

Evaluation of Hydrolysis Conditions for Free Fatty Acid Production Using Immobilized Lipases.

written by:

Lydia Bucher



LUND
UNIVERSITY

Supervised by:

Martin C Johansson

Ariana Causevic

Cecilia Tullberg

Examined by:

Carl Grey

Opposed by:

Mikael Seibold

Abstract

Enzymatic hydrolysis using immobilized lipases for the production of free fatty acids and glycerol is a promising alternative to the current industrial standard. This study investigates a set of hydrolysis reactions catalysed by four different immobilization techniques to increase the knowledge on two-phase systems. The evaluation of how water purity, buffer salts, and water amount affect the reaction rate, the pH profile, and extent of enzyme leaching was conducted. The four immobilization techniques displayed variations in results from the studied conditions. Two enzyme immobilizations, X1 and X3, exhibited higher FFA yields with immobilization X1 producing the highest FFA yields with the three conditions: 5 g enzyme with 0.05 M phosphate, tap water and 0.05 M citrate buffer with 87.4%, 85.7%, and 86.2% FFA yields respectively. Immobilization X3 produced the highest FFA yields when using 5 g of enzyme and 0.05 M phosphate buffer or 10 g of enzyme and DI water to produce 83.2% and 83.2% FFA yields respectively. While immobilization X4, exhibited a pattern similar to the free enzyme as both achieve high initial rates, but sharply curb off around 2 hours achieving much lower final reaction yields compared to the other immobilizations. This effect is likely due to the high concentrations of protein leaching seen by enzyme X4. Through the unique results for each immobilization, more in-depth research can be done to determine significance and determine conditions for further optimization.

Popular Science Summary

Fats are found in almost everything we consume as they are natural constituents of both plant-based and animal-based foods. They are often used for cooking and baking to add flavour, texture or balance acidity. Fats are primarily made up of triglycerides, or molecules consisting of three fatty acid chains attached to a glycerol backbone. While fatty acids are often seen in our diets, their relevance does not end there as they also have important applications in industrial products. Fatty acids are key ingredients in a range of products, including detergents, lubricants, plastics, emulsifiers and even tires and candles. Free fatty acids are produced from animal-based or plant-based oils through a process called hydrolysis where the triglycerides in the oils are split into free fatty acids and glycerol. Traditionally, this is done using high temperatures and pressures, but this method is energy-intensive and has a significant environmental impact.

A more sustainable alternative for the production of free fatty acids is through a type of enzyme called a lipase. Enzymes are found in all living things, and they act as tiny machines to help get work done like digest food, build new cells, and break down waste. A lipase is a type of enzyme that act like scissors to specifically cut the fatty acids from the glycerol backbone and release them as free fatty acids. This process is more sustainable because it can take place at ambient temperatures and pressures, reducing energy use. These enzymes can be immobilized or trapped to a solid surface or in a material. This allows enzymes to be kept active for longer, by easily separating them from the reaction when it is finished and reusing them for multiple reactions.

This project investigated four different enzyme immobilization techniques, to study how they would react to different reaction conditions. The amount of free fatty acids produced, the pH of the reaction, and the amount of enzyme which leaked out of the immobilization were measured for each condition. During this study, there were different reactions to the conditions between the different lipase immobilizations. One lipase (X4) produced free fatty acids at high rate during the first two hours but subsequently slowed down, while two other lipases (X1 and X3) did not produce at such a high rate in the beginning, but their rate did not slow as much after the first two hours. It is interesting to see how the same enzyme can react so differently depending on the type of immobilization used. Additionally, after studying reactions with waters that differed slightly in ion and salt concentrations, patterns emerged between them. The lipases which were in deionized water, or water containing very little ions or salts, seemed to produce fewer free

fatty acids over the reaction time compared to the waters with more ions or salts. From this study, questions were answered as to what factors help the lipases make more free fatty acids and that recycling once is possible, further studies must be conducted to optimize the process before it can become an industrial process.

Acknowledgments

I would like to express my sincere gratitude to my supervisors at AAK, Ariana and Martin, for their invaluable guidance, support, and encouragement throughout this research. Ariana played a key role in facilitating the set-up and initial phase of the project and provided feedback on the written report. Martin contributed significantly through his ongoing support in project planning, regular discussions, and insightful feedback, which helped drive the progress and successful completion of this thesis.

I am also thankful to my university supervisor, Cecilia, at Lund University, for her support during the gas chromatography (GC) analysis and for her continued guidance and encouragement during the thesis.

I would also like to thank my examiner, Carl, for the valuable discussions and for sharing his expertise on the subject, which helped refine and deepen the scientific foundation of this thesis.

Thank you to AAK for providing the necessary lab materials—including enzymes, oils, solvents, and equipment—along with access to lab space, a computer for SAP, Microsoft Teams, and an AAK email account, all of which were essential for carrying out the experimental work.

I also wish to acknowledge Lund University for supplying the GC analysis materials and Chromeleon software, which were crucial for analysing the oil samples.

Lastly, I would like to thank my family and friends for their unwavering support, understanding, and encouragement, which gave me the strength to persevere through the challenges of this journey.

Thank you all for your time, patience, and dedication.

Abbreviations

BSA: Bovine serum albumin

CLEA: Cross-linked enzyme aggregates

DAG: Diglycerides

DI: Deionized

FFA: Free fatty acids

g: Grams

GC: Gas chromatography

HOSO: High oleic sunflower oil

Hrs: Hours

MAG: Monoglycerides

Min: Minutes

MQ: MilliQ

QC: Quality control

Sec: Seconds

TAG: Triglycerides

Table of Contents

Abstract	2
Popular Science Summary	3
Acknowledgments	5
Abbreviations	6
1 Introduction	9
1.1 Background.....	9
1.2 Objective	9
1.3 Methodology.....	10
1.3.1 Lipid Hydrolysis	10
1.3.2 Two-Phase Systems	11
1.3.3 Specific and Non-specific Lipases	12
1.3.4 Immobilized Enzymes.....	13
2 Materials & Methods.....	15
2.1 Solvents and Chemicals.....	15
2.2 Rotating Packed Bed Reactions.....	17
2.2.1 Standard Conditions for all reactions	17
2.2.2 Altered Conditions for Reactions Regarding Enzyme Loading	18
2.2.3 Altered Conditions for Reactions Regarding Water Purity and Buffer Salts.....	18
2.2.4 Altered Conditions for Reaction Regarding Water Oil Ratio	18
2.2.5 Altered Conditions for Reactions Regarding Comparisons with the Free Enzyme.....	19
2.2.6 Altered Conditions for Reaction Regarding Enzyme Reusability	19
2.3 pH Measurement Analysis.....	20
2.4 Acid Value Measurement Analysis	20
2.5 Bradford Assay Analysis	21
2.6 Partial Glyceride Analysis.....	22
2.7 Quality Control Laboratory Contributions	23
3 Results & Discussion	23
3.1 Enzyme Loading.....	23
3.2 Water Purity and Buffer Salts.....	27
3.3 Water Oil Ratio	31
3.4 Free Enzyme Comparison.....	36
3.5 Enzyme Reusability.....	40
3.6 Partial Glycerides	43
3.7 Possible Sources of Error	47

3.8 Next Steps	48
4 Conclusion.....	49
References.....	51
Appendix	54

1 Introduction

1.1 Background

Plant based oils can be utilized to produce many products including free fatty acids (FFAs) and glycerol through hydrolysis. FFAs are an important hydrolysis product used in the oleochemical industry for the production of emulsifiers, detergents, and plastic auxiliaries (Palm Kernel Fatty Acid Distillate, Distilled Coconut Fatty Acid, Oleic/Stearic Acid - Olivia Oleo, n.d.). The by-product from the production of FFA, glycerol, is used widely in pharmaceuticals, biofuels, and food products (Tan et al., 2013). As the demand for alternative methods to fossil fuel derived products increases, plant-based oleochemicals are becoming a popular choice (Salimon et al., 2010).

Traditionally, hydrolysis is done through the use of high temperatures and pressures. The most common method hydrolyses crude oil at 250°C with 50 bar pressure for two hours (Noor et al., 2003). Another method to produce oleic acid utilizes a dienophile and temperatures between 180°C and 300°C (Cosgrove et al., 1991). In both processes, purification and distillation of glycerol and the desired FFAs are necessary. The current processes have a clear negative environmental impact as they consume high amounts of energy, produce chemical solvent waste, and require additional steps for distillation and product purification.

The use of enzymatic catalysts has been growing in popularity as an alternative to traditional production methods, with hydrolases accounting for more than 75% of commercially available enzymes (Jemli et al., 2014). With this alternative, there is potential for more ambient reaction conditions, fewer side reactions, and no harsh chemical solvents or reagents involved. Additional advantages can be had with the use of immobilized enzymes, as they open the door to reuse the enzymes over multiple batches or in continuous processes as well as providing easier separation of the enzymes from the reaction products.

AAK is a leading producer of plant-based oils and with a particular focus on products which are tailor made to their customers' needs. They are invested in the vision of 'Making Better Happen' by researching enzymatic applications in the field of fatty acid production.

1.2 Objective

The aim of this work is to investigate a broad range of aspects for the enzymatic hydrolysis of high-oleic sunflower oil (HOSO) to produce FFAs through a batch process. The evaluation of different lipase immobilization materials under a variety of conditions such as water qualities,

buffer salts, and water amount will be studied. The aimed outcome is the advancement in the knowledge and understanding of immobilized enzymes in a two-phase system and the influences of different carrier materials. From the information gained, next steps can be taken to do more in depth research on candidate reaction conditions. The aim of the project consists of the following tasks:

- Create a working method which utilizes a rotating bed reactor to conduct enzymatically catalysed hydrolysis of HOSO to produce FFAs.
- Determine specific activities for each of the four enzymes from trials conducted with the developed method.
- Evaluation of the oil phase to determine the degree of hydrolysis and determine the extent of enzyme leaching into the water phase.
- Evaluation of how water purity, buffer salts, and water amount affect the reaction rate, the pH profile, and enzyme leaching.
- Evaluation of the lipid profile in the hydrolysed oil with gas chromatography (GC).

1.3 Methodology

1.3.1 Lipid Hydrolysis

Lipid hydrolysis reactions are the cleavage of fatty acid chains from a glycerol backbone to ultimately produce glycerol and FFAs. This reaction can be conducted with high temperature and pressure, but it can also be catalysed enzymatically using more mild conditions. Enzymes are a type of protein which function as a biocatalyst for a reaction, and they often exhibit chemo-specificity, regiospecificity, and stereospecificity ("Enzymes," 2008). According to the International Commission on Enzymes (EC), there are six classes of enzymes, class 3 of which is called hydrolases, or enzymes which react between the substrate and water (Dicko et al., 2022). Within this class of hydrolases, exists lipases (3.1), which cleave ester bonds such as triglycerides (TAGs) as seen in Figure 1. However, in low water conditions they are also capable of catalysing the reverse reaction, esterification, or ester bond synthesis (Balcão et al., 1996). Through the ability to make and break ester bonds along with a few other reactions, lipases are enabled to catalyse a variation of lipid modifications, making them a promising enzyme for a variety of industrial applications (Borrelli & Trono, 2015).

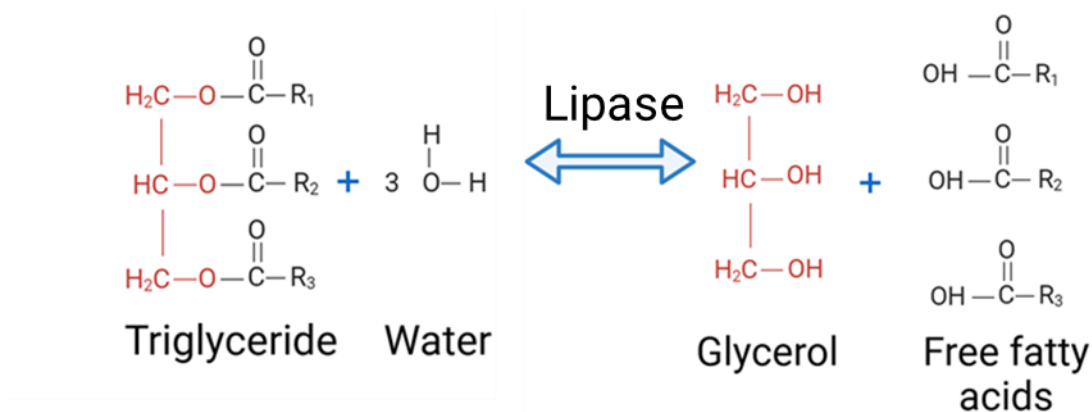


Figure 1. Lipase catalysed reaction from TAGs to FFAs and glycerol. R₁, R₂, and R₃ represent the carbon chains in the fatty acid, which vary depending on the specific fatty acid. (Created with BioRender.com)

1.3.2 Two-Phase Systems

Lipases catalyse reactions at the interface between water and oil. During a hydrolysis reaction, lipases only require a small amount of water to be catalytically active, but once the concentration of water exceeds the solubility limit of the oil substrate, a two-phase system forms and the hydrolysis reaction rate increases substantially (Dicko et al., 2022). This activation on the interphase is associated with a conformational change where a lid opens to reveal the active site of the protein. In a two-phase system, there is an aqueous and oil phase, see Figure 2, where the aqueous phase is made up of water and subsequently glycerol, and the oil phase is made up of oil containing triglycerides (TAGs), diglycerides (DAGs), monoglycerides (MAGs), and free fatty acids (FFAs) (Liu et al., 2007). The free lipase is typically dissolved in the aqueous phase.

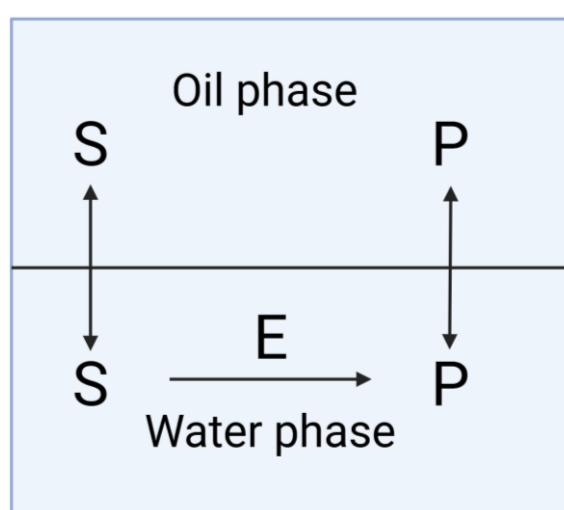


Figure 2. A schematic of an enzymatically catalysed two-phase reaction (S: substrates, E: enzyme, P: product).

(Created with BioRender.com)

Two-phase systems can be advantageous as they allow the products, including DAGs, MAGs, FFAs and glycerol to move into the organic and aqueous phase respectively after they're formed, allowing the concentration of product around the enzyme to remain low, pushing the reaction equilibrium past the natural equilibria of the mono-phase system. In some lipases, the ideal amount of water in a hydrolysis reaction has been studied to find that there is an increase in hydrolysis rate up until a water oil ratio of 1.35:1 (w/w), and after that the rates no longer increased significantly (Liu et al., 2007).

When attempting transesterification enzymatically, two-phase systems are not advantageous due to an increased frequency of hydrolytic side reactions and by-products because water would be a competing substrate, so therefore the water levels are kept minimal (Zhang, 2007). This is not a problem for hydrolysis reactions, so two-phase systems are more frequently implemented. This system allows the glycerol to reside in the water phase and the FFAs to mainly stay in the oil phase. This natural product separation is both advantageous for the yield and for product purification.

Despite being water soluble, lipases enzymatically react at the interface between the aqueous phase and the oil phase. This is due to a conformational change which takes place at the interface, allowing the active site access to the substrate (Borrelli & Trono, 2015). The more interfacial area in the reaction, means that the enzyme will have more active surface area, leading to higher enzymatic activity. The higher the stirrer speed, in a reaction vessel for a two-phase system, the smaller the particle size of oil droplets in the water, and therefore more interfacial area for the enzyme to be active on (Al-Zuhair et al., 2003). The conformational change seen during the activation of lipases plays an important role in their specificity and activity and is seen to vary widely between lipases (Secundo et al., 2006).

1.3.3 Specific and Non-specific Lipases

Lipases tend to be sn1,3-specific lipases meaning they have regioselectivity towards position 1 and 3 on the glycerol backbone, or non-specific lipases meaning they exhibit no preference towards any position on the glycerol backbone is shown. Lipases with high sn1,3-specificity are quite beneficial for a reaction yield when selectivity is desired e.g. acidolysis reactions, but selectivity towards positions 1 or 2 are not particularly beneficial for the yield in hydrolysis reactions (Dicko et al., 2022). In this study the immobilized enzymes are sn1,3-specific lipases.

As shown in Figure 3A and 3C, when using a 1,3-specific lipase, the dominant intermediates are 1,2-DAGs, 2,3-DAGs, and subsequently 2-MAGs, while a non-specific lipase (Figure 3B) will generate a random composition of intermediates (Borrelli & Trono, 2015). Regardless of which type of lipase is used to catalyse the hydrolysis reaction, the intermediates will eventually be hydrolysed to FFAs. Spontaneous acyl migration is likely to occur and is the rearrangement of fatty acid chains to form more stable DAG and MAG intermediates (Dicko et al., 2022). The 2,3-DAG and 1,2-DAG are less stable than 1,3-DAGs, so acyl migration will likely happen altering the composition of intermediates (Zhang, 2007). In the case of hydrolysis, this is expected to cause a *sn*1,3-specific lipase to produce products which appear to be from a non-specific lipase.

For hydrolysis reactions to reach the end products of glycerol and FFAs, lipases must cleave three ester bonds. These three reactions generally take place one at a time, which creates two intermediates, DAGs and MAGs, Figure 3C.

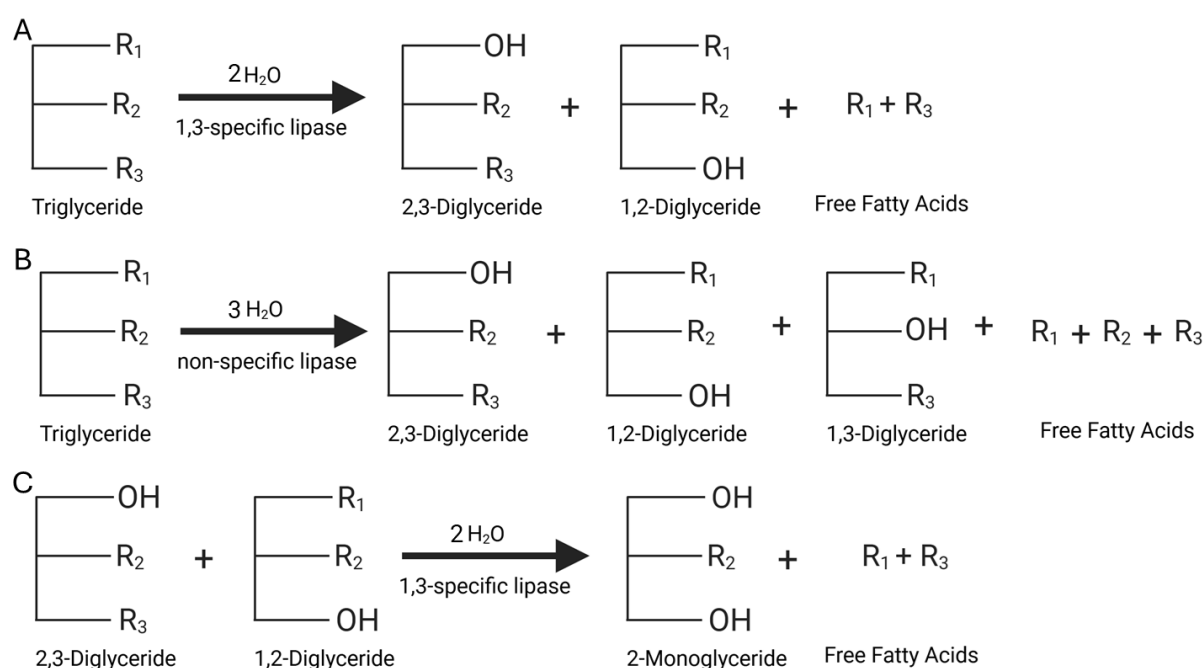


Figure 3. Lipase catalysed hydrolysis reactions with (A) TAGs converted to DAGs with a *sn*-1,3-specific lipase so that only fatty acids are cleaved at position 1 or 3. Alternatively, (B) the conversion of triglyceride to DAG with a non-specific lipase so that fatty acids are cleaved at all three positions randomly. Finally (C) where 2,3-diglycerides and 1,2-diglycerides are cleaved with a *sn*-1,3-specific lipase at positions 1 or 3 to produce 2-MAGs. (Created with BioRender.com)

1.3.4 Immobilized Enzymes

Immobilized enzymes are commonly used in industrial processes (Arbige, 1989). They offer advantages such as the possibility of increased stability and activity, easier separation of

enzymes from the products, and for recirculation possibilities, i.e. the use in multiple batches (Borrelli & Trono, 2015). However, depending on the enzyme and immobilization method, the activity can also be decreased, and usually is when the enzyme is reused. Additionally, immobilizations have an added additional cost, environmental impacts, and mass transport limitations (Dicko et al., 2022).

The three generally acknowledged categories of immobilisation techniques are entrapment, cross-linking, and immobilisation on a carrier (Sheldon et al., 2021). Entrapment can be conducted through the formation of soft membranes or gels, as done by Duan et al. (2024), or hard polymers, as done by Jawale and Bhanage (2024), around the enzyme or the whole cell. This method allows the enzyme, or cell, to keep its original configuration and protect it from destabilizing elements in the reaction medium (Sheldon et al., 2021). One drawback of entrapment is that it often faces mass transfer limitations and in order for the reaction to be successful, the right material must be selected to allow substrate and product diffusion but restricted enzyme diffusion (Imam et al., 2021). The process of entrapping a protein is considered to be relatively easy and simple to do which is considered an additional advantage of the method (Escobar et al., 2018).

The next method, cross-linking, for example, cross-linked enzyme aggregates (CLEAs), is a carrier free enzyme immobilization where covalent bonds form between enzyme molecules (Wang et al., 2025). This method is a two-step process where the enzyme is precipitated using an organic solvent and then cross-linked through the addition of a cross-linking agent (Imam et al., 2021). This was conducted for *Candida antarctica* lipase B (CALB) by Guajardo et al. (2018) by precipitating the enzyme through the addition of ammonium sulphate dropwise and subsequently the addition of glutaraldehyde as the cross-linking agent. Cross-linking is advantageous for being cost-effective partially due to being able to be produced from crude enzyme preparations as well as improved thermal and pH stability (Sheldon et al., 2021). The main limitation of this technique is the variability of the particle size, which can lead to mass transfer limitations, problems with filtration, and inconsistent activities (Velasco-Lozano et al., 2016).

The category, immobilizations on a carrier, can be further broken down and includes adsorption and covalent immobilization. While in both cases the enzymes are bonded to a carrier, in covalent immobilization the bonds are more stable covalent bonds and in adsorption the bonds are typically weaker interactions like van der Waals, ionic bonding, or hydrogen bonding (Causevic,

2022). Adsorption is considered the simplest, fastest and oftentimes the most profitable of the immobilization methods (Hasnaoui et al., 2025). However, in aqueous environments the enzyme is likely to leach from the carrier depending on the pH and ionic strength (Robescu & Bavaro, 2025). On the other hand, covalent attachments are seen to be quite stable with little leaching into the aqueous phase even in harsh conditions (Rivero & Palomo, 2016). The covalent bonds are formed between amino acid side chains, typically amines, thiols and carboxylic groups, and the carrier surface (Robescu & Bavaro, 2025). The bonds are typically formed at several attachment points on the enzyme, limiting the conformational changes which the enzyme typically undergoes. This could be considered a positive, as it improves stability in the enzyme and keeps it from unfolding. However, if this happens to limit the ability of the enzyme to switch from closed to open position, it would lead to inactivity (Adlercreutz, 2013).

When an immobilization method is applied to an enzyme, the microenvironment of the enzyme is altered, often introducing internal and external diffusion effects, where external is the diffusion in the stagnant layer and internal is diffusion through the bead (Dicko et al., 2022). External diffusion is not usually a problem as it can be decreased through proper stirring of the reactor (Dicko et al., 2022). However, internal diffusion is the limitation in substrate concentration at the enzyme's active sites, causing a decreased activity of the enzyme (Dicko et al., 2022). The smaller the particles of immobilized enzyme, the larger the surface area, and the better the diffusion of substrate and product through the carrier to the catalysts, because the distance for the substrate to travel is shorter (Sheldon et al., 2021). The increase in substrate concentration relative to the amount of immobilized enzyme is another tool to decrease the internal as well as external diffusion limitations (T. Zhang et al., 2002).

2 Materials & Methods

2.1 Solvents and Chemicals

All solvents used for the Bradford assay, acid value measurement, and partial glycerides determination are chemical grade or higher and were purchased from ThermoFisher Scientific and VWR. The acids and bases used during the trials were purchased from Merck except for citric acid which was purchased from VWR.

The oil, high oleic sunflower oil (HOSO), used for the duration of the project, was supplied by AAK (Fritex HOSO, Karlshamn). A saponification value of 193 was measured and used to

calculate the FFA percentages, as this value takes into consideration the compounds in the oil which are not able to be hydrolysed. For calculations, the estimated molecular weight of the oil was 282 g/mol as the predominant FFA is oleic acid with more than 80.0% (Table 1). The acid value of the oil before hydrolysis was measured to be 0,04 by the quality control (QC) lab in Karlshamn. The composition of fatty acids in HOSO was provided by AAK and can be seen in Table 1. The deionized (DI) water was supplied by the laboratory, and milliQ (MQ) water was produced with a Thermo Scientific GenPure UV/UF. Tap water used during the trials came from Lund Municipality, see appendix (Table A1) for more information.

Table 1. The composition of fatty acids in high oleic sunflower oil (HOSO), expressed as percent of total fatty acids detected.

Fatty Acid Name	C:D	% in HOSO
Palmitic acid	C16:0	3.5%
Stearic acid	C18:0	3.0%
Oleic acid	C18:1	≥80.0%
Linoleic acid	C18:2	7.0%
α-linolenic acid	C18:3	≤0.5%
Erucic acid	C22:1	≤0.5%

The four immobilized enzymes originated from the same lipase and were provided by AAK as used for the study. The identity of the enzymes along with their formulations and producers are confidential. The amount of enzyme immobilized, and the activity of the immobilizations are unknown. The four immobilizations will be referred to as X1, X2, X3, and X4. All four immobilizations are immobilized on a carrier, but the type of bonds between the enzyme and the carrier will be kept confidential. The porosity of the carriers is not known. The immobilizations increase from hydrophilicity with X1 being the least hydrophilic and X4 being the most hydrophilic. While immobilization X2 can be described as a powder, the remaining three X1, X3, and X4 are a few hundred µm in diameter. Immobilization X1, X3 and X4 are not uniform in size nor shape, and X2 is too fine to distinguish.

2.2 Rotating Packed Bed Reactions

Reactions were conducted in a temperature controlled 200 mL mantled reactor (Spinchem Vessel V2) with a diameter of 100 cm and height of 25 cm, see Figure 4. The reaction vessel is designed for volumes ranging from 120-300 mL. The immobilized enzyme was packed into the rotating bed reactor (Spinchem, RBR S2), which had a height of 3 cm, diameter of 4.5 cm, porosity of 104 μm , and volume of 28 mL. A motor mounted above the reactor rotated the packed bed at 450 rpm. The heating bath (LAUDA) was heated to 50°C. Sampling of the reaction was done with a glass pipet and automatic pipette from the top of the reaction vessel. Each sample was 15 mL in volume and was immediately centrifuged at 2000 rpm for ten minutes and subsequently stored in the refrigerator at 4°C until analysis.

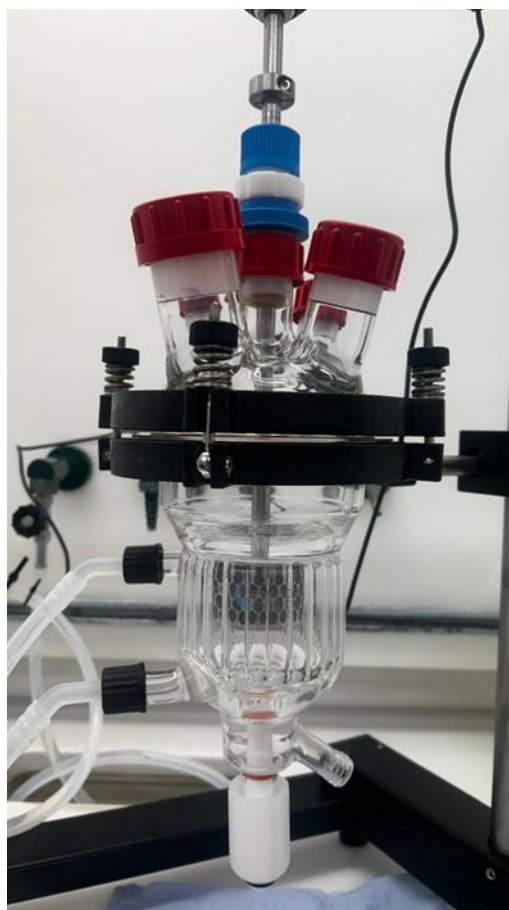


Figure 4. The reaction vessel used for all hydrolysis reactions during this study.

2.2.1 Standard Conditions for all reactions

The standard conditions for all trials conducted for the entirety of this master thesis project, unless specifically stated otherwise, were 200 g of HOSO, 100 g of DI water, and 5 g of immobilized

enzyme were packed into the bed. The water bath was set to heat to 50°C and the rotating bed reactor spun at 450 rpm. The total reaction times were 24 hours, with six samples taken 15 minutes, 1 hour, 2 hours, 4 hours, 6 hours, and 24 hours. In between trials, the rotating bed reactor was fully dismantled, washed with soap and DI water, and dried with paper towels before starting the subsequent trial.

2.2.2 Altered Conditions for Reactions Regarding Enzyme Loading

For reactions which study the variation in enzyme loading follow the standard conditions with the following exceptions. The short normalization trials were conducted over only 1 hour with samples taken at 15 minutes, 30 minutes, and 60 minutes. There were no replicates conducted. For all four enzymes formulations, 5 g of enzyme was tested, and for three of the enzymes (with the exclusion of X3) 1 g was used in the normalization trials.

Additionally, enzymatic trials for 24 hours were conducted to study to variation in hydrolysis extent over a longer reaction period. The reactions containing 5 g of each enzyme were conducted following the standard conditions. The reactions containing 10 g of enzyme varied in that sampling was performed at different time points. An initial sample was taken immediately after the reaction appeared to be homogeneous, which varied in time from 30-60 seconds from reaction initiation. This variation led to significant error among the trials, so this point is excluded from the resulting data set. The remaining 5 samples were collected at 1 hour, 2 hours, 4 hours, 6 hours, and 24 hours, however occasionally a data point was taken at a different time than this and this has been depicted in the figures.

2.2.3 Altered Conditions for Reactions Regarding Water Purity and

Buffer Salts

Another set of trials studied various water purities and buffer salts for three enzymes: X1, X3, and X4 to evaluate the importance of ionic strength for the reactions. The different water purities were DI water (pH 8.25), MQ water, tap water (pH 8.05), 0.05 M phosphate buffer (pH 7.0) and 0.05 M citrate buffer (pH 6.3). Trials containing tap water were replicated as it was seen that there could be the most variation for this water (X1 and X4 n=2, and X3 n=3).

2.2.4 Altered Conditions for Reaction Regarding Water Oil Ratio

The next set of trials studied the ratio of water to oil (w/w) in the reaction and its effect on the reaction. The ratio was altered from standard conditions by the increasing or decreasing the

amount of water in the system. The amount of oil remained 200 g for the trials, as stated in the standard conditions. The ratios studied were water oil ratio 1:1 (200 g DI water) and 1:3 (66.6 g DI water). The data presented for the water oil ratio 1:2 is not data from a separate reaction, instead it is the same data value from the DI water reaction from the trials which studied water purity. This data set is presented as a comparison for the other two water oil ratios. No trials were replicated for this study. The packed bed was vertically adjusted to be in the centre of the reaction at the beginning of the experiment. For the 1:1 ratio the packed bed was moved upwards, and for ratio 1:3 the bed was adjusted downwards to remain in the centre of the reaction at the start of the reaction.

2.2.5 Altered Conditions for Reactions Regarding Comparisons with the Free Enzyme

The set of trials compared data generated using the free enzyme over two batches with data from other trials from this study to compare the reaction patterns based on immobilization type. The reaction data from the three immobilized enzymes, X1, X3, and X4, followed the standard reaction conditions. The first batch contained 0.5% (w/w free enzyme/oil), 200 g of HOSO, and 100 g DI water. The rotating bed remained empty for these trials and simply acted as a stirrer. The free enzyme was studied in two subsequent batches where the reactor was not dismantled or cleaned in between. The contents were drained through a port at the bottom of the apparatus and then centrifuged to separate the water from the oil phase so that the water, containing the free enzyme, could be used again. Some water was lost in the recirculation step, so fresh water was added to the used water to bring the volume to 100 ml. Then the reaction was started again with 100 mL DI water with the remaining enzyme from the first batch and 200 g of fresh HOSO.

The first batch was conducted over 24 hours with samples taken at 1 minute, 1 hour, 2 hours, 4 hours, 6 hours, and 24 hours. The second batch was conducted for only 6 hours with samples at 1 minute, 1 hour, 2 hours, 4 hours, and 6 hours.

2.2.6 Altered Conditions for Reaction Regarding Enzyme Reusability

The set of trials studied the reusability of the enzymes under multiple conditions for the comparison of the reaction rate and hydrolysis yield. The reactions which studied the reusability of 10 g of enzyme were conducted with 200 g of HOSO and 100 g DI water for two batches. The first batch for 24 hours and the second for 6 hours. For the reactions containing 10 g of X1 and X3 enzyme, two subsequent batches where the rotating bed reactor was not dismantled or

cleaned in between were conducted. The reactor contents were drained through a port at the bottle of the apparatus. The enzyme remained in the packed bed, and 200 g of fresh HOSO and 100 g of fresh DI water was added to the reaction. The first batch for X1 was sampled at 1.5 hours, 2 hours, 4 hours, 6 hours, and 24 hours. The second batch for X1 was sampled at 1 hour, 2 hours, 3 hours, 4 hours, and 6 hours. The first batch for X3 was sampled at 1.33 hours, 2 hours, 3 hours, 4.5 hours, and 24 hours. The second batch for X3 was sampled at 1 hour, 2 hours, 3 hours, and 5.5 hours.

The reactions performed to study the reusability of 5 g of X1 or X3 enzyme and either citrate or phosphate buffer were conducted with 200 g of HOSO and 100 g of the respective buffered water for two batches. For these trials, the reactor was dismantled (excluding the packed bed), wiped with a paper towel, and reconstructed. After the reactor was emptied, the bed was spun at 600 rpm for 10 seconds to remove excess oil and water from the packed bed. All batches were conducted for 24 hours and followed the standard sampling procedure stated in the standard reaction conditions.

2.3 pH Measurement Analysis

The pH measurement of the water phase was performed to analyse the pH of the system, and following a protocol provided by AAK. After the samples were centrifuged, two layers formed: an oil layer on top and a water layer on the bottom. The bottom layer was moved into a separate 5 mL Eppendorf tube, so that the water phase was pure and contained as little oil residue as possible. The pH value was measured of the water phase after separation from the oil was complete. No replicate measurements were conducted.

The pH-meter (Accumet AB315 pH/mV meter) and pH electrode (13-620-223A) were purchased from Thermofisher Scientific. The electrode was cleaned using the cleaning solution (D pH cleaner for general cleaning, oil and grease removal Orion Product Thermoscientific) prior to calibration. This was done by placing the electrode in the solution and agitating it for 30 seconds, then rinsing the electrode with DI water. The electrode was then calibrated with buffer solutions at 4.01, 7.00 and 10.01 (Orion Product Thermoscientific).

2.4 Acid Value Measurement Analysis

The assay protocol followed a protocol provided by AAK to measure the acid value in the sample. The oil sample (0.5-1 g) is dissolved into 25 mL of Diethyl ether: absolute ethanol 60:40 (V/V), then three drops of phenolphthalein solution 0.2% in ethanol is added. Sodium hydroxide in

ethanol (0.1 M) is titrated (Eppendorf Top burette M) into the solution until it turns a faint pink colour which persists for 30 seconds. Blank tests were conducted regularly and used to calculate the acid value. Each new day that trials were conducted, a standard containing 50:50 ratio of oleic acid and HOSO were measured to ensure that the analytical error remained within 1% of the calculated acid value. Additionally, the measured values were compared with values measured by AAK's quality control lab in Karlshamn and an error percentage calculated, see appendix. The lower limit for accurate measurements was determined to be acid value 1.00. From the amount of sodium hydroxide titrated, the percent of FFAs in the oil can be calculated using Equation 1.

Equation 1.

$$\text{Percent of Free Fatty Acids (Oleic Acid)} = \frac{((a - b) * N * M)}{10 * i}$$

Key:

a = mL volume of NaOH added until colour change.

b = mL volume of NaOH added to the blank until colour change.

M = average molecular weight of fatty acids. For HOSO, 282 g/mol was used.

i = weight of oil sample in grams

N = concentration of sodium hydroxide (NaOH). For this method, 0.1 M was always used.

2.5 Bradford Assay Analysis

The protein content in the water sample was conducted using a bradford protocol provided by AAK. A five-point calibration curve was made using bovine serum albumin (BSA) (2.0 mg/mL, Thermofisher Scientific). Solutions of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL BSA in 50 mM phosphate buffer were used to create a calibration curve. The absorbance was measured spectrophotometrically at 595 nm (Agilent BioTek Epoch2). A new calibration curve was measured for each 96-well plate (Greiner), see appendix. The lower limit for accurate measurements was determined to be 0.1 mg/mL.

In each well, 6.7 µL of sample or BSA standard and 333 µL of Bradford reagent were mixed and allowed to sit for at least 5 minutes. Triplicates of each BSA standard were conducted with each plate measured and duplicates of all other samples were measured. The spectrophotometer software (Agilent BioTek Gen5) calculated the concentrations of each well dependent on the calibration curve of that plate. From the duplicate concentrations, the mean and standard deviation was calculated and presented. Additionally, the absolute protein content was calculated by multiplying the average concentration of the sample by the estimated amount of water in the

reaction at the time the sample was taken. For reactions initially containing 100 g of water (ratio 1:2 or the standard condition), 4 mL was subtracted from 100 mL for each sample taken, so that the amount of water in the sample at 24 hours was 80 mL. For reactions containing 66.6 g of water (ratio 1:3), 3 mL was subtracted from 66.6 mL for each sample taken, so that the amount of water in the sample at 24 hours was 51.6 mL. For reactions containing 200 g of water (ratio 1:1), 6 mL was subtracted from 200 mL for each sample taken, so that the amount of water in the sample at 24 hours was 170 mL.

It was assumed that no protein was in the oil phase of the sample and that the enzyme was evenly distributed within the water phase of the sample. The density of the water was assumed to be 1 g/mL for the duration of the trial. However, this would lead to some error in the calculation because as the glycerol concentration in the water phase increased, the density of the water would also increase. The concentration of glycerol was not measured, so the concentration was unknown and therefor disregarded in the calculation for the protein assay.

2.6 Partial Glyceride Analysis

The partial glycerides analysis was conducted following the procedure 'Lipid analysis' by silylation (Orata, 2012). The analysis using the gas chromatography (GC TRACE 1300, Thermo Scientific) with flame ionisation detector at the department of PLE at Lund University. The internal standard, 1-Monopentadecanoin (Larodan), was dissolved in MTBE at 20 mg/mL. Subsequently, 50 μ L of internal standard was added to 50 μ L of sample, both were silylated by the addition of MSHFBA and diluted in 1 mL cyclohexane.

The relative area (%) was used to determine the composition of the glycerides and FFAs. While individual peaks for each position specific and fatty acid specific partial glyceride were determined, there were most likely errors between injections or inaccuracies within the peak identification program. So instead of differentiating between each type of fatty acid on each glyceride, all of the relative areas of the glycerides were summed. Additionally, for the analysis of acyl migration, 1,2-DAGs and 2,3 DAGs were summed, and 1-MAGs and 3-MAGs were summed so that these groups are represented together as 1(3),2-DAGs and 1(3)-MAGs respectively. The composition within the glycerides was determined by summing up the relative areas of the glycerides and calculating their percent of the total glycerides.

2.7 Quality Control Laboratory Contributions

The AAK quality control laboratory (Karlshamn, Sweden) contributed to the analysis of this project through the acid value and saponification methods. The purpose of this was to compare with results produced in the AAK Lund laboratory to validate results with the standardized procedures of AAK.

3 Results & Discussion

3.1 Enzyme Loading

Before studying different reaction variables, it was key to determine an amount of enzyme which would produce comparable reaction rates across the studied immobilization methods. It was quickly discovered that a variation of the amount of enzyme in the packed bed had effects on the reaction rate which were difficult to predict. The initial reaction rates, seen in Figure 5, show the difference between 1 g and 5 g of enzyme in the rotating bed. When packing 1 g instead of 5 g, enzymes X1, X2, and X4, showed a decrease in FFA production after the first hour, but X1 and X4 showed more prominent decreases of 10.4% FFAs and 11.2% FFAs respectively while X2 decreased by only 2.98% FFAs. In both cases, enzyme X2 had the lowest FFA yield after 1 hour out of all measured enzymes. If the reactions were to be extrapolated to follow their current trajectories, based on this data X2 would be assumed to continue at the slowest rate of the four enzymes.

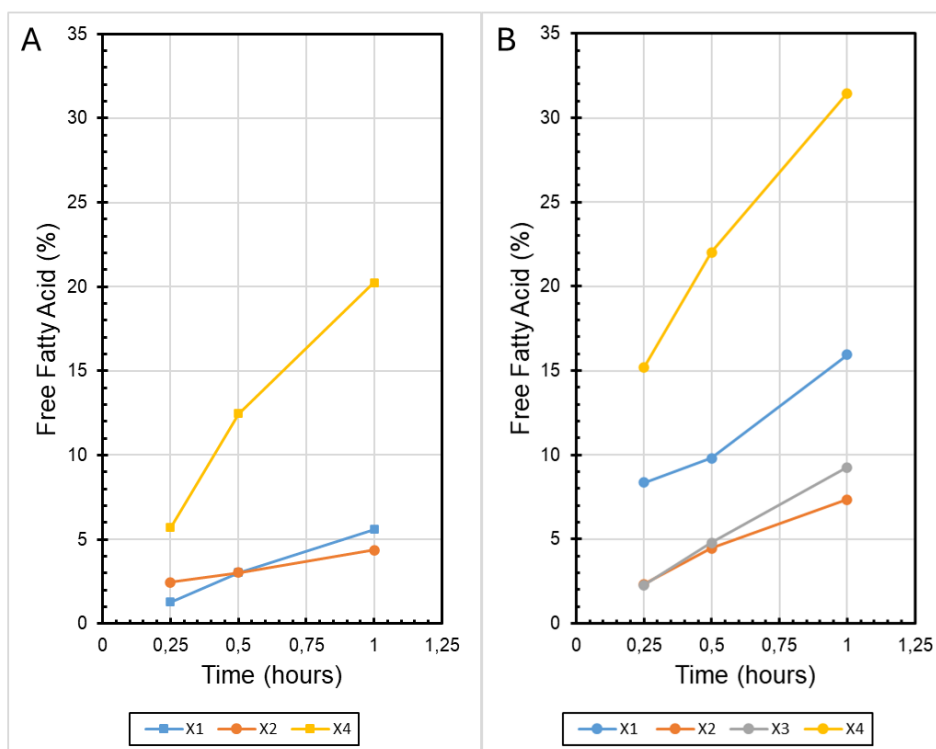


Figure 5. Short term trials to determine initial reaction rates depending on enzyme loading. A. Enzymes X1, X2, and X4 with 1 g loaded into the bed (n=1) B. Enzymes X1, X2, X3, and X4 with 5 g loading into the reactor bed (n=1).

Subsequently, 24-hour reactions were conducted with 5 g and 10 g of enzyme to compare the extent of the hydrolysis reactions, shown in Figure 6. In Figure 6A, it was exhibited that by halving the amount of X1 enzyme from 10 g to 5 g, the FFA percent yield increased from 36.5% to 80.0%.

Inversely, Figure 6C and 6D exhibit a decrease in reaction rate correlating to a decrease in enzyme loading. However, when halving the amount of enzyme packed from 10 g to 5 g, the effect is reversed and to a lesser extent than X1 and the FFA percent at 24 hours for enzymes X3 and X4 only decreased by 17.0% FFAs and 13.3% FFAs respectively. When comparing the reaction rates for 5 g and 10 g of X3, at 4 hours with 5 g of enzyme X3, 23.1% FFAs have been produced, but at 2 hours with 10 g of enzyme X3, 20.7% FFAs have been produced, see Figure 6C. Similarly, this comparison can be made for X4 with 5 g and 10 g of loaded enzymes, see Figure 6D. At 4 hours with 5 g of X4 enzyme, 35.5% FFAs have been produced, while at 2 hours with 10 g of X4 enzyme, 39.0% FFAs have been produced. Based on this, when halving the amount of enzyme loaded, the time it took the enzyme to reach the same FFA percent doubled for the initial phase of the reaction.

Figure 6B contains only a curve for a reaction with 10 g of enzyme X2. It was determined from the results from Figure 5A and 5B, that this reaction set-up was not a good application for enzyme X2. The enzyme exhibited low initial reaction rates at both high and low enzyme concentrations, and it is assumed that the rate would not increase after the first hour. Furthermore, it was observed during the trials that the mixing of the reaction with enzyme X2 was not homogeneous, and this could be because the consistency of this immobilized enzyme formed a paste like sediment. It is hypothesized that the consistency of the enzyme X2 made it difficult for the rotating bed reactor to properly mix the reaction contents, leading to less interaction between the enzyme and the oil, and subsequently less hydrolysis could occur.

Decreasing the amount of enzyme X2 from 10 g to 5 g actually showed a slight increase in reaction rate at 1 hour. This shows that there were internal mass transport limitations, as the decrease in enzyme concentration allowed substrate to more effectively reach the enzyme. If a small amount of enzyme is used, so that there is a high substrate-to-enzyme concentration, and the external mass transfer limitations could be decreased, the reaction rate would remain slow compared to the other enzymes, X1, X3, and X4. Therefore, it was concluded that for this application, enzyme X2 was not a good candidate, and it was excluded from subsequent trials.

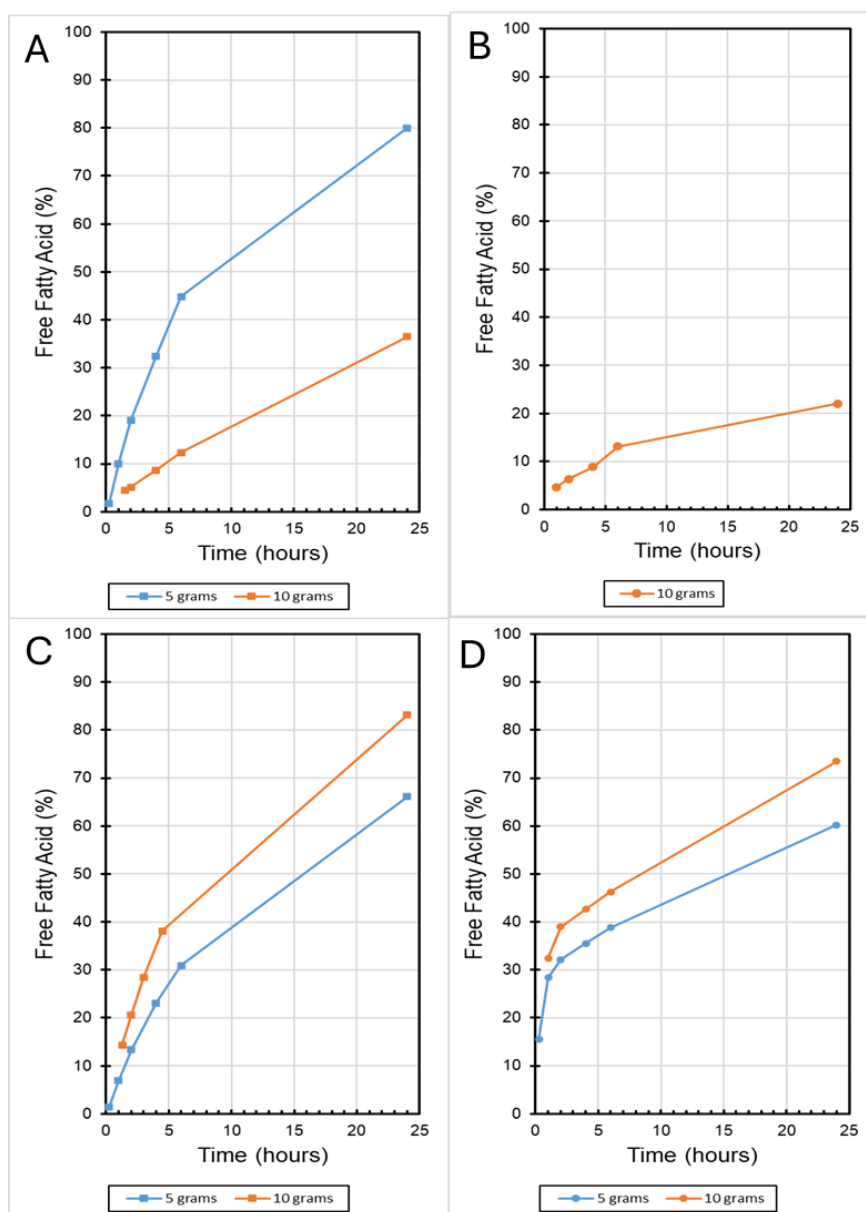


Figure 6. 24-hour Hydrolysis reactions for the determination of hydrolysis extent based on enzyme loading. Graph A: Enzyme X1, (5 g and 10 g, n=1), B: Enzyme X2, (10 g, n=1), C: Enzyme X3, (5 g and 10 g, n=1), and D: Enzyme X4, (5 g and 10 g, n=1).

While enzyme X1 initially exhibited a low reaction rate and low hydrolysis extent with 10 g packed in the bed (Figure 6A), after decreasing the amount of enzyme to 5 g in the packed bed, the yield greatly increased. Similar to enzyme X2, this is most likely due to mass transport limitations of substrate through the packed bed. However, this enzyme differs as it was observed that the particles of the immobilized enzyme X1 expanded after being in the reaction, likely due to the carrier absorbing water during the reaction. This expansion likely made the bed much more tightly packed, making it difficult for the substrate to access the enzyme effectively. While mixing

remained visually sufficient, the decrease in reaction rate is likely due to a decrease in the saturation of substrate on the enzyme due to a lack of effective flow through the entirety of the packed bed.

When halving the enzyme concentration, the initial hydrolysis rate is affected so that it took the reaction twice as long to reach the same FFA yields. This has shown that in the case of enzyme X3 and X4 their specific activities did not change after altering the amount of enzyme packed from 10 g to 5 g, and this indicated that no mass transport limitations are affecting the initial reaction rate. However, after 24 hours, the difference between the two yields (Figure 6C and 6D) for each enzyme became much closer, with X3 only having decreased by 17.0% FFAs and X4 only by 13.3% FFAs, indicating that equilibrium is approaching. When the amount of enzyme loading is decreased, the reaction rate is also decreased, however in the case of both enzymes X3 and X4, the reactions indicated that they will eventually reach the same equilibrium point, which is suspected to be sometime after 24 hours at least for 5 g of enzyme. In this study this point has not been determined, however, it would be interesting future work to determine.

From these trials investigating the effects of enzyme loading, it was concluded that 5 g of enzyme would be best to use for the remainder of the study for the three enzymes: X1, X3, and X4. This was decided because 5 g of immobilized enzyme provided comparable yields across the three enzymes, while retaining high enough activity so that the reaction could reach close to equilibrium by 24 hours. However, if the focus were to be to obtain the highest FFA yield in the shortest time, based on the information from these trials, it would be recommended to use 10 g of enzyme for X3 and X4, and 5 g of enzyme for X1.

3.2 Water Purity and Buffer Salts

During the investigation of water purity, the various water purities (DI water, MQ water, tap water, phosphate buffer and citrate buffer) aligned into two trend groups. This pattern was seen for both enzymes X1 and X3 and persisted for hydrolysis rates and pH curves. The first trend group was DI water and MQ water and the second was tap water, 0.05 M phosphate buffer, and 0.05M citrate buffer.

In Figure 7A presenting enzyme X1, the hydrolysis rates of tap water, 0.05 M phosphate buffer, and 0.05 M citrate buffer overlap. Similarly, the DI water and MQ water overlap for most of the samples, however, sometime after 6 hours, the MQ water decreased in reaction rate. This could

be due to experimental deviation or potentially due to the slight differences in ionic strength between MQ and DI.

Similarly in Figure 7B, the two trend groups form, however, the variation between reactions is wider, with the largest difference in FFAs of 17.0%, is seen between DI and 0.05M phosphate buffer. This indicates that enzyme X3 is more sensitive to the reaction changes in ionic strength than enzymes X1 or X4. Conversely, in Figure 7C, enzyme X4 the two trend groups do not form and very little variation between the three water purities is seen.

The highest FFA yield of 87.4% was achieved with 0.05 M phosphate and catalysed by enzyme X1 (Figure 7A). Of the three enzymes, the initial reaction rate is the highest for X4 (Figure 7C), but its rate decreases sharply after the first 2 hours.

The reaction was conducted with five water purities for enzymes X1 and X3, but only three for enzymes X4. The reason for only using three water purities for enzyme X4 was because there was no effect seen on the hydrolysis extent when using tap water, see Figure 7C. It was assumed that adding buffer would have little to no effect, so the trials were excluded. While for enzymes X1 and X3, an increase in hydrolysis extent was observed with the tap water in Figure 7A and 7B, this was not seen for enzyme X4. If there was more time, it would still be interesting to determine if the buffers would have influenced the hydrolysis extent of X4.

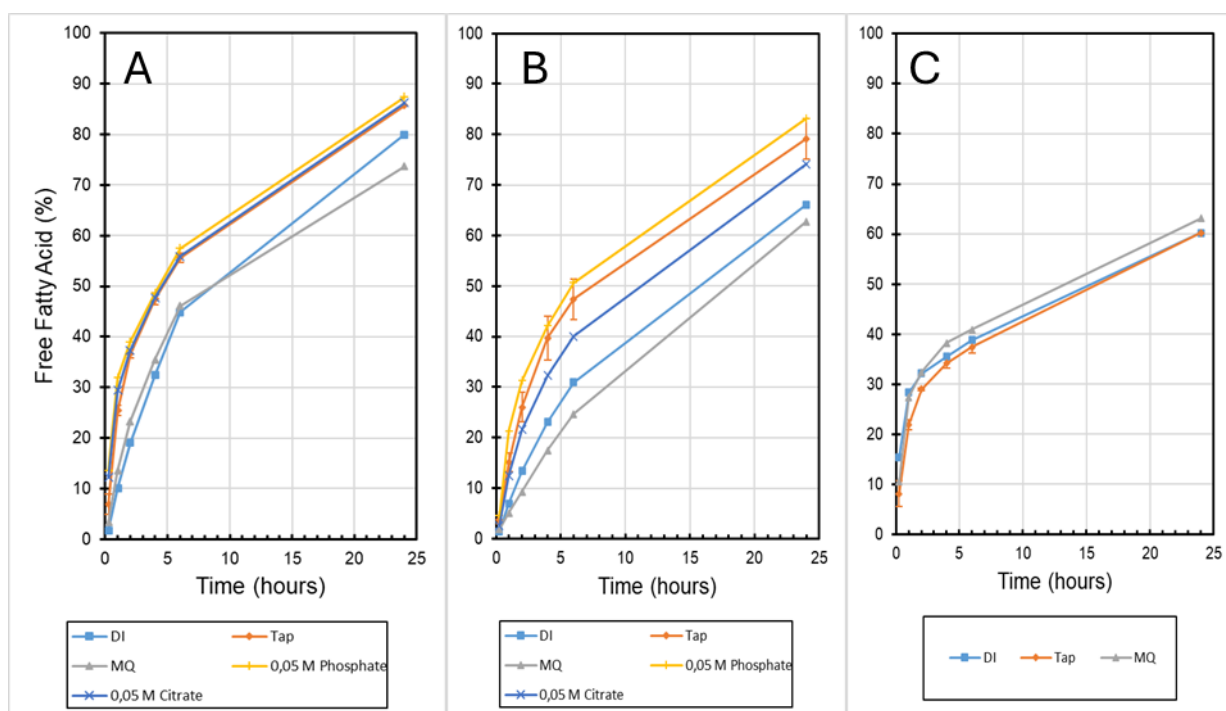


Figure 7. Hydrolysis reactions catalysed enzymatically with varied water purities and buffers. Graph A: Enzyme X1, (DI, MQ, 0.05M Phosphate, and 0.05M Citrate: n=1, Tap: n=2), B: Enzyme X3, (DI, MQ, 0.05M Phosphate, and 0.05M Citrate, n=1, Tap n=3), and C: Enzyme X4, (DI, MQ, n=1, Tap n=2). Error bars are present for replicated reactions and represent one standard deviation.

The two trend groups were likely due to the increase in ionic strength seen in tap water, 0.05 M phosphate buffer and 0.05 M citrate buffer. While DI water and MQ water were intentionally ion free, tap water contained small amounts of Ca and Na ions present at a concentration of 0.5 mmol. Similarly, 0.05 M phosphate and citrate buffers contained buffer salts which have an increased ionic strength compared to DI and MQ water. The trends seen varies from immobilization to immobilization. The difference is seen most stark between X1 where strong correlation between ionic strength is seen and X4 where no correlation is seen. This is likely due to the difference in hydrophobicity between the immobilization processes. The increase in ionic strength or polarity, would create a greater difference between a more hydrophobic support (X1), therefore increasing the partitioning effect. This could increase in partitioning effect could alter the apparent ideal K_m , K_i and pH profile, and in the case of enzyme X1 this benefits the initial reaction rate as well as the yield at 24 hours.

The trends were further validated in the pH measurement, presented in Figure 8A and 8B, as the two distinct groups of water purity form again. For enzymes X1 and X3, the DI water and MQ water showed a slight increase in pH during the first hour of the reaction, but then subsequently decreased until they were seen to reach roughly the same end point as the other water purities. While the tap water maintained a linear pattern similar to the buffered waters, any buffering capacity was quickly depleted as the water quickly became acidic. The pH values for reactions containing tap water are seen to drop the most, and usually quite dramatically during the first 6 hours.

The pH of the buffered waters (Figure 8A and 8B) becomes more acidic, but more gradually. The buffering capacity is depleted over time, as the reaction has a similar pH at 24 hours to the other water purities. This small change in pH could have a larger effect on the stability of the enzyme over time.

The pH measurement of enzyme X4, in Figure 8C, is shown to be an outlier of the three enzymes. There was little variation between the pH values of the three water purities: DI, MQ and tap. Within 15 minutes of hydrolysis, the reaction catalysed by enzyme X4 reached a sub 6.00 pH, while enzymes X1 and X3 require more reaction time for their pH to reach this acidity. The

immobilization of this enzyme could have been conducted at a lower pH than the others, so that it quickly drops to a lower pH than the other immobilizations as contents from the carrier dissolve into the reaction.

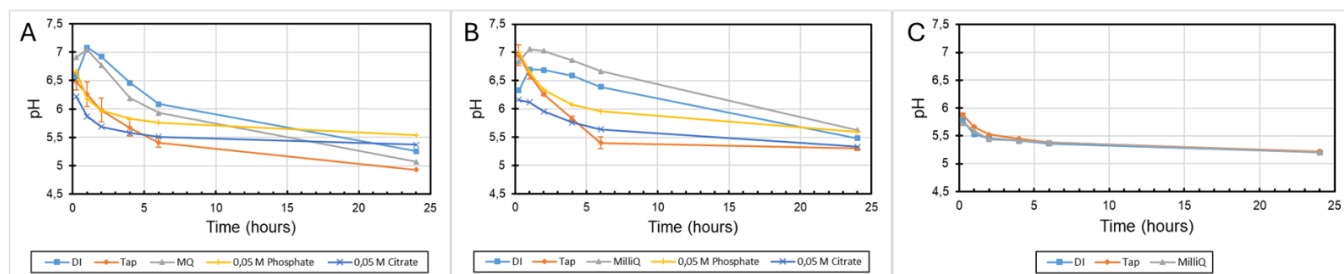


Figure 8. The pH of the reaction over the course of the reaction. Graph A: Enzyme X1, (DI, MQ, 0.05M Phosphate, and 0.05M Citrate: n=1, Tap: n=2), B: Enzyme X3, (DI, MQ, 0.05M Phosphate, and 0.05M Citrate, n=1, Tap n=3), and C: Enzyme X4, (DI, MQ, n=1, Tap n=2). Error bars are present for replicated trials and represent one standard deviation.

The increase, seen by DI water and MQ water for enzymes X1 and X3, after the initial 15-minute sample is possibly due to buffer salts dissolving from the carriers. A reason for this increase is that residue from the immobilization buffer could be left on the carriers and is dissolving into the water causing an initial decrease in pH, pushing the reaction pH towards the pH at which the enzyme was immobilized. This could cause a small buffering effect of the reaction. This effect is not seen by enzyme X4, and this is hypothesized to be because the reaction for enzyme X4 moves too quickly for this effect to be seen. The acid value (Figure 7C) is increasing too quickly and any buffering capacity that the carrier may have had would be depleted by 15 minutes.

A trend is seen between the pH values and the FFA percents regardless of water purity, see Figure 9. This indicates that the acid value is correlated with the pH value. The more FFAs produced as the reaction proceeds, the lower the pH value drops. While all three enzymes follow the same pattern, enzymes X1 and X3 exhibit a larger variation in relationship between pH and acid value between reaction conditions relative to enzyme X4.

The linearity of the correlation is strong for the majority of the reaction time, except for the first sample from enzymes X1 and X3 at 15 minutes for the DI and MQ water, see Figure 9A and 9B. These initial samples deviate from the linearity of the graphs. This could be due to various effects taking hold of the pH in the early reaction. One possible effect is that the enzyme carriers are dissolving carrier material into the water that is altering the pH (see Appendix).

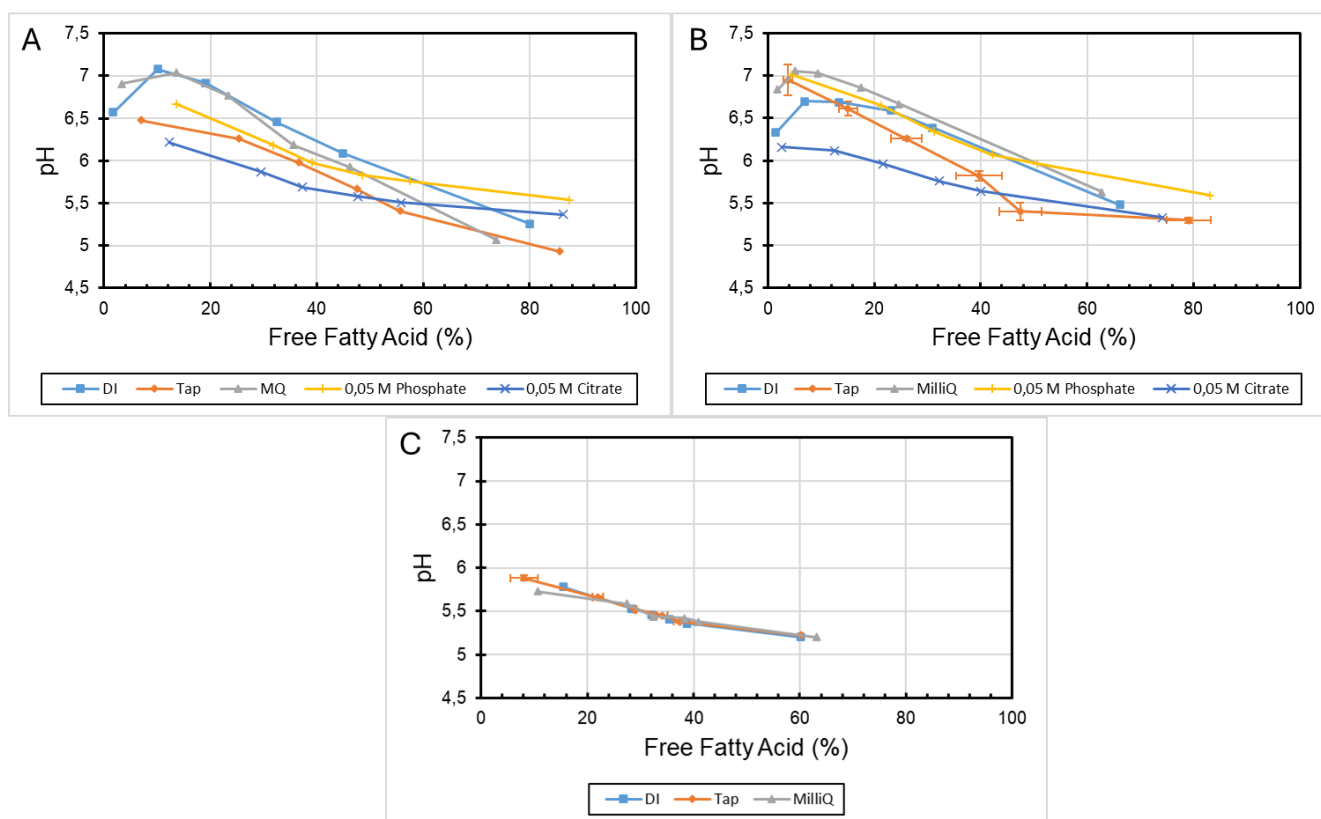


Figure 9. The pH values plotted against the FFA percentage of the reaction. Graph A: Enzyme X1, (DI, MQ, 0.05 M Phosphate, and 0.05 M Citrate: $n=1$, Tap: $n=2$), B: Enzyme X3, (DI, MQ, 0.05M Phosphate, and 0.05 M Citrate, $n=1$, Tap $n=3$), and C: Enzyme X4, (DI, MQ, $n=1$, Tap $n=2$). Error bars represent one standard deviation in the x and y planes.

3.3 Water Oil Ratio

The ratio of water to oil (w/w) was studied to determine if there was a change in hydrolysis extent and pH based on the amount of water added to the system. It is expected for lipases in two-phase systems that with an increase in water, up to a certain point, that the activity should increase by the immobilization. However, it is unknown for this lipase and these immobilizations at what ratio will be the point where the activity no longer increases significantly. Enzyme X1, in Figure 10A, only had a small difference in initial hydrolysis rate and hydrolysis extent between the three ratios (1:1, 1:2, and 1:3). At 24 hours, the water oil ratio of 1:3 was at 82.2% FFAs, while 1:2 and 1:1 were just below at 80.0% FFAs and 76.7% respectively. The results from enzyme X3, Figure 10B, indicated that the 1:2 ratio hydrolysed fewer FFAs than both the conditions of 1:3 and 1:1. While ratios 1:3 and 1:1 followed the same path to 79.2% FFAs at 24 hours, the curve for 1:2 did not display the same initial rate, but between hour 6 and 24, the reaction kept the same reaction rate as 1:1 and 1:3. Finally, enzyme X4, in Figure 10C, shows high similarity between ratios 1:2 and 1:3, and with ratio 1:1 showing an increase of 16.1% FFAs at 24 hours compared

to 1:2 and 1:3. Opposite to the observation of enzyme X3, the 1:1 ratio shares the same initial rate as 1:2 and 1:3 ratios, but after the first hour, the other curves decrease in reaction rate to a greater extent than 1:1.

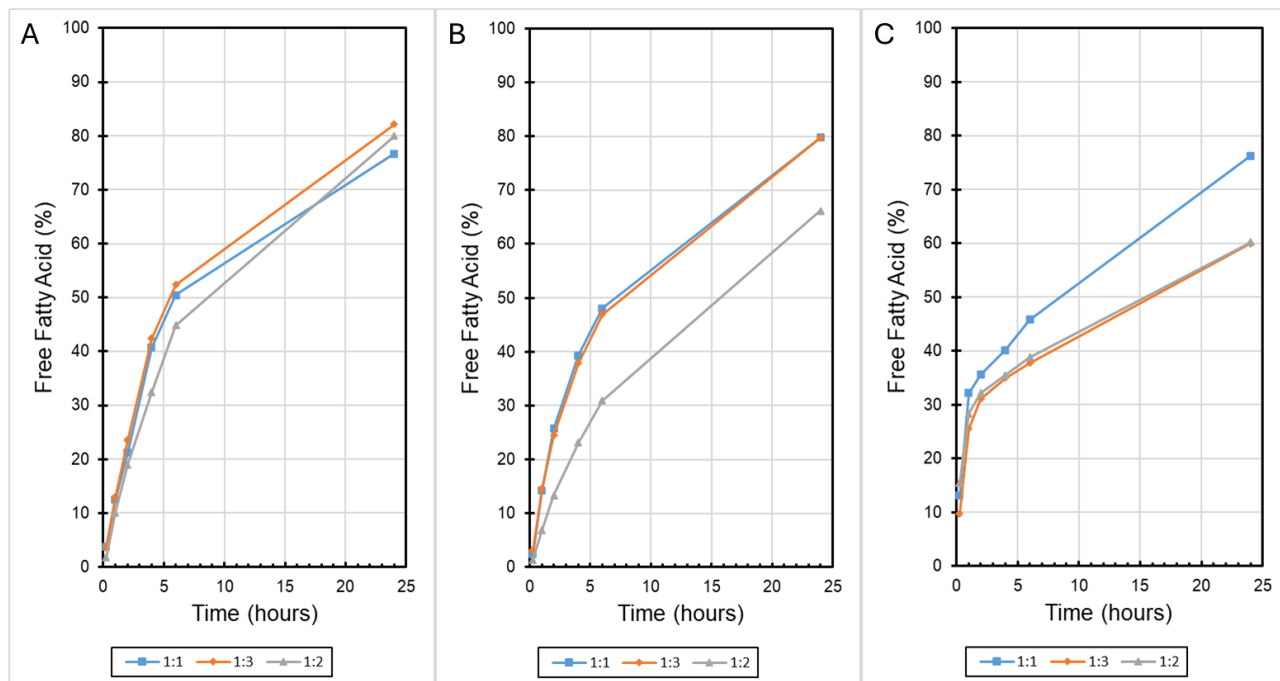


Figure 10. Comparison of hydrolysis over time through the investigation of different water to oil ratios. Graph A: Enzyme X1, (1:1, 1:3, and 1:2 $n=1$), B: Enzyme X3, (1:1, 1:3, and 1:2 $n=1$), and C: Enzyme X4, (1:1, 1:3, and 1:2 $n=1$).

The result in Figure 10B with enzyme X3 with clear differences between the oil ratios investigated was unexpected, but one possible explanation is that ratios 1:1 and 1:3 had better initial hydrolysis rates compared to ratio 1:2 for contradicting reasons. The 1:1 ratio could have had a higher initial hydrolysis rate, due to an increase of water and therefore surface area for the lipase to act on, however, this effect is typically seen for free lipases and cannot be assumed for immobilized lipases. Another possibility for an increased initial rate for ratio 1:1, is that the reaction is less viscous as it is thinned by the increase in water. The lower viscosity could allow the substrate to pass through the reactor more easily offering better access of substrate through the packed enzyme. Meanwhile, the 1:3 ratio could have benefited from the lower reaction volume by having more effective mixing of the substrates thereby decreasing external diffusion limitations. As there is less water in the system, the oil is more highly concentrated, and this could also lead to better substrate access for the enzyme.

The enzyme X4 1:1 ratio, in Figure 10C, reaction broke the trend seen by enzyme X4 previously in Figure 6D, where its high initial reaction rate consistently curbs after 1 hour. This reaction, despite after only the addition of water, held a similar reaction rate to the other enzymes for the duration of the 24-hour reaction. One possible explanation for this is that the higher water concentration could have dissolved more glycerol, allowing the reaction to go in further in the forward direction. It is possible that the difference in hydrophobicity between the immobilizations, could have an effect on how each enzyme behaves when the amount of water increases or decreases. This increase in hydrophobic water could alter the polarity of the reaction conditions. An immobilization that is more protected by the carrier is less likely to show an effect by the reaction, but a immobilization that leaves the enzyme more vulnerable is more likely to seen changes during the change in ratio.

By looking at the pH profiles over the runs in Figure 11A, 11B, and 11C, more clear patterns can be found. As the water in the system decreased, the initial upward curve, which is seen within the first hour, also decreased. The 1:3 ratio for enzymes X1 and X3 is shown to be the most alkaline for the first sample at 15 minutes, however, the difference between the samples is very small. The 1:1 and 1:3 ratios for both enzymes X1 and X3 have the same pH at 24 hours, despite showing different slopes of getting there. Where ratio 1:1 more gradually decreases in pH, the 1:3 ratio decreases quickly during the first 6 hours and then decreases more gradually between 6 and 24 hours. While enzymes X1 and X3 seems to follow similar trends, their absolute pH values differ slightly by an average pH of 0.3. Meanwhile in Figure 11C, the three pH curves for X4 remain tightly correlated with little variation, despite the variation in acid value seen in Figure 10C.

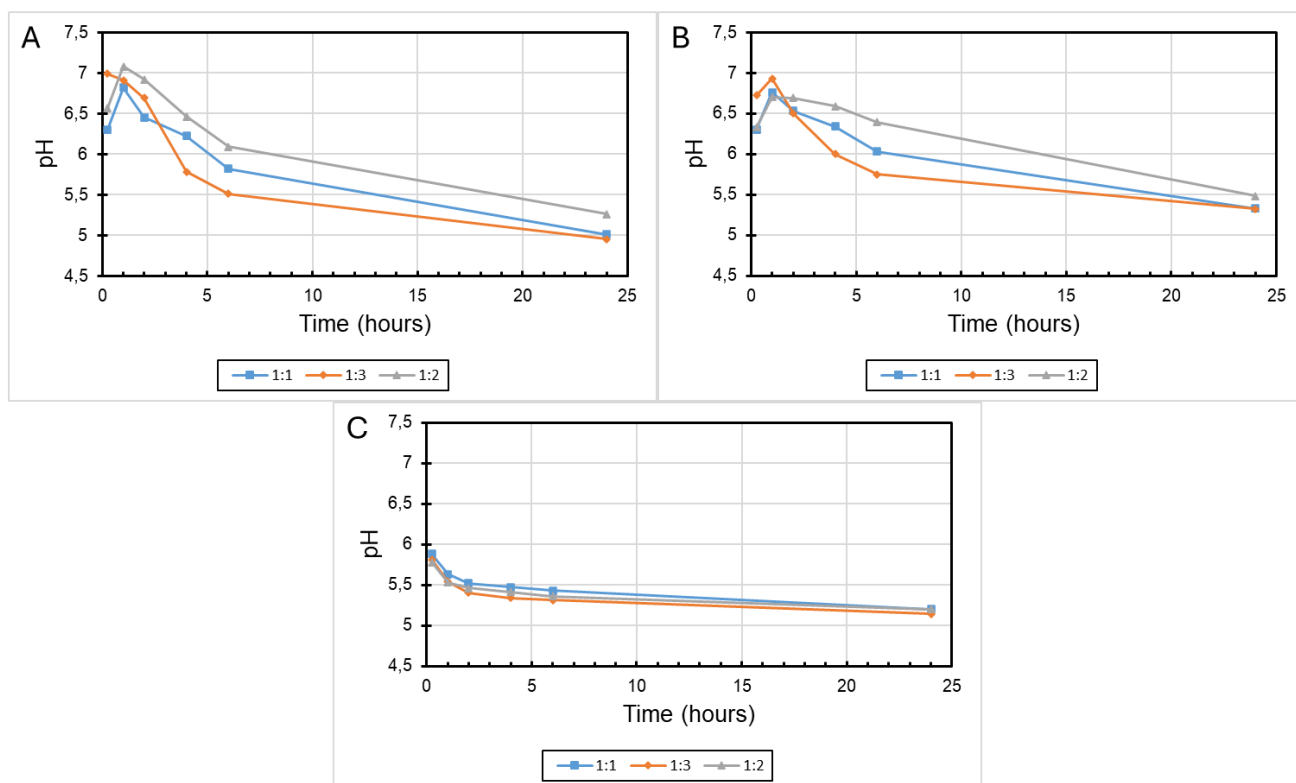


Figure 11. The pH of the water phase over the course of the 24-hour reaction. Graph A: Enzyme X1, (1:1, 1:3, and 1:2 n=1), B: Enzyme X3, (1:1, 1:3, and 1:2 n=1), and C: Enzyme X4, (1:1, 1:3, and 1:2 n=1).

The initial upward curve of pH, presented in Figure 11A and 11B, has previously been seen in all samples from enzymes X1 and X3 containing MQ or DI water. Now this trend has been observed again, but to varying extents. It is possible that the pH curve for ratio 1:3 does not exhibit this to the same extent because of the lower reaction volume. The 1:3 ratio is more effectively stirred than the 1:1 ratio, so the effects seen are not expected to be as prominent. If the effect is due to the leaching of carrier compounds or buffer salts, this explanation would make sense as the better mixed reaction would more quickly even out the contradicting effects.

For all three enzymes, in Figure 11, the 1:3 ratio is seen to start the most alkaline of the three ratios, however, the difference is small and cannot be determined as a significant difference or not due to lack of replicates. One possibility for this is that the higher water concentration could offer slightly more buffering so that in the first 15 minutes, the pH remains a bit higher. However, after this point, the 1:3 ratios decrease at a much faster rate than the other ratios. In Figure 12A and 12B, it can be seen that this is most likely due to the higher FFA percent at that time point, indicating more hydrolysis.

Data for the pH, corresponding to the free fatty acid release, is displayed in Figure 12. At 15 minutes in Figure 12A, the values deviate from one another with ratio 1:3 showing the most linearity between pH and FFA percent for the rest of the plot. Similarly in Figure 12B, the curves show a linear trend, except for the sample at 15 minutes, which does not follow the linearity of the rest of the plot. When comparing Figure 9 with Figure 12, for enzymes X1 and X3 there is less variation between the different the different ratios that between the water purities. inversely, enzyme X4, shows slightly more variation between the linear curves for the different ratios compared to the water purities in Figure 9C. The 1:1 ratio does show a deviation from the other ratios mainly at the highest acid value of 76.3% for enzyme X4.

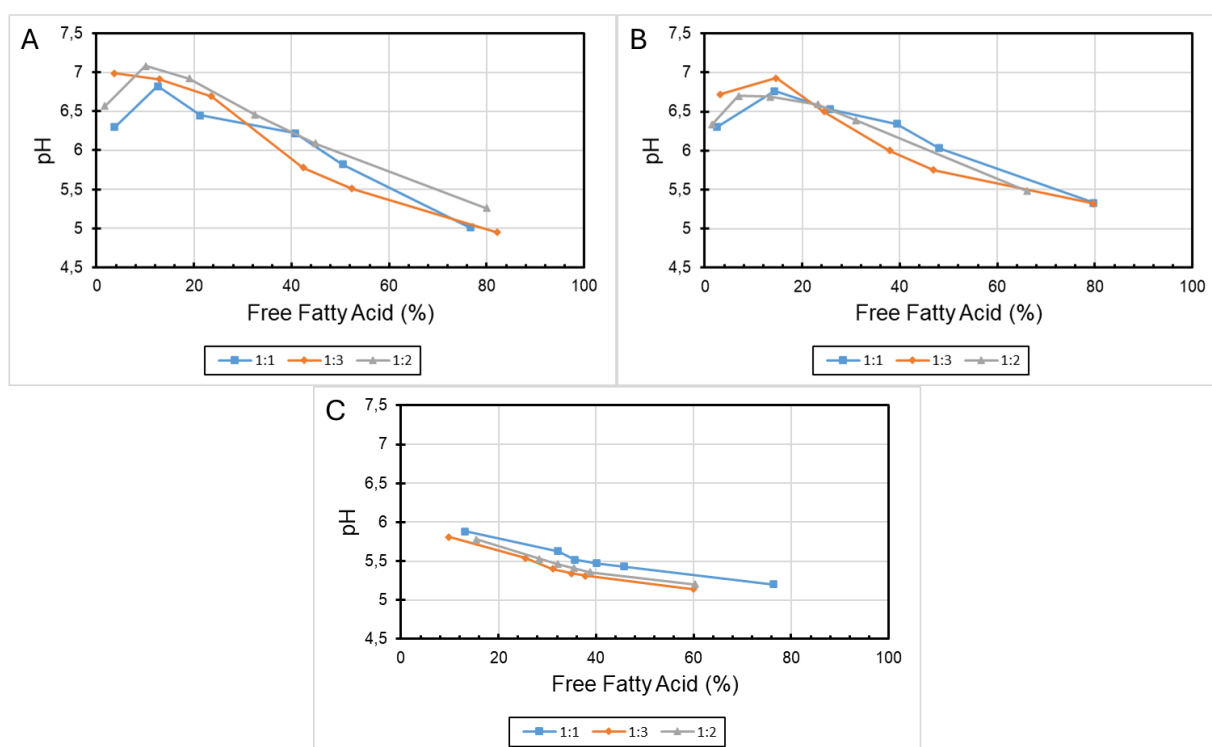


Figure 12. Water and oil ratios compared through the correlation between pH and FFA content. Graph A: Enzyme X1, (1:1, 1:3, and 1:2 n=1), B: Enzyme X3, (1:1, 1:3, and 1:2 n=1), and C: Enzyme X4, (1:1, 1:3, and 1:2 n=1).

The phenomena taking place in the first 15 minutes of the reaction, does seem to be correlated with the amount of water in the reaction or the volume of the reaction. One reason for this is that whatever might have been dissolved into the water phase of the reaction vessel, would be more concentrated in the smaller volume of water. One reason that it could be affected by the volume of the reaction is that the smaller the reaction, the more thoroughly it was mixed. The contradicting effects could possibly have been avoided by mixing more quickly so that the reaction already reached the linear curve by 15 minutes.

Oddly, there is a deviation from the linear curve in Figure 12C, where ratio 1:1 has a relatively alkaline pH for such a high acid value. This could be because of the increased water content in the reaction, as it had a slightly higher buffering effect to the acidic ions. Another possibility is that there could be error in the acid value measurement, and the acid value at 24 hours was not as high as it was measured.

3.4 Free Enzyme Comparison

While the enzymes all have different immobilization techniques in their production processes, they originate from the same free lipase. To understand how the immobilizations have distinguished from the free enzyme, trials have been conducted with the free enzyme, so that a comparison between enzymes X1, X3, X4 and the free enzyme can be done. In Figure 13, the reaction profile of the free enzyme (FE) follows a similar pattern as seen with enzyme X4, as both exhibit an extremely high initial rate followed by a relatively slower rate for the remaining reaction time. Enzyme X4 obtained a FFA yield of 60.3% which is 7.02% FFAs higher than the first batch with the free enzyme. There is little difference between the yields from the first and second batch with the free enzyme. At 6 hours, the two batches of free enzyme differ in FFA yield by 0.53% FFAs. The reaction profiles of enzymes X1 and X3 are shown in Figure 13 to decrease their rates after 6 hours, but to a much less extent than enzyme X4 and the free enzyme. In turn enzymes X1 and X3 have reaction profiles that are more linear while the reaction profiles for enzyme X4 and the free enzyme resemble logarithmic curves.

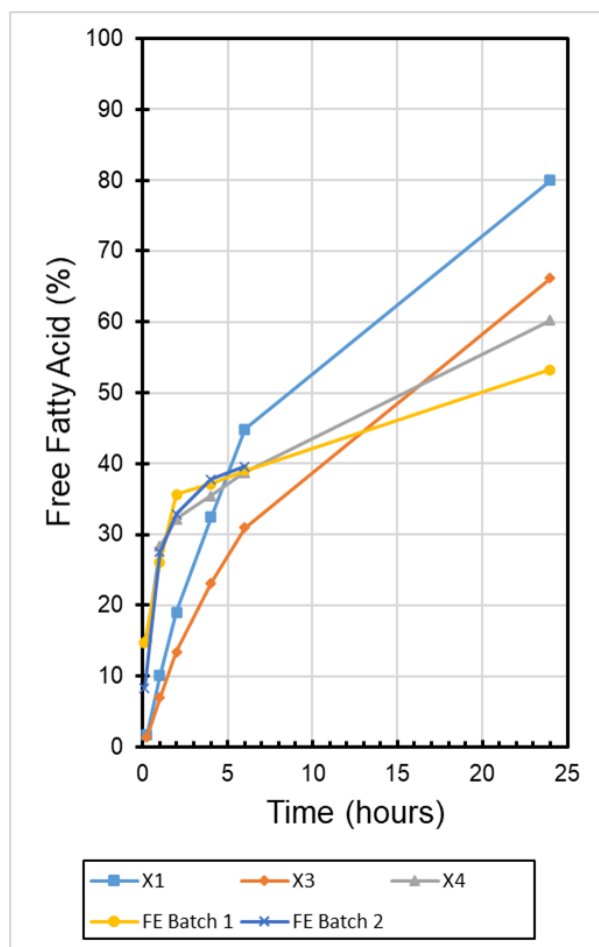


Figure 13. The hydrolysis profile over time for the three immobilized enzymes (X1, X3, and X4) and the free enzyme (FE) in two consecutive batches (FE Batch 1 and 2). Each trial was conducted once and has no replicates (n=1), so no standard error bars are present.

To evaluate the leaching of enzymes, the protein concentration in the water phase was measured, and the protein concentration of the water phase for enzyme X4 is presented in Figure 14. While enzymes X1 and X3 are typically below the limit of quantification for protein concentration (data not presented), enzyme X4 consistently leaches from its carrier and is found to be in the water phase regardless of trial conditions, see Figure 14. There is little variation of protein leaching between the water purities. The protein concentrations vary with ratio 1:1 having a concentration at 24 hours of 0.322 mg/mL and ratio 1:3 having a concentration of 0.881 mg/mL. In ratio 1:1, the leached protein is more diluted in the system than the high concentration seen in ratio 1:3.

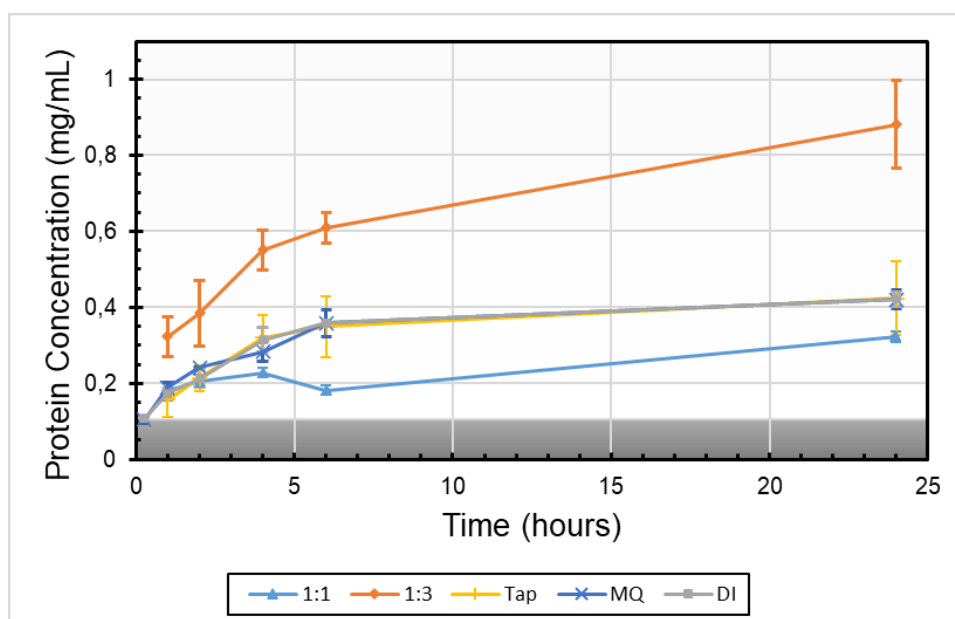


Figure 14. Protein concentration (mg/ml) found in the water phase of X4 catalysed reactions. (DI, MQ, 1:1, 1:3, and 1:2 n=2, Tap n=4). The minimum measurable concentration is 0.1 mg/ml, this is visually represented by the grey background at and below 0.1 mg/ml. In the instance that a measurement is below the limit, there is nothing graphed for that time point.

From the calculations of the absolute protein amounts seen in Table 2, the amount of protein leached from enzyme X4 is highest during the reaction with water oil ratio of 1:1. This indicates that the more water in the reaction, the more leaching is seen. However, a slight increase in the absolute protein amount is seen in the 1:3 ratio despite containing less water in the system.

The protein content measurement for the free enzyme batch 1 showed quite a large deviation in values with the lowest value of 18.72 mg and an upper value of 45.0 mg. The calculated error also shows quite high variation for the free enzyme batch 1. However, the second batch of free enzyme, is much more consistent with a low of 11.5 mg and a high of 15.7 mg. In the switch between first and second batch, some free enzyme was lost but the reaction rate was not affected.

Table 2. Absolute enzyme masses calculated from the enzyme concentrations measured in the water phase. The first five columns represent the absolute enzyme mass (mg) of enzyme X4, the last two columns represent the free enzyme from the first and second trial conducted. The absolute protein content is represented in mg with plus/minus one absolute standard deviation.

Time (h)	X4 DI (mg)	X4 Tap (mg)	X4 MQ (mg)	X4 1:1 (mg)	X4 1:3 (mg)	FE Batch 1 (mg)	FE Batch 2 (mg)
0.25	10,7 ± 0,20	n/a	10,5 ± 0,90	n/a	n/a	No sample	No Sample
1	16,7 ± 1,2	15,0 ± 0,96	18,2 ± 1,2	34,9 ± 4,9	20,5 ± 3,3	43,7 ± 26,1	15,7 ± 0,77
2	19,6 ± 2,0	19,6 ± 0,92	22,2 ± 0,28	38,5 ± 3,2	23,3 ± 5,2	31,8 ± 3,8	15,6 ± 1,6
4	27,6 ± 2,9	28,1 ± 0	25,0 ± 2,2	41,5 ± 2,2	31,7 ± 3,0	31,8 ± 2,7	13,5 ± 0,88
6	30,2 ± 0,59	29,3 ± 1,3	30,1 ± 2,9	32,0 ± 2,1	33,3 ± 2,2	45,0 ± 3,4	11,5 ± 0,84
24	33,8 ± 1,5	33,9 ± 0,40	33,6 ± 2,0	54,7 ± 2,2	45,5 ± 6,0	18,72 ± 0,80	No Sample

The protein leaching by enzyme X4 across all conditions was found to be in the same magnitude as the protein content in the water phase for the free enzyme batches. Additionally, enzyme X4 and the free enzyme batches are found to have similar logarithmic reaction curves. It is difficult to determine what hydrolysis extent can be attributed to the immobilized X4 enzyme and what can be attributed to the leached X4 enzyme. This limits what can be concluded from the enzyme X4 as an immobilized enzyme. Any effects which we observed from the applied conditions were possibly more likely due to the free enzyme rather than the immobilized enzyme. However, an increase in FFA yield at 24 hours is seen by the immobilized X4 enzyme compared to the free enzyme batch 1 (Figure 13). This FFA increase have been hydrolysed by the portion of X4 enzyme which is still immobilized on the carrier. While it is unknown how much enzyme is remaining on the carrier, second batch trials can be conducted to determine if there is still activity remaining on the carrier. A trial with two batches has been conducted with 10 g of enzyme X4, and activity largely remained on the carrier during the second batch, see appendix.

In this study, it was seen that the free enzyme gave comparable results as those of the immobilized enzyme X4, however further attention was not paid to determine what effect the immobilized X4 had on the reaction compared to the leached X4. There are a few possible ways to try and determine this. One way is to conduct multiple batches with the same enzyme, while measuring the amount of enzyme leaching and activity during each batch. If the leaching decreases over several batches but activity remains, then it can be concluded that the remaining activity belongs to the immobilized X4 enzyme. However, the amount of enzyme loaded on the immobilization is not known, so it would not be possible to determine how much enzyme remains immobilized. Another way would be to conduct a reaction with low water activity, as this would inhibit protein leaching. However, this reaction set-up would greatly alter the conditions of the reaction and would likely alter the activity of the enzyme making comparisons to this study difficult.

The question remains as to why both enzyme X4 and the free enzyme show the trends that they do. One possibility is that the free enzyme has lower rate of acyl migration than the immobilizations, and therefore the reaction slows as the preferred substrate concentration decreases. This could be determined through lipid composition analysis of the oil phase from the free enzyme reactions. The free enzyme is also more vulnerable to the reaction conditions, meaning that it may have a higher apparent ideal pH than the immobilizations. If the pH dropped quickly for the reaction containing the free enzyme, as it was seen to do for enzyme X4, the enzyme's activity is likely to decrease. With high levels of enzyme X4 leaching, it can be hypothesized that the activity of this enzyme has decreased for the same reason, however, the remaining activity from the carrier pushes the reaction forward faster than the free lipase.

Glycerol also likely plays a role in enzyme activity. The more hydrophobic immobilizations are likely to see higher yields as glycerol is expelled from the carrier due to opposing forces. Whereas in the hydrophilic immobilizations, the glycerol is more likely to be attracted to remain in the carrier and microenvironment of the immobilization, causing an inhibition to the reaction.

3.5 Enzyme Reusability

While limited trials were conducted to measure the reusability of the immobilizations, they have been presented in Figure 15 and 16 to facilitate the comparison of different conditions and their ability for reuse. Figure 15A and 15B show how the enzyme X1 loses only a small amount of activity between a first (Figure 15A) and second batch (Figure 15B). The reaction containing 5 g of enzyme X1 and 0.05 M phosphate buffer exhibits the best FFA yield across both batches, with only a decrease of 0.884% FFAs at 24 hours. The reaction containing 5 g of X1 and 0.05 M citrate buffer similarly exhibits a high FFA yield but loses slightly more activity for the second batch with a decrease of 2.80% FFAs at 24 hours.

Again, the decrease in enzyme packing from 10 g to 5 g, can be seen to have an improvement on the FFA yield at 24 hours. When utilizing the citrate buffer, the yield has increased 49.8% FFAs at 24 hours, and with the phosphate buffer the yield has increased 50.9% FFAs at 24 hours, compared to the FFA yield of 10 g with DI water (Figure 15A). While during the second batch in Figure 15B, the activity of the 10 g in DI water is retained, it is still 35.0% FFAs less than the phosphate buffer at 6 hours.

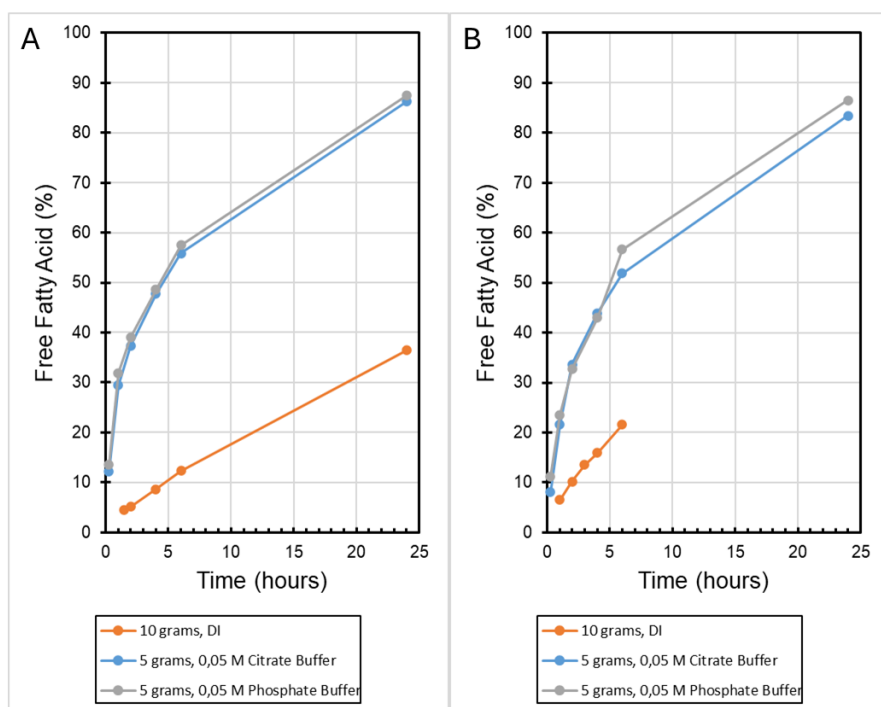


Figure 15. FFA production by enzyme X1 in three conditions for the determination of reusability over two batches

Graph A: Enzyme X1 First batch with packed enzyme (10 g and DI, 5 g and 0.05M Citrate Buffer, and 5 g and 0.05M Phosphate Buffer, n=1), B: Enzyme X1 Second batch with packed enzyme (10 g and DI, 5 g and 0.05M Citrate Buffer, and 5 g and 0.05M Phosphate Buffer, n=1).

Trials containing the X1 enzyme and DI (at both 1:1 and 1:3 ratios), MQ or tap water, show that the protein concentration remains below minimum quantifiable amount of 0.1 mg/ml. However, after the introduction of citrate and phosphate buffers, low levels of protein leaching are measured in the first batch as seen in Figure 16. During the second batch trials, protein leaching is no longer detected above 0.1 mg/ml. When accounting for the standard error bars, the protein leaching concentration does not significantly to increase, but a measurable amount of protein does leach into the water phase and remain to be measurably there for the rest of the reaction.

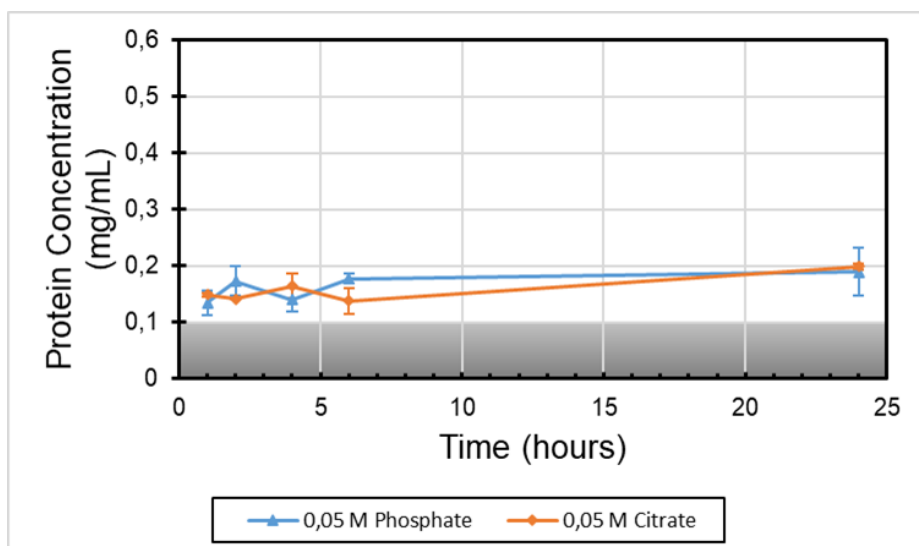


Figure 16. Protein concentration (mg/mL) found in the water phase during first batch reactions catalysed by enzyme X1 containing 0.05 M phosphate and 0.05 M citrate buffer (n=2). Error bars represent one standard deviation.

The decrease in FFA yield during the second batch is likely due to either enzyme inactivation, enzyme leaching, or general variation between trials. In this case, it is likely due to the leaching seen during the first batch (Figure 16). The small amounts of enzyme released into the reaction to act as free enzyme, creates a slightly curved reaction profile in Figure 15A similar to but not to the same extent as seen in Figure 13 where enzyme X4 and the free enzyme exhibit this behaviour.

While with enzyme X1 a large increase in activity between the 5 g and 10 g is seen, with enzyme X3 this is not the case. In Figure 17A, the two conditions of enzyme X3 follow a similar initial rate, but the reaction with 10 g of enzyme X3 retains a higher reaction rate until the 24-hour sample so that it yields 9.02% FFAs more than the reaction with 5 g and 0.05 M citrate buffer.

The enzyme X3 never experienced any leaching above the minimum quantifiable value of 0.1 mg/mL (not presented graphically). The reaction containing 5 g and 0.05 M citrate buffer is consistent with an increase in yield of 1.71% FFAs after 24 hours. Both reactions in Figure 17B retain their activity, but the second batch reaction with 10 g of enzyme only ran for 6 hours, so it is unknown how far the reaction would have gone after 24-hours.

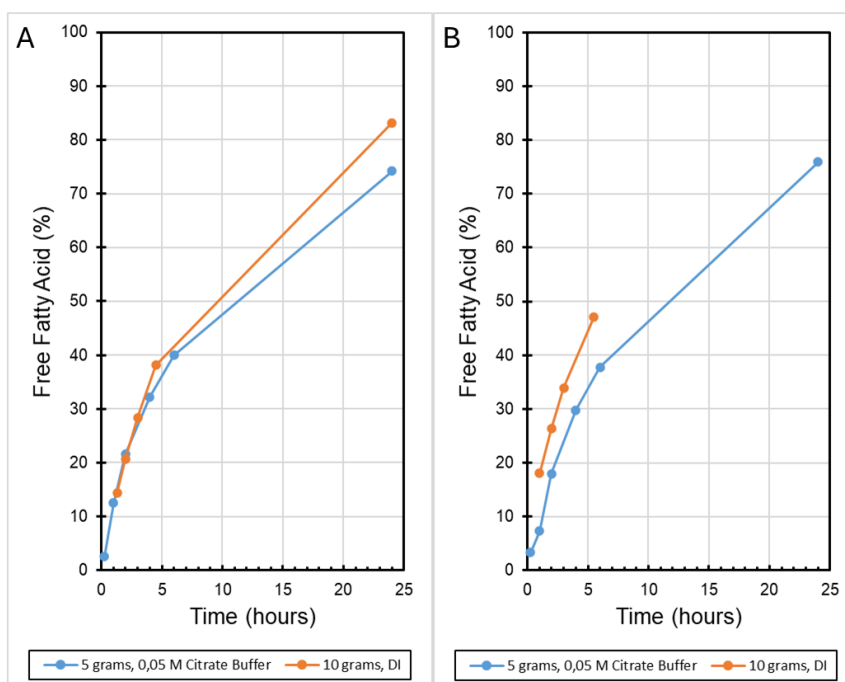


Figure 17. FFA production by enzyme X3 in two conditions for the determination of reusability over two batches. Graph A: X1 First batch with packed enzyme (10 g and DI, 5 g and 0.05M Citrate Buffer, n=1), B: X1 Second batch with packed enzyme (10 g and DI, 5 g and 0.05M Citrate Buffer, n=1).

From this study, it was determined that both X1 and X3 can be reused for at least one batch. While neither enzyme presented a large decrease in FFA hydrolysis at 24 hours, more research is required to determine how many more batches the enzymes retain activity for. Additionally, during the first batch of enzyme X1 in both buffers, low levels of leaching was measured. Enzyme X3 in 0.05 M citrate buffer produced two batches with high FFA yields at 24 hours. This is a promising result as no activity was lost after the first batch. Additionally, the two batches of X3 in 0.05 M citrate buffer can be used a duplicate to show that there is little variation from reaction to reaction with this condition. While this low deviation sample to sample cannot be extrapolated to other conditions, it is a reassurance that the result is not an outlier.

3.6 Partial Glycerides

In Figure 18, the composition of glycerides expressed as percent of total glycerides detected is shown at 6 hours (A) and at 24 hours (B) after reaction with enzyme X1. This presents the composition of only the substrate, TAGs, and the intermediates, DAGs and MAGs. The products glycerol and FFAs are in a separate bar, so that it can be seen how far the reaction has gone. The 2-MAGs (grey) and 1(3)-MAGs (orange) are seen to be evenly represented at 6 hours, but by 24 hours, the 1(3)-MAGs (orange) have increased to be at least double the 2-MAGs (grey). A

similar trend is seen when looking at the composition of DAGs. The 1(3),2-DAGs (yellow) represent the majority of the DAGs at 6 hours, but at 24 hours, the 1,3-DAGs (dark blue) and 1(3),2-DAGs (yellow) evenly represent the DAG composition of glycerides. The percent of TAGs (green) among the glycerides stays consistent at 40-55% between the 6- and 24-hour samples. The percent of FFAs (light blue) expressed as percent of total detected lipids show that at 6 hours, hydrolysis has reached at most 65.7% with tap water, and at 24 hours, hydrolysis has reached at most 85.4% with phosphate buffer.

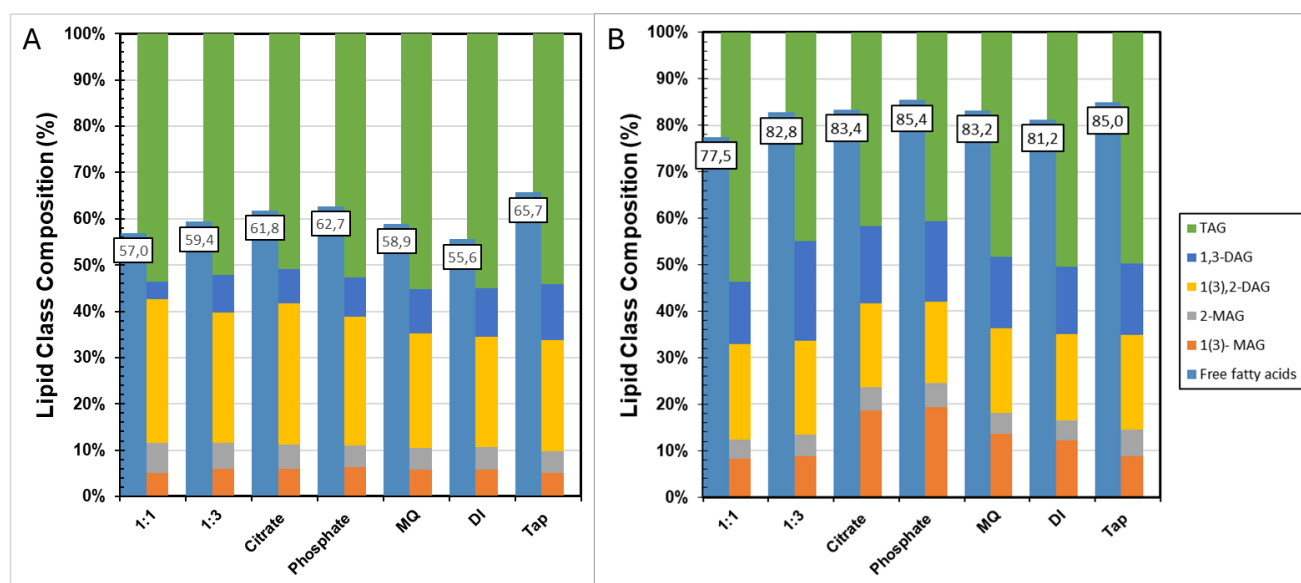


Figure 18. The composition of glycerides relative to total glycerides are shown a one bar equalling to 100% for each reaction condition with reaction using enzyme X1. A second overlapping bar representing the FFA percentage of total detected lipids for that condition at that point in time A. 6 hours with enzyme X1 (ratio 1:1, ratio 1:3, 0.05 M citrate buffer, 0.05 M phosphate buffer, MQ, DI, and tap, n=1). B. 24 hours with enzyme X1 (ratio 1:1, ratio 1:3, 0.05 M citrate buffer, 0.05 M phosphate buffer, MQ, DI, and tap, n=1). Data determined through silylation and GC analysis.

The change in composition in DAGs and MAGs between 6 and 24 hours is most likely due to acyl migration. This effect is seen already at 6 hours as there are even proportions of 2-MAGs and 1(3)-MAGs at that time. As the lipase is sn1,3-specific, it will target only those positions. Therefore, MAGs with cleaved fatty acids at position 2, are not likely from the enzyme but rather from acyl migration. The DAGs have also been affected by acyl migration as the composition between 1(3),2-DAGs and 1,3-DAGs greatly changes. There is little variation in glyceride compositions between sample conditions, and this indicates that the specificity of the lipase does not alter based on conditions like water purity or water quantity, which is expected.

In Figure 19, the results from the reactions catalysed by enzyme X3, analysed in the same manner with the GC to determine the lipid class composition are shown. The patterns regarding MAG and DAG proportions at 6 hours (A) and 24 hours (B) are similar to those of X1. Namely, at 6 hours, the 2-MAGs and 1(3)-MAGs represent the same percent of the glycerides, and the 1(3),2-DAGs represent the majority of the DAGs with 1,3-DAGs only representing about 10% of all glycerides. At 24 hours, the composition of 1,3-DAGs has increased to represent half of the DAGs and about 20% of the glycerides. The composition between MAGs to both DAGs and TAGs alters between 6 hours and 24 hours, with a slight increase in the relative amount of MAGs.

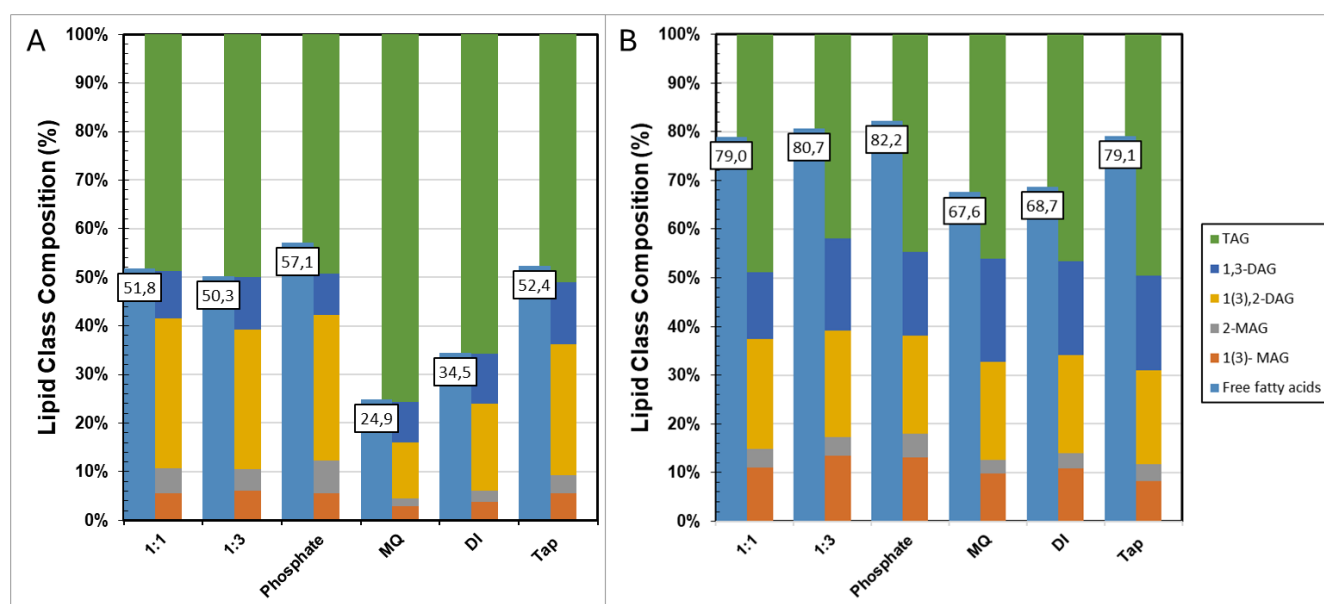


Figure 19. The composition of glycerides relative to total glycerides are shown a one bar equalling to 100% for each reaction condition with reaction using enzyme X3. A second overlapping bar representing the FFA percentage of total detected lipids for that condition at that point in time A. 6 hours with enzyme X3 (ratio 1:1, ratio 1:3, 0.05 M phosphate buffer, MQ, DI, and tap, n=1). B. 24 hours with enzyme X3 (ratio 1:1, ratio 1:3, 0.05 M phosphate buffer, MQ, DI, and tap, n=1). Data determined through silylation and GC analysis.

The increase in composition of 1,3-DAGs at 24 hours is again most likely due to acyl migration. The fatty acids at position 2 of the DAGs appear to have migrated to either position 1 or 3 so that the composition changed between the 6-hour sample and the 24-hour sample.

There is some variation between sample conditions for enzyme X3, particularly for the sample which contained MQ water, compared to the other conditions especially in Figure 19A, showing lipid class composition at 6 hours reaction time. The GC analysis was conducted without replicates, so there is no way to know the reproducibility of the data. The general lack of variation

provides reason to trust the results, however, it is not possible to know whether the 6-hour sample for MQ water is an outlier or not.

Regarding enzyme X4, in Figure 20A and B, the trend in DAG ratios has slightly changed. It appears that while the glycerol compositions at 6 hours for enzyme X4 remained the same as in the 6-hour samples from X1 and X3, the 24-hour sample composition varied. The composition of 1(3),2-DAGs and 1,3-DAGs are not equal, as was observed for the other enzymes at this point. The 1(3),2-DAGs retain a higher composition than the 1,3-DAGs. Another difference is that while the 1(3)-MAGs seem to increase relative to the DAGs and TAGs from 6 hours to 24 hours in enzymes X1 and X3, this is not as strongly seen in enzyme X4 at 24 hours.

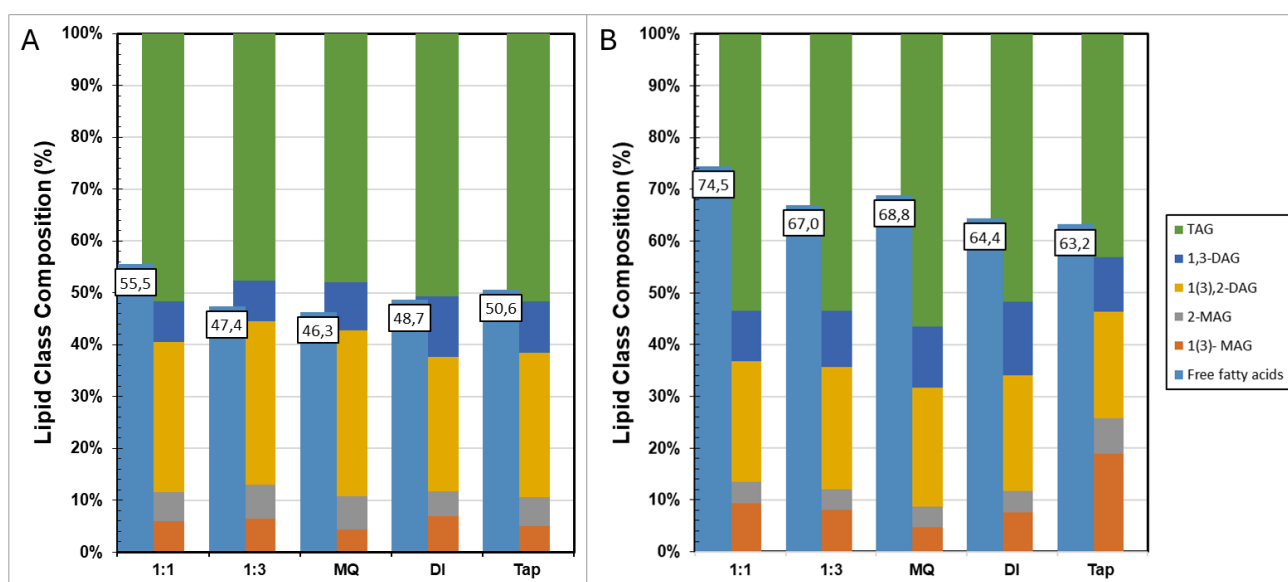


Figure 20. The composition of glycerides relative to total glycerides are shown a one bar equalling to 100% for each reaction condition with reaction using enzyme X4. A second overlapping bar representing the FFA percentage of total detected lipids for that condition at that point in time A. 6 hours with enzyme X4 (ratio 1:1, ratio 1:3, MQ, DI, and tap, n=1). B. 24 hours with enzyme X4 (ratio 1:1, ratio 1:3, MQ, DI, and tap, n=1). Data determined through silylation and GC analysis.

When comparing the three enzymes, X1 and X3 follow similar acyl migration trends. Enzyme X4 also shows to exhibit acyl migration, but seemingly at a slower rate. Alternatively, the enzymatic hydrolysis could be occurring at a faster rate, overshadowing the acyl migration.

From the GC analysis data of partial glycerides, the intention was to measure the concentration of each peak, at various time points so that the amount of each glyceride could be compared. It was expected that the amount of TAGs would decrease throughout the reaction, but this was not

able to be confirmed based on this presentation of the data. Additionally, DAGs and MAGs would initially be at low concentrations then increase and subsequently decrease as the oil in the reaction vessel is hydrolysed fully. The results from Figures 18, 19, and 20 only compare the relative amounts of glycerides, but it would be interesting to continue work further towards optimization of this method so that the concentrations of each component can be accurately determined. The FFA percents found in Figures 18, 19, and 20 can be compared with the calculated FFA percents from the measured acid values, and there is variation between the two values. The GC method is assumed to contain more error in the values than those from the acid values measurements.

3.7 Possible Sources of Error

This project focuses on a broad range of conditions but sacrifices the determination of reproducibility, so the variation due to errors between reaction replicates is largely undefined. Several factors are either observed or hypothesized to have an influence on the variation between samples. The rotating bed reactor is lined with metal mesh to contain the immobilized enzyme but allow reaction medium to pass through. After many batches of inserting, removing, and washing the mesh, it becomes worn, losing its perfect shape against the rotating bed reactor. This allows some particles to escape the mesh and enter the open reaction system. It is not easily observed as the reaction mixture becomes cloudy once the reaction begins, however, so it is unclear how many immobilized particles would have escaped for each trial. However, if there is variation in the amount of enzyme that has escaped based on the newness of the mesh, this could alter the reaction rate, amount of leaching of enzyme, and the pH trend.

According to the Spinchem website, the reactor in this study is suited for reactions between 100-300 mL, however, the reactions conducted in this trial ranged in volume from ~290 mL to ~420 mL, with most trials using a volume of ~320 mL (assuming the density of HOSO is 0.913 g/mL). While this range in volume is only a suggestion, it is possible that the reactions were not mixed as thoroughly as expected. Visually, the majority of the reactions were homogeneously mixed, with the exception being some of the 1:1 ratio trials. The nonhomogeneous mixing could lead to sampling that is not representative of the whole reaction. The lack of optimal mixing could lead to reaction rates that are slower than what would be expected for the different reaction conditions.

The reproducibility of this work is unknown, as so few replicates were conducted. When speaking about the results, nothing can be determined as a significant difference due to the lack of

replicates. The reason so few replicates were conducted, was because the focus of this work was to study a broad range of conditions for several enzyme immobilizations to get a basis of understanding for how the reaction conditions effect the different enzymes. The study can be used as a tool to determine which paths are most interesting to follow in more depth. However, from the limited trials which were replicated, it can be concluded that there is variation between duplicates. This is seen especially for enzyme X3, as the variation between the three trials conducted with tap water vary more than the tap replicates conducted with enzymes X1 and X4.

3.8 Next Steps

The results from this work, as with most, leads to more questions to investigate. In this study, it was found that the immobilized enzymes retain activity during a second batch, however, for this to be a financially feasible industrial process, the immobilized enzymes are required to retain their activity over many batches. Future research into longer reusability studies would be beneficial to determine how many batches each immobilized enzyme can retain activity for. Additionally, the length of the reactions studied in this project were chosen to be 24 hours or less, but longer reaction times could be studied to determine the equilibrium point of the reaction. During trials studying enzyme loading with amounts of 1 g, 5 g, and 10 g, it was not determined if the equilibrium point altered based on the change in enzyme loading. It would be interesting to conduct a study with longer reaction lengths to determine how long each enzyme loading amount requires to reach equilibrium, as well as to determine if there is a difference between them in equilibrium extent. If that were to provide interesting findings, it would be interesting to conduct longer trials for additional conditions to determine if there is further effects on the equilibrium position. For this work however, 24 hours was considered to be close to the equilibrium position of the reaction for the majority of the conditions. Additionally, in this work, no samples were taken between 6 hours and 24 hours, but it would be interesting to study the reaction rate between those time points. The most promising reaction conditions studied in this work were those containing phosphate and citrate buffers, so further research should be done on these buffers. First, the buffered waters should be tested with enzyme X4, as no trials were conducted with this condition. Second, it should be determined whether there is a significant difference in reaction rate between buffered water and non-buffered water for the enzymes. Third, the molarity of the buffered water could be increased to study the influence on enzyme leaching and hydrolysis extent. Further studies could be conducted on the amount of enzyme packing relative to substrate, to determine the fastest reaction rate.

In this study, it was concluded that it is unknown to what extent the hydrolysis of enzyme X4 could be attributed to the immobilized X4 and what could be attributed to the leached X4. Further studies could be conducted to investigate this. One method to try and determine this is to conduct multiple batches with X4 while measuring the amount of protein leaching into the reaction. If the leaching decreases after some number of batches, the remaining, but possibly limited activity, is due to the remaining immobilized X4. The amount of X4 enzyme which is immobilized is unknown, so it would not be possible to know how much is remaining in the immobilization. Another possible method would be to conduct a reaction while maintaining the minimum water activity necessary for the enzyme to be active. This would prevent leaching from happening, and the activity of the immobilization would be measurable.

4 Conclusion

This work focused on the effects of enzyme loading, water purity and pH, water content on FFA production as well as initial hydrolysis rate, pH trends, and enzyme leaching. This work examined these conditions and effects for four enzyme immobilizations, X1, X2, X3, and X4. The results found that each enzyme immobilization had unique responses to the studied conditions.

The first immobilization, X1, produced the highest FFA yields with the three conditions: 5 g enzyme with 0.05 M phosphate, tap water and 0.05 M citrate buffer with 87.4%, 85.7%, and 86.2% FFAs yields respectively. The X1 immobilization exhibited mass transport problems when 10 g was packed into the bed, likely due to swelling of the enzyme causing mass transport limitations. There was an observed change in reaction rate and FFA production when water purity was altered to increase the ionic strength of the aqueous phase, but no effect observed when the water oil ratio was altered. During the reusability trials, X1 with 0.05 M phosphate buffer (pH 7.0) and X1 with 0.05 M citrate buffer (pH 6.3) both retained their activities during a second batch. While leaching was not detected for X1 in DI, MQ, or tap water, low levels of leaching was detected in 0.05 M phosphate buffer and 0.05 M citrate buffer.

The second immobilization, X2, was only studied during the initial phases of the project, as it was subsequently deemed that this reaction method was not ideal for the X2 immobilization.

The third immobilization, X3, produced the highest FFA yields when using 5 g of enzyme and 0.05 M phosphate buffer or 10 g of enzyme and DI water to produce 83.2% and 83.2% FFAs

yields respectively. While determining the best amount of enzyme packing for comparable results, it was determined that X3 does not face major mass transport problems based on enzyme loading. There were observed changes based on the ionic strength of the aqueous phase, with a wider spread between the different water purities than seen for X1 and X4. The trials with tap water were conducted in triplicates, and showed that X3 varied in FFA yield between replicates more than X1 and X4. An increase in hydrolysis extent was observed in both water oil ratios 1:1 and 1:3 compared with 1:1. During the reusability trials, X3 with 0.05 M citrate buffer retained full activity during the second batch. The immobilization, X3 never exhibited measurable amounts of protein leaching, and is seen as the most stable immobilization in this study.

The fourth immobilization, X4, exhibited high initial rates, but for the majority of the conditions could not produce FFA yields higher than about 60% FFAs at 24 hours. The two exceptions where higher FFA yields could be obtained were with 10 g of enzyme with DI water and 5 g enzyme with water oil ratio of 1:1 to produce yields of 73.5% and 76.3% respectively. Similar to X3, the X4 immobilization did not exhibit mass transport limitations during variation in enzyme packing. Through variation in ionic strength of the aqueous phase, X4 showed little change in hydrolysis rate and FFA production at 24 hours. An increase in FFA production at 24 hours, is seen in one instance compared to all other conditions, this instance is when the water oil ratio was changed to 1:1, so that the water in the system is 200 g. However, with this change in FFA production, no change in the pH trend is observed. When comparing X4 with the free enzyme over two batches, it can be seen that X4 and the free enzyme in batches 1 and 2 follow similar trends over the 24 hours. After measuring the amount of protein which leached into the water phase, it was seen that X4 and the batches of free enzyme have similar amounts of free enzyme in the aqueous phase. It was hypothesized that the FFA curve from X4 is more likely shaped by the enzyme which has been leached into the water. The extent to which X4 is dominated by the leached protein, is unknown, however, it is concluded that in order to increase the yields for X4, it must be treated as a free enzyme rather than an immobilized one.

References

- Adlercreutz, P. (2013). Immobilisation and application of lipases in organic media. *Chemical Society Reviews*, 42(15), 6406. <https://doi.org/10.1039/c3cs35446f>
- Al-Zuhair, S., Hasan, M., & Ramachandran, K. (2003). Kinetics of the enzymatic hydrolysis of palm oil by lipase. *Process Biochemistry*, 38(8), 1155–1163. [https://doi.org/10.1016/s0032-9592\(02\)00279-0](https://doi.org/10.1016/s0032-9592(02)00279-0)
- Arbige, M. (1989). Industrial enzymology: a look towards the future. *Trends in Biotechnology*, 7(12), 330–335. [https://doi.org/10.1016/0167-7799\(89\)90032-2](https://doi.org/10.1016/0167-7799(89)90032-2)
- Balcão, V. M., Paiva, A. L., & Malcata, F. X. (1996). Bioreactors with immobilized lipases: State of the art. *Enzyme and Microbial Technology*, 18(6), 392–416. [https://doi.org/10.1016/0141-0229\(95\)00125-5](https://doi.org/10.1016/0141-0229(95)00125-5)
- Borrelli, G., & Trono, D. (2015). Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. *International Journal of Molecular Sciences*, 16(9), 20774–20840. <https://doi.org/10.3390/ijms160920774>
- Causevic, A. (2022). *Lipase-catalyzed production of structured triacylglycerols* [PhD dissertation, Lund University]. <https://portal.research.lu.se/sv/publications/lipase-catalyzed-production-of-structured-triacylglycerols>
- Cosgrove, J. P., Hayden, J. G., Robinson, P. L., & Corp, W. (1991, June 10). *US5194640A - Process for making high-purity oleic acid* - Google Patents. <https://patents.google.com/patent/US5194640A/en>
- Dicko, C., Grey, C., Adlercreutz, P., Larsson, P.-O., & Månsson, M.-O. (2022). *Enzyme technology*.
- Duan, G., Chen, Q., Shao, R., Sun, H., Yuan, T., & Zhang, D. (2024). Encapsulating lipase on the surface of magnetic ZIF-8 nanosphers with mesoporous SiO₂ nano-membrane for enhancing catalytic performance. *Chinese Chemical Letters*, 36(2), 109751. <https://doi.org/10.1016/j.cclet.2024.109751>
- enzymes. (2008). In *International Union of Pure and Applied Chemistry (IUPAC) eBooks*. <https://doi.org/10.1351/goldbook.e02159>
- Escobar, S., Bernal, C., Bolivar, J. M., Nidetzky, B., López-Gallego, F., & Mesa, M. (2018). Understanding the silica-based sol-gel encapsulation mechanism of *Thermomyces lanuginosus* lipase: The role of polyethylenimine. *Molecular Catalysis*, 449, 106–113. <https://doi.org/10.1016/j.mcat.2018.02.024>

- Guajardo, N., Ahumada, K., De María, P. D., & Schrebler, R. A. (2018). Remarkable stability of *Candida antarctica* lipase B immobilized via cross-linking aggregates (CLEA) in deep eutectic solvents. *Biocatalysis and Biotransformation*, 37(2), 106–114.
<https://doi.org/10.1080/10242422.2018.1492567>
- Hasnaoui, I., Mechri, S., Dab, A., Bentouhami, N. E., Abouloifa, H., Bellaouchi, R., Allala, F., Saalaoui, E., Jaouadi, B., Noiriél, A., Asehraoui, A., & Abousalham, A. (2025). Preparation and Biochemical Characterization of *Penicillium crustosum* Thom P22 Lipase Immobilization Using Adsorption, Encapsulation, and Adsorption–Encapsulation Approaches. *Molecules*, 30(3), 434. <https://doi.org/10.3390/molecules30030434>
- Imam, H. T., Marr, P. C., & Marr, A. C. (2021). Enzyme entrapment, biocatalyst immobilization without covalent attachment. *Green Chemistry*, 23(14), 4980–5005.
<https://doi.org/10.1039/d1gc01852c>
- Jawale, P. V., & Bhanage, B. M. (2024). Determination of Stability and activity of immobilized Lipase for transesterification reaction in fluorous solvent and deducing the reaction mechanism by molecular docking study. *Journal of the Indian Chemical Society*, 101(9), 101233. <https://doi.org/10.1016/j.jics.2024.101233>
- Jemli, S., Ayadi-Zouari, D., Hlima, H. B., & Bejar, S. (2014). Biocatalysts: application and engineering for industrial purposes. *Critical Reviews in Biotechnology*, 36(2), 246–258.
<https://doi.org/10.3109/07388551.2014.950550>
- Liu, Y., Jin, Q., Shan, L., Liu, Y., Shen, W., & Wang, X. (2007). The effect of ultrasound on lipase-catalyzed hydrolysis of soy oil in solvent-free system. *Ultrasonics Sonochemistry*, 15(4), 402–407. <https://doi.org/10.1016/j.ultsonch.2007.09.013>
- Noor, I., Hasan, M., & Ramachandran, K. (2003). Effect of operating variables on the hydrolysis rate of palm oil by lipase. *Process Biochemistry*, 39(1), 13–20.
[https://doi.org/10.1016/s0032-9592\(02\)00263-7](https://doi.org/10.1016/s0032-9592(02)00263-7)
- Orata, Francis. (2012). Derivatization reactions and reagents for gas chromatography analysis. *Adv. Gas Chromatogr.—Prog. Agric. Biomed. Ind. Appl.* 83–107.
Palm Kernel Fatty Acid Distillate, Distilled Coconut Fatty Acid, Oleic/Stearic Acid - Olivia Oleo. (n.d.). <https://www.oliviaoleo.com/distilled-fatty-acids.html>
- Rivero, C., & Palomo, J. (2016). Covalent Immobilization of *Candida rugosa* Lipase at Alkaline pH and Their Application in the Regioselective Deprotection of Per-O-acetylated Thymidine. *Catalysts*, 6(8), 115. <https://doi.org/10.3390/catal6080115>
- Robescu, M. S., & Bavaro, T. (2025). A comprehensive guide to enzyme Immobilization: All you need to know. *Molecules*, 30(4), 939. <https://doi.org/10.3390/molecules30040939>

- Salimon, J., Salih, N., & Yousif, E. (2010). Industrial development and applications of plant oils and their biobased oleochemicals. *Arabian Journal of Chemistry*, 5(2), 135–145. <https://doi.org/10.1016/j.arabjc.2010.08.007>
- Secundo, F., Carrea, G., Tarabiono, C., Gatti-Lafranconi, P., Brocca, S., Lotti, M., Jaeger, K., Puls, M., & Eggert, T. (2006). The lid is a structural and functional determinant of lipase activity and selectivity. *Journal of Molecular Catalysis B Enzymatic*, 39(1–4), 166–170. <https://doi.org/10.1016/j.molcatb.2006.01.018>
- Sheldon, R. A., Basso, A., & Brady, D. (2021). New frontiers in enzyme immobilisation: robust biocatalysts for a circular bio-based economy. *Chemical Society Reviews*, 50(10), 5850–5862. <https://doi.org/10.1039/d1cs00015b>
- Tan, H., Aziz, A. A., & Aroua, M. (2013). Glycerol production and its applications as a raw material: A review. *Renewable and Sustainable Energy Reviews*, 27, 118–127. <https://doi.org/10.1016/j.rser.2013.06.035>
- Velasco-Lozano, S., López-Gallego, F., Mateos-Díaz, J. C., & Favela-Torres, E. (2016). Cross-linked enzyme aggregates (CLEA) in enzyme improvement – a review. *Biocatalysis*, 1(1). <https://doi.org/10.1515/boca-2015-0012>
- Wang, N., Wang, W., Su, Y., Zhang, J., Sun, B., & Ai, N. (2025). The current research status of immobilized lipase performance and its potential for application in food are developing toward green and healthy direction: A review. *Journal of Food Science*, 90(2). <https://doi.org/10.1111/1750-3841.70038>
- Zhang, H. (2007). Evaluation of practical process aspects for lipozyme TL IM catalyzed bulk fat modification in a batch reactor. *The Open Biotechnology Journal*, 1(1), 72–80. <https://doi.org/10.2174/1874070700701010072>
- Zhang, T., Yang, L., Zhu, Z., & Wu, J. (2002). The kinetic study on lipase-catalyzed transesterification of α -cyano-3-phenoxybenzyl alcohol in organic media. *Journal of Molecular Catalysis B Enzymatic*, 18(4–6), 315–323. [https://doi.org/10.1016/s1381-1177\(02\)00112-1](https://doi.org/10.1016/s1381-1177(02)00112-1)

Appendix

During the initial phase of the project, control tests was conducted to determine the error between acid values measured in the Lund AAK laboratory and the AAK quality control (QC) laboratory in Karlshamn. The initial acid value measurements of known FFA percents from Lund laboratory and QC laboratory are presented in Table A1. Based on the high error from the measurement containing 0.5% FFAs, the minimum measurement was determined to be 1% FFAs.

Table A1. The determination of error between Lund acid values and QC acid values for known FFA values.

De lobra %	Oleic acid %	FFA %	Syratal	QC Syratal	% error
100	0	0,5	0,967241379	0,03	3124,137931
99	1	1	1,934482759	1,88	2,898019076
95	5	5	9,672413793	9,99	3,17904111
90	10	10	19,34482759	19,9	2,789811125
80	20	20	38,68965517	39,7	2,544949188
60	40	40	77,37931034	80,2	3,517069395
40	60	60	116,0689655	121	4,07523511
20	80	80	154,7586207	159	2,667534157

Additionally, the error between the calculated FFA percent and measured acid value was determined each day prior to the measurement of samples. Table A2 presents a table showing the calculated and measured values. This was used to show that every day that the titrator was used, that the acid value measurement remained accurate. In the case that it was not accurate, troubleshooting including cleaning of the titrator or replacement of the sodium hydroxide solution was implemented.

Table A2. The standards conducted to determine daily variation of the titrator.

HOSO %	Oleic acid %	FFA %	Measured Acid Value
50	50	50,35	100,2
50	50	50,72	100,9
50	50	50,72	100,9
50	50	50,46	100,4
50	50	50,53	100,5
50	50	50,56	100,6
50	50	50,54	100,5
50	50	50,02	99,5
50	50	49,89	99,2

Bradford assays were conducted regularly to measure the protein concentration in the water phase of the samples. The assay was conducted in 96-well plates, and for each plate ran, a 5-point calibration was performed. In Table A3, the slope, Y-intercept, and R^2 value are shown for each date that the Bradford assay was conducted. There was variation in the slope and y-intercept between measurements, but the linearity of the line did not show much variation.

Table A3. Calibration curves from Bradford. The equation including the slope, y-intercept, and R^2 value for each time the Bradford assay was conducted.

Date	A	B	R2	$Y=A*X+B$
02.11	0,494	0,452	0,996	
02.19	0,461	0,47	0,977	
03.07	0,494	0,461	0,993	
03.21	0,507	0,432	0,998	
04.08	0,457	0,466	0,995	
04.16	0,454	0,463	0,997	
05.15	0,435	0,461	1	

During the study on water purity, tap water from Lund was analysed. To assist in the reproducibility of future work related to this project, a translated copy of a quality control test conducted by VA SYD has been presented in Figure A1. The water used in the study labelled 'tap' originated from Lund. Ions vary in tap water depending on its origin, so it is important to include a written document on the contents of the water used for this study.

Water quality in Lund municipality

Places that usually get water from Lake Bolmen.

Lund, Stångby, Odarslöv, Hardeberga, Flyingeby, and most of Södra Sandby.

Drinking water surveys at consumer premises

Subject matter	Unit	Average value Year 2023	Average value Oct-23 to end of year Mar-24	Average Apr-24 to Apr-12 Sep-24	Limit value ¹ National Food Agency
Alkalinity, HCO_3	mg/l	46	46	Ej	Missing
Ammonium, NH_4	mg/l	<0,013	<0,013	<0,013	0,5
Fluoride, F	mg/l	0	0	Ej	1,5
Phosphate phosphorus, PO_4P	mg/l	<0,015	<0,015	Ej	Missing
Color	mg/l Pt	<5	<5	<5	30
Iron, Fe	mg/l	0,03	0,01	0,11	0,2
Calcium, Ca	mg/l	21	19	19	100
Potassium, K	mg/l	1,0	1,0	Ej	Missing
Total chlorine	mg/l	<0,03	<0,03	<0,03	Missing
Chloride, Cl	mg/l	27	29	Ej	250
Coliform baked, 35 °C	cfu/100 ml	0	<1	<1	Demonstrated
Conductivity	mS/m	18	19	20	250
Copper, Cu	mg/l	<0,05	<0,05	<0,05	2
Odor	Missing	No odor	No odor	No odor	Distinct odor
Slow growing bacteria, 7 d	cfu/ml	35	22	61	See ^{below2}
Magnesium, Mg	mg/l	1,5	1,5	1,4	30
Manganese, Mn	mg/l	<0,002	<0,0005	<0,005	0,05
Sodium, Na	mg/l	11	11	13	200
Nitrate, NO_3	mg/l	<0,9	<0,9	Ej	50
Nitrite, NO_2	mg/l	<0,004	<0,004	<0,004	0,5
Culturable microorganisms at 22 °C, 3 d	cfu/ml	8,0	3,4	9,1	See ^{below2}
pH	pH	8,5	8,5	8,5	6,5 - 9,5
Flavor	Missing	No taste	No taste	No taste	Clear taste
Sulphate, SO_4	mg/l	<6	<6	Ej	250
Temperature at sampling	°C	16,0	14,2	18,0	Missing
Total hardness, calculated	°dH	3,2	3,0	Ej	Missing
Total organic carbon, TOC	mg/l	2,2	2,2	Ej	See ^{below2}
Turbidity (cloudiness)	UNF	0,3	0,2	0,3	1,5

The sign < means less than the given value. Only values based on at least two samples are reported.

1) Limit values according to LIVSFS 2022:12

2) No abnormal change - Results are assessed based on VA SYD's normally expected levels.

"Not" means that not enough values are available.

Figure A1. The quality control report of the Lund tap water conducted by VA SYD (Translated by DeepL).

In the interest of determining whether the enzyme carriers effected the pH of water externally to other effects on the pH, a trial was conducted without oil. Trials were conducted in 4 mL glass vials containing 2 g of water and 0.1 g of the respective enzyme. The vials were placed in the Thermoshaker at 50°C and 1200 rpm for 15 minutes, 1 hour, and 2 hours. After removal from the Thermoshaker, the contents were transferred to Eppendorf tubes for immediate measurement of the pH. The resulting pH measurements are displayed in Figure A2. The conclusions from this could be utilized to supplement the pH measurement results from the study.

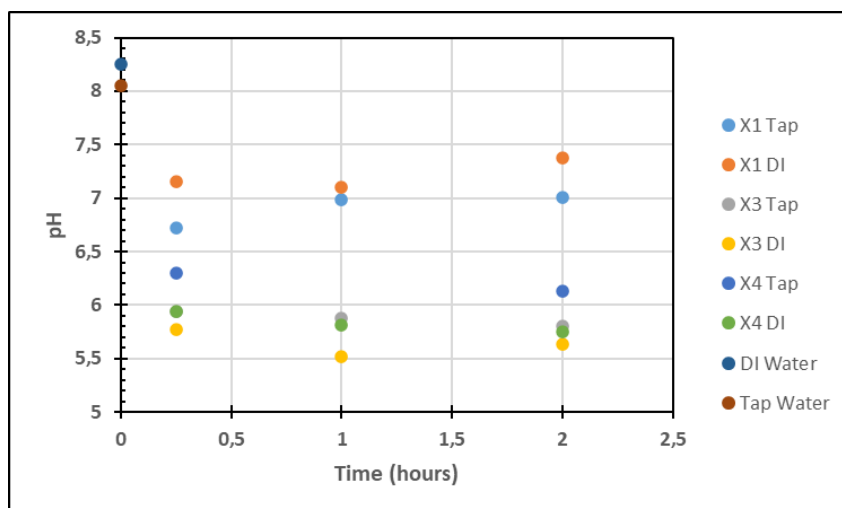


Figure A2. pH measurements of the water after exposure to the enzyme without oil. The sample for X4 with tap water at 1 hour was dropped and no measurement was obtained. For all samples there are no replicates (n=1).

While the reproducibility of X1 and X3 is presented during the study, X4 and X2 were also conducted, however, as there were no other trials conducted to study reuse with those enzymes, this data was not presented as part of the results in the study. In Figure A3, both enzymes show to retain partial activity during the second batch.

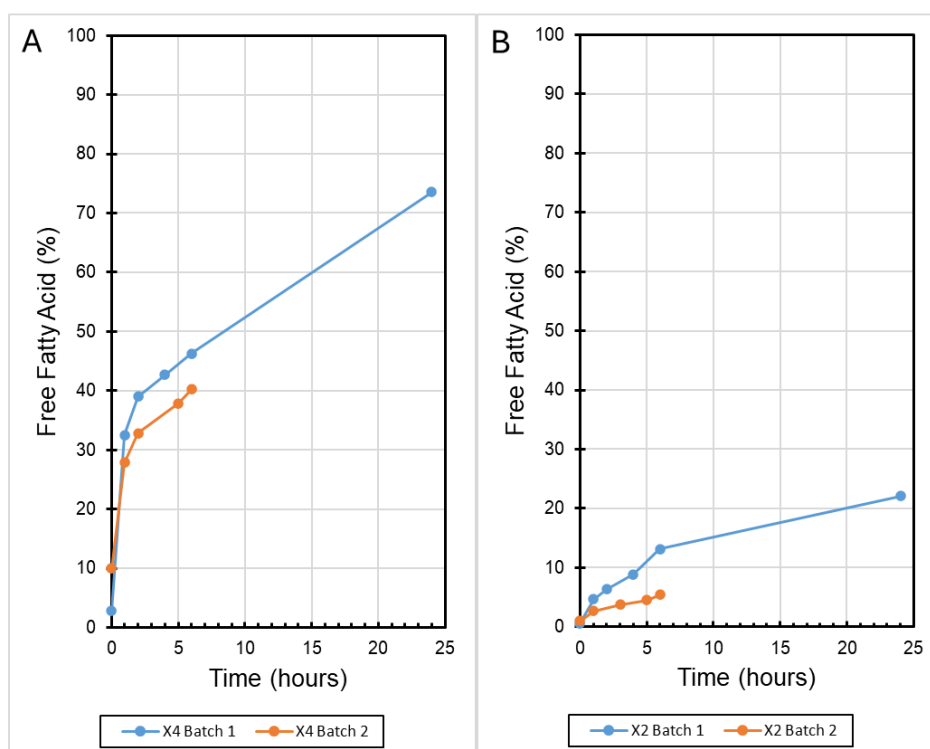


Figure A6. 24-hour Hydrolysis reactions for the determination of hydrolysis extent over two batches. Graph A: X4 (Batch 1 and Batch 2, n=1), B: X2 (Batch 1 and Batch 2, n=1).