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Lipase engineering, production and application

Expanding the lipids tailoring toolbox with applied bioinformatics

Dong, Zehui

2024

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ZEHUI DONG | DIVISION OF BIOTECHNOLOGY | LUND UNIVERSITY

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A better future

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Abstract

Enzymes, as efficient and environmentally friendly catalysts, have gained significant scientific and industrial attention for their mild operating conditions, high substrate selectivity and reusability when immobilized. Among them, lipases dominate the global industrial enzyme market, catalyzing various reactions with a focus on substrate specificity and selectivity. This thesis investigates the optimization of *Rhizopus oryzae* lipase (ROL) for lipid modification, emphasizing its potential for industrial applications.

The research is structured into four key phases aimed at optimizing ROL and its application: 1) Screening the binding interactions between different fatty acid substrates and the ROL molecule, and investigating relevant structural features (**Paper I**); 2) Designing ROL mutants based on structural insights from phase 1, with the objective of altering substrate selectivity (**Paper III**); 3) Developing an efficient and convenient production system for ROL in the oleaginous yeast *Yarrowia lipolytica* (GRAS) (**Paper II**); and 4) Evaluating ROL immobilization using promising methods and carriers (**Paper IV**).

Through the integration of molecular docking, molecular dynamics (MD) simulations and enzymatic experiments, this study explored the interactions between various fatty acids and the ROL enzyme, correlating these interactions with substrate selectivity. Structural insights guided the creation of ROL mutants with targeted mutations in the lid region, leading to the development of a lid-swapped chimera mutant that exhibited increased selectivity for long-chain fatty acids over medium-chain fatty acids – a novel contribution to the field.

Additionally, a high-yield production platform for ROL was developed in *Y. lipolytica* using minimal media, simplifying lipase purification and enabling the evaluation of different expression factors.

Lipase immobilization is crucial for ensuring efficient applications, especially in non-aqueous environments. This study investigated immobilization strategies involving hydrophobic interactions and a combination of hydrophobic interaction and covalent coupling. The results indicate that polyacrylic supports functionalized with a short alkyl chain and a minimal number of epoxy groups are effective for ROL immobilization. Furthermore, the study reveals that ROL immobilization influences its fatty acid selectivity, regardless of whether the process involves pure adsorption or a combination of adsorption and covalent coupling.

Popular science summary

In today's global environment and economy, the pressing challenges of climate change, resource scarcity and environmental degradation have made sustainable solutions more critical than ever. Biological solutions, such as enzymes, offer an innovative and eco-friendly approach to addressing these issues. Enzymes, being highly efficient and specific biocatalysts, can reduce the need for harsh chemicals and energy-intensive processes in industries ranging from agriculture to pharmaceuticals. Enzymes have been performing significant functions as greener alternatives to traditional chemical processes.

Lipase has been one group of enzymes that attracts specific interest due to its versatile applications. Examples include biodiesel (fatty acid methyl esters) synthesis with triglycerides from fats and oils using lipases and biosynthesis of structured lipids with desired properties. As enzymes digesting fats, lipases function by breaking down the ester bonds on lipids mainly existing in the form of triglycerides, which are composed of one glycerol backbone with three fatty acid chains attached.

The structure and composition of triglycerides are versatile due to the diversity of fatty acids (length and saturation) and their distribution on the glycerol backbone (sn1, sn2 and sn3) and this essentially influences the characters of triglycerides and behaviour of lipids. Apart from simply being an energy provider, lipids are also important for food texture and provide health benefits. An example is cocoa butter, which provides the smooth and creamy texture of chocolates and melting-in-mouth feeling, due to the similar melting point as human body temperature contributed by one of its major triglyceride SOS. An isomer of SOS, SSO, which have the same fatty acid composition but different distribution on glycerol, show clear difference in the melting point and therefore will not be able to make this function.

However, not very often, we can find the desired lipids in nature or not in an abundant and sustainable amount. Thus, lipids tailoring using lipases is of specific interest for many food, cosmetics and pharmaceutical applications. The advantages of using lipases instead of a chemical catalyst in industrial processes are specificity, mild conditions and reduced waste. However, this process does face some challenges at the same time, including the production, specificity, selectivity and downstream application. Compared with many other enzymes, lipases face one more application challenge, which is the hydrophobicity of its substrates where lipases are not soluble, and this is normally handled by immobilizing lipases on supports.

Thus, how should we improve the lipase application in industrial processes? It can be tackled from several approaches, including lipase engineering to achieve broader utilizations, optimizing the lipase production process, and improving lipase downstream application via lipase immobilization.

In this thesis, we used a promising microbial lipase, lipase from filamentous fungus *Rhizopus oryzae* (ROL) as a case study to optimize it for a better industrial application.

We investigated the essential structural features of ROL for its substrate selectivity with a bioinformatic approach. Thanks to the modern bioinformatic methods, we can visualize the tiny lipase molecule (30 kDa, radius equal to approximate 2 nm) with our bare eyes on the computer if knowing its DNA sequence information. Furthermore, the algorisms can predict and visualize how a possible substrate molecule may interact with the enzyme molecule during the catalysis, and how stable the interaction might be.

We also investigated the substrate preference of ROL and showed that the unsaturated fatty acid oleic acid (C18:1), which is a long-chain fatty acid with one C=C double bond making it unsaturated, is preferred over its saturated counterpart stearic acid (C18:0), when it is immobilized on hydrophobic particles. However, this preference is different when it is a free enzyme in an aqueous environment, where the enzyme shows the highest preference to saturated middle chain fatty acid, rather than long-chain saturated fatty acid and lastly long-chain unsaturated fatty acid. We managed to alter the substrate selectivity of free form ROL from middle chain length fatty acids to long-chain fatty acids by protein engineering based on the information gained from the bioinformatic approach mentioned above.

An efficient production system of ROL in oleaginous yeast *Yarrowia lipolytica* was also developed in this thesis. The developed production system showed unique advantages, including: 1) high production yield in short time; 2) little byproduct, meaning less work for downstream lipase purification; 3) considered as safe to be used for the food industry.

The produced ROL was immobilized on several supports based on different immobilization principles, and their immobilization results were evaluated. Our results allowed us to suggest characters the immobilization support should have for a better immobilization of ROL.

Holistically, this thesis went through several approaches aimed at improving lipase as a lipid tailoring tool, and for its utilization in industrial applications. Our results provided knowledge and strategies to expand the lipids tailoring toolbox via both lab work and bioinformatic tools.

Enzymes have been discovered and researched for more than 100 years. However, there are still unachieved functions on the wish list. With the development of AI, it would be fantastic if, one day, enzyme molecules with any preferred functions can be designed in a simple click.

Populärvetenskaplig sammanfattning

I dagens globala miljö och ekonomi har de akuta utmaningarna med klimatförändringar, resursbrist och miljöförstöring gjort hållbara lösningar mer nödvändiga än någonsin. Biologiska lösningar, såsom enzymer, erbjuder ett innovativt och miljövänligt sätt att ta itu med dessa problem. Enzymer, som är mycket effektiva och specifika biokatalysatorer, kan minska behovet av starkakemikalier och energikrävande processer i olika industrier, från jordbruk till läkemedel. Enzymer har redan visat sig vara betydelsefulla som gröna alternativ till traditionella kemiska processer.

Lipaser är en grupp av enzymer som väcker särskilt intresse på grund av deras mångsidiga tillämpningar. Exempel inkluderar syntes av biodiesel (fettsyrametylestrar) från triglycerider i fetter och oljor med hjälp av lipaser, samt biosyntes av strukturerade lipider med önskade egenskaper. Som fettnedbrytande enzymer fungerar lipaser genom att bryta esterbindningar i lipider, främst triglycerider, som består av en glycerolryggrad med tre fästa fettsyrakedjor.

Triglyceridernas struktur och sammansättning är varierande på grund av diversiteten hos fettsyror (längd och mättnadsgrad) och deras fördelning på glycerolryggraden (sn1, sn2 och sn3), vilket i sin tur påverkar triglyceridernas egenskaper och lipiders beteende. Utöver att fungera som energikälla, är lipider också viktiga för livsmedelstextur samt ger hälsofördelar. Ett exempel är kakaosmör, som ger choklad dess lena och krämiga konsistens samt den smältande känslan i munnen, tack vare att dess huvudsakliga triglycerid SOS har en smältpunkt nära kroppstemperaturen. En isomer av SOS, SSO, med samma fettsyrasammansättning men olika fördelning på glycerol, visar tydliga skillnader i smältpunkt och kan därför inte uppfylla samma funktion.

Dock är det sällan man finner de önskade lipiderna i naturen eller i en tillräcklig och hållbar mängd, vilket gör att lipidmodifiering med hjälp av lipaser är av särskilt intresse för många livsmedels-, kosmetik- och läkemedelsapplikationer. De specifika fördelarna med att använda lipaser istället för kemiska katalysatorer i industriella processer inkluderar specificitet, milda betingelser och minskat avfall. Samtidigt finns det utmaningar, såsom produktion, specificitet, selektivitet och efterföljande tillämpning. Jämfört med många andra enzymer står lipaser inför en extra utmaning i och med att deras substrat är hydrofoba och inte lösliga, vilket ofta hanteras genom att immobilisera lipaser på bärare.

Hur kan vi förbättra lipasens tillämpning i industriella processer? Det kan angripas från flera håll, inklusive lipasengineering för att uppnå bredare användningsområden, optimering av lipasproduktionsprocessen och förbättring av lipasens efterföljande tillämpning via immobilisering.

I denna avhandling användes ett lovande mikrobiellt lipas, lipas från filamentösa svampen *Rhizopus oryzae* (ROL), som ett fallstudie för att optimera den för bättre industriell tillämpning.

Vi undersökte ROL:s väsentliga strukturella egenskaper för dess substratselektivitet med en bioinformatisk metod. Tack vare moderna bioinformatiska metoder kunde vi visualisera den lilla lipasmolekylen (30 kDa, med en radie på cirka 2 nm) på datorn utifrån dess DNA-sekvens. Vidare kunde algoritmer förutse och visualisera hur en potentiell substratmolekyl kan interagera med enzymmolekylen under katalysen och hur stabil interaktionen kan vara.

Vi upptäckte också att ROL visade en preferens för den omättade fettsyran oljesyra (C18:1), som är en långkedjig fettsyra med en C=C-dubbelbindning som gör den omättad, jämfört med dess mättade motsvarighet stearinsyra (C18:0) när den är immobiliserad på hydrofoba partiklar. Denna preferens skiljer sig dock när det är ett fritt enzym i en vattenmiljö, där det visar högsta preferens för mättade medelkedjiga fettsyror, följt av långkedjiga mättade fettsyror och slutligen långkedjiga omättade fettsyror. Vi förändrade också substratselektiviteten hos den fria formen av ROL från medelkedjiga fettsyror till långkedjiga fettsyror genom molekylärbiologiska metoder (protein engineering) baserat på den information som erhållits från den tidigare nämnda bioinformatiska metoden.

Ett effektivt produktionssystem för ROL med oleaginös (oljerik) jäst *Yarrowia lipolytica* utvecklades också i denna avhandling. Det utvecklade produktionssystemet visade unika fördelar, inklusive: 1) hög produktionsavkastning på kort tid; 2) få biprodukter vilket innebär mindre arbete för efterföljande lipasrening; 3) betraktas som säkert för användning inom livsmedelsindustrin.

Den producerade ROL immobiliserades på flera bärare baserade på olika immobiliseringsprinciper och deras immobiliseringsresultat utvärderades. Våra resultat föreslogvilka egenskaper som immobiliseringsbäraren bör ha för en bättre immobilisering av ROL.

Sammantaget undersökte denna avhandling flera tillvägagångssätt för att förbättra lipaser, som verktyg för lipidmodifiering, och deras användning i industriella tillämpningar. Våra resultat tillhandahöll kunskap och strategier för att utöka verktygslådan för lipidmodifiering med både laboratoriearbete och bioinformatiska verktyg.

Enzymer har upptäckts och forskats på i mer än 100 år, men det finns fortfarande ouppnådda funktioner på önskelistan. Med utvecklingen av AI skulle det vara fantastiskt om enzymmolekyler med önskade funktioner en dag kunde designas med ett enkelt klick.

科普摘要

在当今的全球环境和经济中，气候变化、资源短缺和环境退化等紧迫挑战使得可持续解决方案比以往任何时候都更加重要。生物学解决方案，比方酶，提供了一种创新且生态友好的方法来应对这些问题。酶作为高效且特异性的生物催化剂，可以减少农业到制药等各行业中对烈性化学品和高能耗工艺的需求。酶作为传统化学过程的绿色替代品已经在不同工业应用中发挥了重要作用。

脂肪酶是一类因其多功能应用而备受关注的酶。例子包括利用脂肪酶对来自脂肪和油脂的三酰甘油进行生物柴油（脂肪酸甲酯）的合成，以及具有所需特性的结构脂质的生物合成。作为消化脂肪的酶，脂肪酶通过分解主要存在于三酰甘油形式的脂质上的酯键来发挥作用，三酰甘油由一个甘油骨架和三个连接的脂肪酸链组成。

由于脂肪酸的多样性（包含其长度和不饱和度）及其在甘油骨架上的分布（sn1、sn2 和 sn3），三酰甘油的结构和组成具有多样性，这本质上影响了三酰甘油的特性和脂质的行为。除了作为能源供应者，脂质对于食物质地和提供健康益处也很重要。一个例子是可可脂，它为巧克力提供了顺滑和奶油般的质地以及入口即化的感觉，这归功于其主要三酰甘油 SOS 的熔点与人体温度相近。SOS 的异构体 SSO，虽然具有相同的脂肪酸组成但在甘油上的分布不同，表现出明显不同的熔点，因此无法实现这种功能。

然而，我们并不总是能够在自然界中找到所需的脂质，或存在供应不足且自然资源不可持续的问题。因此通过脂肪酶催化生产结构脂质对许多食品、化妆品和制药应用具有特殊意义。在工业过程中，使用脂肪酶而非化学催化剂的优点是其特异性、反应温和条件和降低污染。然而，它同时也面临一些挑战，包括其大量生产、特异性与选择性和下游应用的优化。与许多其他酶相比，脂肪酶还面临一个实际应用上的难题，即其底物的疏水性导致常见底物与脂肪酶并不相溶，之一难题可以通过将脂肪酶通过不同的相互作用固定在载体上来解决。

那么，我们应该如何改进脂肪酶在工业过程中的应用呢？这可以从多个方法入手，包括脂肪酶工程以实现更广泛的应用、优化脂肪酶的生产过程以及通过脂肪酶固定化改进其下游应用。

在本论文中，我们对一种有前景的微生物脂肪酶——米曲霉（*Rhizopus oryzae*）脂肪酶（ROL）进行案例研究，对其进行优化以实现更好的工业应用。

我们采用生物信息学方法研究了 ROL 对其底物选择性的基本结构特征。得益于现代生物信息学方法，仅需要其 DNA 序列信息，我们便可以使用计算机肉眼可视化微小的脂肪酶分子结构（30 kDa，半径约 2 纳米）。此外，算法可以预测并可视化可能的底物分子在催化过程中如何与酶分子相互作用，以及这种相互作用是否稳定。

我们的研究还发现，当 ROL 固定在疏水颗粒上时，相对于饱和长链脂肪酸硬脂酸（C18:0），固定化的 ROL 对其一种不饱和对应物油酸（具有单个 C=C 双键，C18:1，ω-9）表现出偏好。然而，ROL 在水中以游离状态存在时展现出不同的底物偏好，顺序依次为：对饱和中链脂肪酸显示最高偏好，其次是饱和长链脂肪酸，最后是不饱和长链脂肪酸。我们还基于上述生物信息学方法获得的信息，通过蛋白质工程将游离形式的 ROL 的底物选择性从中链长的脂肪酸改变为长链脂肪酸。

除此之外，本论文还开发了利用产油酵母 *Yarrowia lipolytica* 生产 ROL 的高效生产系统。所开发的生产系统显示出独特的优势，包括：1）短时高产；2）副产物少，减少下游脂肪酶纯化难度；3）可安全用于食品工业。

生产出的 ROL 基于不同固定化原理被固定在多种载体上，同时本论文也评估了其固定化结果。我们的结果对以提高酶固定化效率为目的，固定化载体应具备的特性给出了几点建议。

总体上，本论文通过多种方法，旨在改进脂肪酶作为脂质定制工具及其在工业应用中的利用。通过生物信息学工具导向蛋白质工程和酶生产应用优化，我们的研究结果为进一步优化扩展脂质设计与加工提供了知识和策略。

迄今，虽然人类已经发现并利用酶超过 100 年的时间，但对于酶的功能与应用还有未达成的愿望。随着人工智能的发展，如果有一天能够通过简单的鼠标点击设计出具有任何所需功能的酶分子，酶的巨大潜力还将被进一步发掘以造福于人类。

List of Papers

1.1.1.1 Paper I

Investigation of structural features of two related lipases and the impact on fatty acid specificity in vegetable fats.

Z. Dong, K.Olofsson, J.A. Linares-Pastén and E. Nordberg Karlsson

2022, International Journal of Molecular Science 23(13): 7072

Paper II

Production of *Rhizopus oryzae* lipase using optimized *Yarrowia lipolytica* expression system

L.Vidal*, Z. Dong*, K.Olofsson, E. Nordberg Karlsson and J.M. Nicaud

2023, FEMS Yeast Research 23: foad037

1.1.1.2 Paper III

Exploring the influence of lid region residues on fatty acid selectivity in a lipase orginating from *Rhizopus oryzae*

Z.Dong, M. Haddad Momeni, K. Olofsson, E. Nordberg Karlsson

(Submitted, 2024)

1.1.1.3 Paper IV

Evaluating immobilization preparations of lipase from *Rhizopus oryzae* and their effect to activity and substrate selectivity

Z.Dong, M. Haddad Momeni, K. Olofsson, E. Nordberg Karlsson

(Manuscript, 2024)

* The authors contribute equally to the work.

Author's contribution to the papers

All work described in this thesis was performed under the supervision of *Prof*. Eva Nordberg Karlsson at the Division of Biotechnology, Dr. Kim Olofsson and Dr. Majid Haddad Momeni at AAK AB.

1.1.1.4 Paper I

I designed the study with my co-authors and performed all the experiments. I evaluated the data and wrote the first draft of the manuscript. I revised the manuscript together with my coauthors and submitted the paper as corresponding author.

1.1.1.5 Paper II

I designed the study together with my co-authors. I performed the experiments and analyzed the data including strain engineering (relevant to enzyme production), enzyme production and activity evaluation. I wrote the manuscript covering my part of experimental work and revised the manuscript together with my co-authors.

1.1.1.6 Paper III

I designed the study and performed all the experiments. I evaluated the data and wrote the first draft of the manuscript. I revised the manuscript together with my coauthors and submitted the paper as corresponding author.

1.1.1.7 Paper IV

I designed the study and performed all the experiments. I evaluated the data and wrote the first draft of the manuscript. I revised the manuscript together with my coauthors.

My work is also contributing to

1.1.1.8 Paper I

Characterization of a GH17 laminarinase, MlGH17A, from a laminarin polysaccharide utilization locus in the marine bacterium *Muricauda lutaonensis*.

Leila Allahgholi, Maik G.N. Derks, Zehui Dong, Antoine Moenaert, Javier A. Linares-Pastén, Ólafur H. Friðjónsson, Guðmundur Óli Hreggviðsson, Eva Nordberg Karlsson

Submitted manuscript

1.1.1.9 Paper II

Production and Characterization of Biosurfactants and Emulsifier from Thermophile *Geobacillus 7349*

Roya. R.R.Sardari, Zehui Dong, Carl Grey, Eva Nordberg Karlsson

Manuscript

Abbreviations

sTAGs	Structured triglycerides
GRAS	Generally Recognized As Safe
ROL	Lipase from *Rhizopus oryzae*
RML	Lipase from *Rhizomucor miehei*
SCFAs	Short chain fatty acids
MCFAs	Middle chain fatty acids
LCFAs	Long chain fatty acids
OOO	Triolein
SOO	1-stearin-2,3-olein
PSO	1-palmitin-2-stearin-3-olein
*p*NP	*p*-nitrophenol
MD	Molecular dynamic
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
IDA	Iminodiacetic acid

1 Introduction

Lipases are versatile enzymes which have been drawing attention in different industrial processes, including, but not limited to, the food, nutrition, pharmaceuticals and biodiesel industries. As a biocatalyst, lipase has contributed and still shows a significant potential in pushing the current manufacturing processes to a more environmentally sustainable approach. As one of the most industrially applied enzymes, following carbohydrase (50%) and protease (30%), lipases only account for less than 10% market share [1], which might be due to the cost of production, stability and activity, specificity and selectivity, and application challenges (immobilization is commonly needed because of the hydrophobic nature of lipids), etc. However, lipases play an indispensable role in many industrial processes, and therefore, still continue to be an attractive research topic, as shown by the increasing publication numbers over the years since 1980s, when lipases first became popular in industrial applications (Figure 1). One example process in which the lipases are essential is producing structured triglycerides (sTAGs). Compared with chemical modification, enzymatical modification requires less energy consumption (mild reaction conditions), produces less byproduct and provides higher selectivity and specificity [1–9]. However, the limiting factor here would be that the selectivity of the lipases does not always fit the requirement of the specific processes and the cost of lipases themselves.

Within the global lipase market, 90% of the lipases are produced by microbial organisms recombinantly, even though they are widely presented in versatile organisms in nature, which is due to several advantages such as easy, consistent and cost-effective production.[3,4] The production organisms should fulfil several important criteria: 1) provide high production yield in active form consistently; 2) easy to scale up; 3) the enzyme should be easy to purify from the media; 4) economically feasible; 5) non-pathogenic, and ideally, the organism should be Generally Recognized As Safe (GRAS).

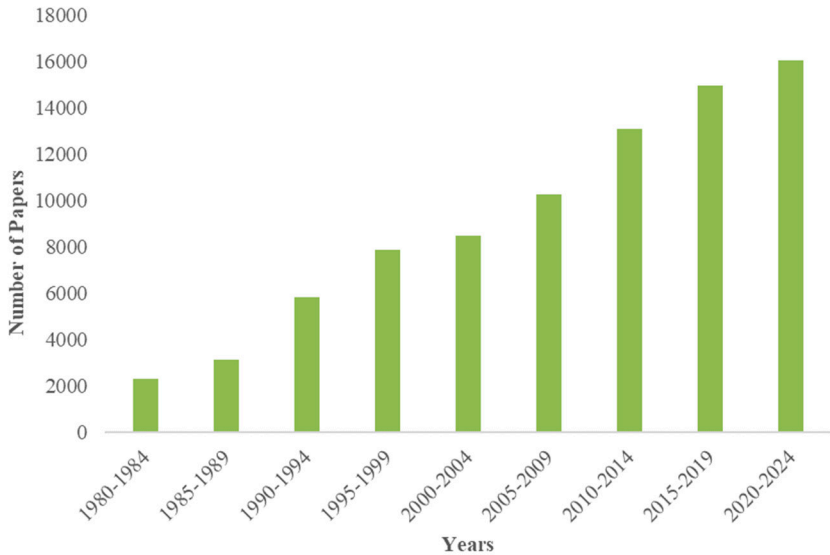

Figure 1. Publication numbers over the years since 1980 with "lipase" as search criteria. Source: Web of Science, via Lund University Library (https://www-webofscience-com.ludwig.lub.lu.se/wos/woscc/basic-search).

Apart from the production, another important aspect in lipase application is the substrate specificity and selectivity. Enzyme-mediated catalysis enables more advanced chemical reactions due to the specific interactions between enzymes and substrates, resulting in the release of selective products. Catalytic environments shaped by the active site periphery have naturally evolved to favour specific or selective reactions. Therefore, changes in an enzyme's structure can significantly alter its catalytic properties, which leads to people's attempts to engineer enzymes by rational design or directed evolution when desired specificity and selectivity cannot be found in nature.

The key point to investigate and to further tailor the enzyme specificity and selectivity, is knowing the biological structure of the enzyme and how the enzyme molecule interacts with the substrate molecules.

Knowing the structure of an enzyme provides essential information on enzyme engineering [10–12] by introducing changes in amino acid sequence on the functional related region, to achieve an improved catalyst for a given reaction or process [13–20]. This approach is called rational design, which is one of the major methods applied in enzyme engineering.

Another important step for lipase application is immobilization. When using lipases in different industrial applications, they are commonly immobilized. Immobilization is generally interesting for many enzymes due to its benefits

including higher stability, reusability, continuous operation and reduced contamination, etc. Lipases immobilization is specifically attractive to the food industry, especially for oil and fat production, due to the hydrophobic nature of the substrates and restrictions on safety. In addition to the usual advantages of enzyme immobilization, it can also result in a significant boost in catalytic activity, hypothetically due to conformational changes in the lipase molecules.

1.1 Aim and Scope of the thesis

The presented thesis concentrated on contributing knowledge and possible strategies to optimize the production and properties of lipase used in industrial applications, initially for the plant-based oil and fat industry by applying bioinformatic tools combined with molecular biology and enzymology. The work utilized a promising lipase in industrial application, lipase from *Rhizopus oryzae* (ROL), as a case study to look into three essential perspectives in lipase application: 1) Developing a convenient production system for lipase in food application (**Paper II**); 2) Investigating the structural features of ROL that are highly relevant to its substrate selectivity, and further engineer it based on the structural information from a rational design approach (**Paper I** and **Paper III**); 3) Evaluating immobilization conditions and their influence on activity and substrate selectivity (**Paper IV**). The workflow is shown in Figure 2. The research aims at investigating and answering how molecular structure of lipase would influence its fatty acid selectivity and if it can be modified via mutagenesis. The study also covered evaluating the impact of immobilization on the lipase activity and selectivity.

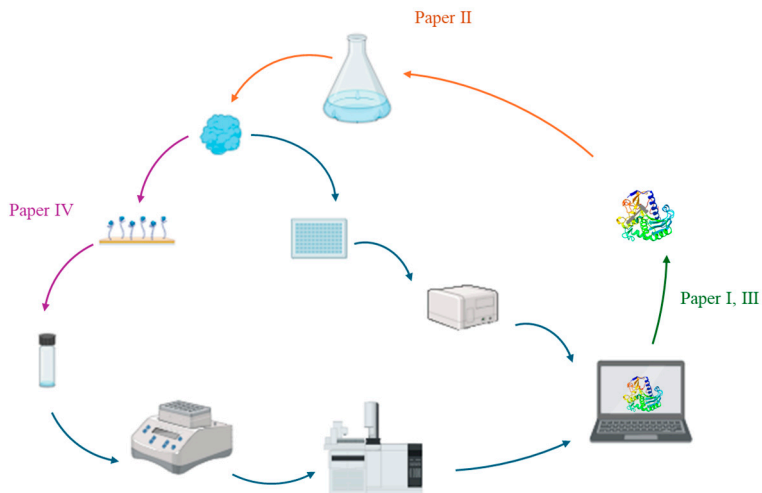

Figure 2. Sketch of the flow of this thesis work. The figure has been generated by using Biorender.

2 Lipase

Lipase (EC 3.1.1.3) belongs to the family of α/β-hydrolases and catalyzes the hydrolysis of ester bonds. It is widely spread across nature to digest oil and fat in plants, animals and microorganisms.

Lipases are one of the most popular industrial enzymes which find extensive use in industries such as food, detergents, biofuel and pharmaceuticals. Especially in the food industry, lipases are used in multiple applications, including but not limited to dairy processing, flavour enhancement, bakery, chocolate and confectionery, and fat modification.

Lipases play an important role in fat modification by tailoring fatty acids on a triacylglycerol backbone to make structured triacylglycerol (sTAG), meeting specific application property or health functions. A variety of fatty acids can locate at different positions of the glycerol backbone including short-chain, mid-chain and long-chain fatty acids.

In this thesis, lipase from *Rhizopus oryzae* (ROL), a promising industrial lipase, caught attention due to its high substrate regio-specificity and stability and versatile applications in different industries, including biodiesel production, structured lipids production and flavour esters production. [10]

2.1 Lipase structure and mechanism

Most of the lipases hold the general α/β-hydrolase fold composed of a central parallel β-sheet consisting of eight β-strands, in which seven β-strands are parallel and one is anti-parallel (β2). The third to eighth β-strands (β3- β8) are packed by six amphiphilic α-helixes. Reactions catalyzed by lipases follow the double displacement mechanism (Ping-Pong mechanism). Several structural features are essential for lipase catalysis, including catalytic triad residues, substrate binding pocket, oxyanion hole and a lid structure functioning as a “switch”.

2.1.1 Catalytic triad and mechanism of lipase

Lipases possess a conserved catalytic triad consisting of a nucleophilic serine (Ser), an acidic residue, which is either an aspartic acid (Asp) or glutamic acid (Glu) and a histidine (His) as base residue [21,22]. The nucleophile Ser is typically located in a conserved consensus motif glycine (Gly)-X1-Ser-X2-Gly in the turn after β5 named "nucleophile elbow", while the acid residue is normally located after β7 and the His is found in the loop after β8 (Figure 3). [23].

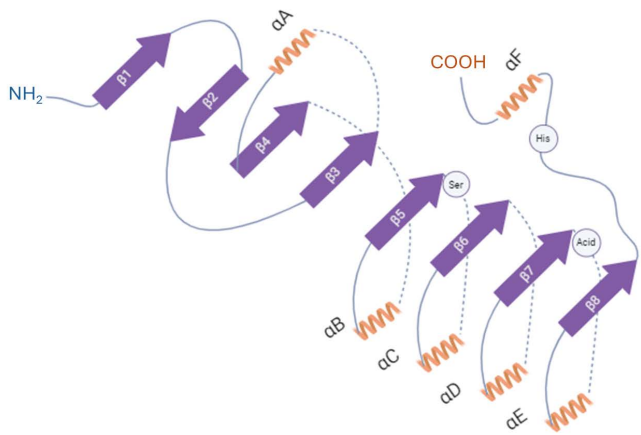

Figure 3. Overview of the structure of α/β-hydrolase. Adopted from David et al. [24]. The figure has been generated using Biorender.

The catalysis of lipases that follows the double displacement mechanism consists of several steps. First, the acid residue forms a hydrogen bond with the imidazole ring of the catalytic His, which increases the pKa of the nitrogen atom and makes the His become a general base, which further deprotonates the hydroxyl group on the nucleophilic Ser. The deprotonated nucleophilic Ser will then attack the ester bonds and form a negatively charged tetrahedral acyl-enzyme intermediate, which is stabilized by the oxyanion hole. The alcoholic group or the glycerol backbone will play as the leaving group. Next, an incoming nucleophile molecule, i.e. a water molecule (hydrolysis) or an alcohol molecule (alcoholysis), which is the acyl acceptor, gets deprotonated by the catalytic His and attacks the acyl-enzyme intermediate, releasing the second product from active site and freeing the enzyme (Figure 4).

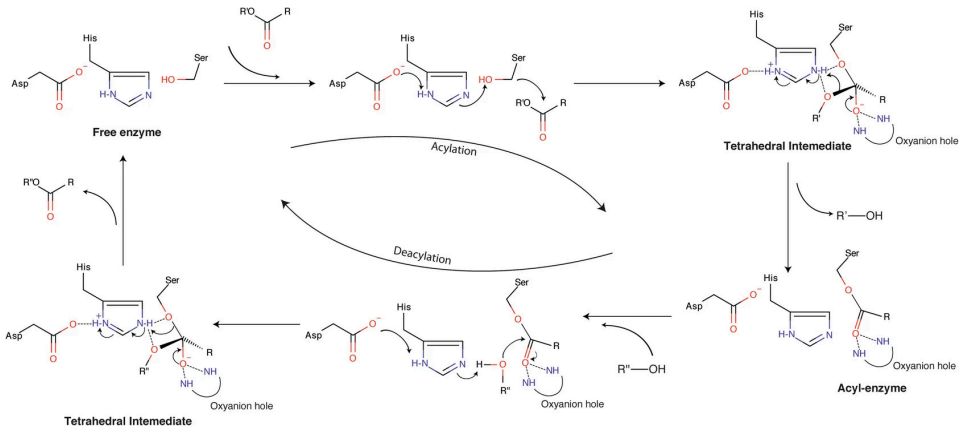

Figure 4. Catalytic mechanism of lipases. Source of illustration: Casas-Godoy, L., et al. (2018) Lipases: An Overview. In: Sandoval, G. (eds) Lipases and Phospholipases. Methods in Molecular Biology, vol 1835, with permission from Springer Nature (License number: 5856530607939).

2.1.2 Lid, the switch of lipases

Lipases show better activity at the aqueous-organic interface compared with a pure aqueous environment with dissolved substrate molecule [25]. The enzymatic activity increases drastically, with a supersaturation level of lipids substrate forming micelles or emulsion [26]. This phenomenon is called "interfacial activation", which can be hypothetically explained by the control of a specific mobile region which is commonly called the lid, located over the catalytic centre of many lipases. The lid domains of different lipases can vary structurally depending on their origin. In a previous review [27], the lid region of 44 lipases, from both eukaryotes and prokaryotes, based on 149 available crystal structures in the Protein Data Bank (https://www.rcsb.org/) have been classified into three different groups: 1) lipases without a typical lid domain; 2) lipases with a lid composed of one loop or helix; 3) lipases with a lid composed of two or more helixes. It has been reported that the lid region is involved in modulating the activity, substrate selectivity and thermostability [27–30]

The lid is amphipathic. When the lid is closed, the hydrophilic side is exposed to the solvent and covers the active site of lipases, preventing the access of the surrounding environment. While interacting with a hydrophobic surface, the lid flaps expose the hydrophobic side and create a large hydrophobic patch named substrate binding pocket, exposing the active site to the substrates.

The shift between open- and closed-conformation of lipases has been supported by their crystal structures. The open-lid crystal structures as complex with substrate-analogue are available in different homologous families. For filamentous fungi

lipases, lipase from *Rhizomucor miehei* (RML) has solved crystal structures in both the open conformation (PDB: 4TGL) and closed conformation (PDB: 3TGL) [31].

However, not all lipases that have open-lid and closed-lid conformations have available 3D structures, due to the practical reasons as described in the introduction. Therefore, the available open-lid crystal structures are valuable templates to generate the homology model of homologous lipases, which does not have the open-lid crystal structure. In this thesis, we generated an open-lid model of ROL (ROLop) with the crystal structure of open-lid conformation of its homologous lipase RML (PDB: 4TGL) (**Paper I** and **Paper III**), which will be further elucidated in Chapter 5. Figure 5 illustrates the conformation change between lid-open and lid-closed ROL and the exposure of active site when the lid is open.

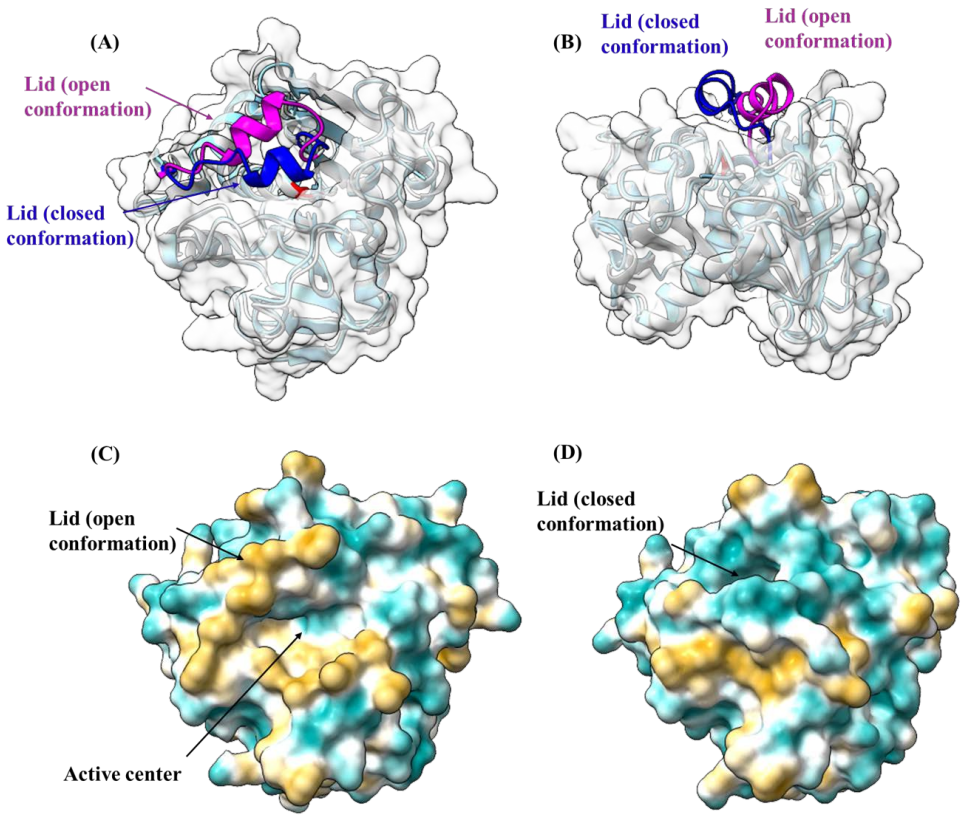

Figure 5. *Rhizopus oryzae* lipase in lid-open and lid-closed conformation.
(A) Front view and (B) side view of superimposed ROL structure in open-lid conformation (homology model, lid coloured in magenta) and closed-lid conformation (PDB:1LGY, lid coloured in blue). The surface characteristics of open (C) and closed (D) conformation where yellow and cyan represent, respectively, the hydrophobic and hydrophilic regions on lipase surface (**Paper III**).

2.1.3 Substrate binding pocket.

In all lipases, the substrate binding centre is located inside a deep elliptical pocket on the top of the central β-sheets. However, the shape of the substrate binding centre varies in different lipases, which relates to their different substrate specificity. Understanding the molecular basis of how substrate molecules may interact and further be catalyzed by lipases is important for choosing appropriate lipases for reactions, and further engineering certain features to get improved enzyme properties[32–35]. Pleiss et.al. compared six representative lipases with different substrate specificities and divided them into three subgroups: 1) lipases with a funnel-like substrate binding region [e.g. lipase B from *Candida antarctica* and mammalian pancreas]; 2) lipases with a tunnel-like substrate binding region (e.g. lipase from *Candida rugosa*); 3) lipases with a crevice-like substrate binding region (e.g. lipases from filamentous fungi like *Rhizopus* and *Rhizomucor miehei*) (Figure 6) [36].

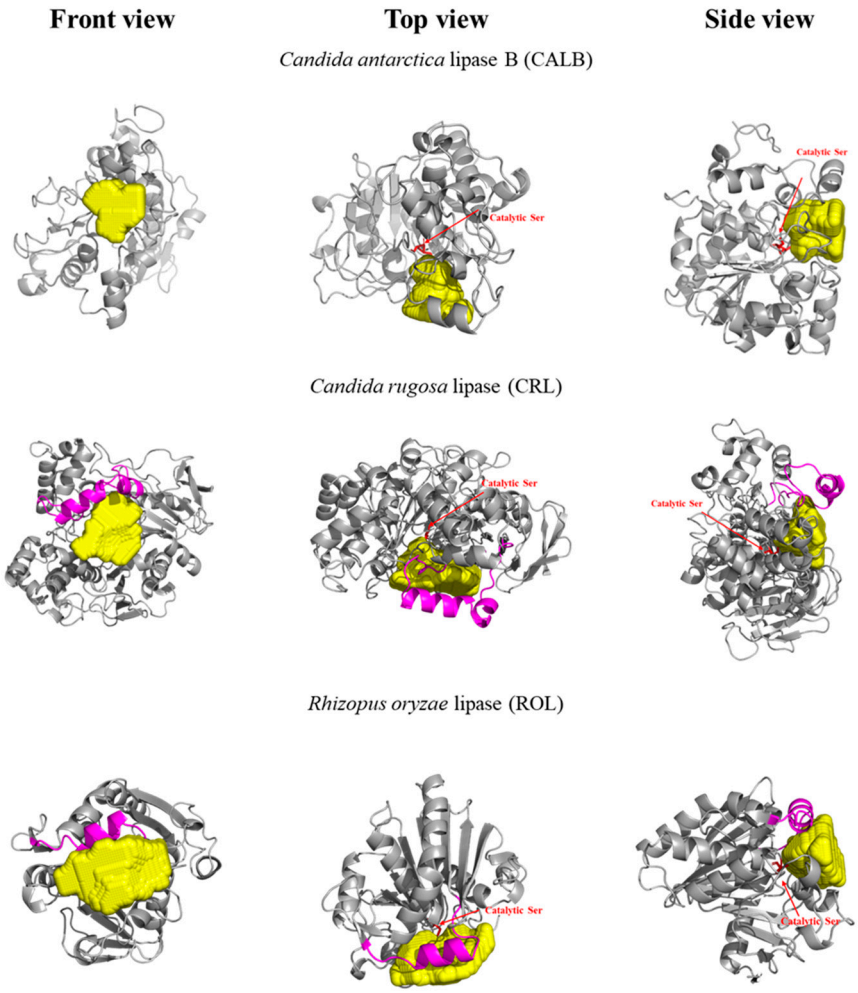

Figure 6. Substrate binding pocket of three classes of lipases.

The shape of the substrate binding pocket (marked in yellow) of lipases are shown from front (left panel), top (middle panel) and side (right panel) views. The lid of different lipases [27] are marked in magenta, and the catalytic serine is marked in red. Lipases with a funnel-like binding site (class 1) are represented by CALB (PDB:5A71 [37]). Lipases with a tunnel-like binding site (class 2) are represented by CRL (PDB: 1CRL, [22]). Lipases with a crevice-like binding site (class 3) are represented by ROL (homology model with template: PDB: 4TGL [21]).

2.2 Lipase catalyzed reactions

Lipases hydrolyze, the ester bonds as the natural function. They can catalyze different reactions depending on the first step acyl donor and the second step nucleophile during the catalysis (Figure 4) [25]. Esters are typical acyl donors, but free carboxylic acid can also serve the function. Depending on the second step nucleophile, the catalyzed reaction can release free carboxylic acid (water as the nucleophile) or a new ester (alcohol as nucleophile). The reaction scheme is elucidated in Figure 7.

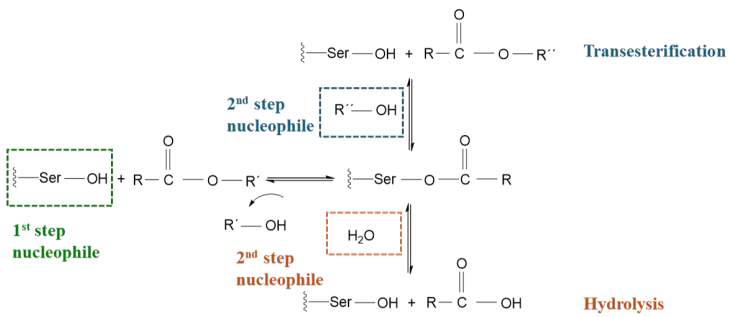

Figure 7. Different reactions catalyzed by lipases. The catalytic serine on a lipase active site can attack the ester bond on esters or carboxylic acid and form an acyl-enzyme intermediate. The intermediate can be attacked by nucleophiles like alcohol or water and release either a new ester (transesterification) or carboxylic acid (hydrolysis).

With this mechanism, lipases can catalyze a bunch of different reactions (scheme shown in Figure 8) based on this mechanism depending on the different substrates. In aqueous media, lipases catalyze the hydrolysis reaction of fats or esters at the interface of water/lipids releasing corresponding fatty acids and glycerol or alcohol. In non-aqueous media, the lipase might catalyze the reverse synthesis reactions, such as esterification, which is the reverse reaction of hydrolysis; and transesterification, which can be further divided into acidolysis (exchange groups between an ester and an acid), alcoholysis (exchange groups between an ester and an alcohol) and interesterification (exchange groups between two esters).

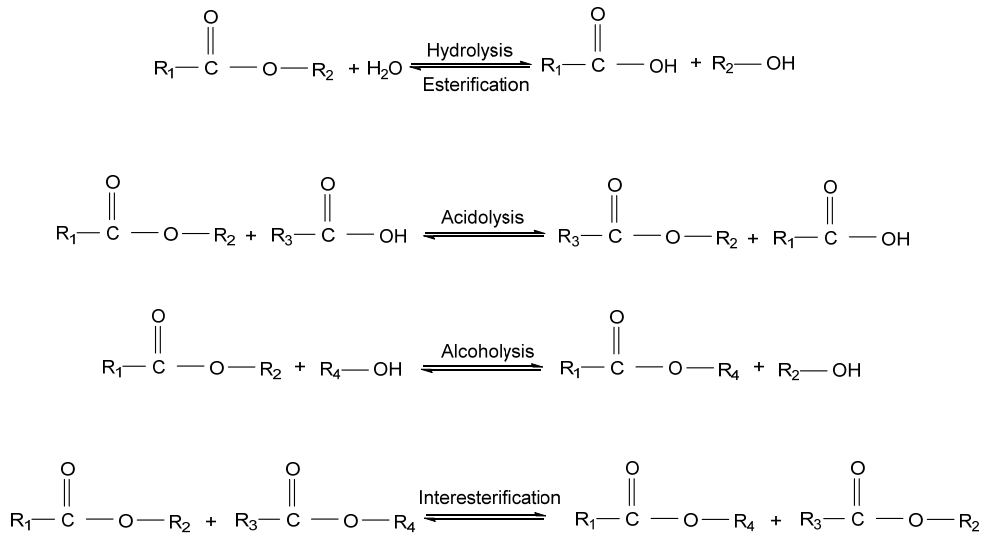

Figure 8. Lipase catalyzed reactions

2.3 Substrate specificity and selectivity of lipases

Lipases' specificity and selectivity are key attributes for their application in structural lipids production for the food and pharmaceutical industry[38–43]. When there is more than one substrate that lipases can act on, i.e. esters of different fatty acids, or ester bonds on different locations on triglycerides, the lipase may have different specificity towards the different substrates, based on the fatty acid types and steric location on triglycerides. The selectivity can be quantified as the ratio between the specificities towards the different substrate [8] (**Paper I and Paper III**). Lipases' specificity and selectivity is highly relevant to their structure. An understanding of the structural features of substrate selectivity could facilitate the production of customized lipases displaying desired substrate specificity and selectivity.

2.3.1 Lipase regio-selectivity

Lipase regio-selectivity is the ability to distinguish between the sn-1,3 (primary) position and sn-2 (secondary) position in a triglyceride molecule, and it is an important character in the manufacture of structured lipids. Based on regio-selectivity, lipases can be divided into non-specific lipases, sn-1,3 specific lipases and sn-2 specific lipases. There has been no strictly sn-2 specific lipases discovered, despite some results showing certain lipases, such as *Candida antarctica* lipase A

[44], behaving with an sn-2 specific character under certain conditions. This is very likely because the sn-2 carbon is difficult to be approached by nucleophile in the enzyme active site due to the steric hindrance. Non-specific lipases catalyze all three ester bonds on the triglycerides, while sn-1,3 specific lipases can specifically recognize and catalyze the ester bonds at the sn-1,3 position. However, a spontaneous reaction, named acyl migration, which happens to diglycerides and monoglycerides during the hydrolysis of triglycerides, should be noticed when defining and utilizing lipases' regio-specificity. Acyl migration transfers the fatty acids on the diglycerides to the adjacent free hydroxyl group, which perform a nucleophilic attack within one diglyceride molecule, very likely from the sn-2 position to sn-1 or sn-3 position due to energy favourability and steric factors. Many lipases, including ROL, are sn-1,3 specific, which makes them a convenient tool for lipids modification.

2.3.2 Lipase chemo-selectivity

Lipase chemo-specificity shows the ability to hydrolyze specific types of fatty acid preferentially or even exclusively, despite lipases showing some activities, regardless of chain length or unsaturation level [6,45]. A lipase may show chemo-selectivity with different specificity towards fatty acids of different length (short, medium and long chain) and saturation level. A fatty acid may contain one or more carbon-carbon double bond(s), making the fatty acid mono- or poly-unsaturated, which introduces essential structural change. An example of C18 fatty acids, stearic acid (saturated) and oleic acid (mono-unsaturated), is shown in Figure 9.

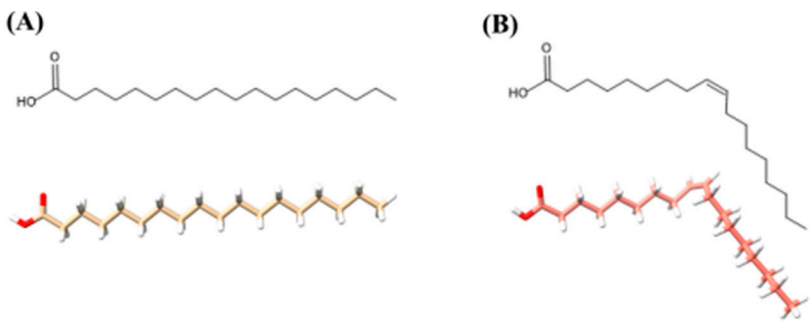

Figure 9. 2D and 3D structure of (A) stearic acid (C18, saturated) and (B) cis-oleic acid (C18, mono-unsaturated). As shown in the figure, the chemical structure of the two fatty acids shows significant difference due to the C-C double bond located between C9 and C10 in oleic acid.

2.4 Characters of lipase from *Rhizopus oryzae*

ROL is highly sn-1,3 specific and natively prefers middle chain rather than long-chain fatty acid substrates. The high regio-selectivity and broad range of substrates makes it convenient to apply in different industrial processes. It has shown great potential as an industrial biocatalyst for lipids modification in different industrial sectors in the past years [46–49], making it a good candidate to be used in a case study to investigate its potential of working even better in industrial applications.

The matured form of ROL (without signal-peptide and pro-peptide) is around 30 kDa. ROL and possesses a typical lid structure, consisting of a short α-helix that covers a crevice-like substrate binding region on the surface of the lipase molecule. It is highly sn-1,3 specific and natively prefers middle chain rather than long-chain fatty acid substrates.

ROL has a homolog from the filamentous fungus *Rhizomucor miehei* (RML), which is also widely utilized in industrial applications for lipid modification. Despite their overall structural similarity, the lid regions of RML and ROL share only 33% sequence identity. In **Paper I**, we constructed an open-lid structural model of ROL using the open-lid crystal structure of RML (PDB: 4TGL) as a template. We then investigated the structural features of ROL and RML that may influence their fatty acid preferences and which contribute to differences in substrate selectivity, using oleic acid and stearic acid esters as substrates. Our results indicated that stearate exhibited less stable molecular interactions within the substrate binding crevice of both RML and ROL, with a higher propensity to exit the binding region, compared to oleate, whose structure is more rigid due to the presence of a double bond. These findings, obtained through bioinformatic analysis, will be further discussed in Chapter 5.

Interestingly, Ala89, located in the centre of the lid structure, and important for substrate entry, is replaced by Trp at the identical location in RML, leading to a hypothesis of an unneglectable effect of this amino acid residue on substrate affinity (**Paper I**). Another residue of interest for substrate selectivity is Phe95, positioned at the end of the lid region, near the terminal part of the substrate binding crevice (**Paper I**) [29,30]. To explore the influence of residue size and polarity on substrate selectivity, site-directed mutagenesis was performed on Ala89 and Phe95 in ROL **(Paper III**). Additionally, to further examine the role of all amino acid residues within the lid structure on substrate binding and selectivity, the entire lid region ROL was swapped to RML, yielding notable effects on substrate selectivity (**Paper III**).

The result indicated that mutations of Phe95, located at the junction of the lid and substrate binding region, to a smaller residue, significantly enhanced the enzyme's selectivity towards medium-chain fatty acids (MCFAs), particularly C8, over short-chain fatty acids (SCFAs, C4) and long-chain fatty acids (LCFAs). Conversely,

increasing the side chain size at position 89 (from Ala89 to Phe or Trp) led to a substantial reduction in overall enzyme activity while also shifting the activity profile towards longer fatty acids. Notably, by swapping the lid region of ROL with that of RML, which naturally contains Trp at the position corresponding to Ala89, the substrate preference of ROL was more effectively adjusted towards LCFAs, away from its original preference for MCFAs (Figure 10).

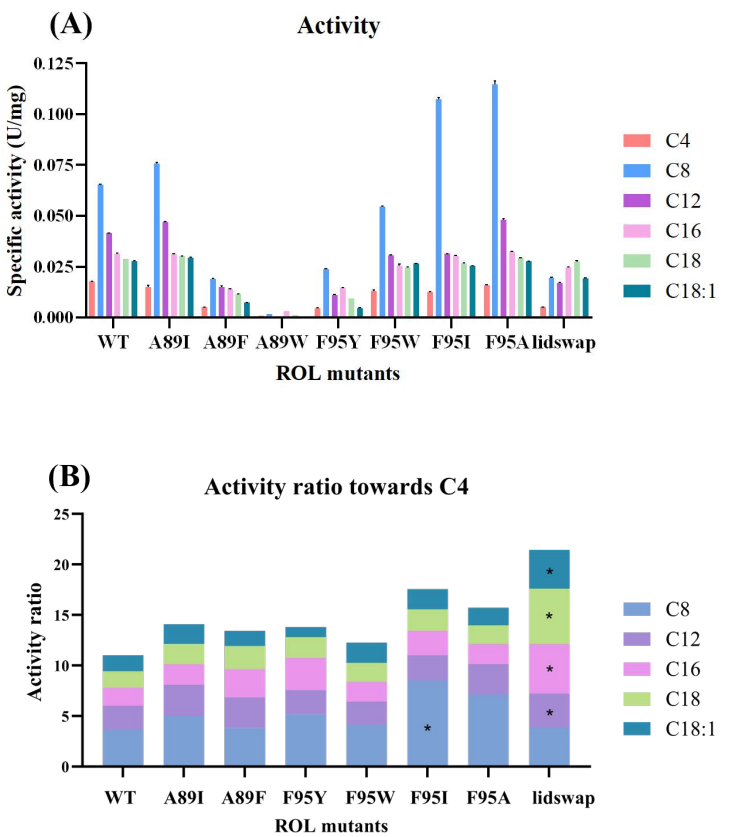

Figure 10. Activity profile of ROL WT and the mutated variants. The substrates used were *p*-Nitrophenyl (*p*NP)-FAs. (A) The specific activities of the produced variants on *p*NP-FAs of different lengths (or saturation). (B) Activity ratio. The activity on *p*NP-mid- (MSFA) and long-chain fatty acid esters (LCFA) are divided by the activity on *p*NP-C4. Adopted from Paper III.

3 Lipase activity assay

3.1 Fast screening method for lipase expressing strain with drop test

The drop test using solid media containing an emulsion of oils and fats is a straightforward and widely used methodology for detecting and screening lipase-producing microorganisms [50]. This qualitative assay involves preparing agar plates with a growth medium that is supplemented with finely emulsified triglycerides, such as triolein (OOO), or simply vegetable oils or fats, such as rapeseed oil, which serve as substrates for lipase activity. Emulsifiers like Tween® (polysorbates) can be also added to stabilize the lipid emulsion, but in small amounts, since they can also act as substrates for lipases.

Microbial strains suspected of producing lipases are inoculated onto the surface of these plates, typically in small drops or streaks, and then incubated under appropriate conditions. During incubation, microorganisms that produce lipases will hydrolyze the emulsified fats, breaking them down into free fatty acids and glycerol. The lipid emulsion typically led to an opaque appearance of the solid media and the lipase activity results in visible clear zones, or halos, around the colonies, indicating lipase production (Figure 11). Using solid media containing different lipids substrate can evaluate the different activities of the produced lipase towards these substrates. For example, as shown in Figure10, the halos that appeared around the positive strain are bigger and clearer when using triolein (OOO) than when using shea olein (mainly POS and SOO) under 28 ºC, which indicates that the produced lipase showed a higher activity towards triolein (**Paper II**).

The inclusion of pH indicators or dyes in the agar can further enhance the detection by causing colour changes in response to fatty acid release. The size and clarity of the halo provide a visual indication of the enzyme's activity level. This method is particularly valuable for initial high-throughput screening and selection of lipase-producing strains in various fields, including environmental microbiology, industrial enzyme production and biotechnological research. Its simplicity, cost-effectiveness and direct visual results make it an essential tool for identifying potential lipase producers.

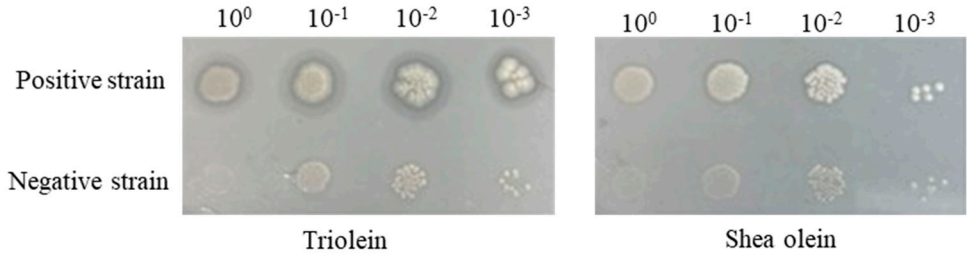

Figure 11. Drop test of ROL production strain of *Y. lipolytica* with solid media containing lipid substrates. Cell suspension was loaded on the solid media with different dilution factors (10^0-10^{-3}).

3.2 Colorimetric assay for lipase free form reactions: hydrolysis with *p*-nitrophenyl substrates

The colorimetric assay using *p*-nitrophenol substrates is a widely utilized and highly effective method for quantifying lipase activity. This assay measures the enzymatic hydrolysis of *p*-nitrophenyl (*p*NP)-esters, where the lipase catalyzes the cleavage of the ester bond, releasing free fatty acid and byproduct *p*-nitrophenol. The released *p*-nitrophenol exhibits a distinct absorbance peak at 405 nm, allowing for its concentration to be measured spectrophotometrically (**Paper II and IV**) [34,51–53].

In this thesis, *p*NP-esters which were composed of fatty acids with different length and saturation levels were used to evaluate the enzyme activity and fatty acid selectivity of different free form (not immobilized) ROL variants. To evaluate the fatty acid selectivity of different ROL variants (**Paper III**), different *p*NP-ester in each hydrolysis were kept in the same molar concentration.

The colorimetric assay using *p*-nitrophenol substrates is widely appreciated for its simplicity and effectiveness, however, it has several drawbacks. One significant limitation is that the assay is restricted to single substrate reactions because the product detected is *p*-nitrophenol, existing in all *p*-nitrophenyl esters. This makes it impossible to simultaneously assay multiple substrates or determine substrate specificity in a mixed-substrate environment. Therefore, if to evaluate the substrate selectivity, the reactions with different *p*NP-esters have to be performed in different individual reactions where it is essential to keep all the reaction conditions and substrate concentration identical, adding experimental complexities and risks of higher operation error.

3.3 Transesterification reaction assay for immobilized lipase

To complement the disadvantage of calorimetric assay with *p*-nitrophenyl substrates as mentioned above, an alternative assay to evaluate lipases´ fatty acid selectivity is hydrolysis/transesterification reaction with mixture of equal amount esters composed of different fatty acids. The quantification of the products, fatty acids or esters, can be achieved through techniques such as gas chromatography (GC) (**Paper I, IV**) or high-performance liquid chromatography (HPLC). This approach allowed the determination of substrate specificity in a mixed-substrate environment, since the determined products were different fatty acid (esters), which can reduce the experimental error caused by running separate reactions.

In this thesis, transesterification between 1-propanol and equal-molar mixed ethyl esters of different fatty acid (SCFAs, MCFAs and LCFAs) was used to evaluate the substrate selectivity of immobilized ROL (**Paper I, IV**). By screening the initial velocity of producing different propyl-esters, the selectivity of immobilized ROL towards different fatty acids can be evaluated by comparing the screened initial production velocity, based on the equation (1) [54]. The quantification of the product propyl-esters was achieved through GC (**Paper I, IV**).

$$\frac{Va}{Vb} = \frac{\left(\frac{Kcat}{Km}\right)a*[A]}{\left(\frac{Kcat}{Km}\right)b*[B]} \quad (1)$$

4 Lipase production with *Yarrowia lipolytica*

An efficient production system of recombinant protein has been gaining increasing economic importance for large scale industrial production of recombinant enzymes or therapeutic proteins [39,55–59]. Apart from the most well-known and well-established efficient working horse *Escherichia coli* (*E. coli*), other recombinant production organisms including other bacteria, yeast, filamentous fungi and mammalian cells were also discovered and developed to be used in various applications with different purposes.

Among different eukaryote production chassis, yeasts, including conventional yeast *Saccharomyces cerevisiae,* and unconventional yeasts, methylotrophic yeast *Komagataella phaffii* (*K. phaffii*, formerly *Pichia pastoris*) and oleaginous yeast *Yarrowia lipolytica* (*Y. lipolytica*) have become popular choices for this purpose, due to their post-translational modification, favourable characteristics and established expression system.

4.1 Lipase production with *Yarrowia lipolytica*

Y. lipolytica is an oleaginous yeast isolated from protein- and lipid-rich environment where the yeast is well adapted to due to abundant genes coding proteases and lipases [55,56,60–62]. The wildtype *Y. lipolytica* possesses 16 gene paralogs for lipases and the main and characterized lipases are encoded by *LIP2*, *LIP7* and *LIP8* [63,64].

Among the three main lipases, the structure of Lip2 has been shown to be similar to ROL [65], which increased the possibility of producing an active form of recombinant ROL. The main promoter used for recombinant production for *Y. lipolytica* includes strong constitutive promoter pTEF, lipids-induced promoters pLIP2 and pPOX2, and erythritol induced promoters. Compared with *K. phaffii*, it has been shown that *Y. lipolytica* performed with higher efficiency in the production of several recombinant proteins, such as the production of another recombinant lipase, CalB. In this thesis, a new expression system for recombinant ROL

production was developed (**Paper II**) and the final yield is 250 times higher than for *K. phaffii* [66].

Several engineered *Y. lipolytica* strains have been developed for recombinant protein production by our collaborator, INRAE (Jouy-en-Josas, France), which provided the platform to develop our production system. The starting strain used in **Paper II** had several modifications to allow the transformation and assessment of recombinant ROL, including: 1) Deletion of three main lipases genes in native *Y. lipolytica* (Lip2, Lip7 and Lip8) [63]; 2) An introduced zeta docking platform to allow the targeted integration of a zeta-based expression cassette [67–69]; 3) Deletion of erythrulose kinase gene *EYK1* for utilization of erythritol inducible promoters [70]; 4) Deletion of *URA3* and *LYS5* to use uracil and lysine as selection markers for gene insertion [70].

4.2 Expression strategies using *Y. lipolytica* for ROL recombinant production

4.2.1 Effect of pre-pro-sequence

ROL, as an enzyme natively from a filamentous fungus, is synthesized as an extracellular lipase precursor bearing N-terminal pre- and pro-sequence before secretion. It has been shown that the pro-sequence of ROL keeps the lipase in a non-destructive form to protect the cell from the toxic consequence of the matured ROL, and also supports the folding of lipase [71]. Therefore, in Paper II, eight different constructs with a different pre- and pro-sequence (depicted in Figure 12) were designed to determine their influence on ROL secretion in *Y. lipolytica*. SP signal sequences are robust signal sequences identified in previous research [72]. SSL signal sequences are signal sequences of *Y. lipolytica* lipase lip2 with modifications.

The eight different constructs were transformed into chromogenic vectors with constitutive promoter, pTEF, and then transformed into *Y. lipolytica*. After 72 hours shake flask incubation, the specific activity of ROL produced by the eight different constructs was evaluated by analyzing the specific activity (mU/mg_{CDW}) towards *p*-nitrophenyl butyrate (*p*-NPB). Our result showed that constructs lacking the pro-sequence resulted in an unstable strain and reduced ROL production. The strong SP signal sequences showed equal efficiency to that of the ROL native signal sequence. The pro-sequence of Lip2 showed a similar protection effect for ROL as that of the native pro-sequence of ROL (Figure 13).

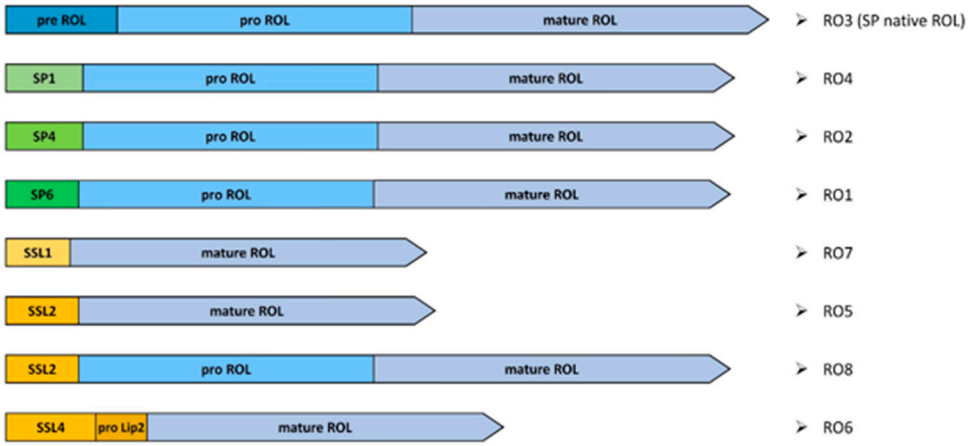

Figure 12. Schematic representation of the different constructions assembled for ROL production in *Y. lipolytica*. These are: the full ROL gene (pre-ROL–pro-ROL–mature-ROL, RO3); three constructions of pro-ROL–mature-ROL using signal sequences previously identified as robust signal sequences [72]—the signal sequence SP1 of the spYALI0B03564g (RO4), the signal sequence SP4 of spYALI0D06039g (RO2), and the signal sequence SP6 of spLip2 (RO1); and different targeting sequences of Lip2 for the expression of the mature-ROL or the pro-ROL–mature-ROL—pre-Lip2 (SSL1)–mature-ROL (RO7), pre-Lip2 with two XA-XA dipeptides (SSL2)–mature-ROL (RO5), pre-Lip2 with two XA-XA dipeptides (SSL2)–pro-ROL–mature-ROL (RO8), and pre-Lip2 with four XA/XP dipeptides (SSL4)–pro-Lip2–mature-ROL (RO6). Adapted from **Paper Ⅱ**.

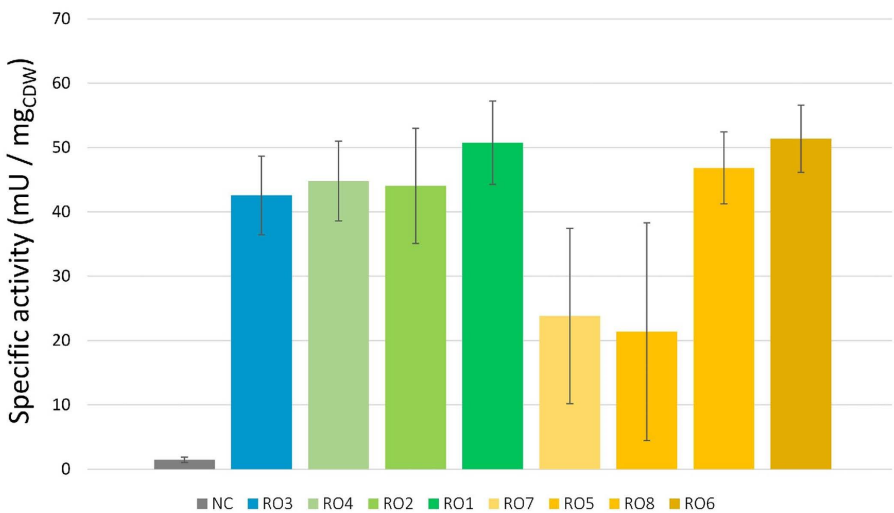

Figure 13. Specific activity of eight constructs with different pre-pro-sequences. NC means negative control which did not possess a ROL expression construct. RO1: SP-proROL-mature ROL; RO2: SP4-proROL-mature ROL; RO3: SPnativeROL- mature ROL; RO4: SP1-proROL-mature ROL; RO5:SSL2-mature ROL; RO6: SSL4-proLip2-mature ROL; RO7: SSL1-mature ROL; RO8: SSL2-proROL-mature ROL.

4.2.2 Effect of promoters

The strength of a promoter is essential for recombinant protein production. However, it has been shown that use of the strongest promoter might not lead to the best protein production, and that the level of produced protein is rather gene/protein dependent [73]. This could be possibly explained by either transcriptional or post-transcriptional regulations, including RNA processing and stability, translation efficiency and the stability and modification of the recombinant proteins [74,75]. Therefore, it is necessary to test several different promoters to get the best production yield.

Four different promoters were tested to choose the most efficient promoter for recombinant ROL production in *Y. lipolytica*: constitutive promoter pTEF, strong constitutive promoter pHp4d, erythritol inducible promoter pHU8EYK and strong erythritol inducible promoter pEYL-5AB. For each construct, four dependent transformants were evaluated for validation purposes. The result showed that erythritol inducible promoters showed significantly higher specific activity compared to the constitutive promoters. The production levels were (88.5 ± 11.1 mU/mg_{CDW} for pTEF, 104.8 ± 10.3 mU/mg_{CDW} for pHp4d, 195.7 ± 19.9 mU/mg_{CDW} for pHU8EYK and 183.4 ± 12.2 mU/mg_{CDW} for pEYL1-5AB) (**Paper II**).

To further increase the ROL yield, a new production strain was constructed by introducing plasmids carrying multi-cassettes that respectively possessed two erythritol inducible promoters pHU8EYK and pEYL1-5AB. The new strain containing a second ROL expression cassette showed significantly higher lipase-specific activity (9 transformants were selected after the transformation for validation purposes, mean of 266.7 ± 19.4 $mU/mg_{CDW.}$). The result is about 1.4 times higher than the mono-copy strain containing pHU8EYK-ROL (185.5 ± 19.4 mU/mg_{CDW}) and about 3.3 times higher ROL expressed than under the pTEF promoter (79.7 ± 12.3 mU/mg_{CDW}) (Figure 14).

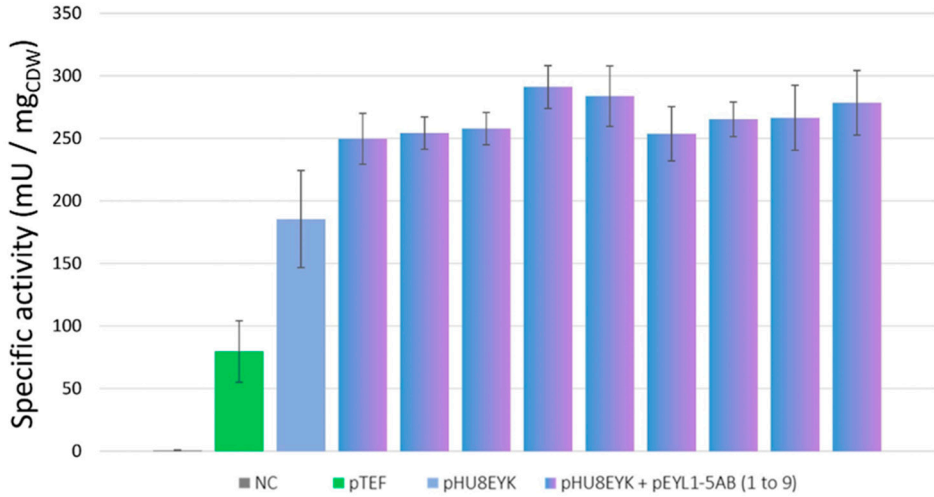

Figure 14. Specific lipase activity of ROL producing strains. Specific lipase activity of ROL of nine independent multi-copy transformants containing ROL under the inducible promoters pHU8EYK and pEYL1-5AB (blue-purple), compared to the mono-copy strain JMY9308 (pHU8EYK, blue), and to JMY9147 (pTEF, green). Strain JMY8671 was used as negative control (NC, grey).

4.3 ROL purification with different strategies

Protein purification strategies are essential in isolating specific proteins from complex mixtures for research or industrial use, especially when using undefined or complex media. Affinity chromatography leverages the specific interactions between a protein of interest and a ligand immobilized on a column matrix, allowing for high specificity and efficiency in purification. Size exclusion chromatography (SEC), also known as gel filtration, separates proteins based on their size and shape, with larger molecules eluting first as they bypass the porous beads in the column, while smaller proteins take longer paths. Ion-exchange chromatography exploits the charge properties of proteins, using either positively (cation exchange) or negatively (anion exchange) charged resins to bind proteins based on their net charge at a given pH, allowing separation through a gradient of salt concentrations. These methods can be individually applied or combined to achieve high purity and yield of the desired protein. In this thesis, affinity chromatography and ion-exchange chromatography were mainly performed.

4.3.1 Affinity chromatography

The purification of recombinant protein with affinity chromatography has been widely applied due to its high efficiency, yield and purity achieved. It is normally achieved by a fusion of target protein and a genetically encoded affinity tag or tail which can be captured by solid matrix. Fusion tags include short sequences such as peptide of six histidine (6xHis), poly-arginine (Arg-tag), FLAG, c-myc and proteins such as maltose-binding protein (MBP). Larger tags have shown positive effect on the solubility of target proteins [76]. In general, the fusion tag can influence the recombinant protein production in different aspects, including the expression levels, solubility, binding between the chromatography ligands, tertiary structure, crystallization and activity, depending on the position (N- or C-terminal of protein sequence), sequence and length of the fusion tag.

In this thesis, ROL purification with his-tag has been attempted on either N-terminal or C-terminal (after the pre-pro-sequence) locations in the expression cassette in *Y. lipolytica*. However, neither design showed specific binding during the protein purification using high-performance immobilized metal affinity chromatography (IMAC) Histrap HP column (Cytiva, Sweden), possibly due to the post-translational modification of *Y. lipolytica*. Therefore, ion-exchange chromatography was applied as a possible substitution.

4.3.2 Ion-exchange chromatography

Ion-exchange chromatography is another common technique for protein purification. It is based on the charge-charge interactions between the proteins in the supernatant and the charges carried by the ion-exchange resins. Resins used in ion-exchange chromatography can be classified into two categories: cation-exchange and anion exchange. Proteins with a net positive surface charge preferentially bind to cation-exchange columns. Successful binding to these columns generally requires that the pH of the binding buffer be lower than the protein's isoelectric point (pI). Cation-exchange resins typically incorporate sulfate-based groups (S-resins), while anion-exchange resins (CM-resins) contain carboxylate groups.

ROL (mature form) has the pI around pH 8.0 predicted by Expasy server (https://web.expasy.org/compute_pi/), and therefore, cation-exchange chromatography was applied for ROL purification. YPD media was used as a complex media for ROL production in the one-step cation-exchange purification trial with HiScreen^{TM} SP HP column (Cytiva, Sweden). The result showed one-step cation exchange gave descent purification result from the raw supernatant from *Y.lipolytica* fermentation with YPD media with a high resolution (Figure.15). However, further purification steps may still be needed if high purity of ROL if necessary.

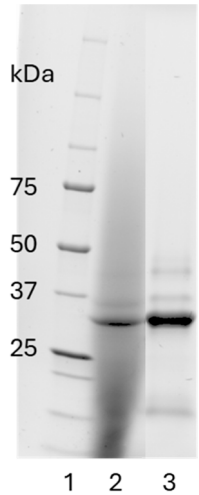

Figure 15. SDS-PAGE showing results of before (column 2) and after (column 3) 1-step cation-exchange purification with SP HP column of ROL WT supernatant using YPD media.

5 Bioinformatic methodology

This chapter will introduce the bioinformatic methods applied in this thesis work. In this thesis, a bioinformatic approach was applied to investigate the structural features of ROL essential for its catalysis function. The aim is to investigate and provide further information on possible strategies to engineer ROL with the intention of increasing its potential for application in lipids tailoring.

As mentioned earlier in the thesis, enzymes are versatile, but unfortunately, natural enzymes do not always meet all the criteria required for different industrial applications, such as high substrate selectivity or selectivity to a certain substrate, long storage time and resistance to sometimes harsh reaction conditions. Therefore, to fulfil these requirements, enzymes are nowadays routinely optimized by enzyme engineering.

There are two main approaches applied in enzyme engineering: directed evolution, and rational design, and they can be combined to form a semi-rational design which is called focused directed evolution [77].

Directed evolution is normally achieved by either randomly recombining several related sequences (gene shuffling) or by random mutation in a single protein sequence (error-prone PCR). However, it is normally labour intensive and time consuming, since several rounds of work with a high number of variants are expected to be screened, and thus it also requires high-throughput screening methods.

Rational design is achieved by making site-directed or random (semi-rational design) mutagenesis on specific functional regions of a protein molecule, based on acquired structural information. It is less demanding on lab labour and assay methods, but it is based on protein crystal structures or reliable models. In this thesis, the rational design approach was applied to the lipase engineering, part supplemented with bioinformatic tools.

Bioinformatic techniques are useful and convenient in providing possible protein engineering strategies; however, to make these tools valuable, they should be combined with traditional wet lab work and chemistry.

In this work, the open-lid model of ROL was built, and different fatty acid ligands were docked into the generated model. The stability and movement of different fatty

acid ligands were screened with MD simulations. This will be explained in the following sections.

5.1 Protein 3D structure modelling

Traditionally, protein structures are solved using methods such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy (cryo-EM). While these techniques are considered the gold standard for determining protein structures, they require substantial experimental effort and can only resolve a small fraction of the billions of known protein sequences.

In addition to the protein structure, understanding the structure of a ligand-protein complex, where the ligand occupies the active site, is crucial for elucidating catalytic mechanisms. However, crystal structures of such complexes are rare for certain lipases due to the elaborate and complex process of crystallization, which requires identifying and applying the correct conditions. Additionally, water is the most common solvent used in crystallization, but lipases often operate in non-aqueous environments like organic solvents due to the hydrophobic nature of their substrates.

Bioinformatic tools offer a promising complement by providing structural information for proteins and protein complexes that are difficult to crystallize or that function in alternative solvents. There are new deep learning methods like AlphaFold that have made it possible to solve protein structures without a known crystal template [78,79]. However, it is not yet straightforward to solve the protein structure in different conformations, such as the lid-open structure of lipases requiring a docked-in substrate/inhibitor.

The traditional homology modelling method is based on physically based models which require a template structure which shares more than 30% sequence identity to the protein whose structure is to be predicted. Building a homology model comprises several key steps: 1) input data; 2) template search; 3) template selection; 4) model building; 5) model quality estimation [80].

The crystal structure of ROL in the lid-closed conformation is available in the PDB database (PDB: 1LGY). However, to investigate the structural features of ROL that are essential for substrate selectivity, it is important to have the ROL with the catalytic centre available for substrate docking, which means the structure of lid-open ROL is required; but unfortunately, there is no available crystal structure.

In **Paper I**, a homology model of open-lid ROL (ROLop) was generated using sequence data from PDB file 1LGY (the structure of the closed-lid ROL, Uniprot P61872) and the open-lid structure of RML (PDB 4TGL) as a template, which has 57.74% identity and 99% coverage, as no crystal structure of ROL in the

open-lid conformation was available in the PDB. The SWISS-MODEL server was chosen for generating the homology model of open-lid ROL, with the reasons provided below:

- Two database searching methods: BLAST [81,82], which is fast and sufficiently accurate for closely related templates, and HHblits [83], which provides sensitivity in case of remote homology, and may increase the coverage of the template options.
- After the search and selection of templates, different models are built according to multiple selected top-ranked templates, which provide the user with the option to use specific template(s) with specific features. An example is, as in **Paper I**, generating the lid-open conformation of lipases, which requires using homologous lipase templates in the lid-open conformation.

The validation of the generated model is important. In **Paper I**, the model was validated with MolProbity on the SWISS-MODEL server. The validation showed a QMEAN value of -1.47 and a MolProbity score of 2.09, which is a log-weighted combination of the clashscore, percentage of Ramachandran not favoured, and percentage of bad sidechain rotamers. Since the MolProbity score of the model was lower than the resolution of the 4TGL template (2.60 Å), the model was considered to be of good quality. This is because a numerically lower MolProbity score, compared to the actual crystallographic resolution, indicates better energy and stereochemical interactions than the average structure at that resolution.

5.2 Substrate docking

Protein-ligand docking is a widely used technique to predict the binding conformation of the small substrate molecule, which is called the ligand, to the protein. It is commonly applied to studies where protein-ligand interactions are of interest, such as drug discovery and enzyme engineering. Docking fatty acid ligands with different length and saturation levels into lipase's active site provides straightforward information on relationships between fatty acid chain specificity and physicochemical properties of the lipase substrate binding site.

In this thesis, the software YASARA was applied for substrate docking simulations, which inhere multiple docking method options for different needs implemented with AutoDock [84]. The covalent docking method was applied in this thesis to mimic a possible covalent intermediate complex of fatty acid ligand and enzyme receptor during catalysis. A few important aspects will be mentioned. First, the enzyme molecule, which is the receptor during the docking simulation, needs to be refined by energy minimization to find a local energy minimum conformation, and cleaned

to add possible missing hydrogen atoms. The water molecules should be removed from the receptor if there are any. Second, the hydrogen that disappears during the bond formation with the ligand should be removed, i.e. in the case of ROLop, the H atom on the hydroxyl group on the catalytic serine should be removed. This should be also applied to the ligand, where the hydrogen disappearing during the bond formation with the receptor should be removed. During the docking, a covalent bond was established between the carboxyl carbon of the fatty acid ligands and the oxygen of the R-chain of the catalytic serine on the lipase molecule. In each docking scenario, both the lipase molecule (receptor) and the fatty acid molecule (ligand) were treated as flexible structures. The generated docking complex was further refined with a short (500ps) MD simulation to get the energy minimum conformation of fatty acid ligand in the enzyme molecule.

By docking fatty acid with different lengths into ROLop, it showed that the size of the substrate binding region fits the best with middle chain fatty acid (C8). When the fatty acid chain is longer than C12, the ligand starts to have conformational change to fit into the substrate binding region (Figure 16-A2 to 16-A3). This provided the structural explanation as to why ROL shows the highest activity towards middle chain fatty acid in the free form (without immobilization).

When docking the saturated C18 fatty acid stearic acid (ROST) and mono-unsaturated C18 fatty acid oleic acid (ROOL) into ROLop, a different binding pattern was observed due to the double bond located in the middle of the oleic acid (Figure 17) (**Paper I** and **Paper III**). However, this significant difference was not observed when docking stearic acid (RMST) and oleic acid (RMOL) into the homologous lipase RML (PDB: 4TGL). The possible reason for this might be the different amino acid components around the lid region (in which RML and ROL share 33% sequence identity) (Figure 17). This is validated by swapping the lid region of RML with ROL, and the result showed that stearic acid and oleic acid showed similar conformation in the substrate binding site in lid-swapped ROLop (lid-swap), which followed the manner of lid donor RML and validated the essential role of the lid region for crevice-binding type lipases.

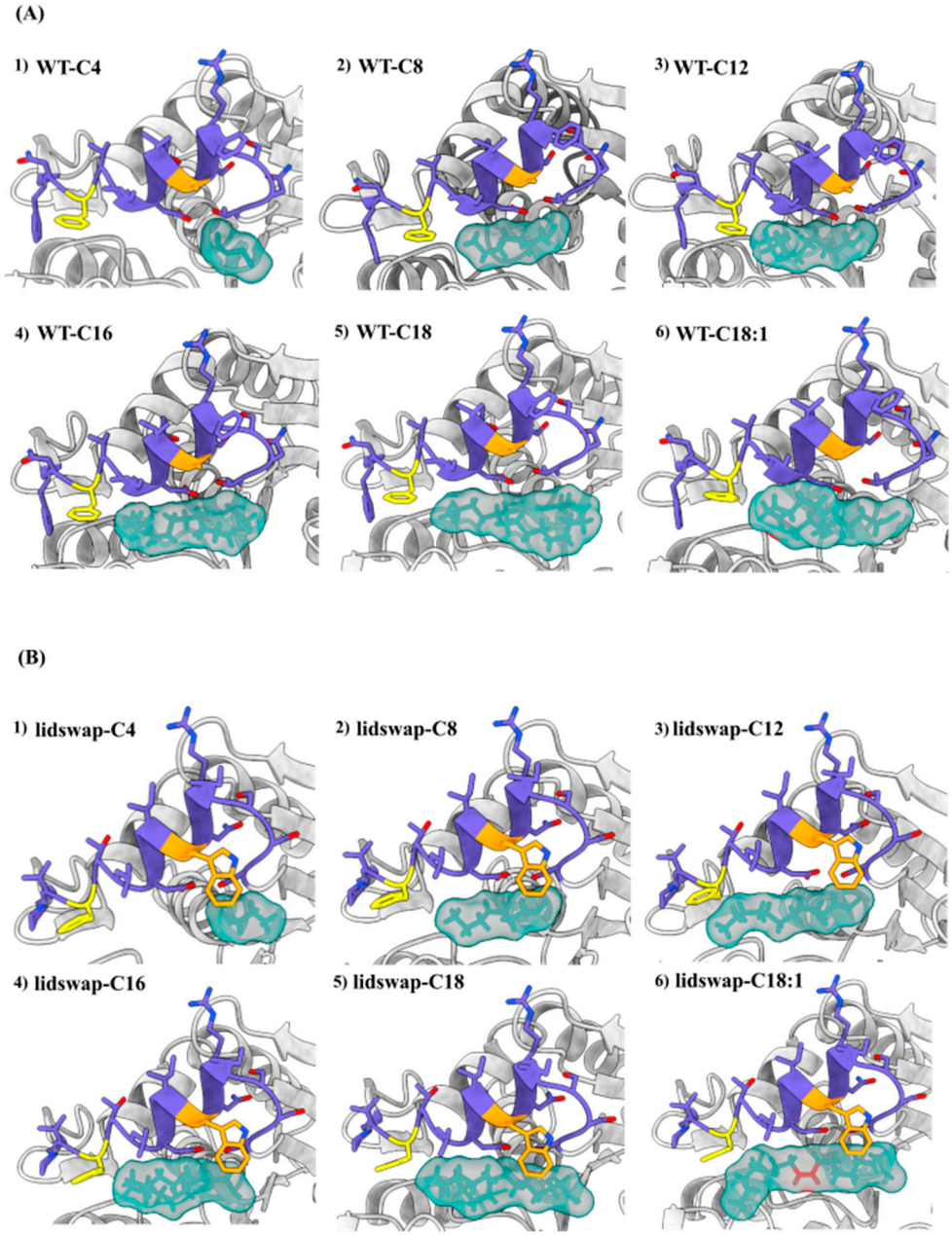

Figure 16. Fatty acid complex with different fatty acid ligands covalently docked in the open-lid conformation in ROL WT (A) and lid-swapped structure (B); modelled using YASARA.

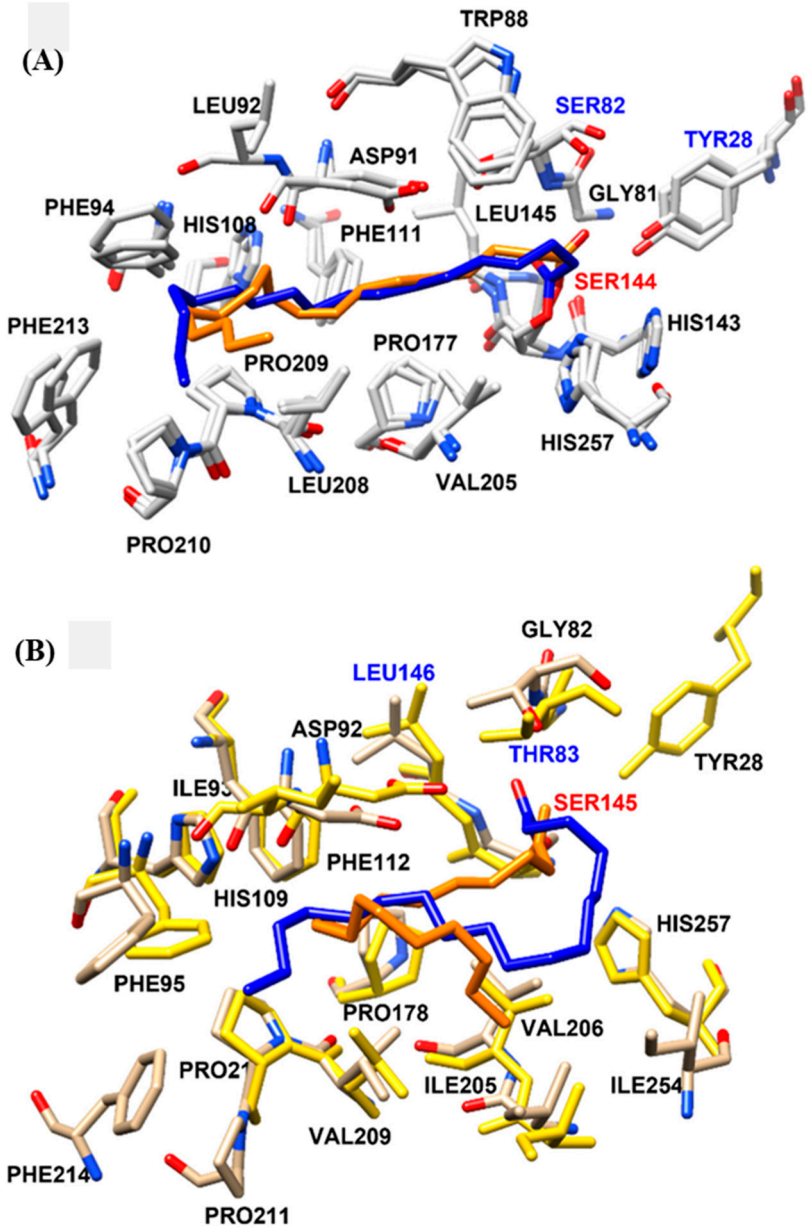

Figure 17. Docking complexes comparison with superposition. (A) Superimposing RML with docked oleic acid (RMOL, amino acid residues marked in dark grey, and the oleic acid ligand marked in orange) and RML with docked stearic acid (RMST, amino acid residues shown in light grey, and stearic acid ligand shown in bright blue). (B) Superimposing ROL with docked oleic acid (ROOL, amino acid residues shown in gold, and the oleic acid ligand shown in orange) and ROL with docked stearic acid (ROST, amino acid residues shown in yellow, and stearic acid ligand shown in bright blue).

5.3 Molecular dynamic simulation

In MD simulation, the conformation change of the protein and ligand molecule and the movement of the ligand molecule inside the binding site are simulated over time. In this thesis, MD simulation was applied in checking the stability of homology models, refining the docking complexes, and screening the stability, movement and conformation change of the ligand in the binding site over the time (50ns). In this thesis, MD simulations were done with YASARA software with AMBER14 force field.

Two important measurements during MD simulations are Root Mean Square Deviation (RMSD), which is defined as the average deviation of all atoms from their starting position, and Root Mean Square Fluctuation (RMSF), a tool which quantifies the dynamics of the polypeptide backbone by finding the extent of movement of each residue around its mean position, throughout the length of the simulation [85]. The ligand movement RMSD and ligand conformation RMSD are useful parameters to screen the ligand status in the receptor during the MD simulation and further evaluate the binding of different ligands [86,87]. In ligand movement, RMSD measures the overall movement or displacement of the ligand relative to its initial position or a reference position during the docking process, while in ligand conformation, RMSD assesses the change in the internal conformation of the ligand itself, irrespective of its position within the binding site. In **Paper I**, a 50 ns MD simulation using cyclohexane as a solvent was applied respectively to the ligand-enzyme complex ROOL, ROST, RMOL and RMST. The result showed that in both ROL and RML complexes, stearate kept a stable conformation but experienced significant movement out from the substrate binding region, while oleic acid experienced higher conformation fluctuation but less movement (higher conformational RMSD).

6 Lipase immobilization techniques

Immobilization is a crucial strategy for improving the performance of lipases under challenging conditions. By attaching lipases to solid supports, immobilization enhances their stability and maintains their activity over extended periods. This process prevents denaturation and aggregation, which are common in non-aqueous media, and helps retain the enzymes' three-dimensional structure, ensuring that the active sites remain accessible to substrates [88,89]. Another advantage of immobilization is easier exploitation of different types of enzyme reactors, especially packed-bed reactors.

The practical benefits of immobilization are numerous. It allows for the recovery and reuse of lipases, making the process more cost-effective and sustainable. However, the performance of immobilization varies with different methods and supports in different conditions. Immobilization can also show a negative effect on enzyme activity, which might be due to four main factors [90]: 1) conformational effects caused by chemical modification of the protein structure during the immobilization; 2) steric effects when enzyme molecules are immobilized in a position whereby the active site is hard to access for substrates; 3) microenvironmental effect caused by partitioning due to the difference between hydrophobicity and electric charge of different components in the reaction mixture; 4) diffusion restrictions that occur when substrates diffuse to immobilized enzymes.

Moreover, immobilization may influence the substrate selectivity of lipases. Changes in the enzyme's microenvironment and spatial orientation due to immobilization can enhance their specificity and efficiency towards substrates. For instance, hydrophobic supports can increase selectivity for hydrophobic substrates by providing a more favourable interaction environment. Additionally, immobilization can lead to conformational changes that stabilize enzyme structures more selectively for specific substrates while also preventing non-target interactions through steric hindrance [88,91–94].

Therefore, it is essential to evaluate different immobilization methods to successfully immobilize the enzyme of interest. Lipases can be immobilized using most of the common methods developed for enzyme immobilization, such as adsorption, covalent coupling, entrapment, and cross-linking, either alone or in combination [25,88,95–97].

6.1 Immobilization with hydrophobic interaction

Physical adsorption, especially with hydrophobic interactions, is frequently used for lipase immobilization, since it is simple to operate, and the condition is mild. Porous hydrophobic polymers like polypropylene, polyethylene or acrylic polymers are traditional common materials for supports maintaining decent activity after immobilization [25,88], while materials like modified silica, resin, agarose and other new materials designed with a different level of hydrophobicity have also been designed and evaluated [88,89,98–106].

The immobilization process relies on hydrophobic interactions between the hydrophobic surface of the polymer and the hydrophobic regions on the enzyme molecule. This not only facilitates the adsorption of the enzyme onto the polymer but could induce the opening of the enzyme's lid under low ionic strength, thereby activating the immobilized lipase. The hypothetical process is based on interfacial activation of lipases, which describes the lid movement exposing the active centre and the large substrate binding pocket, in the presence of hydrophobic structures. In aqueous medium, the open-lid conformation is not stable, and the lipases tend to stay in the closed form. In the presence of a hydrophobic surface, the lipase may become adsorbed on the surface via the large hydrophobic substrate binding pocket (shown in Chapter 2 and 5) as the lipase lid-open form is fixed and activated.

Lipase immobilization on the hydrophobic surface is reversible, which permits the recycling of the immobilization supports by removing the residual enzymes with a moderate concentration of detergents [107].

However, there is also a challenge brought by the reversible interaction. Immobilization by hydrophobic interaction faces the risk of enzyme leakage in highly non-polar reactions, such as biodiesel production, which consequently leads to poor operational stability [25,39].

6.2 Immobilization with covalent binding

A more stable method to improve the enzyme leakage issue is to introduce multi-point covalent coupling for enzyme immobilization [108], where epoxy-activated supports are very promising matrices, since they allow for a very simple procedure for both laboratory and industrial production [49,109]. The epoxy-groups can form stable covalent linkages with different protein groups, including amino, thiol, phenolic, etc., and react with proteins under very mild conditions [109]. However, conventional covalent coupling does present several limitations, which include requiring high salt concentration of protein samples and the possibility of deactivating enzymes, and it may reduce the immobilization efficiency and

performance [25,110–112]. This limitation may be improved by employing a two-step mechanism for protein immobilization to new multifunctional supports (second-generation covalent coupling epoxy supports), which contain two types of functional groups: 1) groups able to facilitate physical adsorption; 2) groups able to covalently immobilize the enzyme. Several different hetero-functional supports have been published, such as amino-epoxy, bononate epoxy, IDA (iminodiacetic acid)-epoxy, IDA-Me-epoxy and di-epoxy, etc. [111–115]. In this thesis, we evaluated immobilization of ROL onto four different commercially available supports, based on hydrophobic interaction with unmodified hydrophobic polymer polypropylene (Accurel MP 1000) and alkyl-modified polyacrylic carrier, or combined hydrophobic interaction and covalent binding with alkyl-epoxy-modified polyacrylic carriers (**Paper IV**).

Concluding remarks and future perspectives

Enzymes, as efficient and organic catalysts, have been attracting both scientific and industrial attention in their current and potential applications, due to their irreplaceable advantages, including milder operation conditions, outstanding substrate selectivity and specificity, easy separation from substrates and reusability (if immobilized), etc. They are playing an important role today in shifting pollutant manufacturing processes to an eco-friendlier approach.

Lipases are the third largest group in hydrolytic enzymes, which is a dominant type in the global industrial enzyme market (>70%). It can catalyze different reactions depending on the environment and the substrates. One of the most interesting and important characters lipases possess is their substrate specificity and selectivity. Efficient production and convenient application methods are also essential for lipase utilization.

This thesis concentrated on investigating knowledge and developing strategies to optimize for better application of lipids modification, with a case study of the lipase from *Rhizopus oryzae* (ROL). ROL has shown its promising potential to be an industry-friendly lipase due to its high specific activity and its wide spectra of substrates. However, there is still the need to further investigate and expand its fatty acid selectivity, to suit more specific target reactions in substrates containing a mixture of fatty acids. For example, vegetable oils, which are common substrates in industrial lipase applications for synthesizing structure lipids.

The work in this thesis aimed at tackling the possibility from four different phases in optimizing ROL and its application: 1) Screening binding patterns between different fatty acid substrates and the ROL molecule and investigating relevant structural features; 2) Designing ROL mutants based on the structural information obtained in phase 1), aiming to alter the substrate selectivity of ROL; 3) Developing an efficient and convenient ROL production system in oleaginous yeast *Y. lipolytica* (GRAS); 4) Evaluating the immobilization of ROL with promising methods and carriers.

Firstly, by combining docking and MD simulation together with enzymatical experiments related to substrate selectivity, the interaction between different fatty

acids, and the structures of the ROL and the related RML molecules were investigated.

Secondly, with the structural information obtained, potentially important residues in the substrate binding site were selected, and single mutations on the lid region were generated to evaluate their effect on substrate selectivity. A lid-swapped chimera mutant was also successfully produced which showed higher selectivity towards LCFAs over MCFAs, which is a novel finding in the field, adding to our knowledge on enzyme substrate interactions. Apart from chemo-selectivity (fatty acids), another essential and interesting substrate selectivity is regio-selectivity. Further focus on bioinformatic methods aiming at investigating the interaction between the lipase molecule, especially the sn-1,3 specific lipases like ROL, and the fatty acid on different locations on triglycerides, can be beneficial for lipase applications in structured triglycerides synthesis, but requires more research.

Thirdly, a convenient production platform for ROL in *Y. lipolytica* was developed in this research with high yield of the enzyme. In our study, minimal media based on yeast nitrogen base and glucose was used for the simplicity of lipase purification and convenience when evaluating different recombination expression factors, including different pre-pro-sequences and promoters. For larger scale production for industrial purposes, other growth media, cheaper and easier to access on an industrial scale, should be tested on the most optimal strain developed in this research.

Finally, lipase immobilization is another essential factor ensuring efficient lipase applications, specifically in non-aqueous environments. In this study we tried immobilization with hydrophobic interactions and mixed hydrophobic interaction and covalent coupling. The findings indicate that polyacrylic supports functionalized with a short alkyl chain and a minimal number of epoxy-groups showed a good result as effective supports for ROL immobilization. Additionally, our study indicates that the immobilization of ROL influences its fatty acid selectivity, irrespective of whether the immobilization process involves pure adsorption or a combination of adsorption and covalent coupling. Future research should aim to explore methods for modulating substrate selectivity between free and immobilized enzymes.

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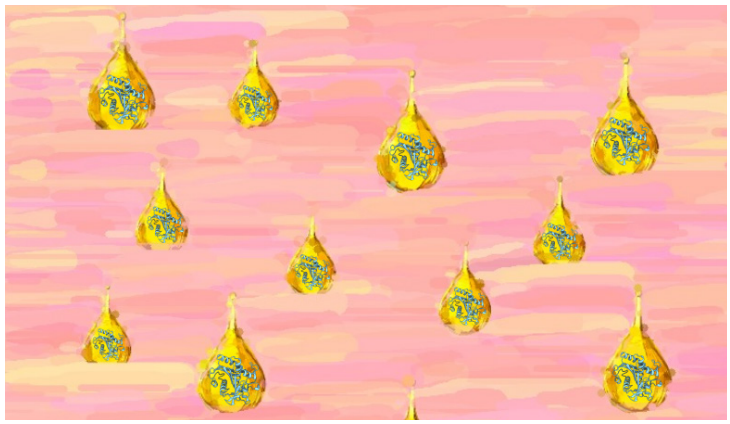

IN TODAY'S GLOBAL ECONOMY, climate change, resource scarcity, and environmental degradation call for sustainable solutions. Enzymes, like lipases, offer eco-friendly alternatives to chemical processes in industries such as agriculture and pharmaceuticals. Lipases are valued for applications like biodiesel production and creating structured lipids. By breaking down triglycerides, they influence lipid behavior, impacting food texture and health benefits, as seen in cocoa butter's smooth texture due to its specific triglyceride composition

The author of this work loves chocolate, so work related with cocoa butter is specifically interesting for her!

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