data. It was found that the quadratic model was required to describe the data and the significance of the variables from most important to least were as follows: $t^2 > t > cat^2 > cat > cat \cdot t$.
- A *GC*-ramp, described in Section 3.3.4, was developed and used to investigate the reaction of vinyl laurate and acetone.

Further Work

1 **Further Investigation** on the reaction to methyl laurate and lauric acid by:

   - looking for other products, especially if the proposed derivates of acetone are present.
   - trying other solvents for the reaction between acetone and vinyl laurate.
   - investigating more parameters, e.g. reaction volume, temperature, enzymes etc.

2 **Synthetization of Galactose Laurate** where parts of the methodology developed here can be used.

3 **Reaction Kinetics** on any of the reactions and to do this a sampling methodology needs to be developed. With kinetics, continuous flow schemes for instance a *packed bed reactor*, can be proposed.

Bibliography

[1] P. Anastas and N. Eghbali, "Green chemistry: Principles and practice," *Chemical Society Reviews*, vol. 39, no. 1, pp. 301–12, 2010, ISSN: 1460-4744. DOI: 10.1039/b918763b.

[2] R. Y. Hayes and G. Douglas, "Bioactive properties of sugar fatty acid esters," *Frontiers in Bioactive Compounds*, vol. 2, pp. 124–145, 2017.

[3] M. Enayati, Y. Gong, J. M. Goddard, and A. Abbaspourrad, "Synthesis and characterization of lactose fatty acid ester biosurfactants using free and immobilized lipases in organic solvents," *Food Chem*, vol. 266, pp. 508–513, 2018, ISSN: 1873-7072. DOI: 10.1016/j.foodchem.2018.06.051.

[4] M. Ferrer, J. Soliveri, F. J. Plou, N. López-Cortés, D. Reyes-Duarte, M. Christensen, J. L. Copa-Patiño, and A. Ballesteros, "Synthesis of sugar esters in solvent mixtures by lipases from thermomyces lanuginosus and candida antarctica b, and their antimicrobial properties," *Enzyme and Microbial Technology*, vol. 36, no. 4, pp. 391–398, 2005, ISSN: 01410229. DOI: 10.1016/j.enzmictec.2004.02.009.

[5] E. R. Monreal, "Síntesis, aislamiento y purificación de análogos antitumorales de origen marino," Universidad Complutense Madrid, Jul. 2017.

[6] F. J. E. Alonso, "Síntesis, aislamiento y purificación de análogos antitumorales de origin vegetal," Universidad Complutense Madrid, Oct. 2017.

[7] E. Abdulmalek, H. S. Mohd Saupi, B. A. Tejo, M. Basri, A. B. Salleh, R. N. Z. Raja Abd Rahman, and M. B. Abdul Rahman, "Improved enzymatic galactose oleate ester synthesis in ionic liquids," *Journal of Molecular Catalysis B: Enzymatic*, vol. 76, pp. 37–43, 2012, ISSN: 13811177. DOI: 10.1016/j.molcatb.2011.12.004.

[8] D. An, X. Zhao, and Z. Ye, "Enzymatic synthesis and characterization of galactosyl monoesters," *Carbohydr Res*, vol. 414, pp. 32–8, 2015, ISSN: 0008-6215. DOI: 10.1016/j.carres.2015.05.011.

[9] J. Liang, W. Zeng, P. Yao, and Y. Wei, "Lipase-catalyzed regioselective synthesis of palmitolyglucose ester in ionic liquids," *Advances in Biological Chemistry*, vol. 02, no. 03, pp. 226–232, 2012, ISSN: 2162-2183 2162-2191. DOI: 10.4236/abc.2012.23027.

[10] H. P. Tai and G. Brunner, "Sugar fatty acid ester synthesis in high-pressure acetone–co2 system," *The Journal of Supercritical Fluids*, vol. 48, no. 1, pp. 36–40, 2009, ISSN: 08968446. DOI: 10.1016/j.supflu.2008.09.009.

[11] S. H. Lee, D. T. Dang, S. H. Ha, W. J. Chang, and Y. M. Koo, "Lipase-catalyzed synthesis of fatty acid sugar ester using extremely supersaturated sugar solution in ionic liquids," *Biotechnol Bioeng*, vol. 99, no. 1, pp. 1–8, 2008, ISSN: 1097-0290. DOI: 10.1002/bit.21534.

[12] I. S. Yoo, S. J. Park, and H. H. Yoon, "Enzymatic synthesis of sugar fatty acid esters," *Journal of Industrial and Engineering Chemistry*, vol. 13, no. 1, pp. 1–6, 2006.

[13] F. Ganske and U. T. Bornscheuer, "Optimization of lipase-catalyzed glucose fatty acid ester synthesis in a two-phase system containing ionic liquids and t-buoh," *Journal of Molecular Catalysis B: Enzymatic*, vol. 36, no. 1-6, pp. 40–42, 2005, ISSN: 13811177. DOI: 10.1016/j.molcatb.2005.08.004.

[14] M. V. Flores, K. Naraghi, J. M. Engasser, and P. J. Halling, "Influence of glucose solubility and dissolution rate on the kinetics of lipase catalyzed synthesis of glucose laurate in 2-methyl 2-butanol," *Biotechnol Bioeng*, vol. 78, no. 7, pp. 815–21, 2002, ISSN: 0006-3592. DOI: 10.1002/bit.10263.

[15] K. Ren and B. P. Lamsal, "Synthesis of some glucose-fatty acid esters by lipase from candida antarctica and their emulsion functions," *Food Chem*, vol. 214, pp. 556–563, 2017, ISSN: 1873-7072. DOI: 10.1016/j.foodchem.2016.07.031.

[16] A. R. Rufino, F. C. Biaggio, J. C. Santos, and H. F. de Castro, "Chemoenzymatic synthesis: A strategy to obtain xylitol monoesters," *Journal of Chemical Technology & Biotechnology*, vol. 84, no. 7, pp. 957–960, 2009, ISSN: 02682575 10974660. DOI: 10.1002/jctb.2117.

[17] M. Habulin, S. Šabeder, and Ž. Knez, "Enzymatic synthesis of sugar fatty acid esters in organic solvent and in supercritical carbon dioxide and their antimicrobial activity," *The Journal of Supercritical Fluids*, vol. 45, no. 3, pp. 338–345, 2008, ISSN: 08968446. DOI: 10.1016/j.supflu.2008.01.002.

[18] S. Šabeder, M. Habulin, and Ž. Knez, "Lipase-catalyzed synthesis of fatty acid fructose esters," *Journal of Food Engineering*, vol. 77, no. 4, pp. 880–886, 2006, ISSN: 02608774. DOI: 10.1016/j.jfoodeng.2005.08.016.

[19] M. K. Walsh, R. A. Bombyk, A. Wagh, A. Bingham, and L. M. Berreau, "Synthesis of lactose monolaurate as influenced by various lipases and solvents," *Journal of Molecular Catalysis B-Enzymatic*, vol. 60, no. 3-4, pp. 171–177, 2009, ISSN: 1381-1177. DOI: 10.1016/j.molcatb.2009.05.003.

[20] M. Enayati, Y. Gong, and A. Abbaspourrad, "Synthesis of lactose lauryl ester in organic solvents using aluminosilicate zeolite as a catalyst," *Food Chem*, vol. 279, pp. 401–407, 2019, ISSN: 1873-7072. DOI: 10.1016/j.foodchem.2018.12.027.

[21] M. Ferrer, M. Angeles Cruces, M. Bernabé, A. Ballesteros, and F. J. Plou, "Lipase-catalyzed regioselective acylation of sucrose in two solvent mixture," *Biotechnol Bioeng*, vol. 65, pp. 10–16, 1998.

[22] L. Casas-Godoy, J. Arrizon, D. Arrieta-Baez, F. J. Plou, and G. Sandoval, "Synthesis and emulsifying properties of carbohydrate fatty acid esters produced from agave tequilana fructans by enzymatic acylation," *Food Chem*, vol. 204, pp. 437–43, 2016, ISSN: 1873-7072. DOI: 10.1016/j.foodchem.2016.02.153.

[23] N. d. A. S. Neta, J. C. S. d. Santos, S. d. O. Sancho, S. Rodrigues, L. R. B. Gonçalves, L. R. Rodrigues, and J. A. Teixeira, "Enzymatic synthesis of sugar esters and their potential as surface-active stabilizers of coconut milk emulsions," *Food Hydrocolloids*, vol. 27, no. 2, pp. 324–331, 2012, ISSN: 0268005X. DOI: 10.1016/j.foodhyd.2011.10.009.

[24] S. E. H. J. Van Kempen, C. G. Boeriu, H. A. Schols, P. De Waard, E. Van Der Linden, and L. M. C. Sagis, "Novel surface-active oligofructose fatty acid mono-esters by enzymatic esterification," *Food Chemistry*, vol. 138, no. 2-3, pp. 1884–1891, 2013, ISSN: 0308-8146. DOI: 10.1016/j.foodchem.2012.09.133.

[25] D. W. Templeton, M. Quinn, S. Van Wychen, D. Hyman, and L. M. L. Laurens, "Separation and quantification of microalgal carbohydrates," *Journal of Chromatography A*, vol. 1270, pp. 225–234, 2012, ISSN: 0021-9673. DOI: 10.1016/j.chroma.2012.10.034.

[26] K. A. Jung, S.-R. Lim, Y. Kim, and J. M. Park, "Potentials of macroalgae as feedstocks for biorefinery," *Bioresource Technology*, vol. 135, pp. 182–190, 2013, ISSN: 0960-8524. DOI: 10.1016/j.biortech.2012.10.025.

[27] J. Berg-Nilsen, "Production of micro algea-based products," Algetech Produkter AS, Report, 2006.

[28] M. Šoštarič, D. Klinar, M. Bricelj, J. Golob, M. Berovič, and B. Likozar, "Growth, lipid extraction and thermal degradation of the microalga chlorella vulgaris," *New Biotechnology*, vol. 29, no. 3, pp. 325–331, 2012, ISSN: 1871-6784. DOI: 10.1016/j.nbt.2011.12.002.

[29] R. P. John, G. S. Anisha, K. M. Nampoothiri, and A. Pandey, "Micro and macroalgal biomass: A renewable source for bioethanol," *Bioresource Technology*, vol. 102, no. 1, pp. 186–193, 2011, ISSN: 0960-8524. DOI: 10.1016/j.biortech.2010.06.139.

[30] W.-C. Huang, T.-H. Tsai, L.-T. Chuang, Y.-Y. Li, C. C. Zouboulis, and P.-J. Tsai, "Anti-bacterial and anti-inflammatory properties of capric acid against propionibacterium acnes: A comparative study with lauric acid," *Journal of Dermatological Science*, vol. 73, no. 3, pp. 232–240, 2014, ISSN: 0923-1811. DOI: 10.1016/j.jdermsci.2013.10.010.

[31] C. B. Huang, Y. Alimova, T. M. Myers, and J. L. Ebersole, "Short- and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms," *Archives of Oral Biology*, vol. 56, no. 7, pp. 650–654, 2011, ISSN: 0003-9969. DOI: 10.1016/j.archoralbio.2011.01.011.

[32] T. Watanabe, S. Katayama, M. Matsubara, Y. Honda, and M. Kuwahara, "Antibacterial carbohydrate monoesters suppressing cell growth of streptococcus mutans in the presence of sucrose," *Current Microbiology*, vol. 41, no. 3, pp. 210–3, 2000, ISSN: 0343-8651.

[33] J. Marchetti, V. Miguel, and A. Errazu, "Possible methods for biodiesel production," *Renewable and Sustainable Energy Reviews*, vol. 11, pp. 1300–1311, 2007. DOI: 10.1016/j.rser.2005.08.006.

[34] National Center for Biotechnology Information. (). Pubchem database. dodecanoic acid, source=hsdb. Accessed on Dec. 9, 2019, [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/source/hsdb/6814#section=Environmental-Fate-%26-Exposure.

[35] N. Bouzaoui and C. Bidjou-Haiour, "Response surface methodological study of glucose laurate synthesis catalyzed by immobilized lipase from candida cylindracea," *Biological Forum*, vol. 8, pp. 420–427, 2016, ISSN: 2249-3239.

[36] L. Li, F. Ji, J. Wang, Y. Li, and Y. Bao, "Esterification degree of fructose laurate exerted by candida antarctica lipase b in organic solvents," *Enzyme and Microbial Technology*, vol. 69, pp. 46–53, 2015, ISSN: 1879-0909. DOI: 10.1016/j.enzmictec.2014.12.003.

[37] E. Abdulmalek, H. S. Mohd Saupi, B. A. Tejo, M. Basri, A. B. Salleh, R. N. Z. Raja Abd Rahman, and M. B. Abdul Rahman, "Improved enzymatic galactose oleate ester synthesis in ionic liquids," *Journal of Molecular Catalysis B: Enzymatic*, vol. 76, pp. 37–43, 2012, ISSN: 13811177. DOI: 10.1016/j.molcatb.2011.12.004.

[38] D. Prat, J. Hayler, and A. Wells, "A survey of solvent selection guides," *Green Chemistry*, 2012. DOI: 10.1039/x0xx00000x.

[39] B. Ndaba, I. Chiyanzu, and S. Marx, "N-butanol derived from biochemical and chemical routes: A review," *Biotechnology Reports*, vol. 8, pp. 1–9, 2015, ISSN: 2215-017X.

[40] T. Cheng, Y. Zhao, X. Li, F. Lin, Y. Xu, X. Zhang, Y. Li, R. Wang, and L. Lai, "Computation of octanol-water partition coefficients by guiding an additive model with knowledge," *Journal of Chemical Information and Modeling*, vol. 47, no. 6, pp. 2140–2148, 2007, ISSN: 1549-9596. DOI: 10.1021/ci700257y.

[41] R. Sharma, Y. Chisti, and U. C. Banerjee, "Production, purification, characterization,and applications of lipases," *Biotechnology Advances*, vol. 19, pp. 627–662, 2001, ISSN: 0734-9750.

[42] D. Derawi, "Enzymatic glycerolysis of methyl laurate utilizing candida antarctica lipase b," *Malaysian Journal of Analytical Science*, vol. 20, no. 6, pp. 1365–1372, 2016, ISSN: 13942506. DOI: 10.17576/mjas-2016-2006-15.

[43] H. P. Yennewar, N. H. Yennewar, and G. K. Farber, "A structural explanation for enzyme memory in nonaqueous solvents," *Journal of the American Chemical Society*, vol. 117, pp. 577–585, 1995.

[44] E. Wehtje, J. Kaur, P. Adlercreutz, S. Chand, and B. Mattiasson, "Water activity control in enzymatic esterification processes," *Enzyme and Microbial Technology*, vol. 21, pp. 502–510, 1997, ISSN: 0141-0229/9.

[45] A. M. Kibanov, "Improving enzymes by using them in organic solvents," *Nature*, vol. 409, pp. 241–246, 2001.

[46] A. M. Gumel, M. S. M. Annuar, T. Heidelberg, and Y. Chisti, "Lipase mediated synthesis of sugar fatty acid esters," *Process Biochemistry*, vol. 46, no. 11, pp. 2079–2090, 2011, ISSN: 13595113. DOI: 10.1016/j.procbio.2011.07.021.

[47] NovozymesA/S, *Immobilized lipases for biocatlysis*, Catalog, 2016.

[48] V. Ferreira-Leitão, M. Cammarota, E. Gonçalves Aguieiras, L. Vasconcelos de Sá, R. Fernandez-Lafuente, and D. Freire, "The protagonism of biocatalysis in green chemistry and its environmental benefits," *Catalysts*, vol. 7, no. 12, 2017, ISSN: 2073-4344. DOI: 10.3390/catal7010009.

[49] N. Rami, S. Rai, D. Basu, S. Patel, Y. Patel, A. Sanyal, and D. J. Sen, "Scale ranging 1-20 of surfactant decides the solubility of water into oil or oil into water to produce monophasic formulation," *International Educational Scientific Research Journal*, vol. 3, no. 2, pp. 32–35, 2017, ISSN: 2455-295X.

[50] S. J. Choi, J. W. Won, K. M. Park, and P.-S. Chang, "A new method for determining the emulsion stability index by backscattering light detection," *Journal of Food Process Engineering*, vol. 37, no. 3, pp. 229–236, 2014, ISSN: 01458876. DOI: 10.1111/jfpe.12078.

[51] V. S. Edmond de Hoffmann, *Mass Spectrometry Principles and Applications*, Third Edition. John Wiley & Sons, Ltd, 2007, ISBN: 978-0-470-03310-4.

[52] D. C. Montgomery, *Design and Analysis of Experiments*, 5th edition. Arizona State university: John Wiley & Sons, INC, 2001, ISBN: 0-471-31649-0.

[53] D. Baş and İ. H. Boyacı, "Modeling and optimization i: Usability of response surface methodology," *Journal of Food Engineering*, vol. 78, no. 3, pp. 836–845, 2007, ISSN: 02608774. DOI: 10.1016/j.jfoodeng.2005.11.024.

[54] C. Ortiz, M. L. Ferreira, O. Barbosa, J. C. S. Dos Santos, R. C. Rodrigues, Á. Berenguer-Murcia, L. E. Briand, and R. Fernandez-Lafuente, "Novozym 435: The "perfect" lipase immobilized biocatalyst?" *Catalysis Science & Technology*, 2019, ISSN: 2044-4753. DOI: 10.1039/c9cy00415g.

Appendix

Appendix A: 12 Principles of Green Chemistry

Appendix B: Reactions Conducted

Appendix A

The 12 Principles of Green Chemistry

These 12 points were introduced in 1998 by Anastas and Warner [1]

1. **Prevention.** It is better to prevent waste than to treat or clean up waste after it is formed.

2. **Atom Economy.** Synthetic methods should be designed tomaximize the incorporation of all materials used in the process into the final product.

3. **Less Hazardous Chemical Synthesis.** Whenever practicable, synthetic methodologies should be designed to use and generate substances that pose little or no toxicity to human health and the environment.

4. **Designing Safer Chemicals.** Chemical products should be designed to preserve efficacy of the function while reducing toxicity.

5. **Safer Solvents and Auxiliaries.** The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary whenever possible and, when used innocuous.

6. **Design for Energy Efficiency** Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.

7. **Use of Renewable Feedstocks.** A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.

8. **Reduce Derivatives** Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.

9. **Catalysis** Catalytic reagents /as selective as possible) are superior to stoichiometric reagents.

10. **Design for Degradation.** Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist int he environment.

11. **Real-Time Analysis for Pollution Prevention** Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.

12. **Inherently Safer Chemistry for Accident Prevention** Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Appendix B

Conducted Reactions

All the reactions conducted to investigate the *SFAE* production, reproducibility of trials and investigation of side reactions are presented in Table B1. The reactions used to build a conversion based model with *DOE* are presented in Table B2. The tables are on the following pages due to size.

Table B1: Reactions conducted before the *DOE* analysis

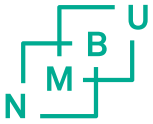
#	**Raw Material**	**MR**	**T** [$^{\circ}C$]	**P**[bar]	**Solvent**	**Enzyme**	**E** $wt\%$	**Reactor**	**Time** [h]
1.1	LA+G	1:1	60	0.5	-	-	-	Parr	6
1.2	LA + G	1:1	60	0.5	t-BuOH	N435	15	Parr	6
1.3	LA + G	1:1	60	7	t-BuOH	N435	15	Parr	6
1.4	LA + G	1:1	60	7	t-BuOH	-	-	Parr	6
1.5	LA + G	1:1	60	0.5	t-BuOH	-	-	Parr	6
1.6	LA + G	1:1	60	1.4	Acetone	N435	15	Parr	6
1.7	LA + G	1:1	60	1	MeCarb	N435	15	Parr	6
1.8	LA + G	1:1	60	1.4	Acetone	N435	15	Parr	6
1.9	VL + G	1:1	60	AP	t-BuOH	N435	15	Vial	6
1.10	VL + G	1:1	60	AP	Acetone	N435	15	Vial	6
1.11	VL + G	1:1	60	AP	VL	N435	15	vial	6
1.12	LA + G	1:1	60	AP	Acetone	N435	15	Parr	6
1.13	VL + G	1:1	60	AP	Acetone	N435	15	Parr	6
1.14	VL + G	1:1	60	AP	Acetone	N435	15	Quark	6
1.15	LA + G	1:1	60	AP	Acetone	N435	15	Quark	6
1.16	LA + G	1:1	60	AP	t-BuOH	N435	15	Quark	6
1.17	VL + G	1:1	60	AP	t-BuOH	N435	15	Quark	6
1.18	OA + G	1:1	60	AP	t-BuOH	N435	15	Quark	6
2.1	VL + G	1:1	60	AP	Acetone	N435	30	Quark	2
2.2	VL + G	3:1	60	AP	Acetone	N435	30	Quark	2
2.3	VL + G	5:1	60	AP	Acetone	N435	30	Quark	2
2.4	VL + G	1:1	60	AP	Acetone	N435	15	Quark	6
2.5	VL + G	1:1	60	AP	Acetone	N435	15	Quark	3
3.1	VL + G	1:1	50	AP	Acetone	N435	15	EF	3
3.2	VL + G	1:1	50	AP	Acetone	N435	15	EF	3
3.3	VL + G	1:1	50	AP	Acetone	N435	15	EF	3
3.4	VL + G	1:1	50	AP	Acetone	N435	15	EF	3
3.5	VL + G	1:1	50	AP	Acetone	N435	15	EF	3
3.6	VL + G	1:1	50	AP	Acetone	N435	15	EF	3
3.7	VL + G	1:1	50	AP	t-BuOH	N435	15	EF	3
3.8	Gal	1:1	50	AP	Acetone	N435	15	EF	3
3.9	VL	1:1	50	AP	Acetone	N435	15	EF	3
3.10	LA	1:1	50	AP	Acetone	N435	15	EF	3
5.0	VL	1:1	50	AP	Acetone	-	-	EF	3

G is galactose, **LA** is lauric acid, **VL** is vinyl laurate, **OA** is oleic acid and **AP** is ambient pressure. The volumes used were 100, 5, 200 and 40 ml in the *parr batch reactor*, *vials*, *quark glass reactor* and *Erlenmeyer flasks*, **EF**, respectively. Generally 1 g of the galactose was used.

Table B2: Reactions conducted to build the *DOE* analysis.

#	**Encoding**	**Actual Molar Ratio**	**Enzyme**	**Used in DOE**
Central Points	(Cat,Time)	VL:Acetone	$wt\%$	
4.1	(0 , 0)	1:1.21	17.8 %	No
4.2	(0 , 0)	1:1.08	13.4 %	No
4.3	(0 , 0)	1:1.03	13.4 %	No
4.4	(0 , 0)	1:0.98	13.4 %	No
4.5	(0 , 0)	1:1.00	15.3 %	No
DOE 1	(0 , 0)	1:1.01	15.8 %	Yes
DOE 2	(0 , 0)	1:1.01	(13.5,17.2) %	No
DOE 3	(0 , 0)	1:1.01	16.27 %	Yes
DOE 4	(0 , 0)	1:1.01	15.0 %	Yes
DOE 1.R	(0 , 0)	1:1.01	15.8 %	Yes
DOE 2.R	(0 , 0)	1:1.00	~ 16.8 %	No
DOE 3.R	(0 , 0)	1:1.01	14.5 %	No
DOE 4.R	(0 , 0)	1:1.01	15.2 %	No
Linear Points				
5.1	(-1 , -1)	1:0.98	11.8 %	No
5.2	(1 , -1)	1:0.99	12.4%	No
5.3	(1 , 1)	1:1.00	23.5%	No
5.4	(-1 , 1)	1:0.99	25.3%	No
DOE 5	(-1 , -1)	1:1.01	9.7%	Yes
DOE 6	(1 , -1)	1:1.02	19.2%	Yes
DOE 7	(1 , 1)	1:1.02	20.4%	Yes
DOE 8	(-1 , 1)	1:1.00	10.8%	No
DOE 8.R	(-1 , 1)	1:1.02	9.6%	Yes
Quadratic Points				
DOE 9	$(-\alpha$, 0)	1:1.02	8.3%	Yes
DOE 10	(α , 0)	1:1.01	21.8%	Yes
DOE 11	(0 , $-\alpha$)	1:1.01	15.4%	Yes
DOE 12	(0 , α)	1:1.00	15.6%	No
DOE 12.R	(0 , α)	1:1.02	15.9%	Yes
DOE 12.R2	(0 , α)	1:1.02	15.4%	No

For all the reactions the raw material and solvent were vinyl laurate and acetone in the presence of *Novozyme 435*. Stirring, molar ratio and temperature were set to constant values of 400 rpm, 1:1 and 50 $^{\circ}C$ respectively. The volume of solvent in each reaction was 40 ml and the weight of vinyl laurate was 1 g.



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway